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The Binding Characteristics of Diphenylhydantoin Sodium to Serum Albumin in Relation to Its Pharmacological Implications

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THE BINDING CHARACTERISTICS OF DIPHENYLHYDANTOIN SODIUM
TO SERUM ALBUMIN IN RELATION TO ITS PHARMACOLOGICAL
IMPLICATIONS

BY

Ronald K. Browne

A Dissertation Submitted to the Graduate School Faculty
of Loyola University - Stritch School of Medicine
in Partial Fulfillment of the Requirement
for the Degree of Doctor of Philosophy

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Finally, I dedicate this dissertation to my wife, Karen, for her love, devotion, spirit, and understanding and to my children, Michael, Sandra and Kimberly who love and perservered a sometimes cantankerous and often part-time father.
BIOGRAPHY

Ronald K. Browne was born in Chicago, Illinois on October 21, 1934. He married Miss Karen E. Cremieux in June of 1962. They have three children, Michael, Sandra, and Kimberly.

Mr. Browne attended Proviso Township High School in Maywood, Illinois, and then Kelvyn Park High School in Chicago from which he was graduated in June, 1952. He attended and was graduated from Wright Junior College in June, 1954. In the fall of 1954, he entered the U.S. Army and served as supply and missile radarman of a classified Nike Missile system. After honorable discharge, he resumed his education at the University of Illinois, majoring in the biological sciences, and was graduated with a B.S. degree in June 1960.

Later in 1960, he joined Armar-Stone Laboratories in Mt. Prospect, Illinois as a junior pharmacologist. He was advanced to senior pharmacologist in 1964. At Armar-Stone Laboratories, he has been engaged in and has responsibility for the supervision of programs involving local anesthetics, catecholamines, central nervous system stimulants and depressants, and the screening and toxicological evaluation of new drugs.

In the fall of 1965, he began graduate studies in pharmacology with the Department of Pharmacology at the Stritch School of Medicine, Loyola University. He received a Master of Science degree in pharmacology from Loyola University in February of 1969.
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CHAPTER 1
INTRODUCTION

The importance of drug-serum protein interaction was vividly demonstrated by Sellers and Koch-Weser in 1970. They showed an enhancement of the hypoprothrombinemic action of warfarin following simultaneous daily administration of warfarin and chloral hydrate over a period of seven consecutive days. Sellers and Koch-Weser indicated that the mechanism involved was the displacement of warfarin from serum-albumin binding sites by trichloracetic acid, a metabolite of chloral hydrate. The authors provided in vivo evidence as well as in vitro warfarin-human serum albumin binding data to support their conclusions. Prior to this study, chloral hydrate was thought to alter the hypoprothrombinemic effect of warfarin by induction of the hepatic enzymes required for the metabolic inactivation of warfarin. The Sellers and Koch-Weser study represents an important contribution to the field of drug-serum protein interaction as it demonstrates a direct relationship between the clinical effectiveness of a drug and its binding to serum proteins.

There are two excellent articles which survey the years of research associated with drug-serum protein interactions. An in depth review by Goldstein (1949) concerning drug and plasma protein interactions focused upon the consequences of the drug-protein complex on efficacy, half-life, distribution, and excretion of drugs. Goldstein indicated that much of the drug-protein interaction work in application to the in vivo situation had been "conceptual and inconclusive, leading to confusion rather than clarity". This indictment was intended to fall upon the mass of literature published
prior to 1942. Although the effect of drug-protein binding in the clinical situation is still a complex question, the confusion as to drug inactivation by its interaction with serum proteins has been answered and positive steps have been taken to clarify the relationship between drug-protein interaction and clinical drug activity. Goldstein's statement that "the capacity of drug molecules to enter into specific combinations with proteins poses for pharmacology its most fundamental task - to comprehend the intimate nature of drug action in terms of these molecular interactions" emphasizes the importance of drug-protein interaction.

The Goldstein review contributed to an increased awareness of, and stimulated many investigators to study, the phenomenon of the drug-protein complex. The resurgence of interest was greatly aided by Cohn et al. (1946) who, through alcohol fractionation and electrophoretic analysis, developed a system for separating protein and lipoprotein fractions of biological tissues and fluids. Since 1949, considerable effort has been spent in describing and clarifying the binding of a considerable portion of the new drugs now available.

In 1968, Meyer and Guttman reviewed drug-protein binding research for the period between 1949 and 1968. In very brief tabular form, they summarized the interaction between serum proteins and hypnotics, anticoagulants, CNS stimulants, anti-infectives, cardiovascular agents, psychotropics, etc. Their review indicated that in vitro and in vivo evidence amply demonstrated that the serum protein binding can influence the distributional, pharmacological, and pharmacokinetic properties of certain drugs. The drug-protein interaction phenomena is now being more carefully scrutinized in terms of possible clinical implications. Meyer and Guttman caution that "evidence
exists that only in the case of highly bound agents will binding be important in a practical sense. They conclude: "A number of important drugs do, however, fall in the category of 'strongly bound' and they serve as examples which emphasize the need to at least consider protein binding as a necessary parameter in the characterization of drug behavior".

The phenomena known as drug-serum protein binding has been a subject of research and speculation for seventy-three years. The primary impetus for stimulating this interest may be attributed to four investigators. In 1898, Mathews reported some of the first laboratory observations involving the interaction of proteins with chemical agents. Mathews indicated that food stuffs combined chemically with coagulated egg albumin in solution. Heidénhain (1902) added further evidence when he reported that aniline dyes combined chemically with egg albumin. At approximately the same time, Moore and Roaf became involved with the drug-serum interactions. These two investigators demonstrated in 1904 that chloroform and ether had a greater solubility in serum than in saline. They attributed the increased drug solubility to "substance interaction with proteins". Since 1904, the literature is replete with references to various aspects of drug-protein interaction.

The implications inherent in the initial findings of Mathews, Heidénhain, Moore and Roaf did not become readily apparent until the 1920's. Between 1920 and 1942, a host of investigators began to delve into the questions posed by the interaction of drugs with serum and serum proteins. Due to limited instrumentation and technology, research was limited to dyes, plant extracts and the few pure drugs readily available. Nevertheless, answers as to which proteins were combining with drugs, what occurs after the union, and what effect does a change in hydrogen ion concentration have upon the
drug–protein complex, were being provided.

In 1913 Oppenheimer determined that the addition of serum to digitoxin solutions markedly diminished the digitoxin toxicity to the isolated frog heart. The time lag, apparently due to technology, was evident since it was not until 1931 that Hoekstra, and Brücke in 1934, confirmed and extended Oppenheimer's work, finding that rabbit serum could completely protect the isolated frog heart from digitoxin poisoning. It was not until 1944 that it was determined what constituent of serum was responsible for the digitoxin inactivation. Fawaz and Farah found that the protection against digitoxin (1:100,000) poisoning in the isolated frog heart appeared to be due to the albumin fraction. Furthermore, this protective action was directly proportional to the protein content of the solution. Two percent rabbit serum globulin, lipids, and soluble proteins extracted from the heart, liver and striated muscle, were not found to decrease digitoxin toxicity.

A major contribution to the knowledge of drug–protein interaction was made by Storm Van Leeuwen (1921) who studied the effects of pilocarpine on isolated cat gut preparations. He found that pilocarpine solutions in rabbit serum required forty times more pilocarpine to produce gut contractions compared to those observed with pilocarpine alone dissolved in saline. The inactivation of the drug was attributed to a physical combination or adsorption to the "Colloids" or some substance in serum, rather than a chemical change. He stated that a chemical change would cause drug destruction which had not occurred. To determine the possible in vivo significance of this drug–serum interaction, cats were treated with pilocarpine solubilized in serum. The animals, injected i.v. with 10 mg./Kg. non-serum pilocarpine solutions, exhibited salivation, dyspnea, vomiting and sometimes death, while cats treat-
ed with serum solutions containing the same amount of pilocarpine had less intense symptoms with only slight dyspnea. No deaths occurred.

Continuing with his work, Storm Van Leeuwen teamed with Zeigner (1921) to repeat the prior work but included atropine. They stated: "Very often we found that a solution of 1 mg. of atropine in 5 cc. of rabbit serum showed only one-thirtieth or one-fortieth the original strength, so that about 96 or 98 percent of the atropine had been brought into an inactive form." However, they also found that cat serum, human serum, milk, etc. had little or no adsorbing power for atropine. Since at that time the presence of atropine-esterase in certain rabbit strains was not known, they concluded that the difference in adsorbing power could be ascribed to differences in "colloidal nature" of the various serums. It took another thirty-six years before the interaction of atropine with the plasma proteins of other species was documented. Tonnesen (1956) reported that 50% of a therapeutic dose of atropine was absorbed onto the plasma proteins in man. He postulated that the slow elimination of the drug in man might be due to its interaction with plasma protein. Tonnesen surmised that the plasma proteins served as a carrier by which atropine was transported to various organs, and intimated that the drug-protein binding may slow the elimination of atropine in man.

In 1920, Dale reported that histamine was about ten times more toxic for cats anesthetized with ether or chloroform than for the unanesthetized animal. This work spurred Storm Van Leeuwen and von Szent-Györgyi (1921) to investigate the influence of anesthetics and other drugs upon the pilocarpine-serum-protein relationship. Using the isolated gut preparation, they determined that pilocarpine mixed with serum from anesthetized rabbits had less binding power than normal serum. This was shown by the greater ability of the latter
to cause a much stronger gut contraction. Ether decreased the pilocarpine binding power of serum more than chloroform. Peptone had a similar effect whereas urethane, magnesium sulfate, starch and lecithin had no influence. The theory concerning pilocarpine inactivation was modified to reflect this new evidence. They concluded that serum contained substances which inhibit atropine and pilocarpine by some physio-chemical process, presumably adsorption.

In 1924, Storm Van Leeuwen wrote a series of short articles reflecting upon the possible relationship between drug antagonism, drug sensitivity, and the drug-protein complex. He theorized that the reaction between a drug and the dominant receptor at the site of action was not a simple one, and that it was greatly influenced by the chemical and physical composition of the blood and other tissues. He stated that when two drugs are introduced into the body concomitantly, "it is likely that one drug will be more easily adsorbed than the other one". This, he continued, was especially true if both drugs had the same dominant receptors. The concomitant administration of drugs having similar effects might be antagonistic due to receptor competition. In this case, one drug acting alone might be more effective than if used in combination.

Storm Van Leeuwen (1924) also offered the drug-protein interaction phenomena as a possible explanation for certain cases of drug hypersensitivity in man. He indicated that drug hypersensitivity appears to be both qualitative and quantitative in nature. "Although the chemical constitution and the 'normal' pharmacological actions of a drug, which may cause allergic symptoms, vary very considerably, the symptoms of hypersensitiveness are as a rule similar inter se." Moreover, they differ entirely from symptoms which
these drugs induce in normal men." He asserted that in "quantitative hypersensitivity", patients react the same as normals except at lower doses.

Drug toxicity in normal individuals simply requires higher doses. In "qualitative hypersensitivity", he placed those patients who respond selectively to different drugs, i.e. some respond to aspirin, others to boric acid.

Storm Van Leeuwen presented the following example of the effect of protein binding power on patients hypersensitive to salicylates. In normal patients, 0.76 - 0.90 mg. of salicylate/5 cc was found bound to serum. In hypersensitive asthmatics, 0.45 - 0.56 of the salicylate was bound. In an attempt to desensitize, patients were treated with various proteins, and salicylate binding to serum achieved levels of 0.78 mg./5 cc. In his conclusions, Storm Van Leeuwen hypothesized that qualitative hypersensitivity may be associated with the inhibitor action of normal sera and that possible augmenting influences may occur due to the presence of other drugs.

Thus, between the years 1921 and 1924, Storm Van Leeuwen asked questions and postulated answers to problems concerning the influence of protein binding upon the pharmacological activity of drugs. Although some of his theories have borne fruit, such as the interference with the drug-protein complex by the presence of other drugs, the relationship between patient variation in drug dosage effectiveness and drug-protein interaction is still unresolved.

From 1923 until 1942, research in this field appeared to be concerned primarily with the novelty of drug-protein binding. Which drugs are bound? how do they form a protein attachment? and what is the strength of this association? Oliver and Douglas (1922-1923) investigated protein binding of arsphenamine. These researchers found that gum arabic, egg albumin, plasma proteins, and specifically the globulins, form complexes with arsphenamine
and inorganic arsenates both in vitro and in vivo. The arsphenamine content of the drug-protein complex was found to vary with the hydrogen ion concentration. They observed that the degree of binding was increased as the pH of the medium increased. They also determined that protection afforded by hydrophilic colloids against the agglutination of red blood cells by arsphenamine was due to the union of the former with the latter, which prevented the binding of the arsphenamine by the cells. The change in the coagulating properties of the plasma proteins after the injection of arsphenamine was explained by the assumption that the compound with arsphenamine was soluble at certain high concentrations.

Until 1925, investigation of the drug-protein complex was of a semi-quantitative nature. In 1925, it appears that Grollman may have published the first really quantitative work, studying the combination of phenolsulphonphthalein (phenol red) with blood serum and albumin of various animal species. Using an ultrafiltration method, he determined that as the percentage of albumin was increased, the percent of uncombined dye decreased. Grollman reported that the binding of the dye followed the equation $X/m = Kc^{1/n}$, in which $X =$ millimoles of dye absorbed by $m$, gram of adsorbent, and $c$ is the concentration of dye in millimoles per liter. $K$ and $n$ are constants with $n$ being equal to 1 for proteins. Grollman also found species difference in drug-protein binding. He reported that phenol red was bound by the blood proteins of the rabbit 95%, dog 75%, pig 75%, hen 54%, duck 53%, and frog 20%.

Beutner (1925), while discussing drug-protein interaction, recognized that "the binding power of the serum is an important physiological phenomenon and certainly plays a role in all pharmacological actions". He cites
Jendrassiks' procedure (1922) for equilibrium dialysis as a tool to test the serum binding power of drugs. In 1926, Beutner determined that rabbit serum bound more pilocarpine than the serum of cattle. However, he reported "the binding is looser", or more easily reversed, for rabbit serum.

Rosenthal (1925) studied the dissociation of drugs bound to protein. He reported that the union between dog serum proteins and tetrachlorotetraiodofluoresein (rose bengal) or bromosulphalein were completely bound in vitro to proteins of the blood. He found that the presence of bile salts caused a liberation of the dye stuff which was not due to protein precipitation. Phenolsulphonephthalein, which was partially bound by blood proteins, was also dissociated by bile salts. Subsequently, Rosenthal (1926) reported that undiluted dog serum bound 65% of the phenol red in the solution, while a 0.1% serum solution bound the same quantity of rose bengal. He determined that molecular aggregation occurred in very dilute aqueous solutions. Using strongly acid (pH 2) solutions of serum albumin, gelatin, globulin, and dog serum, he found that the amount of dye bound to the colloid followed the classical Freundlich absorption isotherm equation

\[ \log \frac{x}{m} = k + \frac{1}{n} \log P \]

where \( x \) is the mass absorbed, \( m \) is the absorbent mass, \( k \) and \( n \) are experimentally determined constants, and \( P \) is the pressure. Finally, the author found that the addition of 25 mg. of Na Oleate/cc. of whole dog blood increased the non-protein filtrates 20-55%. Rosenthal attributed this increase to the liberation of non-protein nitrogenous substances which ordinarily remain attached to the proteins and do not appear in the filtrates.

Thus, during the years 1925 and 1926, recognition of drug-protein affin-
ity, drug interference or receptor site competition, and species differences in the drug-protein complex became apparent. Undoubtedly, the efforts of Grollman, Beutner, Rosenthal and others has led to the present day speculation and emphasis on the importance of drug-protein association constants. By 1926, the era of qualitative drug-protein binding research has begun and the importance of the strength of the drug-protein bond (affinity) has been recognized.

The attraction of plasma proteins for sterols was studied by Gardner and Gainsborough (1927). These workers found a close association between cholesterol and serum proteins, the most dramatic combinations being with euglobulin. They indicated that it was not possible to conclude that the cholesterol-protein complex was chemical in the ordinary sense, but that there was really no sharp distinction between so-called physical absorption and chemical union of associated substances. Gardner and Gainsborough reported that changes in the globulin albumin ratio in plasma most likely have some influence on the variations in sterol content of the plasma under normal physiological conditions and in disease.

In 1927, Hewitt investigated complex between proteins and phthalein-fluorescein dyes, such as rose bengal, bromophenol blue, eosin, fluorescein, erythrosin, phenolsulphonephthalein, and phloxin. He reported that the colors which change on the acid side of the isoelectric point of the proteins were not discharged or altered with acidification after being mixed with egg or serum albumin. This non-responsiveness to pH changes was attributed to a dye-protein combination phenomena. Hewitt states that this dye-protein association was not "surface absorption" but a chemical combination.

In 1930 Hershfelder began investigations to determine which specific
serum proteins were binding drugs and the extent of the affinity. He reported that coagulated solutions of egg albumin had a greater affinity for antiseptic dyes than serum protein solutions. He reinforced Rosenthal's findings by determining that the interaction between solutions of purified egg albumin and triphenylmethane dyes, crystal violet, malachite green, and brilliant green obeyed the Freundlich absorption isotherm equation. Finally, Hershfelder found that the affinity of the dyes for the protein was increased by the introduction of alkyl groups into the dye molecule.

Within seven years, investigators were studying the relationship between drugs and individual serum protein entities. Gutman and Gutman (1937) studied clinically the relationship of serum calcium to total serum protein and serum protein fractions. They discovered a direct proportionality between protein and total calcium in sera from normal and nephrotic patients. However, calcium levels were not found to rise above normal in hyperproteinemia. They concluded that serum calcium was composed of (1) calcium bound to and proportional to the albumin concentration; (2) calcium bound to a globulin fraction which remains relatively constant in amount regardless of the total globulin level; (3) a small fraction of calcium bound to another globulin fraction which increases with the total globulin level but becomes significant only in marked hyperglobinemia; (4) calcium not bound to protein.

Immunologists became increasingly interested in the relationship between the drug-protein complex and antigen formation. In 1937, Mulinos and Schlesinger cite the 1917 research of Landstiner and Lampl as a stimulus for their antigen work. Landsteiner and Lampl showed that new protein antigens could be formed through the chemical union between chemically simple drugs such as aniline and a protein. This finding led Mulinos and Schlesinger to diazotize
antipyrine and couple it to rabbit serum and egg white albumin. Using the isolated guinea pig uterus preparation, they demonstrated that by this means antipyrine could be coupled to proteins to form new antigens.

During the years 1940-1943, the true pharmacological implications of drug-protein binding as envisioned by Storm Van Leeuwen and Beutner began to unfold. Butler and associates (1940) reported on their studies in synthetic immunochemistry. They indicated that an aspirin-serum globulin (horse and rabbit) complex produced a powerful antigen. These investigators then obtained an aspirin-protein complex antisera by injecting pyretic rabbits with this antigen. After treating normal rabbits with the antisera, they observed that the antipyretic effect of aspirin was diminished. Butler et al state that this "demonstrates the possibility of neutralizing the effect of a pharmacologically active group with an antiserum against a protein in which this group is acting as a haptene, even though the group in question has no chemical connection with protein itself." The attachment of the haptene to the antibody appeared to be preventing the former from reaching its normal site of action in the body and therefore from exercising its usual physiological effects.

In 1941, Bassett, Salter and co-workers did an extensive study of the blood protein-iodine complex. These investigators determined that the albumin fraction of both horse and human serum was the major iodine binding protein. Their results suggested that blood plasma-protein bound-iodine might serve as a useful index for circulating thyroid hormone. Later in the year this group demonstrated clinically that plasma-bound iodine appeared to be a highly reliable and sensitive criterion for diagnosing hypothyroidism.

While working with sulfonamides, Davis made a major contribution involv-
ing the direct effect of the drug-protein complex in relationship to pharma-
cological activity. During the years 1942-1943, Davis reported that sulphon-
amide, sulfapyridine, sulfadiazine serum protein binding was due to albumin. He indicated that the degree of binding accounted for the distribution of the drugs in the body fluids and their increased solubility in plasma. He found that over a pH range of 6.0 - 8.5, sulfa drug binding increased with increasing alkalinity, suggesting that anionic dissociation of the sulphonamides was a factor in binding. More significantly, Davis tested the bacteriostatic activity of these sulphonamides in the presence of albumin. Davis was able to conclude that protein-bound sulphonamides appeared to be bacteriostatically inactive. Finally, Davis and Wood (1942) found a quantitative correlation between sulphonamide-protein binding tendency and bacteriostatic activity.

Protein binding work, like most basic investigation, was curtailed between 1941 and 1946 due to war. After World War II, advanced technology enabled investigators with drug-protein complex interests to become more active. The application of new methodology and instrumentation allowed both qualitative and quantitative measurement of drug-protein binding sites, estimation of drug-protein affinity through the use of association constants, and drug inactivation after protein binding.

Contrasting results were reported concerning the effects of serum, blood, living tissues or their autolytic products on the bacteriocidal activity of penicillin. Chow and McKee (1945) reported that penicillin combined with human serum albumin but did not appear to complex with the globulins. They stated that, unlike the albumin-sulphonamide complexes, the penicillin-albumin complex possessed antibiotic activity. Thus, doubt was cast upon the theory that pharmacological inactivation occurred after the formation of a drug-
serum protein complex. However, in a thorough study, Eagle (1947) demonstrated that penicillins F, G, K, and X were all rendered biologically inactive by human and rabbit serum. The author stressed that in vivo ineffectiveness of penicillin may be due not only to rapid excretion of the drug but also to the fact that as blood-drug concentration falls, serum inactivation of penicillin increases via an increase in binding.

The definition, determination, and application of binding site class identification and association constants, as an expression of drug-protein affinity in the modern context, began in 1946. Klotz and his associates (1946-1948) attempted to clarify drug-protein interaction quantitatively in terms of the statistical and electrostatic factors which influence binding. These investigators were able to determine the number of serum bovine albumin binding sites and their association constants for organic ions (methyl orange, azosulfathiazole and some mono- and di-sulfonated dyes) using equations derived from the law of mass action. Varying the pH of the buffer solutions, they determined that the binding of organic ions as well as copper diminished as the medium became more acidic. From the pH data, it was concluded that the ammonium group of lysine was strongly involved in the binding of organic ions while the protein carboxyl groups were the primary factor in the copper-albumin complex. Finally, Klotz emphasized that commonly used buffer systems such as phthalate, veronal, and citrate affect proteins by forming complexes with the protein. These investigators state: "In view of the frequent use of these electrolytes in protein studies, particularly in investigations of electrophoretic mobilities, it is essential that their affinity for the protein molecule by recognized and accounted for in any interpretation of the physio-chemical behavior of these large molecules."
Following the Klotz lead, George Scatchard (1949) made a significant contribution in the area of binding site and association constant identification. Using calculations based on the law of mass action, Scatchard determined that plotting \( \frac{r}{c} \), the ratio of the moles of drug bound per mole protein \( (r) \) to the concentration of free drug \( (c) \) against \( r \), would permit the resolution of the binding sites \( (n) \) and their intrinsic association constants \( (K) \). The values of \( n \) and \( nK \) were determined by extrapolation of the resultant plot to the appropriate x-y intercepts. He observed that should the plot result in a curved line, there were more than one class of binding sites with a separate intrinsic association constant for each class of sites. Although published in 1949, the Scatchard plot is presently the major technique for the determination of binding sites and their association constants. If the plot is not a straight line but a curve, the curve is resolved into its linear components, each of which are then extended to their appropriate intercepts. Using this tool, Scatchard et al (1950) reported two classes of binding sites - \( n_1 = 10, K_1 = 44 \), and \( n_2 = 30, K_2 = 1.1 \) for chloride ion on human serum albumin at pH 4.9 - 5.2. They also found that the results were well described by the law of mass action.

