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Binding of Phenobarbital Sodium by Human Serum Albumin

Ronald K. Browne

Loyola University Chicago

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BINDING OF PHENOBARBITAL SODIUM

BY HUMAN SERUM ALBUMIN

by

RONALD K. BROWNE

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a Thesis Submitted to the Graduate School Faculty of
Loyola University - Stritch School of Medicine
in Partial Fulfillment of the Requirement
for Master of Science Degree
DEDICATION

This thesis is dedicated to those who have given the needed guidance, spirit, patience and understanding necessary to such work. Above all stand Dr. Y. T. Oester and Dr. John F. Zaroslnski, whose comprehension, encouragement and stability provided stimulus for this research. A special tribute to my wife, Karen, for her love, tolerance and perseverance.
BIOGRAPHY

Ronald K. Browne was born in Chicago, Illinois on October 21, 1934. He 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 of Chicago in June, 1954. In the fall of 1954, he volunteered for service in the U. S. Army and served as supply and missile radarmen 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 Arnar-Stone Laboratories in Mt. Prospect, Illinois as a junior pharmacologist. He was advanced to senior pharmacologist in 1964. At Arnar-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.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>INTRODUCTION AND STATEMENT OF PROBLEM</td>
<td>1 - 8</td>
</tr>
<tr>
<td>II</td>
<td>MATERIALS AND METHODS</td>
<td>9 - 17</td>
</tr>
<tr>
<td>III</td>
<td>RESULTS</td>
<td>18 - 30</td>
</tr>
<tr>
<td>IV</td>
<td>DISCUSSION AND SUMMARY</td>
<td>31 - 39</td>
</tr>
<tr>
<td>V</td>
<td>APPENDIX</td>
<td>40 - 47</td>
</tr>
<tr>
<td>VI</td>
<td>BIBLIOGRAPHY</td>
<td>48 - 50</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Among the first reports involving serum protein binding of drugs is one covering work done by Moore and Roaf (1904). They demonstrated that several volatile compounds, such as ether and chloroform, had a greater solubility in serum than in saline. This was attributed to "substance interaction with proteins". Historically, a significant advance in the era of modern drug-protein interaction was contributed by Davis (1942, 1943). His research related the binding of sulfonamides by plasma proteins to drug distribution in body fluids and indicated that the bound portions were bacteriostatically inactive. Within eight years, several investigations describing serum-drug interaction of sulfonamide, digitoxin, organic ions, penicillin, inorganic ions, and serum albumin binding sites were published (Derouaux, 1943; Farwaz and Farah, 1944; Klotz and Walker, 1947; Eagle, 1947; Scatchard, 1949, 1950; and Karush, 1949, 1950).

A classic review by Goldstein (1949) regarding drug and plasma protein interactions focused upon the consequences of drug binding on half-life, efficacy, distribution, and excretion of drugs. His 