During this early post World War II era, Fred Karush also became interested in protein binding. In 1949, Karush and Seonenberg reported on the interaction of alkyl sulfates and bovine serum albumin. The authors had thought sodium octyl, decyl and dodecyl alkyl sulfate to be bound in a homologous manner until they subjected bovine serum binding data to the Scatchard plot interpretation. "Deviation of the binding curves from the simple theory based on the mass action law have shown to be inexplicable on the basis of electrostatic effects. A new heterogeneity theory based on a particular
distribution of the intrinsic binding constants has been proposed and has been found to account quantitatively for our results." Continuing his investigations, Karush (1950) reported on the binding of an anionic azo dye, p-(2-hydroxy-5-methylphenyl-azo)-benzoic acid, and competitive interactions by dodecyl sulfate with bovine serum albumin. Karush found that bovine serum albumin binding sites for the azo dye were heterogeneous, group 1 binding sites had a much greater affinity for both drugs than the group 2 sites, and that as the concentration of the sulfate was increased, the amount of dye bound was diminished. Karush concluded on the basis of binding studies that his results served to support the concept that the binding properties of serum albumins were associated with the configurational adaptability of a number of regions of the protein. He reasoned that the same sites (group 1) strongly bound the two structurally different anions, suggested that the binding sites had a high degree of structural adaptability, i.e., they could assume structures which were complementary to a wide range of configurations. He continued that it was this property which conferred upon the albumins the distinctive ability to form complexes with a wide variety of anions. Finally, Karush indicated that the secondary sites were restrictive in adaptability, having smaller association constants which lead to selectivity in association.

Weiner et al. (1950) investigated the physiological disposition of dicumarol clinically. Using in vitro dialysis experiments and blood taken from patients, they determined that ninety-nine percent of the compound was bound by plasma constituents. When dicumarol was mixed separately with beta and gamma globulins, alpha globulin, and albumin, these plasma proteins bound the drug 20, 50 and 99% respectively. The low free plasma level, slow rate of metabolism, slow rate of biotransformation, and slow elimination rate of the
anticoagulant were attributed to its extensive binding by plasma proteins.

The physiological disposition of procainamide and its interaction with plasma proteins was investigated by Mark et al (1951). These workers determined that procainamide was principally localized in kidney, liver, spleen, lung, etc. They found that 15% of the drug was bound to non-diffusible elements of plasma. After allowing for a 1-hour drug metabolism-excretion period, the authors found that: "Organ tissues reversibly localize considerable amounts of the drug which are released to the plasma as the drug is lost by metabolic transformation or urinary excretion."

Scrutiny of the barbiturate-serum protein complex began about 1950 when Goldbaum and Smith investigated the binding of various barbiturates by tissue homogenates. Their results showed that the order of tissues binding capacity was liver, kidney, plasma, brain, red cells, and muscle. Theophylline was bound to the greatest degree, followed by seconal, pentobarbital, phenobarbital, and barbital. After developing a more sensitive ultraviolet spectrophotometric assay (Goldbaum, 1952), these authors investigated the binding of this series of barbiturates with bovine serum albumin (BSA) and its possible relationship with pharmacological activity. In 1954, Goldbaum and Smith reported that decreasing the substituted alkyl side chain length from six to two carbons diminished the percentage of drug bound. The barbiturates having the greatest fraction bound also had the shortest duration of activity. They determined that the binding of barbiturates was related to pH, protein concentration and drug concentration. Goldbaum and Smith observed partial displacement of the barbiturates from the drug-protein complex with the addition of other barbiturates of dissimilar organic anions. Finally, they estimated the number of barbiturate binding sites per molecule of BSA. The
authors concluded: 1) the extent of binding in vitro appeared to be partially related to the distribution of drugs in vivo; 2) the degree of protein binding correlated fairly well with the known pharmacological properties of the drugs. In vitro work by Brown (1969), using phenobarbital Na at concentrations approximating those reported clinically by Sunshine for sedation, confirmed the pH and protein concentration relationship to drug binding reported by Goldbaum and Smith. However, human serum albumin (HSA) was employed. Browne found that HSA provided approximately 15 phenobarbital binding sites per molecule of albumin as compared to 21.7 for BSA reported by Goldbaum.

Taylor et al. (1954) studied in vivo and in vitro binding of thiopental by rabbit plasma. Their results also corroborated the data of Goldbaum and Smith showing that the amount of drug bound in vitro closely resembled the amount bound in vivo. It was demonstrated that as the concentration of thiopental increased, the percentage of drug bound diminished, resulting in a prolongation of sleeping time duration. Finally, Taylor found a positive correlation between bovine and human serum albumin binding and the enhancement of sleeping time.

Examining the fate of barbital, phenobarbital, and allypropymal in man, Lous (1954) determined that the percentage of barbiturate bound to plasma was 95% (88-104) for barbital, 50% (33-71) for phenobarbital, and 60% (54-65) for allypropymal. These values also approximate the values reported by Goldbaum and Smith. Unfortunately, Lous did not determine nor identify the serum protein or pH of his samples. Wadell and Butler (1957) further examined the distribution and excretion of phenobarbital in dog, mice and men. These authors found that the percentage of drug bound in vitro was minimally affected by pH variation. In contrast to in vitro findings, in vivo experiments
in dogs, mice and man revealed that after causing alkalosis induced by intravenous infusion of NaHCO₃ or hyperventilation and acidosis from CO₂ inhalation, plasma phenobarbital concentrations rose with the elevation of pH. Alkalosis was found to lighten phenobarbital anesthesia by diminishing drug concentrations in the brain.

Markus and Karush (1958) investigated the effects of various structures of the anionic azo dyes on human (HSA) and Bovine (BSA) serum albumin. Employing equilibrium dialysis, optical rotation, rotatory dispersion, and viscosities, they determined that these dyes affected the optical rotation patterns of HSA and BSA differently. Their results were interpreted as structural changes in the albumin molecule resulting from interaction with dye molecules, involving the stabilization of configurations varying in helical content. The diversity in the rotatory patterns indicated flexibility in the albumin molecules and provided further indirect evidence for the Karush concept of configurational adaptability.

McMenamy and his associates (1963), while studying HSA binding and related thermodynamic values of L-tryptophan and its analogues, added weight to the theory of HSA configurational adaptability. These authors postulated that three points of reference were necessary for a compound to bind to a site in a stereospecific manner. These could be either positions of attachment of the 2 interacting molecules or adapted spaces which permit the molecules to come together. Examples given for reference points were the indole ring contribution of energy from the indole ring which provided attachment by van der Waals forces, an ionizable group on the protein having an association constant in the range of the tryptophan imidazole or N-terminal groups, and spatial tolerance as represented by the distance separating the protein
N-terminal and \( \alpha \)-amine groups. This spatial distance must be small enough to be able to provide a minimal repulsive force equivalent to 2 kilocalories when both groups are positively charged. The authors also determined that tryptophan was bound primarily by one site, the binding being pH dependent. The association of the indolylethylene group was "attributed to entropy changes at the protein site, presumably due to the loss in freedom of the hydrophobic groups on the protein".

The work of Spratt and Okita (1958) provided some information concerning the use of radioisotope tracers in drug-protein binding. Earlier, Okita (1953) determined the renal excretion pattern of digitoxin employing randomly labeled \( ^{14} \text{C}-\)digitoxin in arteriosclerotic heart disease patients with congestive heart failure. Familiarity with radioisotope procedures provided the stimulus and means for Spratt and Okita to investigate the binding of digitoxin by rat serum proteins and compare their findings with those of other investigators. They found that 1 mg. of rat serum protein bound 0.01 mcg. of digitoxin when therapeutic doses were employed. These findings were in contrast to those of Rothlin and Kallenberger (1950) who found that human serum albumin bound 2.2 mcg. of digitoxin per milligram of protein. Spratt and Okita indicated that in vivo application of the Rothlin and Kallenberger data "is not feasible since it might imply that a normal man could bind 300 mg. of the drug without significant pharmacological effect."

With the advent of improved liquid scintillation counters, spectrophotometry, new techniques for estimating drug-serum protein binding, i.e. centrifugation, Sephadex, etc., exponents of protein binding began investigating the newer drugs, hormones, and began re-examining the binding of drugs previously investigated, especially in terms of drug-protein interaction in the
presence of competing ligands. Information concerning multiple drug vis-a-vis interaction with proteins was not exactly new as indicated by studies previously discussed; however, new methodology as well as reports of enzyme induction and the therapeutic use of drug mixtures caused a renewed interest in binding, especially in terms of possible clinical consequences.

Like Chow and McKee (1946) who indicated that penicillin bound to protein was pharmacologically active, Paul et al (1960) published a contradictory note concerning the effect of protein binding upon a drug's activity. Paul and his collaborators, working with nitrofurans, determined that plasma proteins bound 50-90% of the anionic compounds (nitrofurantoin) while the non-ionized drugs (nitrofurazone) were bound approximately 30%. Cationic drugs such as furaltadone were not bound to any appreciable extent. However, the authors were unable to establish any correlation between plasma binding of the drugs examined and their known systemic effects. Unfortunately, neither the assay method employed nor their detailed results were presented. Although the nitrofurans might be the exception, it still remains the author's opinion that drug-plasma protein complexes are therapeutically inactive.

Anton (1960,1961) reported that the plasma binding of sulfonamides varied markedly between animal species. He quantitatively confirmed that albumin binding of sulfaethylthiadiazole (SET), sulfisoxazole, sulfamethoxy-pyridazine and sulfadiazine inhibited the antibacterial activity of these drugs. Anton found that phenylbutazone displaced SET. The author indicated a possible practical application for drug displacement; by releasing the active drug from its binding, the inactive agent might promote the penetration of the active drug to a particular site. He theorized that not only might it be possible to increase an agent's effectiveness by this procedure, but that
the availability of a higher concentration of the active drug at the site of action might help to decrease the incidence of resistance to chemotherapeutic agents. Anton then presented in vivo evidence showing that the distribution of SET in the rat could be altered by competitive inhibition of its binding by plasma proteins through the use of phenylbutazone, ethylbiscoumacetate, sulfinpyrazone and iophenoxic acid. "The net effect of displacement in vivo was a drop in plasma concentration and an increase in the unbound fraction, resulting in an increased concentration of sulfonamide (unbound) in tissue." Finally, Anton and Boyle (1964) reported that the albumin binding of sulfamethoxypyridazine interfered with the enzymatic alteration of the drug. This effect, they continued, was a specific one for this particular drug and was not observed with sulfanilamide which was insignificantly bound to albumin.

Clausen (1966) established the molecular relationship between a sulfonamide and serum proteins. He quantitatively approximated the total number of binding sites and the intrinsic association constants per molecule of albumin for 3-sulfamido-6-methoxypyridazaine. He also demonstrated that prealbumin, glycoproteins, and alpha 2-macroglobulin possessed secondary binding properties for the drug. He concluded that the binding of sulfonamides may have biological importance for the elimination and distribution of the drug.

In 1970, Walker examined the influence of protein binding on the metabolism and excretion of several sulfonamides clinically in man. He determined that sulphasomidine, a short-acting sulfonamide, was bound to a lesser extent to plasma albumin than the longer acting agents, i.e. sulphorthodimethoxine. Walker found that the binding of the sulfonamides investigated "appeared to be an important factor in determining their excretion rate". Small structur-
al changes in a drug apparently alters drug-protein binding, i.e., substitution of a methoxyl group for a methyl group in sulphasomidine in the "4" position results in a large increase in the percentage of drug bound.

Earlier, Storn van Leeuwen and Zeijdir (1921), Beutner (1926), and Tonnensen (1956) considered the plasma protein binding of atropine in terms of inactivation, transport, and elimination. In 1962, Oroszlan and Maegwyn-Davies published more detailed studies relating to the atropine-albumin complex and the effect of acetylcholine upon this interaction. Oroszlan and Maegwyn-Davies reported that the number of atropine binding sites per molecule of albumin increased from 20 at pH 6 to 100 at pH 8, with only a slight increase in association constant at pH 8. They observed that electrostatic and configurational changes on the protein would explain the pH dependent binding site alterations. The authors demonstrated that the protein binding of atropine inhibited the drug's ability to antagonize the effect of acetylcholine (ACh) upon the in vitro rat colon preparation. On the other hand, the addition of cysteine interfered with the atropine-protein complex, restoring atropine's ACh antagonistic potency. Finally, the author demonstrated that ACh facilitated the binding of atropine between pH 5-7, but had little effect upon the atropine-albumin complex between pH 7-8. The facilitating activity of ACh was attributed "to an alteration in the protein-SH group reactivity".

The interaction between chemically inert anesthetic gases and albumin was studied by Featherstone et al (1961). These workers found an increase in the solubility of cyclopropane with increasing concentrations of serum albumin. They state that Pauling's microcrystal hydrate theory of anesthesia is consistent with data presented concerning gas-protein interaction.

Eichman and his colleagues (1962) worked with xanthines (caffeine, theo-
phylline, theobromine and structurally related compounds). Although some of
the derivatives were bound up to 98%, the binding of caffeine, theophylline
and theobromine was slight to negligible, and a gradual decrease in binding
occurred as the pH was increased from 4.8 - 8.5. The authors provided evi-
dence suggesting that in the protein the (-amino group of lysine was respon-
sible for xanthine-albumin interaction.

Between 1964 and 1966, several researchers re-examined the interaction of
the plasma proteins with various penicillins. Lithander (1964) observed a
greater percentage of penicillin absorbed by protein when lower concentrations
of the drug were administered. Also the capacity to complex penicillin was
reduced as the plasma was diluted. Parallel investigations by Robinson and
Sutherland (1965) demonstrated that the binding of penicillin by human serum
was reversible and independent of antibiotic concentrations below 100 mcg./ml.
Above 100 mcg./ml, penicillin binding diminished with increased drug concen-
tration. They found that temperatures between 4 - 37°C. had little influence.
Robinson and Sutherland also revealed that phenylbutazone, sodium salicylate
and sulfonamides interfered with the penicillin-protein complex. Finally,
they demonstrated direct proportionality between reduction in penicillin anti-
bacterial activity and the extent of its binding by serum. Kunin (1964, 1965)
published work involving radio-isotope labelled penicillins and various other
antibiotics, as well as known displacing agents was effectively inhibited,
resulting in an enhancement of microbial activity of the penicillins in the
presence of human serum. The author indicated that during the equilibration
period, the rate of drug diffusion from the blood into extravascular tissues
is controlled by the amount of free drug. Kunin reiterates that the free drug
may indirectly control the amount of penicillin available for antimicrobial
activity. Thus, binding of the drug and alterations in the drug-protein complex could lead to (1) lower serum levels as the relative volume of distribution increases and, thereby, allows a greater fraction of the free active drug to be available in the extravascular, extracellular space; and (2) lower serum levels by increasing renal excretion as the filterable fraction is expanded.

Finally, the author presented evidence showing a definite relationship between serum binding and the side chain or R groups of the penicillins. He also found that drugs reacting with these R groups were effective inhibitors of penicillin-serum binding.

Reviewing current studies concerning the penicillins, Warren (1965, 1966) discussed the prognostic significance of penicillin serum levels in clinical medicine in terms of drug-protein interaction. He indicates that drug serum levels can't be relied upon exclusively for predicting efficacy. Warren emphasized that the extent of binding, such as percentage bound, should be distinguished from degree because the degree or affinity appears more related to activity. The author theorizes that "therapeutic efficacy is probably less dependent on serum levels and extent and degree of serum binding than on distribution in tissues, parenchymatous organs and body fluids". Yet, he neglected to consider points stressed in the work reviewed. One of these major points is that protein binding, because of its reversibility, not only influences drug distribution but affects diffusibility, tissue accumulation, and rate of inactivation. And, it is these very factors which Warren deems more relative to therapeutic efficacy than serum binding.

The degree or affinity to which penicillins interact with serum and serum albumin was reported by Keen (1965, 1966a, 1966b). In 1965, Keen reported on preliminary experiments in which he demonstrated considerable species varia-
tion in the binding of phenethicillin, phenoxymethylpenicillin, and benzylpenicillin. The highest percent of penicillin was bound by horse plasma, followed by goat, ox, sheep and pig. Keen attributed the differences in amount of drug bound to serum albumin concentrations, differences in drug molecule affinity and variations in plasma ionic concentrations together with differing competing substances present. Keen (1966a) determined that phenoxy-methylpenicillin has a primary binding site number and association constant of 0.78 and 2376, with secondary sites numbering 86.8 and an association constant of 16.2. These values, Keen continues, predict that 64% of the drug would be bound in vivo which agrees nicely with the extent of binding in vitro. The reason for one site having a predominant affinity was explained through reinforcement by van der Waal's forces, while albumin flexibility accounted for binding of numerous different agents with a high degree but little selectivity. In this work, Keen carefully and clearly described the methodology and mathematic formulae necessary to conduct drug-protein binding studies. He discussed the influences of unbound ions of buffer solutions, electrostatic forces, Donnan equilibrium effects and space occupied by solutes upon the drug-albumin complex. Keen also reported (1966b) that phenol red, phenoxy-methylpenicillin and sulphamethoxypyrindazine could be effectively displaced by several anionic drugs such as phenylbutazone, salicylate or tolbutazamide. The anionic displacing agents showed some selectivity. In his conclusion, the author states: "If a substance is highly bound in plasma, the displacement of only a small percentage of it from the proteins will bring about a significant increase in free drug concentration". He cites bilirubin-albumin binding displacement by sulfisoxazole, resulting in increased incidences of kernicterus in premature infants as one of several examples. The publications by Keen are
among the very few good papers involving the interaction of drugs with serum proteins.

To re-emphasize the transport-distribution role played by serum proteins, Mirkin et al (1966) studied the binding of tritiated norepinephrine by plasma. After examining the results of their in vitro work using blood taken from healthy volunteers, this group suggested that a specific protein, possessing electrophoretic migration characteristics quite distinct from albumin in human plasma, transported some circulatory catecholamines from sites of release to their sites of action by binding the catecholamine to the protein. This catecholamine-protein complex in blood serves as a protective mechanism, making the catecholamines unavailable for biotransformation.

Krasner and McMenamy (1966) studied the binding of indole compounds to bovine serum albumin. They found a definite change in the binding of indole ligands which was consistent with the simple ionization process of the protein between pH 7.5 and 9.5. They also reported a reduction of albumin-indole binding in the presence of fatty acids. However, they found no discernible differences in the binding affinity of crystalline and Fraction V albumin. Krasner and McMenamy, however, pointed out specific differences in the binding properties of human (HSA) and bovine serum albumin (BSA), dependent upon the drug employed. The acetyl-l-tryptophan-BSA complex was stronger than the HSA complex, while the reverse was observed with l-tryptophan and skatole. On the other hand, BSA bound skatole and acetyl-l-tryptophan with essentially the same degree, suggesting that the acetylamino and the carboxylate ion (-COO⁻) groups of these drugs are better accommodated at the BSA binding sites than at the HSA sites.

L-tryptophan is an essential amino acid partially responsible for the
maintenance of nitrogen equilibrium in young humans, growth of human tissue
culture cells, and for growth in the rat. Because there is no mechanism for
storing nitrogen, the essential amino acids must undergo degradation or be
used for protein synthesis within a short time after ingestion (Geyer, 1965).
The previous work by McMenamy and his associates led investigators to contemplate the possible relationship between the l-tryptophan-albumin complex and disease.

In 1969, McArthur and Dawkins examined the effect of salicylate on the binding of l-tryptophan to human serum proteins and bovine albumin. They found that the presence of increasing concentrations of salicylate proportionally elevated the concentration of free, unbound amino acid. The course of their investigations led them to consider the possibility that a portion of salicylates' anti-inflammatory activity was mediated by tryptophan. Pursuing this avenue of thought, McArthur et al (1971) studied the association between l-tryptophan-albumin binding and rheumatoid arthritis. Their work demonstrated that the concentrations of bound and unbound tryptophan was significantly reduced in the serum of patients with active rheumatoid arthritis receiving antirheumatic drugs as compared to normals. After removing the antirheumatic drugs and amino acids from pooled patient sera by ultrafiltration, they added tryptophan. Then they determined the binding of the amino acid, alone and in the presence of salicylate, to the sera. When compared to normal sera, they found that the tryptophan bound to the serum proteins in the patients sera was less easily displaced by salicylates. Phenylbutazone, indomethacin, prednisolone, chloroquine and gold were also reported to interfere with the tryptophan-protein complex. From these results and other findings, the authors theorized: "When drugs bind to circulating albumin, they displace other bio-
logically active small molecules from their binding sites on serum proteins. The present work shows that this displacement extends to dipeptides which bind to human serum proteins. It is suggested that the unbound forms of some peptides may exert a protective effect against the actions of mediators of chronic inflammatory insults. The binding of these hypothetical peptides to circulating proteins may be abnormally strong in patients with the rheumatic diseases because in such patients the circulating albumin possesses an abnormal amino acid composition. The fraction of the peptides present in the free form would then be insufficient to exert a protective role. It is proposed that the antirheumatic drugs act by increasing the proportion of free peptides in the blood." Although this theory may be controversial, it is interestingly novel.

The plasma binding effect of proteins on insulin activity in patients with acute epidemic hepatitis as compared to normal subjects was discussed by Leibush (1966). The author found potentiation of "free' insulin-like activity" in patients with acute hepatitis traced to an increase in the "free" insulin blood fraction coupled with a reduction in the fraction bound. Leibush carefully noted that the insulin activity changes were the result of fraction redistribution as opposed to total plasma elevation. Leibush suggests that plasma of patients with acute hepatitis is capable of increasing the activity of "free" insulin. Hence, Leibush has presented an example in which a disease has altered the pharmacological effects of a hormone by influencing the normal drug-protein relationship.

Curry (1970) examined the protein binding of chlorpromazine, employing plasma obtained from circulating blood of dogs, rabbits, and rats. He also conducted in vivo and in vitro binding experiments employing psychiatric pa-
patients suffering from schizophrenia. Curry found that therapeutic concentrations of chlorpromazine were highly bound to plasma proteins and that the variation between different humans was marked, 91-99.0%. As drug concentrations were increased, Curry observed an increase in the unbound chlorpromazine fraction, indicating binding site saturation. Finally, Curry found significant species differences in chlorpromazine binding in the descending order of dogs, rabbits, and rats. However, the percentage of chlorpromazine bound to human plasma proteins spanned the range of the percentage drug bound by dogs, rabbits, and rats. As previously indicated, some investigators have shown species differences in drug binding; however, here we see a rare report of intraspecies drug-protein variation. Unfortunately, Curry did not attempt to explain the reason for this variability.

There seems little question that drug interaction with serum proteins, i.e., protein binding, can alter or modify drug distribution clinically, thereby influencing the dose-response relationship and the rate of drug elimination. Based upon theoretical models derived mathematically, Martin (1965) presented a simplified, quantitative visualization for estimating the influence of drug-protein interaction of pharmacological activity. Martin feels that the extent to which a drug is bound is dependent upon concentration and affinity. He indicates that the affinity value or apparent association constant (k) should be greater than $1 \times 10^4$ before binding will have an appreciable effect upon drug distribution. Using $K 10^4$, he indicates that although as much as 83% of the drug in the plasma may be bound, 73% of the total drug dose may be in the unbound form in both plasma and body water compartments. Although Martin's work provides a convenient yardstick, unfortunately he did not consider the binding of extravascular drug by other proteinaceous elements, pH variations,
or changes in albumin concentration. However, he was careful to emphasize that there is a dosage-range within which small increase in the dose could produce relatively large increase in the concentration of free drug which could result in a potentiated biological response.

Weder and Bickel (1970) studied the interaction of imipramine, desipramine, and 3-chlorodesmethylimipramine with human and bovine plasma proteins. According to their results, the drugs were bound primarily by albumin up to 70\% using drug concentrations within the therapeutic range. They estimated the affinity of imipramine to be $10^4$. Following Martin's outline, Weder and Bickel have stated the improbability that protein binding would influence the pharmacodynamics of imipramine. Again, it is regrettable that the aforementioned parameters were not considered prior to drawing conclusions.

Extensive studies of the coumarin anticoagulant drugs and their pharmacological activity have been published by O'Reilly, Aggeler and their collaborators from 1962 through 1968. In 1962 and 1963, O'Reilly et al determined that the gastrointestinal absorption of the drug was rapid and complete. They found a relationship between warfarin plasma concentrations and the prothrombinopenic activity together with an exponential decay for drug disappearance from plasma. O'Reilly and associates found a correlation between biological activity and elimination rate - the slower the rate of elimination, the more pronounced was the depression of prothrombin complex activity. Prothrombin time variations among normal subjects receiving similar doses appeared to be conditions of elimination rate differences, while the volume distribution (approximately 13\% of body weight) of the drug was equal with that found for pure albumin. Employing $^{14}$C-labeled warfarin, they found that approximately 97\% of the drug was bound to the plasma albumin fraction. Their results
concerning the association between warfarin and albumin led O'Reilly and Kovitz (1967) to investigate specifically the human serum albumin warfarin complex. The authors found a single strong albumin binding site for warfarin, plus a secondary class of several sites having a much lower degree of affinity. Since circulating albumin acted as a temporary depot which is in equilibrium with the unbound-active drug, the authors postulated that the warfarin-albumin interaction influenced the degree and duration of drug activity. Furthermore, binding was thought to protect the body against a sudden and full pharmacological effect. In the absence of binding, the unmetabolized drug would be more rapidly cleared by glomerular filtration and metabolism, thereby decreasing the duration of activity. O'Reilly suggested that the degree or affinity of the albumin binding process accounted for the absence of warfarin from erythrocytes, CSF, and urine. Warfarin had a volume distribution identical to that of albumin.

In 1967 and 1968, Aggeler, O'Reilly et al reported on the clinical potentiation of the warfarin prothrombinopenic effect by phenylbutazone. Earlier, Weiner (1964), Burns (1965), and Cucinell et al (1965) proposed that the inhibition of coumarin hepatic metabolizing enzymes by pyrazolone compounds might enhance the anticoagulant effects of coumarin drugs, while Brodie (1964) theorized about the displacement of warfarin from plasma proteins. This displacement, Brodie stated, would make more free warfarin available at its site of action. Aggeler and associates, using equilibrium dialysis experiments, provided evidence showing phenylbutazone displacement of warfarin from albumin binding. Aggeler indicated that this data supported Brodie's hypothesis. In 1966, Cucinell and associates reported on the death of a cardiac patient being treated with bishydroxycoumarin, who was also treated with chloral hydrate.
The authors postulated that chloral hydrate caused an induction of the enzymes which metabolized the anticoagulant. They assumed that the hypnotic stimulated enzyme induction similar to that reported by Conney et al (1960). They attributed the death of the patient to the discontinuance of chloral hydrate therapy which reduced the bishydroxycoumarin catabolizing enzymes. This, they reasoned, led to elevated anticoagulant activity, subsequently resulting in massive hemorrhage. Prior to this, no decrease in bishydroxycoumarin effect was noted during chloral hydrate therapy.

A thorough in vitro and in vivo study examining the interaction between warfarin, chloral hydrate, and human serum albumin was conducted by Sellers and Koch-Weser (1970). Their data contradicted the conclusions drawn by Cucinell et al and indicated that the enhancement of coumarin activity was most likely due to displacement from binding sites. They emphasized that the important difference between enzyme induction and displacement from protein-binding sites as causes of a shortened half-life of warfarin is that enzyme induction must be accompanied by a decreased hypoprothrombinemic action. In contrast, displacement of warfarin from protein-binding sites during therapeutic use will cause potentiation of its action. This crucial observation represents a milestone in the field of drug-serum protein interaction. Sellers and Koch-Weser were able to associate directly the clinical effect of a drug to its serum protein interaction.

In response to the Sellers and Koch-Weser findings, Dr. Garnham, Vice President-Medical Affairs of Abbott Laboratories, wrote physicians apprizing them of this information. In his letter he indicated the necessity to carefully monitor patients receiving warfarin, especially whenever any drug is added or withdrawn from the regimen of a patient. Dr. Garnham stressed that
the controlled study of drug interactions was relatively new and that the scope and importance of the drug interaction problem becomes more evident daily. (The complete text of Dr. Garnham's letter may be found in the appendix.)

In the presence of the extensive work which has been done in the field of drug-protein interactions, an attempt has been made to outline the appropriate events in some chronological order. Protein binding has been reviewed from the initial observations, through basic research, to the application of its influence directly in the individual patient. Some of the theories of Storm van Leeuwen have borne fruit; work by McMenamy suggests that amino-acid-protein interaction may be a factor in certain growth processes. Now in the field of drug-serum protein interaction, an even more complex and perplexing challenge is further stimulating investigation. The new problem deals with the effects of multiple drug therapy in patients. This new challenge has been best summed up by Weiner (1970). He suggests that many drugs are effective by being a catalytic reagent or, through a chemical interaction with endogenous hormones, neurohumoral transmitters, enzyme co-factors, etc. He stresses that both the equilibrium status and kinetics of the binding competition influence the pharmacological activity of a drug. In this age of drug therapy, few patients escape simultaneous treatment with more than one drug. Weiner concludes: "We are still a long way from accomplishing optimal treatment for many patients with a single, perfectly tailored drug. Thus, our goal cannot realistically be 'one patient, one drug' but, rather, an ever increasing knowledge of how drugs might interact so that we can tailor our therapy accordingly."
CHAPTER 2

STATEMENT OF THE PROBLEM

During the years 1937 and 1938, Merritt, Putnam and Schwab introduced the hydantoin group of anticonvulsant-antiepileptic drugs. Of this group, diphenylhydantoin was considered the most promising. The authors studied the anticonvulsive properties of diphenylhydantoin against electrically induced convulsions in cats. They reported that 200 mg. of diphenylhydantoin administered orally provided protection against such convulsions. Very impressive was the fact that this provided protection without causing symptoms of narcosis.

Since its initial debut, diphenylhydantoin (DPH) has been found effective in grand mal, focal or Jacksonian, and psychomotor seizures. This broad therapeutic usefulness has led many experienced clinicians to accept DPH as "the drug of first choice in all forms of epilepsy except those of the petit mal group" (Toman, 1970). Due to its widespread use, DPH has been the subject of intensive research and speculation, and this has been discussed both in scientific and lay literature. Recently, Bojach and Dreyfus (1970) published a comprehensive bibliography and review entitled "The Broad Range of Use of Diphenylhydantoin". This review cites some 750 references covering the clinical applications of DPH. The review includes chemistry, absorption and excretion, biochemical and neurophysiological mechanisms of action, together with the entry and binding of DPH in the brain.

Among investigators active in DPH research have been Dill and associates (1955), Plaa and Hine (1955), Svensmark and Kristensen (1963), Marino (1963),
and Wallace (1966, 1968). These workers developed chemical and spectrophotometric analytical procedures for the determination of DPH by itself and in DPH-phenobarbital mixtures both in vivo and in vitro. Noach et al. (1958) studied the distribution and fate of DPH in rat. Using $^{14}$C-labelled DPH, they determined the order of drug-tissue concentration in terms of total dose administered 3 minutes after intravenous injection. This was - liver 19.2%, brain 15.9%, kidney 14.8%, plasma 14.4%, salivary gland 13.5%, fat 11.5% and muscle 10.6%. After 4 hours, the drug concentrations remained essentially the same except that liver, kidney, muscle concentrations rose, plasma remained stable, while the other tissues declined somewhat. The authors reported that DPH and its metabolites were both absorbed and excreted by the gastrointestinal tract. In 1961, Loeser studied the metabolism of DPH clinically. Blood samples from patients receiving a constant regimen of DPH averaged 9.5 $\mu$g./cc., with blood levels varying directly with dose. Kemp and Woodbury (1971) examined the subcellular distribution of $^{14}$C-Diphenylhydantoin in the rat brain, specifically the cerebral cortex. These investigators found that DPH easily gained access to the cerebral cortex with the greatest amounts being bound to the microsomal fraction 12 hours after intrathecal administration.

The field of drug bioavailability has become of major governmental interest within the last few years. Irrespective of this, the attempt to relate drug action with plasma levels has been an ever-present scientific interest. Duchtal et al. (1960) reported a serum level requirement of 10-20 $\mu$g./ml. DPH for clinical control of seizures in grand mal patients. Acute pronounced side effects were noted in patients with serum levels above 30 $\mu$g./ml., while none were seen in concentrations below 14 $\mu$g./ml. Kutt and co-workers (1964) made
a more detailed account of the relationship between DPH blood levels and toxic symptomatology in epileptic patients who were displaying signs of DPH intoxication. The authors reported observing nystagmus when DPH blood levels approached 20 γ/ml., ataxia at 30 γ/ml., and mental slowing at 40 γ/ml. Although blood levels in excess of 60 γ/ml. were obtained without observing disorientation, Kutt et al. warned that accumulation of the unmetabolized drug systemically should be avoided since reports in the literature indicate that large doses of diphenylhydantoin may have produced permanent nerve cell injury in the cerebellum. Damage to neurons has been observed in experimental animals who were given large amounts of diphenylhydantoin, i.e., up to 20 mg./Kg. or more. Emphasizing this point, Laubscher (1966) reported the death of a 4-1/2 year old girl due to the ingestion of 2 grams of DPH. A blood sample taken 24 hours after onset of symptoms revealed a DPH level of 94 γ/ml. The average suggested oral therapeutic dose for DPH is 300-400 mg. per day for adults, while one-third of this dose is considered safe in children under 4 years of age (Toman, 1970). Adults receiving 600 mg. per day very often exhibit signs of drug intoxication. Buchthal et al. (1960) reported "that 5-8 mg./Kg. was necessary to obtain a serum level of 10 γ/ml. - 20 γ/ml."

These levels were obtained in patients who had been receiving diphenylhydantoin at a daily dose of 350-550 mg. per 70 Kg. for two to three weeks. On the other hand, hospitalized patients receiving DPH for six months maintained the same blood levels taking 3-5 mg./Kg. per day. This dosage incongruity was attributed to the considerable time necessary to attain steady DPH serum concentration.

In view of this well documented DPH serum level-therapeutic-toxicity relationship, the natural chain of events has led investigators to inquire
about the relationship between serum proteins and diphenylhydantoin. Unfortunately work involving the association between serum proteins and DPH has just begun. To date a review of the literature has revealed few detailed investigations concerning the binding of diphenylhydantoin Na by serum albumin. Lightfoot and Christian (1966) using radial immunoelectrophoresis determined that diphenylhydantoin is bound to albumin and 2-globulin. Barlow et al., in 1962, studied the usefulness of gel filtration techniques as a tool to investigate plasma binding of diphenylhydantoin. In vitro dialysis work by Loeser (1961) indicates that a 1% human serum albumin solution binds DPH proportional to the albumin concentration. Unfortunately, Loeser's work did not indicate the drug concentration or the buffer used. Firemark et al (1963) found that equilibrium was rapidly achieved between the unbound concentration of DPH in cerebral spinal fluid, plasma and brain fluids. Conard et al (1970-1971) reported that human plasma bound 90-95% of the DPH present in the serum fraction while rat plasma bound 88-90%. These authors used total DPH concentrations of 5 to 125 mcg./ml. Finally, Rudman et al (1971) attempted to characterize the binding of DPH by bovine, rabbit and human serum albumin. Unfortunately, the solubility characteristics of the drug in their dialysis system allowed these investigators to determine only the nK intercept.

Concomitant phenobarbital-diphenylhydantoin therapy is clinically justified and has not resulted in increased toxicity or loss of control of the epileptic patient. Conversely, Soloman and Schrogie (1967) reported an increase in the mean biological half-life of DPH from 25 to 55 hours in patients receiving DPH and phenylramidol concurrently. These authors stated: "In patients taking both drugs simultaneously, elevated plasma concentrations of diphenylhydantoin could account for the increased anticonvulsant effects observed". The authors indicated that phenylramidol interfered with DPH metabolism. According to the findings of Scriabine et al (1970), DPH protected dogs from digoxin-induced ventricular arrhythmias without significantly modifying digoxin lethality. On the other hand, digoxin protected the animals from DPH-induced myocardial depression and hypotension.

Careful search of the literature has not disclosed any studies involving the binding of DPH to serum proteins in the presence of other drugs. In fact, relatively few researchers have studied the effects of drug mixtures upon serum albumin binding of the individual drug entities. Among the drug pairs that have been examined are barbiturates (Goldbaum and Smith, 1954), tolbutamide and insulin (Haselblatt, 1963), penicillins and phenylbutazone, salicylate and sulfonamides (Robinson and Sutherland, 1965), sulfonamides (Anton, 1961), penicillin G with probenecid (Fishman, 1964), penicillin with sulfonamides, salicylates, etc. (Kamin, 1965), and warfarin with salicylates (Zarosinski et al, 1971). Most of these investigators reported displacement of one drug entity from albumin by the presence of the second drug. However, the in vitro work of Zarosinski and collaborators revealed broader phenomena. These investigators found "that simultaneous therapeutic plasma levels of warfarin and salicylates would result in doubling their free or active
concentrations". Zaroslniski et al reported a decrease in the number of warfarin-albumin binding sites and association constants in the presence of salicylate.

A majority of the protein binding work involving drug mixtures has concerned antibiotics. In addition to single drug dosages, DPH is available therapeutically for epilepsy in a combination of DPH 100 mg. - phenobarbital 30 and 60 mg., and DPH has been used as an adjunct with trimethadione.

Goldstein (1949) and Meyer and Guttman (1968) include over 850 references in their reviews on drug-protein interaction. Of all the work done in the field, only a small percentage was concerned with a comparison of drug-protein binding between mammalian species. Much less work has been done to investigate the in vivo therapeutic potency of drugs during physiological states of alkalosis or acidosis. Anton (1960) and Genazzani and Pagnini (1963) determined species differences in the binding of sulfonamides. In 1962, Wishinsky et al noted species differences in binding of 4 sulfonylureas by bovine, human, dog, horse, pig and rat albumin. The percentage of a given concentration of drug bound to plasma of various mammalian species for three penicillins was reported by Keen in 1965. Diphenyl, phenoxyethyl, and benzyl penicillin were shown by Kunin (1965) to be more highly bound to human serum than rabbit serum. Robinson and Sutherland (1965) determined species differences in serum binding of benzylpenicillin and cloxacillin. Conard and his associates (1970, 1971), while comparing the percentage of DPH bound by human and rat plasma, reported that species difference resulted in higher free drug levels in rat plasma than in human plasma. They attributed this to the fact that the percentage of albumin in the two plasma sources employed differed. The rat plasma protein fraction consisted of approximately 31% albumin while
the human plasma protein fraction was approximately 43%.

Rudman et al (1971) determined the binding site characteristics of salicylate on human and bovine serum albumin. Their work revealed a similar number of binding sites and binding site classes for both albumins; however, the association constants for each class of sites for salicylate on bovine serum albumin was from four to ten times greater than that for human serum albumin. Although only able to determine nK intercepts for DPH, Rudman and associates showed that the total nK values for the albumins differed, being 11,000 for HSA and 14,500 for BSA.

While studying the effects of pH on drug action, Waddell and Butler (1956) were able to show that the median anesthetic dose for phenobarbital in mice was 20% higher after NaHCO₃ induced alkalosis. Waddell and Butler also stated that phenobarbital blood plasma concentrations rose as the pH rose, and fell as the pH fell.

The purpose of this study is fourfold. The work will endeavor to determine and compare

I. The binding characteristics of human and mouse serum albumin in relation to diphenylhydantoin sodium (DPH). This work appears to be the first attempt to investigate thoroughly the binding characteristics of DPH to serum albumin and compare binding differences between two species, providing they exist. The data obtained together with information in literature should be useful in providing insight to a better understanding regarding the importance of the characterization of primary and secondary binding sites. Sunshine (1969) in "Handbook of Analytical Toxicology" provided data and numerous references which show the association between
blood concentrations of phenobarbital, diphenylhydantoin, and other drugs, and pharmacological and toxicological effects. Normally, binding site classifications and association constants are determined from a graphical presentation of the data obtained from plotting a range of drug concentrations. The drug concentrations usually range between a serum plasma level which has no pharmacophysiological effect to levels far in excess of those found to cause death. If the data plot is a curve, then more than one class of binding sites is considered involved. Work done to date has not considered the fact that drug binding to serum proteins can only have pharmacological implications when the drug concentrations used to characterize binding remain in a range which is less than drug blood levels found at death. If the binding data results in a plot which is a straight line at serum levels below or at those found at death, then it is likely that only one class of binding sites prevails. In this situation the primary or single class of binding sites and its association constant might conceivably determine the pharmacological-toxicological implications between a drug and its binding characteristics in situ.

II. The work will attempt to show changes in serum drug levels, brain drug levels and drug availability relative to DPH anticonvulsant activity in mice during normal, acidotic, and alkalotic states. Hopefully, the pH-drug potency data can be reconciled with the effects of pH upon DPH binding to albumin, determined during in vitro binding experiments.

III. Experiments will be conducted to obtain information concerning human serum albumin binding of DPH in the presence of other drugs which
might be related to its therapeutic availability or potency. For this purpose, four drugs have been selected which should generate information concerning the effect of drug combinations upon serum albumin binding of DPH. These drugs are phenobarbital, trimethadione, phenylbutazone and warfarin. Phenobarbital is a drug possessing similar therapeutic activity and is used clinically in combination with diphenylhydantoin. Trimethadione is a drug used for petit mal epilepsy and has been employed therapeutically in combination with diphenylhydantoin in petit mal seizures where the EEG contains multiple spikes in the spike and wave pattern. Since epileptic patients sometimes have been given drugs possessing analgesic, antipyretic and anti-inflammatory activity, and since many patients do suffer from cardiovascular diseases, phenylbutazone and warfarin have been included. Both of these drugs are highly bound to albumin which indicates that they could possibly compete with DPH for binding sites on the albumin molecule. These two drugs have not been reported to possess anti-epileptic properties.

IV. Finally, it is the object of this study to discuss the serum albumin binding of diphenylhydantoin in connection with its pharmacological utility and consider it in relation to its \textit{in vivo} anti-convulsant activity.
CHAPTER III
MATERIALS AND METHODS

In this study, in vitro and in vivo experiments were designed to explore the possible pharmacological and toxicological implications inherent in the binding of diphenylhydantoin sodium (DPH) with serum albumin. The binding characteristics of albumin for DPH were determined by varying specific physical and chemical conditions. All binding experiments were conducted in an in vitro system devised to minimize the binding of ions other than DPH to the albumin. Although in vivo experiments do not deal directly with drug-serum protein, and/or tissue binding, they were planned so that results would reflect indirectly upon in vivo drug-protein binding and pharmacological activity.

The concentration, amount, and percentage of drug bound to serum albumin were determined using a modification of the equilibrium dialysis procedures described by Karush (1958) and Zaroslnski et al (1969, 1971). In this equilibrium dialysis procedure, the protein-drug component was confined in one chamber and separated by a semi-permeable membrane, cuprophan, from the buffer, non-drug component. The cuprophan allowed unbound drug molecules to diffuse freely between the drug-albumin retentate chamber and the buffer diffusate chamber until equilibrium was attained. When unbound, free drug concentrations in opposing chambers became equal, drug increments in protein compartment were considered to represent the amount of drug bound to protein. The DPH in the buffer retentate was considered unbound, free drug. (See page 48 for details.)

The concentration and percent of drug bound was determined as follows:

1) Concentration of bound DPH = Concentration of DPH in the serum albumin retentate chamber minus the DPH concentration in the buffer retentate chamber.
(2) \% \text{Bound DPH} = \frac{\text{Concentration of bound DPH}}{\text{Concentration of DPH in serum albumin retentate}} \times 100

The distribution of DPH between the dialysis chambers, due to the presence of albumin, will be slightly influenced by the Donnan effect and the space occupied by proteins and other solutes. Because of these effects, the concentration of the drug in the buffer retentate fraction over-estimates the concentration of free drug in the albumin retentate fraction. Therefore, the Keen (1966 b) procedure was employed to correct for these variables. Consequently, equation (1) becomes:

(3) \text{DPH bound} = \text{DPH}_{\text{albumin}} \times f - \frac{\text{DPH buffer}}{r} \times 1.004

where \( f = \frac{100}{(99.6 - 0.75) \text{ (g. albumin/100 ml)}}.\)

"This assumes that the specific gravity of albumin is 1.33 and that 0.4\% of the volume of the solution is occupied by solutes other than protein." Similarly, DPH in the buffer retentate was multiplied by \( \frac{100}{99.6} = 1.004 \) to correct for solute space.

Finally, \( r \) represents the Donnan correction factor which was determined from the distribution of sodium between the albumin retentate fractions.

(4) \( r = \frac{\text{concentration Na}^+_{\text{albumin}}}{\text{concentration Na}^+_{\text{buffer}}} \)

Keen as well as several other investigators does not adjust binding data to account for electrostatic interaction. Keen (1966 b) suggests, as does Scatchard (1949), that electrostatic correction might be inapplicable because anions displace buffer anions from binding sites rather than reacting with uncombined albumin. Thus the binding data generated in this work was not corrected for electrostatic interaction.

It is the consensus that this method of equilibrium dialysis, in which
equal volumes of solution are dialyzed, provides one of the most accurate and uncomplicated methods for the study of drug protein interaction. Although this technique of dialysis is time consuming, requiring 12-16 hours to attain equilibrium, it allows for the convenience of an overnight binding reaction.

Diphenylhydantoin was assayed using the counting procedure of Zarosinski and associates (1969, 1971) and Oester et al (1971). An aliquot of 5,5'-diphenylhydantoin-4-14C was added to the albumin-DPH solution in the protein chamber just prior to dialysis. After equilibrium was attained, a 2 ml. aliquot of the protein retentate was added to one counting vial while a like aliquot of its corresponding buffer diffusate was added to another counting vial. In all experiments, counting vials were prefilled with 10 ml. of Insta-Gel, maintaining a 10:2 ratio between emulsion-fluor and sample. For the in vivo experiments, the blood was centrifuged at 5000 g., using an International Equipment Co. Model PR-6 refrigerated centrifuge. One ml. serum was introduced into a counting vial together with 1 ml. buffer. Brain tissues were solubilized over a 24 hour period in 6-7 ml. Soluene-100, then made up to 10 ml. with the soluent. Two ml. of the digested material were added to a counting vial. All samples were counted in triplicate for 10 min. in a Packard Model 3380 Liquid Scintillation Counter. The counts were averaged and corrected for quenching. This procedure combined precision, accuracy and reproducibility with specificity and simplicity for the in vitro and in vivo measurement of minute quantities of DPH in albumin, buffer, serum and brain tissue solutions. To minimize binding study experimental variance, resulting from extraneous variables and pipetting errors, each experimental point was established on the basis of 2 separate experiments. Since the variation in results between duplicate experiments was minimal, two experiments per point were considered suffi-
cient and the results were averaged. (See Appendix, page 118 for more details)

Estimation of Sodium. Sodium concentrations were determined in a Coleman Model 21 flame photometer interfaced with a Coleman Junior Model 6C spectrophotometer for percent transmittance readout. (See Appendix, page 121).

Measurement of pH: The pH of aqueous solutions were determined with a Corning Model 7 pH meter. The measurement of blood pH was ascertained with an Instrumentation Laboratories Model 113 ultra-micro blood pH/gas analyzer system.

Estimation of Protein Concentration: Serum albumin concentrations were determined following the method described by Gornall et al (1949). A Beckman DU-2 spectrophotometer was used to measure optical density (see Appendix, page 120).

SYSTEM COMPONENTS AND PROCEDURE

Unless specified, all solutions were constituted with glass distilled water. The binding experiments were conducted using Sorenson's M/15 (0.067M) phosphate buffer. Sorenson's phosphate buffer provides little or no competition between drugs and their binding with serum albumin (Keen, 1966 b). All compounds were weighed on a Mettler Model H20T analytical balance to the nearest 0.01 mg.

Dialysis Membrane (Cuprophan, Edwards Laboratories): The semi-permeable, dialysis membrane was prepared from sheet material. The sheet material is available commercially as Cuprophan and is used clinically for renal dialysis.

Cuprophan was chosen because the high quality and uniformity of the material appear to provide the attainment of equilibrium in a shorter period of time as compared with Visking tubing. Zaroslinski et al (1969) found that equilibrium occurred in approximately 12 hours using cuprophan in the modified
Karush dialysis system. In contrast, they found that attainment of equilibrium required approximately 24 hours when Visking was employed as the semi-permeable membrane.

Discs of cuprophan (40 mm. diameter) were thoroughly rinsed in glass distilled water, then placed in the appropriate buffer and refrigerated overnight the evening before each experiment. The washing and soaking was done to eliminate substances in or adhering to the membrane which interfere with the experiment. The discs were placed between the dialysis chambers and trimmed immediately prior to introduction of buffer and albumin solutions.

Dialysis Chambers: Equilibrium dialysis was accomplished employing paired 5 ml. modified Karush dialysis chambers (Bellico #3221). The two chambers were paired by briskly milling the wide flanged ends together. If this matching of the chambers is not done, they have a tendency to leak after being placed into the spring clip holder (Bellico #3217).

Dialysis System Assembly and Procedure (Figure 1)

1. A wet cuprophan disc was placed between paired dialysis chambers, excess material trimmed to the chamber flanges, and the coupled chambers fitted into the spring clip holder.

2. Using a 5 ml. pipette assembly, 4.6 ml. of the appropriate albumin solution was introduced into the albumin retentate chamber through the porthole. Employing a clean pipette assembly, 4.8 ml. of the appropriate buffer solution was added to the buffer retentate chamber.

3. A 0.1 ml. aliquot of the concentrated DPH solution and 0.1 ml. $^{14}$C-labeled DPH solution was then introduced into the albumin chamber using a Hamilton Microliter pipetting syringe.

4. A 20 mm. square section of Parafilm "M" closure material (American Can
FIG. 1
DIALYSIS SYSTEM

Spring Clip Holder

Dialysis Chambers

Parafilm

Cuprohane

Entry Ports
5. The dialysis system assembly was then placed into a metabolic shaker and agitated at 80 strokes/min. using a 1-1/2 inch stroke. The contents were thus dialyzed for 24 hours at room temperature (25 ±1°C).

6. At the end of the dialysis period, 2 ml. of the albumin chamber contents were added to a counting vial containing 10 ml. Insta-Gel, emulsion-solvent fluor. A 2 ml. pipette assembly under vacuum was employed to accomplish sample transfer. This procedure was also followed with contents from the matching buffer chamber.

7. The paired counting vials were then identified and the contents thoroughly mixed by gently swirling.

8. All samples were then stored at 1°C. for approximately 24 hours prior to counting. If counted prior to the storage time, the proteins have a tendency to interact with the emulsion-solvent system, producing excessive chemoluminescence which results in erroneously high counts (Gibbs, 1969). For additional details, see page 118.

Preliminary Experiments. Pilot experiments were conducted to determine the length of time necessary to achieve system equilibrium and to assure that no interference in binding resulted from the system itself. In one set of binding experiments, various concentrations of DPH up to 50 x 10^-5 M were placed in the human serum albumin (HSA) retentate chamber while the opposite chamber contained buffer only. In a second set of experiments, the procedure was reversed and duplicate concentrations of DPH were placed in the buffer retentate chamber. After 24 hour dialysis, the solutions in both retentate chambers were analyzed for DPH concentration (see Appendix page 118 for more details).

Further experiments ascertained the amount of time necessary to attain
system equilibrium. The aforementioned procedure was employed and the duplicate systems assayed after 2, 4, 8, 12 and 24 hours. Minimal equilibrium time was estimated to be greater than 12 hours but less than 24 hours. Additional work was performed to insure that neither drug concentrations nor their vehicles when added to chamber contents produced any alterations in pH.

Experiments were conducted to observe in vivo blood pH alterations in mice receiving nothing, drug vehicle, DPH plus vehicle, NaHCO₃ or NH₄Cl. Neither the drug vehicle nor the DPH plus vehicle produced any alterations, while NaHCO₃ induced alkalosis and NH₄Cl caused acidosis.

**HSA and MSA Diphenylhydantoin Binding Experiments**

The effect of drug concentration variation upon diphenylhydantoin binding to albumin was studied to estimate the number of binding sites per molecule of human serum albumin (HSA) and mouse fraction V serum albumin (MSA) available to the drug. Information from this data provided a basis to determine the intimacy of the drug-albumin association. Drug concentrations ranging from below those considered therapeutic to those producing severe drug intoxication when measured clinically in serum were investigated as follows:

1. \(1.60 \times 10^{-5} \text{M}\)  
2. \(3.16 \times 10^{-5} \text{M}\)  
3. \(6.29 \times 10^{-5} \text{M}\)  
4. \(12.54 \times 10^{-5} \text{M}\)  
5. \(25.04 \times 10^{-5} \text{M}\)  
6. \(50.04 \times 10^{-5} \text{M}\)  
7. \(100.05 \times 10^{-5} \text{M}\)  
8. \(125.09 \times 10^{-5} \text{M}\)  
9. \(200.04 \times 10^{-5} \text{M}\)

The \(6.29 \times 10^{-5} \text{M}\) concentration was employed in all other HSA and MSA experiments. At equilibrium, the \(6.29 \times 10^{-5} \text{M}\) pre-dialysis concentration resulted in a serum albumin concentration in the HSA retentate chamber comparable to that considered efficacious (14-17 mcg./ml.) as reported by clinical
investigators (see pages 36-37). This concentration also produced a drug concentration in the MSA retentate chamber equivalent to those in mouse serum during those experiments where DPH was found to antagonize maximal electro-shock one hundred percent in mice.

A ratio between the moles of ligand bound per mole of albumin (\( \bar{r} \)) and moles per liter of unbound drug (C) was plotted against \( \bar{r} \). From this curve, the number of binding sites (n) and their association constants (K) were calculated according to the equation formulated by Scatchard et al (1957).

The basic equation is:

\[
\bar{r} = \sum_{i} \bar{r}_i = \sum_{i} \frac{r_i K_i C}{1 + K_i C}
\]

where \( \bar{r} \) is the average molar ratio of bound ligand for the ith binding site of the protein, n is the number of sites in the ith class of site, and K is the association constant for the interaction of the class of site with the ligand. The individual \( n_i \) and \( n_i K_i \) for each class of binding sites were derived graphically following the method described in 1950 by Karush. (See Appendix, page 123 for further details.)

The normal clinical range for blood pH is 7.35 - 7.45 in humans; "any pH below 7.35 or above 7.45 is generally defined acidosis and alkalosis respectively" (Woodbury, 1965). In contrast, the normal range for blood pH in mice appears to be dependent upon the strain of the animals used. Although the standard errors were small, Bernstein (1966) has reported pH differences in right ventricular venous blood ranging from 7.26 - 7.428 in 16 different strains of mice. Thus, it was necessary to fix the age, weight and determine the blood pH of the strain of mice employed prior to conducting any in vitro MSA experiments or in vivo acidosis/alkalosis experiments. The effects of pH variation upon serum albumin-DPH interaction were investigated because many
acute and chronic disease states clinically produce an acid and/or base
disturbance. HSA and MSA equilibrium dialysis systems incorporating the fol-
lowing pH variations were employed:

1. 7.0
2. 7.2
3. 7.4
4. 7.6

All other in vitro HSA and MSA experiments were conducted at a pH 7.4.

Since there are clinical conditions which cause blood serum albumin con-
centrations to deviate from the norm, the effects of HSA and MSA variations
on the DPH-albumin complex were also examined. The following concentrations
of HSA and MSA were utilized:

<table>
<thead>
<tr>
<th>HSA</th>
<th>MSA</th>
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<tr>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>2%</td>
<td>2%</td>
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<tr>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>6%</td>
<td></td>
</tr>
</tbody>
</table>

Due to the difficulty in obtaining large quantities of MSA, the 6% concen-
tration was omitted. All other experiments were conducted using the 4% 
albumin concentration.

Effects of Ligand Competition upon DPH-HSA Interaction

Seldom does a patient receive a single drug entity to combat illness. Thus, it was decided to study diphenylhydantoin-human serum albumin inter-
action in the presence of other drugs. During these experiments, the secondar-
ary drug was placed in the buffer diffusate chamber while DPH was placed in
the HSA chamber. The following drugs and initial drug concentrations were 
employed for the drug combination experiments.

a. Phenobarbital Na:
These concentrations were chosen because it is estimated that their range would produce drug levels in the albumin compartment equivalent to drug concentrations found in serum when therapeutically effective. Due to the scarcity of mouse fraction V serum albumin, the experiments were conducted with HSA only.

Anticonvulsant Effect of Diphenylhydantoin

Maximal electroshock (MES) experiments were chosen to determine the effect—acidosis or alkalosis—might have upon the protection afforded by DPH, because of end point reproducibility and sharpness. This technique also provided a suitable measurement to which drug serum and brain levels could be related.

MES experiments were conducted using the method of Swinyard et al. (1952). The electrical stimulus was applied through paired, silver corneal electrodes employing the electroshock seizure apparatus described by Woodbury and Davenport (1952) as modified in 1961 by Sittig (see Appendix, page 132 for details).
Eight, Charles River CD<sub>1</sub> strain, male albino mice, aged 6-7 weeks, were used per group. The animals weighed approximately 30 gm. each (30.54 ±0.17 S.E.).

The diphenylhydantoin was administered i.p. one hour prior to MES (50 milliamp for 0.2 second duration). Each animal received approximately 20.79 x 10<sup>3</sup> cpm/gm. of weight; therefore, a 30 gm. animal received approximately 623,700 cpm. Protection against MES was defined as the abolition of the tonic extensor reflex.

Serum and brain DPH concentrations in normal, alkalotic and acidotic mice were determined. It was necessary to pool the blood from each experimental group to obtain the appropriate serum volume. DPH brain concentrations were determined for each individual animal. Alkalosis was induced by intraperitoneal injection of NaHCO<sub>3</sub>, 1 gm./Kg., fifteen minutes after DPH administration. Immediately after application of MES, the animal was exanguinated by decapitation and the blood collected in a centrifuge tube. Approximately 10 large drops of blood per animal were obtained. Brains were removed and weighed on a Federal Pacific roller balance to the nearest 1.0 mg. Then, the brain was placed in the digesting solvent (see page 46). The entire procedure from electroshock application to the placement of brain tissue into the solvent took 2-3 minutes. Animals were treated individually at 6 minute intervals to provide sufficient time. To limit the effect of circadiun rhythms and seasonal variables, each series of in vivo experiments was conducted between 9 A.M. and 3 P.M. within a two week period. The median protective doses (PD 50) for DPH under the various conditions were calculated in the manner described by Litchfield and Wilcoxon (1949).
Human Serum Albumin Solution (Research Products Division, Miles Laboratories, Inc.; Pentex Lots #27 and 28).

Crystalline human serum albumin, assayed 100% pure electrophoretically, was dissolved in the appropriate M/15 (0.067M) Sorensen's phosphate buffer. The human serum albumin solutions were freshly reconstituted for each experiment.

Mouse Serum Albumin Solution (Research Products Division, Miles Laboratories, Inc.; Pentex Lot #7).

Mouse albumin, fraction V powder, assayed electrophoretically to be 99% pure albumin, was dissolved in the appropriate M/15 (0.067M) Sorensen's phosphate buffer. Miles-Pentex states that alpha globulins are the major contaminant but alpha-1 acid glycoprotein may also appear. The mouse serum albumin solutions were freshly reconstituted for each experiment.

5,5-Diphenylhydantoin Sodium (Sodium DilantinR) - Parke, Davis and Company, Lot #407265).

The appropriate amounts of powder were dissolved in freshly prepared 0.01 NaOH solution prior to each experiment (Conard, 1969). Then, an aliquot was pipetted into the proper dialysis chamber at the concentrations indicated on page 51. The drug was also dissolved in those buffer solutions which would permit drug solubility while conducting experiments to determine system equilibrium.

5,5-Diphenylhydantoin-4-C\textsuperscript{14} (New England Nuclear, Lots 506-130 and 583-056).

The labeled powder was dissolved in 0.01 NaOH and stored refrigerated as a stock solution (Conard, 1969). An appropriate aliquot of the solution was pipetted into the proper dialysis chamber just prior to each experiment.
Drugs Employed in DPH-Albumin Interaction Antagonism Experiments

Phenobarbital Sodium (Mallinckrodt, Lot #TBP), Phenylbutazone (Geigy, Lot #SN 44921), Warfarin Sodium (Abbott Laboratories, Lot #789-3613), and Trimethadione (Abbott Laboratories, Lot # 788-7314). Each drug was dissolved in the Sorensen's pH 7.4 buffer solution and a 4.8 ml. aliquot of the appropriate solution introduced into the buffer diffusate chamber of the dialysis system.

Sodium Hydroxide Solution (Harleco, #13092).

Sodium hydroxide solutions were prepared by diluting commercially available 10 N analytical reagent grade sodium hydroxide solution with glass distilled water. A 0.01 N solution was employed to dissolve diphenylhydantoin.

Sorensen's M/15 (0.067 M) Phosphate Buffer (Hepler, 1960)

Sorensen's M/15 Phosphate Buffer solutions were prepared at the proper pH by mixing the appropriate quantities from stock solutions of:

1. M/15 Potassium Acid Phosphate (KH₂PO₄), 9.08 gm. qs. to 1 liter.
   (Mallinckrodt, Lot #TSB).

2. M/15 Disodium Phosphate (Na₂HPO₄), 9.47 gm. qs. to 1 liter
   (Mallinckrodt, Lot #XCV).

Biuret Reagent (Harleco Lot #9024P)

The reagent was prepared in accordance with the procedure described by Gornall et al (1949). The preparation consisted of a modified alkaline copper tartrate stabilized with potassium iodide.

Ammonium Chloride Solution (NH₄Cl) (Mallinckrodt, Lot #LUX)

The ammonium chloride solution was prepared by dissolving 400 mg. NH₄Cl in glass distilled water qs. to 10 ml.
Emulsifier-Solvent-Fluor System

Insta-Gel (Packard Instrument Co., Inc., Lots #19-K-69-08 and 27 K 7008). For counting a constant ratio of 10 ml. of Insta-Gel to 2 ml. aqueous sample was employed.

Tissue Sample Solubilizer

Soluene TM 100 (Packard Instrument Co., Inc.). Description of procedure is given on page 46. Although heating of tissue and solubilizer speeds up the digestive process, most animal tissues require 24 hours for total digestion. Since digestion speed is a function of exposed surface, the brain was cut into three sections and totally immersed in the solubilizer. Brain tissue was usually solubilized within 18-20 hours.

Flame Photometry Chemicals

Sodium Flame Photometry Standard "A", 250 mEq./1000 ml. (Harleco, Lot #60301) and Sterox SE (Harleco) were employed in the determination of albumin and buffer chamber sodium concentrations. Both chemicals were diluted to the proper concentrations using glass distilled water.

Special Pipette Assemblies

Individual Hamilton microliter pipetting syringes fitted with Chaney adapters (accuracy ±0.1%, reproducibility ±0.01%) were employed to deliver or withdraw solutions of 0.0 ml. Syringes were marked and used for a specific task, i.e. the introduction of hot DPH into the albumin chamber, to minimize pipetting and counting errors. Pipetting assemblies had to be constructed to allow introduction and withdrawal of solutions greater than 1 ml. through the narrow pipe-like chamber ports of the dialysis chambers. The assemblies consisted of 5 ml. Mohr measuring pipettes (accuracy ±0.02 ml.) or 2 ml. volumetric pipettes (accuracy ±0.006 ml.) to which were attached 20 gauge x 1 inch
monoject, polypropylene Luer-Lok hub, sterile hypodermic needles (Scientific Products - Sherwood Medical Industries).
CHAPTER IV
RESULTS

Prior to beginning in depth diphenylhydantoin (DPH) binding experiments, a series of preliminary experiments were conducted to determine drug recovery and efficiency of the dialysis system. It was found that the buffer solutions produced no quenching. The recovery was 101.9% (±0.23 S.E.) of theoretical. Only a very slight quenching effect was observed with the albumin solutions. The cpm values obtained with 4% albumin in buffer were less than 1.8% of those obtained in buffer alone.

Experiments were also conducted to insure that the administration of NaHCO₃, 1 gm./Kg. and NH₄Cl, 400 mg./Kg. would produce alkalosis and acidosis in mice. Table 1 shows that the blood pH of saline injected control animals was 7.39 ±0.0. Administration of DPH in 0.01 N. NaOH did not significantly alter blood pH whereas NaHCO₃ significantly increased the blood pH to 7.55. NH₄Cl significantly reduced pH to 7.23 when compared with the saline control group. The NaHCO₃ findings were in agreement with those reported by Waddell and Butler (1957).

To estimate the number of binding sites per mole of albumin, the moles of DPH bound per mole of albumin (r) was determined for several concentrations of the drug. The possibility that serum albumin of individual species might bind DPH differently was explored by determining r for both human serum albumin (HSA) and mouse, fraction V, serum albumin (MSA). The r ratios were then related to the molar concentration of unbound (free) drug (c) present by plotting r/c vs. r. The molecular weight for HSA was taken as 69,000 (Phelps and Putnam, 1960). To our knowledge, the molecular weight for MSA has not been determined; however, Charlwood (1959) approximated a molecular weight of 69,000.
for both rat and rabbit serum albumin. Both Keresztes-Nagy (1971) and Basset (1971) have estimated that MSA should have a molecular weight of approximately 69,000. In accordance with these estimates, a molecular weight of 69,000 was used in all calculations involving MSA. The ionic strength of the phosphate buffer was calculated at 0.17 arbitrary units (Martin, 1962). The concentrations of HSA and MSA were 57.97 x 10^{-5} M (4%). Many investigators, among them McMenamy and Oncley (1958), Anton (1960-1961), Eichman et al (1962), Kunen (1965), Conard et al (1971) and Rudman et al (1971), have not made data adjustments for Donnan equilibrium effects or the space occupied by proteins and other solutes (solute space); However, Keen (1966b) stressed that these corrections were necessary for data accuracy. Therefore, it was decided to compare the number (n) and classes of binding sites and their association constants (K) prior to and after making the data corrections discussed by Keen.

The values utilized for the characterization of DPH binding sites and association constants for HSA are presented in Tables 2 and 3, while those for MSA are on Tables 4 and 5. Examination of the tables reveals that MSA bound approximately 96-83% of the DPH present in the albumin retentate, compared to 85-74% for HSA, with increasing drug concentrations. This species difference in serum albumin binding ability is further emphasized by the fact that MSA bound 28-35% more DPH per mole of albumin (r) than HSA. Another striking difference in the binding of DPH by the two albumins is illustrated in Figure 2. At low initial drug concentrations, the moles of DPH bound per mole of albumin for HSA and MSA are approximately equal, resulting in comparable fractions of unbound (free) DPH. However, as the initial DPH concentrations are increased, the concentrations of free drug in MSA protein compart-
Scatchard plots of diphenylhydantoin binding site data with HSA are illustrated in Figure 3 and 4; those with MSA are presented in Figure 5 and 6. Karush (1950), Scatchard (1949, 1950), and others have reported that homogeneity of binding sites (a single class) produces a straight line plot. When the plotted points are extrapolated to the abscissa and ordinate, the intercepts equal $n$ and $nK$ respectively (see Appendix). If a curved line is plotted from the $r/c$ vs. $r$ relationship, the binding sites are considered heterogeneous (more than 1 class of sites).

The plots of these data produced curves; hence, it was assumed that more than one class of binding sites were responsible for binding DPH. Resolution of the curves into two asymptotes revealed that two classes of binding sites, $n_1$ and $n_2$, were available to DPH on both MSA and HSA. Table 6 contains the values calculated for the albumin binding sites and their association constants.

Resolution of the HSA and MSA curves provides data indicating that both albumins contain approximately 7-8 DPH binding sites. Each species of albumin appears to contain a small primary number of binding sites having high DPH affinity, as represented by $K_1$, and a much larger number of secondary sites with lesser affinity. Comparing $n$ and $K$ values, the HSA primary binding site class values are much smaller (0.03 - 0.04) than for MSA (0.6); however, the association constants for HSA (139,613 - 137,317) are approximately twice that of MSA (56,084 - 56,988). On the other hand, the number of secondary binding sites for HSA ranged from 7.3 to 7.9 and are slightly larger than for MSA (6.9 - 7.4); but the MSA association constant is about 65% greater (822-806 vs. 1349-1348).

Finally, examination of Table 6 indicates that data correction for changes
produced by Donnan equilibrium effects and solute space apparently had little influence on the results. Nevertheless, all results have been adjusted to reflect the effects of these parameters for accuracy.

Figure 7 illustrates the effects of varying the DPH concentration upon the concentration of the unbound fraction (Tables 3 and 5). As the concentration of DPH in the albumin retentate doubles, the free drug concentration approximately doubled. This rapid, large increase in free drug availability becomes more important since this effect was achieved with only small alterations in the percent of DPH bound. Figure 8 shows that minor shifts in the percent of drug bound also resulted in a doubling of free DPH concentration. Thus, at serum concentrations considered most desirable therapeutically (10-20 mcg./ml. as represented by the albumin retentate fraction), only a small fraction of the drug was unbound. However, as DPH albumin fractions increased, the unbound fraction rapidly increased. By increasing the initial DPH concentration from 6.29 to $12.54 \times 10^{-5} \text{M}$, the drug concentration in the HSA retentate was equivalent to serum concentrations (30 mcg./ml.) measured when drug toxicity was observed in patients as reported by Buchthal et al. (1960) as well as Kutt and coworkers (1964). These alterations occurred with only a very slight reduction, from 83.3 - 81.6, in the percent of drug bound.

The effects produced by pH alteration upon the concentration of unbound (free) diphenylhydantoin in 4% HSA and MSA are shown in Figure 9, Tables 7 and 8. Reduction of pH from 7.6 to 7.0 produced parallel linear increases in the unbound DPH fraction from 2.24 - 2.67 mcg./ml. (19.2%) with HSA, and 0.63-0.97 mcg./ml. (54.0%) with MSA. When considering the moles of drug bound/mole of albumin (r), the fraction of DPH bound decreased as the pH was reduced. Increasing the pH to 7.6 from 7.4 revealed a decrease in the percent of free
drug in HSA amounting to approximately 12.6%. Decreasing the pH from 7.4 to 7.0 increased the unbound fraction 3.0 - 6.6%. In contrast, increasing the pH from 7.4 decreased the free fraction in MSA 14% while a reduction elevated the unbound concentration from 16.3 - 34.9%. Thus, pH changes appeared to have little effect on unbound DPH in HSA whereas a reduction in pH appeared to significantly affect the unbound DPH concentration in MSA. Again, examination of the alterations occurring in the percent of drug bound show that the changes discussed result from only a small deviation (2%) in the percent of DPH bound.

Alterations in the binding of diphenylhydantoin as the result of varying the concentration of albumin are presented in Figure 10 and Tables 9 and 10. As expected, an increase in albumin concentration was accompanied by a decrease in the free drug fraction and an increased DPH concentration in the albumin retentate. The free drug concentration in HSA retentate decreased from 5.13 mcg./ml. in 1% HSA to 1.67 mcg./ml. in 6% HSA. At a 1% concentration of MSA, the unbound DPH fraction was 2.74 mcg./ml. and 0.74 mcg./ml. at 4% MSA. Conversely, diphenylhydantoin in the HSA (1-6%) retentate rose from 12.26 - 16.28 and from 14.68 - 17.08 in MSA (1-4%) respectively. These findings, however, were in contrast to those where drug concentrations were varied and free drug concentration increased as the drug concentration in the albumin retentate rose. In the albumin variation experiments, there was a reduction of unbound (free) diphenylhydantoin of up to 67.4% in HSA and 73% in MSA even as the drug concentrations in the albumin retentate rose. Therefore, the moles of drug bound per mole of albumin (r) had to increase as a result of elevated albumin concentration. The actuality of this phenomenon and a comparison between the effect in HSA and MSA are shown in Figure 11. Examination
of the data revealed that increasing the HSA concentrations from 1 to 4% produced a 77.3% (0.0449-0.0796) increase in r, while increasing the HSA concentration to 6% produced an overall 105% (0.0449-0.0919) increase in r. Similarly increasing MSA concentrations from 1-4% caused an overall 37% (0.071-0.1028) increase in r. In all of the DPH-albumin interaction experiments, MSA was found to bind more DPH than HSA. The moles of drug bound per mole of MSA were almost twice that of HSA at 1 and 2%, while at 4% a 29.1% differential existed. Thus, it would appear that the number of binding sites unmasked per mole of albumin would be dependent upon albumin concentration, amount of drug present, and the total configuration of the albumin molecules at the time.

The effect of ligand competition on the binding of diphenylhydantoin to human serum albumin is presented in Figure 12, Tables 11 and 12. When compared to control values, the presence of phenobarbital caused some variation but had generally little effect on the DPH-albumin complex or on the concentration of free DPH. Warfarin, reported by O'Reilly (1967) to be bound to albumin 80 to 90%, appeared to cause a slight reduction in the unbound DPH concentration. When the warfarin pre-dialysis concentrations were increased, this influence was diminished. Similarly, the presence of trimethadione also produced a decrease in unbound DPH. However, in contrast to warfarin, increasing the predialysis concentrations of trimethadione further augmented the binding of DPH as compared to control values. The various changes observed in the unbound (free) DPH fraction due to the presence of phenobarbital, warfarin, and trimethadione occurred without influencing the concentrations of diphenylhydantoin in the albumin retentate fraction or unduly altering the moles of drug bound per mole of albumin ratio. On the other hand, addition of phenylbutazone, reported to be bound by albumin 95% (Anton, 1961), to the
dialysis system antagonized the binding of diphenylhydantoin to HSA. When compared with control values, phenylbutazone appeared to inhibit the interaction between DPH and albumin, increasing the concentration of free diphenylhydantoin from approximately 6 to 49% (2.68 - 3.78 mcg./ml.). The alteration in free DPH was apparently due to a reduction in the moles of DPH bound per mole of albumin (r). The presence of phenylbutazone decreased the r value between 2.5 and 20.2% (0.0776 - 0.0635) when compared to 0.0796 for the DPH control.

Experiments were conducted to determine whether diphenylhydantoin serum and total brain concentrations could be correlated with the protection afforded against maximal electroshock (MES) convulsions in mice. Additional MES experiments were designed in an attempt to relate indirectly and generally, the results obtained from the diphenylhydantoin-mouse serum albumin binding experiments to alterations in pharmacological activity of the drug by inducing acidosis and alkalosis in mice. Preliminary experiments revealed that injections of NH₄Cl or NaHCO₃ to induce acidosis and alkalosis respectively, did not alter the incidence of tonic extensor seizures or appear to change the seizure pattern produced by MES when compared to controls. Table 13 shows that the i.p. median protective dose (PD₅₀) for DPH against MES was 10.4 mg./Kg. in normal animals. In alkalotic mice, the PD₅₀ was not altered, while acidosis appeared to induce an increased sensitivity to the drug. The PD₅₀ for diphenylhydantoin in acidotic mice shifted from 10.4 mg./Kg. for the controls to 8.4 mg./Kg. This alteration in PD₅₀ was statistically significant (P < .01).

Table 14 together with Figures 13 and 14 compare diphenylhydantoin serum and brain concentrations with the percentage of animals protected by each DPH
dosage. The percentage of mice protected against MES in all experiments appeared to be related to both serum and brain levels. As DPH concentrations in serum and brain rose, the percentage of animals protected increased. When compared with control animals receiving comparable doses, i.e., 10 mg./Kg., the acidotic animals had lower DPH serum concentrations (7.74 mcg./ml. vs. 8.43) but slightly higher brain concentrations (11.64 vs. 10.82 mcg./gm.). In association with this shift in drug concentrations, the acidotic animals were afforded a higher degree of protection, 75.0% vs. 37.5%. On the other hand, the changes observed in the alkalotic mice were much less clearly defined. The diphenylhydantoin anti-MES protective dose 50% in normal mice was equal to that in alkalotic mice. At 10 mg./Kg., the serum concentration in the alkalotic mice was higher (8.58 vs. 8.43 mcg./ml.) and the brain concentration lower (9.67 vs. 10.82) than in normals. Associated with this change was a decrease in the percentage of alkalotic animals protected. In contrast, at 12.5 mg./Kg. there were decreases in both serum (9.44 vs. 11.24) and brain (13.7 vs. 14.17 mcg./ml.) DPH concentrations of the alkalotics when compared with the normals; but, 85.7% of the alkalotic animals were protected while 75% of the normals were protected. In general, the brain concentrations were lower in the alkalotic mice while serum concentrations were mixed when compared to controls. This combination of differences in the alkalotic mice appeared to produce a mixed MES antagonism pattern, resulting in a PD50 identical with that of the control animals.
TABLE I

Effect of Diphenylhydantoin, NaHCO₃ or NH₄Cl upon Blood pH

of CD₁ Strain Mice

<table>
<thead>
<tr>
<th>pH</th>
<th>Saline Control</th>
<th>Diphenylhydantoin (12 mg./Kg.)</th>
<th>NaHCO₃ (1 gm./Kg.)</th>
<th>NH₄Cl (400 mg./Kg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>7.39</td>
<td>7.41</td>
<td>7.55</td>
<td>7.23</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>S. E.</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Level of Significance vs. Controls</td>
<td>-</td>
<td>N. S.</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Level of Significance vs. DPH treated</td>
<td>-</td>
<td>-</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

*Seven mice were used per group.*
<table>
<thead>
<tr>
<th>Initial Drug Conc. (10^-5M)</th>
<th>Drug Concentration in Albumin Renate (Moles x 10^-8)</th>
<th>Final Free Drug Conc. (c) (10^-5M)</th>
<th>Moles Drug Bound (x 10^-8)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound/Mole HSA (r)</th>
<th>r/c (x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.60</td>
<td>6.62</td>
<td>0.2180</td>
<td>5.58</td>
<td>84.23</td>
<td>0.0200</td>
<td>9.174</td>
</tr>
<tr>
<td>3.16</td>
<td>12.99</td>
<td>0.4560</td>
<td>10.80</td>
<td>83.14</td>
<td>0.0388</td>
<td>8.509</td>
</tr>
<tr>
<td>6.29</td>
<td>25.68</td>
<td>0.9379</td>
<td>21.18</td>
<td>82.40</td>
<td>0.0761</td>
<td>8.114</td>
</tr>
<tr>
<td>12.54</td>
<td>50.45</td>
<td>2.0275</td>
<td>40.72</td>
<td>80.71</td>
<td>0.1463</td>
<td>7.216</td>
</tr>
<tr>
<td>25.04</td>
<td>99.75</td>
<td>4.2584</td>
<td>79.31</td>
<td>79.51</td>
<td>0.2850</td>
<td>6.693</td>
</tr>
<tr>
<td>50.04</td>
<td>195.03</td>
<td>9.4084</td>
<td>90.08</td>
<td>76.84</td>
<td>0.5386</td>
<td>5.725</td>
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<tr>
<td>100.05</td>
<td>386.98</td>
<td>14.4370</td>
<td>293.68</td>
<td>75.89</td>
<td>1.0554</td>
<td>5.430</td>
</tr>
<tr>
<td>125.09</td>
<td>478.63</td>
<td>25.3458</td>
<td>356.97</td>
<td>74.58</td>
<td>1.2830</td>
<td>5.062</td>
</tr>
</tbody>
</table>

aData not corrected for Donnan equilibrium effect or space occupied by solutes.
## TABLE 3

Diphenylhydantoin Binding Site Data With HSA (Corrected)^a

<table>
<thead>
<tr>
<th>Initial Drug Conc. (10^-5 M)</th>
<th>Drug Concentration in Albumin Retentate (Moles x 10^-8)</th>
<th>Final Free Drug Conc. (c) (10^-5 M)</th>
<th>Moles Drug Bound (x 10^-8)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound/Mole HSA (r)</th>
<th>r/c (x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.60</td>
<td>6.86 (3.92)^b</td>
<td>0.2144 (0.59)^b</td>
<td>5.83</td>
<td>84.99</td>
<td>0.0209</td>
<td>9.748</td>
</tr>
<tr>
<td>3.16</td>
<td>13.44 (7.68)</td>
<td>0.4500 (1.23)</td>
<td>11.28</td>
<td>83.93</td>
<td>0.0405</td>
<td>9.000</td>
</tr>
<tr>
<td>6.29</td>
<td>26.58 (15.18)</td>
<td>0.9229 (2.53)</td>
<td>22.15</td>
<td>83.33</td>
<td>0.0796</td>
<td>8.625</td>
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<tr>
<td>12.54</td>
<td>52.22 (29.83)</td>
<td>1.9958 (5.47)</td>
<td>42.64</td>
<td>81.65</td>
<td>0.1532</td>
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<td>25.04</td>
<td>103.24 (58.97)</td>
<td>4.1917 (11.49)</td>
<td>83.12</td>
<td>80.51</td>
<td>0.2987</td>
<td>7.126</td>
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<tr>
<td>50.04</td>
<td>201.86 (115.31)</td>
<td>9.2600 (25.38)</td>
<td>157.41</td>
<td>77.98</td>
<td>0.5657</td>
<td>6.109</td>
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<tr>
<td>100.05</td>
<td>400.52 (228.79)</td>
<td>19.1330 (52.44)</td>
<td>308.68</td>
<td>77.07</td>
<td>1.1093</td>
<td>5.798</td>
</tr>
<tr>
<td>125.09</td>
<td>495.38 (282.98)</td>
<td>24.9230 (68.31)</td>
<td>375.75</td>
<td>75.85</td>
<td>1.350</td>
<td>5.417</td>
</tr>
</tbody>
</table>

^a Data corrected for Donnan equilibrium effect and space occupied by solutes.

^b Values in parentheses represent DPH concentrations in mcg./ml.
<table>
<thead>
<tr>
<th>Initial Drug Conc. ($10^{-7}$M)</th>
<th>Drug Concentration in Albumin Retentate (Moles x $10^{-8}$)</th>
<th>Final Free Drug Conc. (c) ($10^{-5}$M)</th>
<th>Moles Drug Bound ($x 10^{-3}$)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound/Moles MSA (r)</th>
<th>$r/c$ ($x 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.16</td>
<td>14.59</td>
<td>0.1229</td>
<td>14.00</td>
<td>95.96</td>
<td>0.0503</td>
<td>40.927</td>
</tr>
<tr>
<td>6.29</td>
<td>28.90</td>
<td>0.2667</td>
<td>27.62</td>
<td>95.57</td>
<td>0.0992</td>
<td>37.195</td>
</tr>
<tr>
<td>12.54</td>
<td>57.26</td>
<td>0.6083</td>
<td>54.34</td>
<td>94.90</td>
<td>0.1953</td>
<td>32.106</td>
</tr>
<tr>
<td>25.04</td>
<td>113.32</td>
<td>1.4312</td>
<td>106.45</td>
<td>93.94</td>
<td>0.3826</td>
<td>26.733</td>
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<tr>
<td>50.04</td>
<td>221.56</td>
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<td>202.93</td>
<td>91.59</td>
<td>0.7293</td>
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<td>200.04</td>
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<td>83.27</td>
<td>2.4600</td>
<td>8.582</td>
</tr>
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</table>

aData not corrected for Donnan equilibrium effect and space occupied by solutes.
## TABLE 5

**Diphenylhydantoin Binding Site Data With MSA (Corrected)**

<table>
<thead>
<tr>
<th>Initial Drug Conc. $(10^{-5} M)$</th>
<th>Drug Concentration In Albumin Retentate (Moles x $10^{-8}$)</th>
<th>Final Free Drug Conc. (c) $(10^{-5} M)$</th>
<th>Moles Drug Bound $(x 10^{-8})$</th>
<th>%Drug Bound</th>
<th>Moles Drug Bound/Moles MSA (r)</th>
<th>r/c $(x 10^{-3})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.16</td>
<td>15.10 $(8.62)^b$</td>
<td>0.1246 $(0.34)^b$</td>
<td>14.50</td>
<td>96.04</td>
<td>0.0521</td>
<td>41.814</td>
</tr>
<tr>
<td>6.29</td>
<td>29.91 $(17.08)$</td>
<td>0.2708 $(0.74)$</td>
<td>28.61</td>
<td>95.65</td>
<td>0.1028</td>
<td>37.962</td>
</tr>
<tr>
<td>12.54</td>
<td>59.26 $(33.85)$</td>
<td>0.6167 $(1.69)$</td>
<td>56.30</td>
<td>95.00</td>
<td>0.2023</td>
<td>32.804</td>
</tr>
<tr>
<td>25.04</td>
<td>117.29 $(67.00)$</td>
<td>1.4500 $(3.97)$</td>
<td>110.33</td>
<td>94.06</td>
<td>0.3965</td>
<td>27.345</td>
</tr>
<tr>
<td>50.04</td>
<td>229.31 $(130.99)$</td>
<td>3.9312 $(10.78)$</td>
<td>210.44</td>
<td>91.77</td>
<td>0.7563</td>
<td>19.238</td>
</tr>
<tr>
<td>100.05</td>
<td>443.72 $(253.47)$</td>
<td>10.8750 $(29.81)$</td>
<td>391.52</td>
<td>88.24</td>
<td>1.4070</td>
<td>12.938</td>
</tr>
<tr>
<td>125.09</td>
<td>548.17 $(313.10)$</td>
<td>14.9125 $(43.61)$</td>
<td>476.59</td>
<td>86.94</td>
<td>1.7128</td>
<td>11.486</td>
</tr>
<tr>
<td>200.04</td>
<td>851.02 $(486.14)$</td>
<td>29.0400 $(72.59)$</td>
<td>711.61</td>
<td>83.62</td>
<td>2.5570</td>
<td>8.805</td>
</tr>
</tbody>
</table>

*a Data corrected for Donnan equilibrium effect and space occupied by solutes.

*b Values in parenthesis represent DPH concentrations in mcg./ml.
### TABLE 6

Values Determined for the Number of Diphenylhydantoin Binding Sites (n) per Molecule HSA and MSA, and Their Association Constants (K)

<table>
<thead>
<tr>
<th></th>
<th>Human Serum Albumin (HSA)</th>
<th>Mouse Serum Albumin (MSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Uncorrecteda-</td>
<td>- Correctedb-</td>
</tr>
<tr>
<td>Sites</td>
<td>n  ( \times 10^3 )  nK    K</td>
<td>n  ( \times 10^3 )  nK    K</td>
</tr>
<tr>
<td>Primary</td>
<td>0.03 4188.4 139,613</td>
<td>0.04 5492.7 137,317</td>
</tr>
<tr>
<td>Secondary</td>
<td>7.30 6000.0 822</td>
<td>7.90 6369.0 806</td>
</tr>
<tr>
<td>Totals</td>
<td>7.33 10189.0</td>
<td>7.94 11861.7</td>
</tr>
</tbody>
</table>

**a** Values were **not** corrected for Donnan effect or space occupied by solutes.

**b** Values **were** corrected for Donnan effect and space occupied by solutes.
TABLE 7

Effect of pH on the Binding of Diphenylhydantoin to HSA

<table>
<thead>
<tr>
<th>pH</th>
<th>Drug Conc. in HSA Retentate (mcg./ml.)</th>
<th>Conc. Free Drug (mcg./ml.)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound/ Moles HSA (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.6</td>
<td>15.48</td>
<td>2.24</td>
<td>85.53</td>
<td>0.0833</td>
</tr>
<tr>
<td>7.4</td>
<td>15.18</td>
<td>2.53</td>
<td>83.33</td>
<td>0.0796</td>
</tr>
<tr>
<td>7.2</td>
<td>15.10</td>
<td>2.59</td>
<td>82.85</td>
<td>0.0785</td>
</tr>
<tr>
<td>7.0</td>
<td>15.06</td>
<td>2.67</td>
<td>82.27</td>
<td>0.0780</td>
</tr>
</tbody>
</table>

TABLE 8

Effect of pH on the Binding of Diphenylhydantoin to MSA

<table>
<thead>
<tr>
<th>pH</th>
<th>Drug Conc. in MSA Retentate (mcg./ml.)</th>
<th>Conc. Free Drug (mcg./ml.)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound/ Moles MSA (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.6</td>
<td>17.18</td>
<td>0.63</td>
<td>96.33</td>
<td>0.1041</td>
</tr>
<tr>
<td>7.4</td>
<td>17.08</td>
<td>0.74</td>
<td>95.69</td>
<td>0.1028</td>
</tr>
<tr>
<td>7.2</td>
<td>16.94</td>
<td>0.85</td>
<td>94.98</td>
<td>0.1013</td>
</tr>
<tr>
<td>7.0</td>
<td>16.84</td>
<td>0.97</td>
<td>94.24</td>
<td>0.0999</td>
</tr>
</tbody>
</table>

a A molecular weight of 69,000 was used for human serum albumin (HSA) and for mouse serum albumin (MSA).
b The albumin concentration was 4% and the initial DPH concentration of 6.29 x 10^-5M.
### TABLE 9
Effect of Protein Concentration (HSA) on the Bonding of Diphenylhydantoin

<table>
<thead>
<tr>
<th>% HSA Conc.</th>
<th>Drug Conc. HSA Compartment (mcg./ml.)</th>
<th>Conc. Free Drug (c) HSA Compartment (mcg./ml.)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound per Mole HSA (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.26</td>
<td>5.13</td>
<td>58.16</td>
<td>0.0449</td>
</tr>
<tr>
<td>2</td>
<td>13.76</td>
<td>3.68</td>
<td>73.25</td>
<td>0.0634</td>
</tr>
<tr>
<td>4 (Normal)</td>
<td>15.18</td>
<td>2.53</td>
<td>83.33</td>
<td>0.0796</td>
</tr>
<tr>
<td>6</td>
<td>16.28</td>
<td>1.67</td>
<td>89.74</td>
<td>0.0919</td>
</tr>
</tbody>
</table>

**TABLE 10**
Effect of Protein Concentration (MSA) on the Binding of Diphenylhydantoin

<table>
<thead>
<tr>
<th>% MSA Conc.</th>
<th>Drug Conc. MSA Compartment (mcg./ml.)</th>
<th>Conc. Free Drug (c) MSA Compartment (mcg./ml.)</th>
<th>% Drug Bound</th>
<th>Moles Drug Bound per Mole MSA (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.68</td>
<td>2.74</td>
<td>81.34</td>
<td>0.0751</td>
</tr>
<tr>
<td>2</td>
<td>16.07</td>
<td>1.44</td>
<td>91.04</td>
<td>0.0921</td>
</tr>
<tr>
<td>4 (Normal)</td>
<td>17.08</td>
<td>0.74</td>
<td>95.69</td>
<td>0.1028</td>
</tr>
</tbody>
</table>

---

*a* The pH was 7.4 and the initial DPH Concentration was $6.29 \times 10^{-5}$M

*b* A molecular weight of 69,000 was used for the serum albumin
<table>
<thead>
<tr>
<th>Ligand Concentration (10^{-5}M)</th>
<th>DPH Conc. HSA Compartment (mcg./ml.)</th>
<th>Conc. Free DPH HSA Compartment (mcg./ml.)</th>
<th>% DPH Bound</th>
<th>Moles Drug Bound/Mole HSA</th>
<th>% Change of Free DPH from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH Control</td>
<td>15.18</td>
<td>2.53</td>
<td>83.33</td>
<td>0.0796</td>
<td>-</td>
</tr>
<tr>
<td>DPH + Phenobarbital Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>14.99</td>
<td>2.31</td>
<td>84.60</td>
<td>0.0798</td>
<td>- 8.70</td>
</tr>
<tr>
<td>12.50</td>
<td>15.09</td>
<td>2.62</td>
<td>82.64</td>
<td>0.0784</td>
<td>3.56</td>
</tr>
<tr>
<td>25.00</td>
<td>14.91</td>
<td>2.78</td>
<td>81.35</td>
<td>0.0763</td>
<td>9.88</td>
</tr>
<tr>
<td>50.00</td>
<td>15.31</td>
<td>2.41</td>
<td>84.25</td>
<td>0.0812</td>
<td>- 4.74</td>
</tr>
<tr>
<td>DPH + Trimethadione</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100.00</td>
<td>15.25</td>
<td>2.46</td>
<td>83.87</td>
<td>0.0805</td>
<td>- 2.77</td>
</tr>
<tr>
<td>200.00</td>
<td>15.47</td>
<td>2.26</td>
<td>85.39</td>
<td>0.0831</td>
<td>-10.67</td>
</tr>
<tr>
<td>400.00</td>
<td>15.48</td>
<td>2.24</td>
<td>85.53</td>
<td>0.0833</td>
<td>-11.46</td>
</tr>
</tbody>
</table>

a The HSA concentration was 4%, the pH 7.4, and the initial DPH concentration 6.29 X 10^{-5}M.
### TABLE 12

Effect of Ligand Competition on the Binding of Diphenylhydantoin\(^a\)

<table>
<thead>
<tr>
<th>Ligand Concentration (10(^{-5})M)</th>
<th>DPH Conc. HSA Compartment (mcg./ml.)</th>
<th>Conc. Free DPH HSA Compartment (mcg./ml.)</th>
<th>%DPH Bound</th>
<th>Moles Drug Bound/Mole HSA</th>
<th>% Change of Free DPH From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH Control</td>
<td>15.18</td>
<td>2.53</td>
<td>83.33</td>
<td>0.0796</td>
<td>-</td>
</tr>
<tr>
<td>DPH + Phenylbutazone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>15.02</td>
<td>2.68</td>
<td>82.16</td>
<td>0.0776</td>
<td>5.93</td>
</tr>
<tr>
<td>50.0</td>
<td>14.96</td>
<td>2.74</td>
<td>81.68</td>
<td>0.0768</td>
<td>8.30</td>
</tr>
<tr>
<td>100.0</td>
<td>14.25</td>
<td>3.41</td>
<td>76.07</td>
<td>0.0682</td>
<td>34.80</td>
</tr>
<tr>
<td>200.0</td>
<td>13.87</td>
<td>3.78</td>
<td>72.75</td>
<td>0.0635</td>
<td>49.41</td>
</tr>
<tr>
<td>DPH + Warfarin Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.60</td>
<td>15.41</td>
<td>2.32</td>
<td>84.94</td>
<td>0.0823</td>
<td>-8.30</td>
</tr>
<tr>
<td>3.12</td>
<td>15.33</td>
<td>2.39</td>
<td>84.41</td>
<td>0.0814</td>
<td>-5.53</td>
</tr>
<tr>
<td>6.25</td>
<td>15.33</td>
<td>2.39</td>
<td>84.41</td>
<td>0.0814</td>
<td>-5.53</td>
</tr>
<tr>
<td>12.50</td>
<td>15.31</td>
<td>2.42</td>
<td>84.19</td>
<td>0.0811</td>
<td>-4.35</td>
</tr>
</tbody>
</table>

\(^a\) The HSA concentration was 4\%, the pH 7.4, and the initial DPH concentration \(6.29 \times 10^{-5}\) M.
# TABLE 13

Brain and Serum Concentrations of Diphenylhydantoin in Normal, Acidotic and Alkalotic Mice Following Convulsions Induced by MES

<table>
<thead>
<tr>
<th>Diphenylhydantoin Dose (mcg./Kg.)</th>
<th>Normal</th>
<th>Acidotic</th>
<th>Alkalotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>-</td>
<td>1/8</td>
<td>-</td>
</tr>
<tr>
<td>8.0</td>
<td>1/8</td>
<td>3/8</td>
<td>2/8</td>
</tr>
<tr>
<td>10.0</td>
<td>3/8</td>
<td>6/8</td>
<td>2/8</td>
</tr>
<tr>
<td>12.5</td>
<td>6/8</td>
<td>-</td>
<td>6/7</td>
</tr>
<tr>
<td>15.6</td>
<td>8/8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Calculated Median Protective Doses ±95%, Confidence Limits (mg./Kg.)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Acidotic</th>
<th>Alkalotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.4 ±1.6</td>
<td>8.4 ±1.2c</td>
<td>10.4 ±1.6</td>
<td></td>
</tr>
</tbody>
</table>

a MES, maximal electroshock (50 ma.-0.2 sec. duration), was administered one hour after i.p. injection of DPH. NH₄Cl (400 mg./kg.) or NaHCO₃ (1 gm./kg.) was injected i.p. 45 min. prior to MES to induce acidosis of alkalosis.

b Fraction indicates number of mice protected/total number of mice.

c Value significantly altered (P < .01) when compared with normal controls.
TABLE 14
Diphenylhydantoin Antagonism of Maximal
Electroshock Seizures in Normal, Acidotic, and Alkalotic Mice\textsuperscript{a}

<table>
<thead>
<tr>
<th>Diphenylhydantoin (mg./Kg.)</th>
<th>% Protected</th>
<th>Brain Serum</th>
<th>% Protected</th>
<th>Brain Serum</th>
<th>% Protected</th>
<th>Brain Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>12.5</td>
<td>7.33 ±0.16</td>
<td>5.06</td>
</tr>
<tr>
<td>8.0</td>
<td>12.5</td>
<td>8.45 ±0.09</td>
<td>7.58</td>
<td>37.5</td>
<td>8.83 ±0.18</td>
<td>6.62</td>
</tr>
<tr>
<td>10.0</td>
<td>37.5</td>
<td>10.82 ±0.17</td>
<td>8.43</td>
<td>75.0</td>
<td>11.64 ±0.11\textsuperscript{b}</td>
<td>7.74</td>
</tr>
<tr>
<td>12.5</td>
<td>75.0</td>
<td>14.17 ±0.20</td>
<td>11.24</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>15.6</td>
<td>100.0</td>
<td>17.22 ±0.31</td>
<td>13.91</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

\textsuperscript{a} MES, maximal electroshock (50 ma-0.2 sec. duration), was administered one hour after i.p. injection of DPH. \( \text{NH}_4\text{Cl} \) (400 mg./kg.) or \( \text{NaHCO}_3 \) (1 gm./kg.) was injected i.p. 45 min. prior to MES to induce acidosis or alkalosis. Brain concentrations are given in mcg./gm. wet tissue ± standard error.

\textsuperscript{b} When compared with controls, \( P < 0.01 \).
FIG. 2

COMPARISON OF HSA AND MSA CAPACITY TO BIND DIPHENYLHYDANTOIN (DPH)¹

1. Values in parenthesis represent initial DPH concentrations (x 10⁻⁵ M) prior to dialysis.
FIG. 3
SCATCHARD PLOT OF DIPHENYLHYDANTOIN BINDING SITE DATA WITH HSA (UNCORRECTED)*

- Scatchard Plot of Data
- Resolution of $n_1$ and $n_1K_1$
- Resolution of $n_2$ and $n_2K_2$

$r$ = Moles Drug Bound/Mole HSA
$c$ = Unbound Drug Concentration

* = Not Corrected for Donnan Effect and Solute Space
FIG. 4
SCATCHARD PLOT OF DIPHENYLHYDANTOIN BINDING SITE DATA WITH HSA (CORRECTED)

Scatchard Plot of Data
Resolution of $n_1$ and $n_1K_1$
Resolution of $n_2$ and $n_2K_2$

$r = \text{Moles Drug Bound/Mole HSA}$
$c = \text{Unbound Drug Concentration}$
$\star = \text{Corrected for Donnan Effect and Solute Space.}$
FIG. 5

SCATCHARD PLOT OF DIPHENYLHYDANTOIN BINDING SITE DATA WITH MSA (UNCORRECTED)*

Scatchard Plot of Data

Resolution of $n_1$ and $n_1K_1$

Resolution of $n_2$ and $n_2K_2$

$r = \text{Moles Drug Bound/Mole MSA}$

$c = \text{Unbound Drug Concentration}$

* = Not Corrected for Donnan Effect or Solute Space.
FIG. 6
SCATCHARD PLOT OF DIPHENYHYDANTOIN BINDING SITE DATA WITH MSA (CORRECTED)*

Scatchard Plot of Data

Resolution of $n_1$ and $n_1K_1$

Resolution of $n_2$ and $n_2K_2$

$\frac{r}{c} = \text{Moles Drug Bound/Mole MSA}$

$c = \text{Unbound Drug Concentration}$

$\star = \text{Corrected for Donnan Effect and Solute Space.}$
FIG. 7

EFFECT OF DIPHENYLHYDANTOIN (DPH) CONCENTRATION ON THE UNBOUND FRACTION

UNBOUND DPH (mcg/ml)

DPH IN ALBUMIN RETENTATE (mcg/ml)
FIG. 8
RELATIONSHIP BETWEEN THE BOUND DIPHENYLHYDANTOIN FRACTION AND UNBOUND DRUG CONCENTRATION
FIG. 9
RELATIONSHIP BETWEEN pH AND UNBOUND DIPHENYLHYDANTOIN

Human Serum Albumin ○ Mouse Serum Albumin ★★★ Initial DPH concentration in all experiments was 6.29 x 10⁻⁵ M which produced a 15-17 mcg./ml. DPH concentration in albumin retentate fraction; 4% solution of albumin was used. The values in parenthesis represent the percent change in free DPH concentration when compared with the value obtained at pH 7.4.
FIG. 10
RELATIONSHIP BETWEEN ALBUMIN CONCENTRATION AND UNBOUND DIPHENYLHYDANTOIN

% ALBUMIN

UNBOUND DPH (mcg/ml)

Human Serum Albumin — Mouse Serum Albumin — Initial DPH concentration in all experiments was 6.29 x 10^{-5}M at pH 7.4.
FIG. 11
EFFECT OF ALBUMIN CONCENTRATION ON $r^*$

$\%$ ALBUMIN

- $\circ \circ$ Human Serum Albumin
- $\star \star \star$ Mouse Serum Albumin

Initial DPH concentration was $6.29 \times 10^{-6}$M in all experiments.

$r = \frac{\text{Moles DPH Bound}}{\text{Mole Albumin}}$
FIG. 12
EFFECT OF LIGAND COMPETITION ON UNBOUND DIPHENYLHYDANTOIN

UNBOUND DPH (mcg/ml) vs SECONDARY LIGAND CONCENTRATION \( \times 10^{-5} \) M

PHENOBARBITAL

TRIMETHADIONE

PHENYL BUTAZONE

WARFARIN

DPH CONTROL

6.25 12.5 25 50 100 200 400

200 100 50 25

1.6 3.12 6.25 12.5
FIG. 13

RELATIONSHIP BETWEEN DIPHENYLHYDANTOIN SERUM LEVELS AND ANTI-MES ACTIVITY IN NORMAL, ALKALOTIC, AND ACIDOTIC MICE

% PROTECTED

SERUM DPH mcg/ml

Normal

NaHCO₃ 1.0 gm./Kg. (Alkalotic)

NH₄Cl 400 mg./Kg. (Acidotic)

MES Maximal Electroshock (50 ma - 0.2 Sec. Duration)
FIG. 14
RELATIONSHIP BETWEEN DIPHENYLHYDANTOIN BRAIN LEVELS AND ANTI-MES ACTIVITY IN NORMAL, ALKALOTIC, AND ACIDOTIC MICE

% PROTECTED

BRAIN DPH mcg/gm WET TISSUE

- Normal
- NaHCO₃ 1 gm./Kg. (Alkalotic)
- NH₄Cl (400 mg./Kg.) (Acidotic)

MES Maximal Electroshock (50 ma - 0.2 Sec. Duration)
CHAPTER V
DISCUSSION

The responses of living systems to drugs vary widely, especially in the area of therapeutics. Much of this species specific, intra-species and intra-individual drug-response variation may be attributed to the biochemical, physiological, and pathological conditions which prevail during drug administration. The involvement of serum albumin in drug-response variation was clinically documented when Sellers and Koch-Weser (1970) demonstrated that a metabolite of chloral hydrate displaced albumin bound warfarin to an extent which produced a potentiation in the hypoprothrombinemic effect of warfarin. The serum protein-bound fraction of a drug is considered inactive until released. Thus, the binding of drugs by serum albumin may share in drug interactions, alter biotransformation and/or excretory rates, thereby modifying the therapeutic effectiveness of a drug. Once the binding protein becomes saturated, or if competition between drugs or their metabolites for serum albumin binding sites becomes excessive, an increase in side effects or toxicity may appear, especially if drug administration is continued. Sustained therapy, in contrast, may be the result of prolonged blood levels due to the slow release of drugs from the drug-albumin complex. (Storm van Leeuwen, 1924; Davis, 1942; Goldstein, 1949; Taylor, 1954; Anton, 1960; Keen, 1966a,b; Goth, 1966; Meyer and Guttman, 1968). The relationship between drug-serum albumin binding, pH, albumin concentrations, and drug concentrations with effectiveness has been discussed and examined by Goldstein, 1949; Goldbaum and Smith, 1954; Waddell and Butler, 1957; and Browne, 1969. These investigators demonstrated that the response of an organism to drugs may also be influenced by alterations in serum pH or albumin content which may result from changes in the milieu interieur.
of an organism. Thus, any physiological, biochemical, or pathological condition which might alter the drug-serum albumin complex might modify the pharmacological effects of a drug, especially if the drug is highly bound by serum proteins.

Diphenylhydantoin in anticonvulsant doses acts principally on the motor cortex, inhibiting the cortical spread of seizure activity (Toman, 1965). The drug acts to reduce the post tetanic potentiation of synaptic transmission within the spinal cord, thereby interfering with the development of high frequency transmission of impulses in the cerebral excitatory feedback circuits. The high intensity of these feedback circuits apparently results in grand mal seizures. Toman indicates that DPH appears to stabilize excitable membranes and this activity is apparently accompanied by a reduction of intracellular sodium. Goldstein (1949) has emphasized that the free unbound form of the drug is responsible for the pharmacological effects of drugs. Thus, before DPH can exert its anticonvulsant action, an adequate quantity of the drug in its unbound form must be available to interact with receptor sites.

The work by Lightfoot and Christian (1966), Loeser (1961), Conard et al (1970), and Rudman et al (1971a,b) demonstrated that diphenylhydantoin is bound to a great extent by serum albumin. Conard indicated that 90-95% of the DPH in human serum was bound by serum proteins. The present work is in agreement since approximately 74-85% of the DPH present in the protein retentate chamber was bound to human serum albumin.

Prior investigators have documented the binding of DPH to serum proteins and albumin. However, they failed to determine binding sites. In this work, binding sites and association constants on both human serum albumin (HSA) and mouse serum albumin (MSA) have been characterized. Although restricted to
using only low concentrations of DPH, because of their dialysis system, Rudman and associates (1971 a,b) did make a Scatchard plot of their results. Since the information was limited, Rudman did not attempt to characterize DPH binding sites; however, their information was sufficient to determine an $nK$ value. They determined that the human serum albumin $nK$ was 11,000 for diphenylhydantoin. Their $nK$ value for HSA confirms the $nK$ values of 10,189 (uncorrected) and 11,861.7 (corrected), obtained during this research.

Examination of the Scatchard plots suggests that the secondary group of binding sites may play a greater role in the binding of DPH by HSA than MSA. The apparent contribution of the primary and secondary binding site groups to the total amount of drug bound has been estimated using the following equation (Mais, 1971):

$$F_B = F_1 + F_2 = \frac{P_c (n_1K_1)}{T (1 + K_1c)} + \frac{P_c (n_2K_2)}{T (1 + K_2c)} (1)$$

where $F_B$ is the total fraction bound; $F_1$ represents the fraction bound by the primary group of sites and may be substituted by $\frac{P_c (n_1K_1)}{T (1 + K_1c)}$; $F_2$ represents the fraction bound by the secondary group of sites and may be substituted by $\frac{P_c (n_2K_2)}{T (1 + K_2c)}$; $P$ is the protein concentration; $T$ equals the amount of drug bound plus $c$; $K$ is the association constant; and $c$ represents the concentration of free or unbound drug. Using this formula, it was determined that DPH was mostly bound by the secondary sites on HSA. At concentrations in the albumin retentate chamber corresponding to serum values reported as therapeutic in humans, the secondary group of sites accounted for approximately 72% of the DPH bound. Laubsher (1966) reported a serum level of 94 mcg./ml. just prior to the death of a child. Buchthal et al. (1960) and Kutt and associates (1964)
related the extent of toxic symptomology in epileptic patients with DPH serum levels. When concentrations equivalent to those causing toxicity were reproduced in the albumin retentate chamber, the secondary group of binding sites was responsible for binding up to 81% of the DPH bound. When the DPH present in the protein retentate chambers was comparable to concentrations reported at death, the secondary sites accounted for approximately 94% of the drug bound. Thus, it appears that the secondary group of binding sites are extremely important in the binding of DPH to human serum albumin. This assumption is in agreement with finding reported by Oester et al (1971) for the binding of warfarin. While examining the effect of salicylates on warfarin binding by HSA, they reported that the secondary group of binding sites played an important role in the binding of warfarin. They also indicated that the secondary group of binding sites might play an important role in displacement reactions.

In contrast, the secondary group of binding sites only accounted for approximately 25% of the DPH bound when the albumin retentate chamber contained MSA concentrations equivalent to that determined in the serum of mice which were completely protected against tonic convulsions induced by electroshock. However, the role of the secondary group of sites became increasingly important as drug concentrations were increased. When albumin retentate chamber concentrations were equivalent to those approximately sub-lethal levels (67 mcg./ml.), the secondary group of sites accounted for approximately 34.4% of the drug bound. When the DPH concentration in the protein retentate chamber was increased to 253 mcg./ml., the secondary group of sites was responsible for approximately 65% of the drug bound. Therefore, it would appear that the secondary group of binding sites on MSA might play a major role in
the binding of diphenylhydantoin if drug serum concentrations exceeded therapeumatic levels.

When discussing the number of binding sites per group, the following question has often been expressed: how can there be a fractional number of binding sites, such as ammonium or carboxyl ion groups, be present on a molecule of albumin? The results obtained via the Scatchard plot are in actuality an average, calculated through statistical computation. Thus, if a small $n_1$, such as that calculated for DPH with HSA (0.04) is obtained, then theoretically the steric conformation of the protein molecules may restrict the binding of DPH. In the situation when $n_1 = 0.04$, four binding sites out of one hundred molecules may be available to DPH. This explanation is strengthened by the fact that as the concentrations of diphenylhydantoin were increased, a smaller proportion of the drug was bound per mole of albumin, at a given protein concentration. This suggests that the molecular configuration of the drug-protein complex has become more intricate, exposing fewer binding sites. Thus, as the drug concentration was increased, binding site saturation also increased while the fraction of undissociated DPH rapidly increased.

To date, little investigation has been given to the possible effects of albumin variation on binding of drugs. It has become the custom to characterize the binding sites and association constants of a drug, using low concentrations of albumin, 1% or less. Goldbaum and Smith (1954), Zaroslinski et al (1969) and Browne (1969) have demonstrated that the moles of drug bound per mole of albumin ($r$) are increased as the concentration of albumin was increased. The work presented here provided additional evidence concerning this effect. The moles of drug bound per mole of albumin were observed to
shift from 0.0449 to 0.0919 when concentrations of HSA were increased from 1 to 6%. A similar pattern was seen when MSA albumin concentrations were increased from 1 to 4%. However, the r/c ratios were not increased proportionally, 2400 to 15,056 for HSA and 7527 to 37,962 for MSA respectively. Thus, if a Scatchard r/c vs. r plot would be made, these points would shift drastically upward and to the right. It has been suggested that drugs may increase the number of available binding sites by changing the molecular conformation of the protein (Markus and Karush, 1958; McMenamy, 1963, Keen, 1966a). Assuming a continuation in the r/c shifts at a given albumin concentration, using varied drug concentrations, it would appear that the number of binding sites, their association constants and concentration of unbound drug might easily be altered if a change does occur in the albumin concentration.

Black (1970) furnished information showing that the albumin concentration in the serum of Charles River strain mice ranged from 3.76 to 5.06%, depending upon the age and sex of the animal. Bernstein (1966) reported that the serum protein content in 15 strains of mice ranged from 3.99 - 6.83%. He also showed that the serum albumin concentration in three strains of these mice ranged from 2.17 to 3.38%. One of the strains had an intra-strain difference ranging from 2.17 to 3.15%. In 1966, Mills reviewed the literature concerning circadian rhythms in man. He mentions that Forsgren (1935) reported that albumin concentrations in rodents were subject to circadian rhythms. Frankel (1963) reported that the concentration of albumin in the serum of normal humans ranged from 3.5 to 5.6%. The work reported by Bose et al (1960) adds an additional complication to the DPH-albumin binding picture. They demonstrated that oral administration of 10 mg./Kg. DPH to
rabbits resulted in a reduction of serum albumin content 19% in acute experiments. They also determined that the chronic administration of 10 mcg./Kg. DPH resulted in a maximal reduction of serum albumin amounting to 28%. The peak effect occurred within eight days and values returned to normal by the fifth day after discontinuing the drug. These variations in the concentration of albumin in serum attain greater significance when recalling that in vitro studies demonstrated that decreases of less than 2% (83.3 - 81.6) in the percentage of albumin bound DPH could possibly double the concentration (15.2 - 29.8 mcg./ml.) of pharmacologically active DPH in the serum. Moreover, the concentration of albumin as well as the type of buffer system employed may also partially account for differences in binding sites and association constants obtained for the same drug by separate investigators.

Administration of drugs such as puromycin affect the metabolism of amino acids necessary for the anabolism of serum albumin by interfering with protein synthesis at the assembly state on the ribosome. Physiological conditions such as renal impairment, liver disease, malnutrition and dehydration may produce variations in serum albumin. Odar-Cederlöf et al (1970) reported that epileptic seizures in a uremic patient were controlled by DPH at plasma concentration of less than 2 mcg./ml. They found that the capacity of the plasma proteins to bind DPH was greatly reduced. Reidenberg and coworkers (1971) found a significant correlation between unbound DPH and azotemia, and suggested that the low binding of DPH in uremia was due to a qualitative change in the drug-binding proteins. They indicated that uremic patients would most likely respond favorably to DPH at low plasma concentrations and experience toxicity at serum levels ordinarily considered non-toxic. Reidenberg concluded: "The alteration of drug binding by pathologic states should
be considered when values of total plasma concentrations of protein-bound drugs are used to establish or modify drug dosage regimens."

Therefore, not only drugs and abnormal disease states but the normal everyday biochemical reactions within an individual can and do produce wide fluctuations of serum albumin. The data summarized indicate that changes in serum albumin concentrations may be a participating factor in the regulation of the diphenylhydantoin dose-response relationship. The serum concentration of albumin controls the number of molecules present for binding DPH, thereby regulating the concentrations of unbound drug available for pharmacological activity.

As with albumin, little work has been published discussing the effects of hydrogen ion variation, within physiological limits, upon the binding of drugs by serum proteins. In the in vitro MSA experiments, decreasing the pH from 7.4 to 7.2 produced a 16% increase in the concentration of free DPH; a reduction in pH from 7.4 to 7.0 increased unbound DPH 35%. When the pH was increased from 7.4 to 7.6, the unbound DPH concentration was reduced by 14%. In an effort to determine whether the in vitro alterations in DPH binding might, with changes in pH, be reflected in vivo, normal, acidotic, and alkalotic mice, treated with DPH, were subjected to maximal electroshock (MES). The administration of NH₄Cl reduced in vivo blood pH to approximately 7.23. Induction of acidosis shifted the diphenylhydantoin PD50 against tonic convulsion induced by MES from 10.4 mg./Kg. in normal animals to 8.4 mg./Kg. The administration of NaHCO₃ raised the in vivo blood pH to approximately 7.55. This induction of alkalosis, however, did not alter the PD50 when compared to normal mice. In the in vitro HSA experiments, reducing the pH from 7.4 to 7.0 produced only a 6.6% change in the concentration of unbound DPH.
However, reducing the pH from 7.6 to 7.0 did cause an overall increase in free DPH approximating 19%.

The results concerning the alterations in DPH-MSA binding and the in vivo anti-MES effectiveness of the DPH due to pH changes are consistent with work done by Goldbaum and Smith (1954) and Waddell and Butler (1957). Goldbaum and Smith reported that the binding of phenobarbital and other barbiturates was "related to pH" and that maximal binding occurred at pH 7.8. Waddell and Butler demonstrated that the anesthetic dose 50% for phenobarbital was shifted from $158 \pm 3$ mg./Kg. in mice treated with NaHCO$_3$. Treatment of the mice raised the blood pH 0.2 units when compared to normals.

It could be argued that NH$_4$Cl competed with DPH for albumin binding sites or altered the electro-shock pattern. The affinity for DPH as determined by the association constants ($K$) was approximately 65% greater for MSA than for HSA. If binding site competition is involved, one would have expected the HSA retentate to contain a concentration of unbound DPH comparable to that found in the MSA retentate. Had NaHCO$_3$ been in competition with DPH for serum albumin binding sites, the dosage of DPH necessary to provide protection should have been reduced. Preliminary experiments demonstrated that the administration of NH$_4$Cl or NaHCO$_3$ failed to alter the incidence of tonic extensor seizures or overtly change the MES seizure pattern. These results were consistent with those reported by Hendley et al. (1948) who reported that mild metabolic acidosis and alkalosis, induced by NH$_4$Cl and NaHCO$_3$ respectively, had no effect on the electro-shock seizure threshold in rats. They demonstrated that severe metabolic acidosis caused an increase of 14% in electro-shock threshold. Finally, Hendley reported that severe metabolic acidosis or alkalosis had no effect "upon the maximal seizure pattern of rats, or
upon the resting electroencephalogram, the subconvulsive Metrazol EEG discharge and the threshold for evoked cortical potentials in rabbits". Hence, it would appear that the increased effectiveness of DPH might be due to the physiological condition of acidosis and/or displacement through drug binding site competition and not to alterations in the MES seizure pattern. Although it is apparent that blood pH changes in the mouse may alter the effectiveness of DPH, the significance of hydrogen ion changes clinically would appear to be more dependent upon the overall condition of the patient at the time of drug administration.

The binding of an anionic drug molecule by serum albumin may be attributed to the accessibility of the reactive prototropic groups on the protein. Therefore, the prototropic groups of the amino acids on the albumin molecule should provide the best DPH binding sites. Steinhardt and Beychok (1964) have reported that the histidine-imidazole, lysine (\(-\text{amino}\)) side chain, and the arginine-guanidyl groups appear to be the active sites available for anionic binding.

The negative log of the dissociation constant (pKa) for diphenylhydantoin, a weak acid, is 8.31 (Sunshine, 1969). White and associates (1964) have listed the pKa for the histidine-imidazole group as 5.6 - 7.0, the lysine-\(\text{amino}\) as 9.4 - 10.6, and the guanidyl group of arginine as 11.6 - 12.6. Examination of the pK values of drugs and amino acids, as well as the pH of the solvent system, allows one to estimate the extent of drug and amino acid ionization. This information, in turn, provides a basis for conjecture concerning the prototropic groups responsible for binding diphenylhydantoin. The extent of ionization may be determined by substitution in the following equations:
HA represents a weak acid and $R=NH_2$ the protein with $-NH_2$ the prototropic group.

As the hydrogen ion concentration in a solution containing diphenylhydantoin is increased, the amount of drug ionized is decreased (equation 1). Therefore, the anionic form of the DPH molecule becomes less available for binding as the pH is decreased from pH 7.6 to 7.0. In contrast, decreasing the pH from 7.6 to 7.0 increases the positive charge on the arginine, lysine and histidine prototropic groups (equations 2 and 4). A decrease in the amount of anionic charged DPH molecules in the albumin solution results in an increase in the free, undissociated drug available for pharmacological activity. At a pH 7.4, the albumin molecule contains many more ionized lysine-$\alpha$-amino and the arginine-guanido groups than histidine imidazole groups. Therefore, these two basic amino acids provide the most likely candidates responsible for binding DPH.

Assuming that lysine and arginine are the principal sites on the albumin molecule responsible for binding DPH, the chemical interaction at pH 7.0 to 7.6 may be described as follows:
A comparison of the amino acid composition of human serum albumin reveals that the protein contains 12.3% lysine and 6.15% arginine (Phelps and Putnam, 1960). This suggests that the arginine-guanido groups may comprise the primary group of binding sites on serum albumin, and lysine the secondary. However, the steric conformation of the molecule may also dictate the extent to which DPH is bound to each prototropic group.

The addition of phenobarbital, warfarin, and trimethadione, using predialysis concentrations up to 12.5, 50.0 and $400 \times 10^{-5}$M respectively, had little effect on the binding of diphenylhydantoin. In contrast, the addition of phenylbutazone in pre-dialysis concentrations, ranging from 25 to $200 \times 10^{-5}$M, increased unbound DPH up to 50%.

When alterations in drug activity occur in the presence of other agents, it is tempting to attribute the change to enzyme induction. Conney et al (1960), Cucinell et al (1962), as well as Kato and Chiesara (1962) have demonstrated that animals pretreated with phenobarbital and pentobarbital, as well as several other centrally acting compounds, are capable of inducing enzymes which metabolize drugs in the liver microsomes. This enzyme induction has led to the diminished pharmacological activity of diphenylhydantoin, several
barbiturates, warfarin, tubocurarine, chlorpromazine, etc. On the other hand, the work of Sellers and Koch-Weser (1971) cautions against assuming that a decrease in drug activity is due to enzyme induction in the absence of enzyme data. Protein binding experiments which show an absence of binding site displacement should preclude the hypothesis that alterations in drug-serum protein binding are responsible for changes in pharmacological activity. Oester et al. (1971) showed that pentobarbital at concentrations less than $5 \times 10^{-4}$M failed to produce any appreciable displacement of warfarin from binding sites on HSA. The phenobarbital, DPH-HSA interaction experiments conducted in this study revealed that phenobarbital did not appreciably alter DPH binding.

These results might have been anticipated since DPH is bound to HSA approximately 80% while phenobarbital is bound approximately 50%. Thus, the experiments reported in this work add further value to the theory, that in vivo diminution of DPH and warfarin activity in the presence of pentobarbital and phenobarbital is due, by exclusion, to induction of drug metabolizing enzymes.

In 1965, Yu et al. reported that pretreatment of mice and rats with trimethadione decreased pentobarbital sleeping time and increased the rate of pentobarbital disappearance. They indicated that these changes in pentobarbital activity were due to enzyme induction of metabolizing enzymes. There are no studies known to this author which indicate that trimethadione at concentrations reported as therapeutic produces any reduction in the pharmacological action of DPH. If the pharmacological activity of DPH were to be decreased during concomitant administration with trimethadione, then the trimethadione-DPH interaction results with HSA in this work would tend to support an enzyme induction theory.

The drug interaction experiments with warfarin produced unexpected
results. Drugs which are highly bound to serum albumin, such as phenylbutazone, warfarin, and salicylate, have often been found to compete with and displace other ligands from binding sites (Anton, 1960, Zaroslinski et al., 1971, and Oester and co-workers, 1971). O'Reilly (1967) reported that 80-90% of the warfarin present in the protein retentate was bound to HSA. Thus, the extent to which warfarin and DPH were bound to HSA are comparable. This lack of competition by warfarin for DPH binding sites on HSA may be attributed to the roles played by the binding site groups on the albumin molecule and the capacity of each drug to alter the molecular configuration of the protein.

Oester et al. (1971) demonstrated that although the secondary group of binding sites on HSA became more important to the binding of warfarin as the drug concentrations were increased, the primary group of binding sites still continued to be dominant. Keen (1966b) has indicated that the affinity of an antagonist for albumin might "vary according to the drug with which it was competing because the antagonist's affinity would depend on its ability to distort the albumin molecule in the presence of the other drug".

Phenylbutazone has been reported to displace a host of drugs from serum albumin binding sites. Among some of the drugs displaced were sulfonamides, oral anticoagulants, phenoxymethylpenicillin, L-tryptophan, and phenol red (McArthur et al., 1971; McQueen, 1959; Anton, 1960; Aggerler et al., 1967; and Keen, 1966a). As anticipated, phenylbutazone produced an appreciable displacement of DPH from HSA binding sites. Phenylbutazone has a pKa approximating 4.5 (Sunshine, 1969), and would be almost entirely ionized at a pH 7.4. The dissociated phenylbutazone should be very strongly attracted by the highly charged prototropic groups of arginine and lysine. Phenylbutazone is bound approximately 95% by HSA (Anton, 1961). This strong affinity of serum albumin
for phenylbutazone may partially account for the displacement of DPH. On the other hand, Anton, while studying the displacement of sulfonamides by phenylbutazone, phenylbutazone analogs, aminopyrine, thiopental, probenecid, suramin, salicylate, etc., demonstrated that the pKa of a drug and the percent that it is bound by albumin cannot be considered positive indicators of displacement activity.

However, he did indicate that a drug that was highly bound to albumin would have greater potential for displacement activity. Anton also reported that poly aromatic compounds such as phenylbutazone and DPH appeared to have a more optimal displacement potential. Since DPH is both highly bound and is poly aromatic, this might be an additional factor for the lack of competition by warfarin. On the other hand, phenylbutazone is bound to a greater extent by HSA than DPH and is also poly aromatic which might account for the increase in unbound DPH in the presence of phenylbutazone. Anton demonstrated that only the drugs interfering with sulfonamide binding in vitro were active in vivo and showed that the extent of displacement in vivo followed the relative order observed in vitro. Therefore, it is conceivable that the concomitant administration of phenylbutazone with diphenylhydantoin could potentiate the action of diphenylhydantoin. Aggeler et al (1967) reported that the administration of phenylbutazone with warfarin in normal adult men potentiated the hypoprothrombinemic action of warfarin. Using equilibrium dialysis, they reported that the presence of phenylbutazone diminished the association constant of warfarin for HSA approximately tenfold. Potentiation of the hypoprothrombinemic effect by phenylbutazone was attributed to the displacement of warfarin from serum albumin binding sites. Phenylbutazone has been reported to stimulate hepatic drug-metabolizing enzymes (Conney et al, 1960). However,
Aggeler and associates discounted enzyme induction because the hypoprothromb-inemic effect of warfarin was elevated, not reduced.

When one drug reduces the serum concentration of another, it may indicate that the first drug has stimulated the drug-metabolizing enzymes in liver microsomes. However, this phenomenon often requires several days. Enzyme induction causes a decrease in the pharmacological activity of a drug because its rate of degradation reduces the concentration of free drug at the biologic site of action (Aggeler et al., 1967). In contrast, displacement of a drug from its serum protein binding sites produces "the apparent paradox of lower total blood levels with exaggerated pharmacological response" (Weiner, 1971). The release of a drug from its binding sites, whether due to competition or steric conformation, immediately increases the concentration of free, active drug "at the site of action, but thereafter the drug is also eliminated more rapidly from the body" (Sellers and Koch-Weser, 1970). The induction of acidosis by the administration of NH₄Cl both increased diphenylhydantoin anti-MES activity and reduced the concentration of the drug in serum. Thus, it would appear that the increase in the pharmacological activity of DPH may be attributed to the displacement of the drug from protein binding sites. This hypothesis is supported by the in vitro MSA binding data where unbound DPH concentrations were observed to increase as the pH was decreased.

Weiner (1970) emphasized that both the equilibrium status and kinetics of binding competition influence pharmacological activity. He suggested that binding competition at fixed tissue structures may not parallel the pattern observed in serum. This observation appears to be consistent with the brain-DPH concentrations, when compared with controls, was paralleled by a slight increase in brain concentration in acidotic mice. Although these changes
were not statistically significant, an obvious trend toward an increase in total brain DPH concentration was revealed, and diphenylhydantoin anti-MES activity was increased. A closer relationship between brain drug levels and pharmacological activity may have been established had just the cortex been assayed for DPH. Nevertheless, there was an apparent correlation between DPH brain and serum levels with the anti-MES activity. In general, an increase in pharmacological activity was attended by an increase in both brain and serum DPH concentrations.

As in the case of many investigations, the work accomplished provides the answers to some questions, raises others, and opens additional avenues for scientific probing. It would be interesting to compare diphenylhydantoin binding data, obtained with serum albumin from patients afflicted with grand mal, to results reported in this work. Furthermore, drug interaction studies should be conducted in epileptic patients. DPH serum levels should be determined in these subjects prior to and during concomitant administration of anticoagulants, analgesics, antibiotics, anesthetics, hypnotics, etc.

Since disease states can alter the binding characteristics of drugs, (McArthur et al, 1971; and Reidenberg et al, 1971), it is possible that the binding of DPH to albumin may be influenced by the disease for which DPH is indicated. The importance of in vitro drug binding experiments would be more clearly defined if done using serum proteins from the species in which associated in vivo experiments are conducted. This work has indicated the desirability of conducting DPH-ligand competition experiments in vivo and in vitro using mouse serum albumin. Since only the unbound form of a drug is considered pharmacologically active, reports of in vivo drug-serum levels should also include the concentration of unbound drug present.
CONCLUSIONS

The results obtained in this work caution against the sole use of serum drug levels to determine the bioavailability of a drug. It would appear that the concentration of the unbound drug in the serum as well as blood pH and serum albumin content would provide a better basis by which to estimate the pharmacological activity of a drug.

Conard (1971) stated that species differences in drug-plasma protein interaction could account for species specific adverse drug reactions. The in vitro DPH-albumin binding information, together with the in vivo results, strongly suggest that the interaction between DPH and serum albumin may contribute to differences in the pharmacological and toxicological activity observed between and within species as well as within individuals. At the anti-MES protective dose 100 in mice, the DPH serum concentration was approximately 14 mcg./ml. The in vitro binding experiments, in which the MSA retentate contained approximately 17 mcg./ml., estimate that approximately 95% of the in vivo serum concentration appeared to be bound by albumin. In contrast, the therapeutic concentration of DPH in human serum is reported to be about 15 mcg./ml., and in vitro experiments indicate that approximately 83% of the drug could be bound to HSA. Theoretically, due to the greater capacity of MSA to bind DPH, it would require a comparatively larger dose to cause death in mice as compared to man. As an example, Laubscher (1966) ascribed the death of a 4-1/2 year old girl to the oral ingestion of 2 g. DPH. Unfortunately, the weight of the victim was not reported. However, if her weight is estimated at 20 Kg., then a dose of 100 mg./Kg. DPH was required to cause death. Fink and Swinyard (1959) reported that the oral LD50 for DPH was 490 mg./Kg. in mice. Wey (1946) reported an intraperitoneal LD50 of 200 mg./Kg. in mice.
Thus, diphenylhydantoin appears to be at least twice as toxic in man as compared to mice.

It is the consensus in the great majority of the references cited previously that drugs bound to serum proteins are inactive. Even though a larger dose of drug might be required to stimulate a response in one species as compared to another, it would appear that the amount of unbound drug present would provide a better indicator of drug potency. The affinity ($K$) of the primary group of binding sites on HSA was approximately twice that of MSA; the binding site availability was fifteen to twenty times greater on MSA. The affinity of the secondary group of binding sites for DPH was approximately 65% greater in MSA than for HSA. Furthermore, as the moles of drug bound per mole of albumin ($r$) increased, the unbound DPH fraction available for activity in HSA solutions was approximately twice that found in mice. Therefore, concentration of free drug available for pharmacological action at the site of activity should be greater in the human.

The *in vitro* and *in vivo* results presented in this work suggest that a species requiring a larger drug dosage to cause a response may not only be as sensitive, but possibly more sensitive than a species requiring less drug. If the drug-serum protein interaction is greater, and the pharmacological response equivalent in the latter, less drug would be available to produce the response. Thus, the organism's sensitivity to the drug could be greater.

The present work shows that the pharmacological response to DPH is closely related to albumin concentration, pH, and drug concentration. Changes in these parameters were associated with only small adjustments in the percentage of bound DPH, but were accompanied by significant alterations in the unbound, active drug concentration. *In vitro* drug interaction experiments
indicate that binding studies can provide evidence against the assumption that the potentiation of a response may be due to drug displacement from serum protein binding sites, and suggest that the increase may be attributed to enzyme induction. On the other hand, drug serum protein binding studies can also suggest that the potentiated activity may be the consequence of drug binding site release.

In conclusion, it is the opinion of this author that in vitro drug-protein binding studies provide a valuable tool by which to evaluate the pharmacological and toxicological action of a drug in disease states and in the study of drug interaction. This is especially true in cases where drugs are highly bound, such as diphenylhydantoin.
CHAPTER VI

SUMMARY

1. Diphenylhydantoin was demonstrated to be highly bound by both human serum albumin (HSA) and mouse serum albumin (MSA). A total number of 7-8 binding sites per molecule of both HSA and MSA was estimated for diphenylhydantoin (DPH). Two groups of sites were determined on both species of albumin, a primary group with high affinity and a secondary group with much less affinity.

2. Major differences were revealed in the binding of DPH by the two types of albumin. HSA was less effective than MSA in the binding of diphenylhydantoin. At concentrations in the albumin retentate corresponding to serum values reported as therapeutic in humans, MSA bound approximately 96% of the DPH present, and HSA 83%. The secondary group of binding sites on HSA appear to bind most of the DPH present, while the primary group of sites were dominant on MSA. The association constant for the primary group of sites on HSA was approximately twice that for MSA, whereas the reverse held true for the secondary group of sites. The lysine-α-amino and arginine-guanido groups were postulated as being the prototropic sites on albumin responsible for binding DPH.

3. At a given concentration of DPH in a 4% albumin solution, the concentration of free drug increased as the pH was reduced, while the fraction of drug bound per mole also diminished. However, the increment in unbound DPH as a function of pH was markedly greater with MSA as compared to HSA.

4. At a given pH and albumin concentration, the fraction of drug bound diminished while the moles of drug bound per mole of albumin (r) rose as the
concentration of DPH was increased. The increases in \( r \) in this and the albumin concentration experiments suggested that the presence of the drug may produce alteration in the steric configuration of the albumin molecule.

5. In ligand competition experiments, phenobarbital, trimethadione and warfarin at concentrations in the HSA retentate corresponding to serum values in the therapeutic range failed to displace DPH from binding sites. In contrast, phenylbutazone readily displaced DPH from HSA binding sites. As the concentrations of phenylbutazone were increased, the concentrations of free DPH were also elevated.

6. The antagonism of maximal electroshock seizures (MES) in mice by DPH was potentiated in acidotic mice as measured by a shift in the PD50. This effect was attributed to the displacement of DPH from binding sites by the acidotic condition and/or binding site competition with \( \text{NH}_4\text{Cl} \). No change in DPH effect was observed during \( \text{NaHCO}_3 \) induced alkalosis.

7. A positive relationship was demonstrated between an increase in serum and brain DPH levels and an increase in DPH anti-MES activity in mice.

8. Alterations in the pharmacological response to DPH due to the effects of disease, drug concentration, pH, and albumin concentration were indirectly and directly related, both through this work and that of others, to the displacement of DPH from serum protein (albumin) binding sites. In vivo displacement from binding sites produced an increase in free DPH and, consequently, an increase in activity.

9. The results from in vitro binding site experiments both in this work and in the literature were indirectly related to results observed in vivo.
CHAPTER VII
APPENDIX

Open Letter to Physicians Concerning Panwarfin\textsuperscript{R} (Abbott)

Data published by Sellers and Koch-Weser (1970) indicated that clinical enhancement of coumarin activity in the presence of chloral hydrate was probably due to the displacement of the coumarin from binding sites. In view of this information, Dr. Garnham, Vice President, Medical Affairs, of Abbott Laboratories, sent an open letter to physicians concerning Panwarfin therapy. The full text of the letter is as follows:

"Dear Doctor:

As manufacturers of Panwarfin\textsuperscript{R} (sodium warfarin), we are writing to you to bring to your attention a possible problem with concomitant use of coumarin anticoagulants and chloral hydrate. This information is of particular importance because it is diametrically opposite to that previously believed.

Following a publication by Cucinell \textit{et al.} in 1966, it had been considered that use of chloral hydrate with coumarin anticoagulants could lead to inhibition of the anticoagulant effect. It was observed that, like certain barbiturates, chloral hydrate shortened the serum half-life of bishydroxycoumarin. A similar action, enzyme induction, was postulated as the cause of this reduction of the half-life. New evidence suggests that this may not be so.

Very recently, Sellers and Koch-Weser\textsuperscript{2} published an article showing enhancement of the hypoprothrombinemic effect of warfarin sodium. Increases of as much as 40 to 80 percent in this effect were noted
following concomitant administration of sodium warfarin and 1 gm. of chloral hydrate daily for one week. The authors studied 237 hospitalized patients receiving both drugs and observed only potentiation of warfarin-induced hypoprothrombinemia, and no inhibition.

This recent article suggests that the mechanism involved is the displacement of warfarin by a metabolite of chloral hydrate (trichloracetic acid) from serum albumin-binding sites. If chloral hydrate is added to the therapy of a patient maintained on sodium warfarin, the authors recommend that a reduction in anticoagulant requirement should be anticipated to avoid excessive hypoprothrombinemia with risk of bleeding. On the other hand, discontinuation of chloral hydrate therapy in patients on warfarin may make it necessary to increase warfarin dosage to maintain adequate anticoagulation.

Every effort is made to assure that our product literature reflects current medical knowledge, and our package insert for Panwarfin has been changed to indicate the above findings regarding concomitant therapy with sodium warfarin and chloral hydrate.

While this new information applies only to chloral hydrate, the scope and importance of the drug interaction problem becomes daily more evident. The controlled study of drug interactions is a relatively new field, and only a portion of the possible combinations have yet been examined.

It is, therefore, likely that additional knowledge in this area will continue to accrue. The importance of careful monitoring of prothrombin time whenever any drug is added to or withdrawn from the regimen of a patient on any of the coumarin anticoagulants cannot be
OVER-EMPHASIZED."

(Signed) John G. Garnham
Diphenylhydantoin (DPH) concentrations were determined using the radioisotope dilution technique. DPH is considered only sparingly soluble in distilled water; sparingly soluble was defined as 30-100 parts of solvent to 1 part of solute or 10-30 mg./ml. (Sunshine, 1969). However, it was determined that the solubility of the drug is drastically reduced in buffer solutions of pH 7.0 - 7.6. Conard (1970) attempted to characterize the binding of diphenylhydantoin to rat and human serum while Rudman (1971) tried to determine DPH binding sites on both human and bovine serum albumin. However, both investigators were thwarted by the insolubility of the compound at experimental conditions employed. Conard partially solved this problem by solubilizing and concentrating the drug in 0.01 N NaOH. Then he added an aliquot of the concentrate to the buffer diffusate prior to dialysis. In the present study, it was determined that Sorensen's buffer solution (pH 7.0 - 7.6) would hold approximately 0.07 mg./ml. DPH which had been previously solubilized in 0.01 N NaOH, while a 4% buffered albumin solution would retain DPH solubility up to 0.6 mg./ml. Thus, in these investigations, the solubility problem was suitably solved by dissolving DPH in 0.01 N NaOH and adding a 0.1 ml. aliquot of the DPH concentrate to a serum albumin chamber containing 4.6 ml. of a 4.17% albumin solution. Then 0.1 ml. diphenylhydantoin-4-C¹⁴ (approximately 10,000 cpm) was added to the albumin chamber. In total, the albumin retentate chamber contained 4.8 ml. of an albumin solution consisting of 4% albumin and the appropriate concentration of radioisotope labeled DPH. Experiments were conducted to assure that neither the drug nor the NaOH vehicle altered the pH of the solutions or the counts.
Samples were handled as indicated on page 46 and quenching corrections were made following the ratio of channels ratio method described by Zaroslinski and Mais (1969), Zaroslinski et al (1971) and Oester et al (1971). Preliminary experiments revealed that the counting efficiency of the instrument was approximately 100 ± 2% when the labeled sample in the buffer alone was counted. Only a slight decrease in efficiency was observed with the addition of albumin. With the 4% albumin present, the counting efficiency dropped to approximately 98 ± 2%. Therefore, counts taken from the buffer retentate samples were assumed to be 100%. Both buffer diffusate and albumin retentate samples were counted using two different windows to provide for the determination of a channels ratio value for each sample. A quench correction factor was obtained by dividing buffer diffusate channels ratio by its associated albumin retentate channels ratio (B/A). The albumin counts from the primary window were then corrected for quenching by multiplying those counts by the B/A ratio factor. When this method was compared to the quench curve technique, corrected values were comparable.

By following the techniques described, the percentage of drug bound deviated by less than 0.6% between duplicate dialysis experiments. Therefore, two experiments per point were considered sufficient.
Protein Determination

The method described by Gornall et al. (1949) is based on the formation of a violet color when a strongly alkaline solution of copper sulfate reacts with proteins or polypeptides having a peptide or amide linkages.

Reagent: Gornall et al. biuret reagent contains 6.0 gm. potassium tartrate (NaKC\(_4\)H\(_4\)O\(_6\).4H\(_2\)O) and 1.5 gm. cupric sulfate (CuSO\(_4\).5H\(_2\)O) dissolved in approximately 500 ml. of distilled water to which has been added 300 ml. of 10% NaOH. This solution was then qs. to 1 liter with distilled water.

A 0.1 ml. aliquot of the matching protein and buffer retentate solutions was pipetted into separate test tubes using a Hamilton microliter syringe. To each of the test tubes was added 0.9 ml. of the appropriate Sorensen's buffer using a Mohr measuring pipette. To a separate test tube was added 1 ml. Sorensen's buffer (reagent blank). Four ml. of the Gornall reagent was pipetted into each tube and the mixture allowed to stand for 30 minutes at room temperature. The optical densities of the solution were determined in a Beckman DU-2 using one cm. cells at 540 nm.

Both human serum albumin and mouse fraction V serum albumin standards were used as controls. Various concentrations were employed to plot standard curves from which protein concentrations were determined. This assay was accurate enough to detect protein changes of 0.1 percent.
Determination of Sodium

Sodium concentrations were measured using flame photometry. In flame photometry, the radiation (optical density) of a known amount of a solution of sodium is measured and compared to the radiation of a comparable solution containing an unknown amount of sodium under identical conditions. All samples were diluted freshly with triple distilled water.

Blank, albumin retentate, and buffer diffusate samples were analyzed for sodium ion content using a dilution ratio of 1:200 as follows:

I. Preparation of Stock Reagents

a) Reagent 1: five ml. of 100% Sterox SE was pipetted into a 500 ml. volumetric flask which contained approximately 490 ml. distilled water. Then the flask was filled to the mark with distilled water.

b) Reagent 2: fifty ml. of Sodium Flame Photometry Standard "A" was measured into a 500 ml. volumetric flask and the flask filled to volume.

c) Reagent 3: (0.75 mEq/l. sodium and 0.02% Sterox SE): fifteen ml. of Reagent 2 and 10 ml. of Reagent 1 were pipetted into a 500 ml. volumetric flask and the flask filled to volume.

d) Reagent 4 (0.5 mEq/l. sodium and 0.02% Sterox SE): ten ml. of Reagents 1 and 2 were pipetted into a 500 ml. volumetric flask and the flask filled to volume.

e) Reagent 5 (0.02% Sterox SE, reagent blank): ten ml. of Reagent 1 was pipetted into a 500 ml. volumetric flask and the flask filled to volume.

Note: all of these stock reagents were stored in polyethylene bottles.
II. Sample Preparation and Analysis

a) A sample aliquot of 0.25 ml. was pipetted into a 50 ml. volumetric flask containing 45 ml. distilled water, using an Ostwold-Folen pipette.

b) One ml. of Reagent 1 was added to each flask; then the flask was filled to volume.

c) After the solutions had been well mixed, the pre-labeled 10 ml. pyrex beakers were filled approximately 4/5 full with the appropriate solution.

d) Separately labeled beakers were filled with Reagents 3, 4, and 5.

e) All samples were atomized and the percent transmittance recorded. Reagent 5 served as the reagent blank and also to flush the atomizing device clean between experimental samples. A 10 - 15 second flushing time was utilized.

f) The percent transmittance values recorded with Reagent 3 and 4, representing 150 and 100 mEq./l. sodium respectively, were used for preparing calibration graphs.

g) The ratio of the amount of sodium in the albumin retentate solution to the sodium content of its sister buffer solution was employed to correct DPH dialysis values for Donnan effect.
Estimation of Protein Binding Sites and Association Constants-

Method of Karush (1950) and Scatchard et al (1957)

The mathematical analysis of binding data has been thoroughly discussed by Scatchard (1949), Scatchard et al (1950, 1957), Karush and Sonenberg (1949), Karush (1950), Edsall and Wyman (1958), Goodman (1958), Keen (1966), and Rudman et al (1971). Browne (1969) summarized the manner in which these investigators estimated the number of binding sites per molecule of albumin and their association constants with regard to 1 or 2 classes of binding sites as follows:

1. The moles of drug bound per mole of albumin \( r \) is determined for several concentrations of the drug.

2. Then \( r \) is related to the molar concentration of the free drug present \( c \) by preparing a plot of \( r/c \) vs \( r \) values.

3. The curve resulting from the \( r/c \) vs. \( r \) relationship is extrapolated to the ordinate and the abscissa. The authors state that the values at the intercept points on the ordinate and abscissa equal \( nK \) and \( n \) respectively. \( K \) is equal to the association constant and \( n \) equals the number of binding sites.

According to these authors, a plot of the \( r/c \) vs. \( r \) values produces a straight line when a single, homogeneous group of sites is responsible for drug binding. Scatchard points out that the relationship between the concentrations of bound and free drug is expressed by the formula:

\[
(1) \quad \frac{r}{c} = nK - nr
\]

Should the \( r/c \) vs. \( r \) plot produce a curved line, the deviation from a straight line may be due to electrostatic interaction. Electrostatic interaction is attributed to the repulsion of approaching drug ions by those already bound.
An electrostatic correction factor may be applied by plotting $r/c \ (f)$ vs. $r$, where $f$ equals $e^{2w(Zp+r)}$. "$e$" is the natural log, "$w$" the Debye-Huckie parameter, and $Zp$ is the net charge on the protein. The relationship between the concentrations of bound and free drugs, corrected for Donnan effect and solute space, are represented by the formula (Keen, 1966b):

$$ (2) \ \frac{r}{c} (f) = nK - rK $$

If a curved plot persists after $r/c$ correction for electrostatic interaction, heterogeneity of binding sites is assumed. The experimental plot is extrapolated to graphically estimate the total number of binding sites, the number of binding site groups, and their affinity for the agent being studied. "Assuming that there are $n$ binding sites of two types, $n_1$ and $n_2$ with association constants $K_1$ and $K_2$, and $A$ is the limiting value of $r/c$ as $c$ approaches zero", the equation for the relationship between free and bound drug concentrations is:

$$ (3) \ \frac{r}{c} = \frac{n_1 K_1}{1 + K_1 c} + \frac{n_2 K_2}{1 + K_2 c} $$

where $n = n_1 + n_2$, and line $r/c = n_1 K_1 + n_2 K_2 = A$ (Karush, 1950; Goldbaum and Smith, 1954). The association constant ($K$) for each class of sites is determined by dividing the class $nK$ value by its $n$ value.

The graphically determined $n$ and $K$ values are substituted into the appropriate equation to solve for $r/c$. The calculated $r/c$ ratio vs. $r$ values are plotted. The plot should produce a curve similar to that originally drawn and extrapolated (Keen, 1966b).

Should the use of equation (3) fail to resolve the curve into 2 separate components, then it is probable that more than two classes of binding sites exist. In 1957, Scatchard and associates foresaw this inevitability and math-
ematically calculated a formula to determine multiple binding site classes. When the curve is "concave upwards" or as we have found is more arc shaped than "L" shaped, multiple binding sites (>2) exist. The basic equation in the Scatchard analysis is:

\[ r = \sum_i r_i = \sum_i \frac{n_i K_i c}{1 + K_i c} \]

The curve resulting from the Scatchard type plot for the diphenylhydantoin binding site data was resolved by IBM 360 computer for equation (3) employing the Non-linear Least Squares technique. The program utilized was obtained by Conard (1971) from the Vogelback Computing Center, Northwestern University, Library Number NU CC 260, Subroutine NLSQ. This computer program originating from work published by Hartley (1961), Marquardt (1963), and Draper (1966) was refined and updated by Stein and Cohen (1971). The general description of the program is described by the Vogelback Computing Center as follows:

"The subroutine finds the constants of the equation,

\[ y = f(X, b) = f(x_1, x_2, \ldots, x_p ; b_1, b_2, \ldots, b_m) \]  

(1)

Where X is the matrix of independent variables, y is the vector of dependent variables and b is the vector of coefficients to be estimated.

\[
\begin{pmatrix}
  y_1 & x_{11} & x_{12} & \cdots & x_{1p} \\
  \vdots & \vdots & \vdots & \ddots & \vdots \\
  y_n & x_{n1} & x_{n2} & \cdots & x_{np}
\end{pmatrix}

\]

must be given.

The coefficients, \( b = (b_1, \ldots, b_m)' \),

are computed by minimizing the least squares function,
\[ s = \sum_{i=1}^{n} \left[ y_i - f(x_i, b) \right]^2 \]  

\textbf{Computational Procedure}

The classical least-squares problem (3) is solved by a modified Gauss-Newton method using the Marquardt scaling factor \( \lambda \). Let \( h \) be the index of the \( h \)-th observation and expand \( f(x_h; b) \) in a first order Taylor series about \( b_0 \), an initial estimate of \( b \),

\[ f(x_h; b) = f(x_h; b_0) + \left[ \frac{\partial f(x_h; b_0)}{b} \right] (b - b_0) \quad \forall h = 1, \ldots, n \quad (4) \]

Writing \( Z = (f(x_1; b), \ldots, f(x_n; b))' \)

\[
Z_0 = (f(x_1; b_0), \ldots, f(x_n; b_0))' \\
D_0 = \begin{bmatrix}
\frac{\partial f(x_1; b_0)}{b_1} & \cdots & \frac{\partial f(x_1; b_0)}{b_p} \\
\vdots & \ddots & \vdots \\
\frac{\partial f(x_n; b_0)}{b_1} & \cdots & \frac{\partial f(x_n; b_0)}{b_p}
\end{bmatrix}
\]

we have, in matrix form

\[ Z = Z_0 + D_0 (b - b_0) \quad (6) \]

What the program does in the first iteration is to find the difference \( d_0 = (b - b_0) \) such that,

\[ (y - Z_0)' (y - Z_0) = s(b_0) \quad (7) \]

is minimum. Substituting (6) into (3) and minimizing with respect to \( d_0 \) yields a system of normal equations

\[ d_0 = (D_0' D_0)^{-1} D_0' (y - z_0) \quad (8) \]

a new solution \( b_1 \) is found such that \( b_1 = b_0 + d_0 \).
So that, in general, we have at the \((K + 1)\)-th iteration

\[
\begin{align*}
    b_{K+1} &= b_K = d_K \\
    d_K &= (D_K' D_K - I)^{-1} D_K'(y - Z_K)
\end{align*}
\]  

(9)  
(10)

The iteration process is terminated in any one of the following ways.

1. The number of iterations exceeds a pre-specified limit.
2. The absolute relative change in the sum of squares from any two successive iterations is less than or equal to a prescribed constant \(\varepsilon\), i.e.,

\[
\frac{S_{K+1} - S_K}{S_K} < \varepsilon
\]  

(11)

3. The absolute relative change in any one of the parameters in any two successive iterations is less than or equal to a prescribed constant \(\varepsilon\), i.e.,

\[
\frac{b_{iK+1} - b_i}{b_i} < \varepsilon \quad \text{for all } i
\]  

(12)

Marquardt's modification to the Gauss Newton method represents a compromise between linearization (via Taylor series) and the steepest descent method and accelerates convergence, the computation of \(d_K\) is given by

\[
d_K = (D_K' D_K - I)^{-1} D_K'(y - Z_K)
\]  

(13)

where \(\lambda\) is Marquardt's scaling factor.

Because of attenuation in the \(s(b)\) contours, we may get \(S_{K+1} > S_K\), in which case \(\lambda\) is multiplied by an adjustment factor \(\alpha\).
Usage

Calling Sequence

CALL NLSQ (FUN, FGRAD, X, Y, F, B, EPS, GAA, V, WORK1, WORK2, NOB, NP, MIT, IDIMX, IER) where,

FUN - Name of user supplied subroutine that computes the value of the function, VAL, for values of the coefficients b and independent variables X. The form of the subroutine is,

SUBROUTINE FUN (B, S, VAL)

DIMENSION B (1), X (1)

COMPUTE VAL = f[b(1), ..., b(NP), X(1), ..., X(NX)]

FUN must be declared in an external statement in the calling program.

FGRAD - Name of user supplied subroutine that computes the gradient values. The form is,

SUBROUTINE FGRAD (B, X, GRAD)

DIMENSION B(1), X(1), GRAD(1)

COMPUTE GRAD(I) = \frac{\partial f[b(1), ..., b(NP), X(1), ..., X(NX)]}{\partial b(I)}

for I from 1 to NP. FGRAD must be declared in an external statement in the calling program.

X - 2 dimensional array of row size NX (the number of independent variables) and column size NOB (the number of observations) specifying the values of the independent variables. The variables X(J, I) contains the value of j-th variable on the i-th observations.
Y - 1 dimensional array of size NOB that contains the observed dependent variable.

F - 1 dimensional array of size NOB that contains the final function values computed at each set of independent variables.

B - 1 dimensional array of size NP that contains the resultant constants. Initial estimates of B must be specified prior to the call.

EPS - Specifies the desired accuracy ($\varepsilon$). Suggested value = $10^{-8}$.

GAA - Marquardt scaling factor ($\lambda$). For a detailed explanation of this variable see reference 1. If the choice must be made arbitrarily the recommended value is 2.0.

V - Adjusted factor. Again see reference 1 for a detailed explanation. If the choice must be made arbitrarily the recommended value is 1.5.

WORK1 - Work area of size $2\cdot(NP^2)$. It is used internally as a storage area. It may be used in the calling program for other purposes, but its contents will be destroyed when NLSQ is called.

WORK2 - Work area of size $4\cdot NP$. The same statements about WORK1 apply.

NOB - Integer specifying the number of observations (n).

NP - Integer specifying the number of constants to be computed (m). NP must not be greater than NOB.
MIT - Integer specifying the maximum number of iterations the subroutine may perform.

IDIMX - Integer specifying the row dimension of the array X. It must be at least NX (the number of independent variables).

IER - Error parameter. Upon return this indicates success or failure of the computation.

IER = 1 indicates that convergence was not obtained in MIT iterations.

IER = 2 indicates that NP > NOB and no computation was performed.

IER = 3 indicates that a singular matrix $D_k^T D_k - I$ was encountered and the computation was aborted at that point.

The variables X, Y, b, EPS, GAA, V, NOB, NP, MIT, and IDIMX, all must be initialized prior to calling NLSQ.

Coding Information

The statement,

\[
\text{EXTERNAL FUN, FGRAD}
\]

MUST appear in the calling program.

Required Subprograms

FUN and FGRAD

Common Storage

None

Example

The following program finds the values of $b_1$, $b_2$, and $b_3$ for any
set of observations points. The function is,

\[ f(x_1, x_2, x_3, b_1, b_2, b_3) = b_1 + \frac{x_1}{b_2 x_2 + b_3 x_3} \]  \hspace{1cm} (14)

The gradient vector is,

\[ \frac{\partial f}{\partial b_1} = 1, \quad \frac{\partial f}{\partial b_2} = \frac{-x_1 x_2}{(b_2 x_2 + b_3 x_3)^2}, \quad \frac{\partial f}{\partial b_3} = \frac{-x_1 x_3}{(b_2 x_2 + b_3 x_3)^2} \]  \hspace{1cm} (15)

A program to read the observation points and find the corresponding constants is given below. (TH is equivalent to our B)

PROGRAM NLSQEXP (INPUT, OUTPUT)
DIMENSION X(3,15), Y(15), F(15), TH(3), WORK1(18), WORK2(12)
EXTERNAL FUN, FGRAD
NP = 3
READ 100, NOB
100 FORMAT (15)
READ 101, (TH(J), J = 1, NP)
101 FORMAT (6F10.3)
DO 2 I = 1, NOB
2 READ 101, Z(I), (X(J, I), J = 1, 3)
CALL NLSQ (FUN, FGRAD, X, Y, F, TH, EPS, GA, V, WORK1, WORK2, NOB, NP, MIT, 3 1, IER)
PRINT 102, IER, (TH(J), J = 1, NP)
102 FORMAT (1X, 8HERROR = , 11, 11H RESULTS = , 3(F10.7, 1X))
STOP
END

SUBROUTINE FUN (TH, X, F)
DIMENSION TH(1), X(1)
F = TH(1) + X(1)/(TH(2)*X(2)+TH(3)*X(3))
RETURN
END

SUBROUTINE FGRAD (TH, X, GRAD)
DIMENSION TH(1), X(1), GRAD(1)
GRAD(1) = 1.
FACTOR = -X(1)/(TH(2)*X(2)+TH(3)*X(3))**2
GRAD (2) = FACTOR*X(2)
GRAD (3) = FACTOR*X(3)
RETURN
END
Maximal Electroshock Apparatus (Woodbury and Davenport, 1952)
as Modified by Sittig, R.A. (1961)

Prior to 1952, the methods for electrically inducing seizures in experi­mental animals for studying anticonvulsants varied widely, resulting in contrast­ing results between investigators. The instrument originally designed by Woodbury has become a standard piece of pharmacological equipment because it provided controlled types of electrically induced seizures and reproducibility of results. Instrumental modifications were incorporated by Sittig to further sophisticate the instrument and to simplify its operation. The circuit diagram is illustrated in Figure 15. The parts list for the electroshock apparatus is as follows:

\[ S_1 \] Interlock switch on cabinet door
\[ S_2 \] Master Switch
\[ S_3 \] Push button to initiate timer; normally connected to timer
\[ S_4 \] Timer switch 5 pole, single circuit wafer type
\[ S_5 \] Current range switch, porcelain base, high voltage
\[ T_1 \] Small replacement type transformer 350-0-350 volts
\[ T_2 \] Variable autotransformer, 3 ampere capacity
\[ T_3 \] Plate supply transformer, primary 110 volts, secondary 2000 volts, 400 ma
\[ C \] Timer condenser, 5 mfd, 1000 volts
\[ R_y_1 \] Sensitive relay, 10,000 ohm winding
\[ R_y_2 \] Heavy duty, double pole relay or contactor, 110 volt coil
\[ R_1 \] Adjust to give 25-30 ma through regulator tubes; approximately 2,000 ohms 10 watts.
\[ R_2 \] 10 K 10 turn precision potentiometer
R3 5000 ohm 1 watt resistor
R4-R8 Wire wound, 100 watt variable resistors with sliding taps. Adjust to give desired full scale current. The following are approximate:

R4 120,000 ohms
R5 40,000 ohms
R6 30,000 ohms
R7 16,000 ohms
R8 4,000 ohms

Meter (Indicated by circle-enclosed arrow in lower left part of diagram). Any meter capable of reading rms a-c volts; scale may be hand-calibrated to give desired current ranges.

Any desired additional information may be obtained from the Woodbury and Davenport reference.
CIRCUIT DIAGRAM FOR MES APPARATUS
CHAPTER VIII

BIBLIOGRAPHY


APPROVAL SHEET

This dissertation submitted by Ronald K. Browne has been read and approved by five members of the Faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

This dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 9, 1972

Date

[Signature of Advisor]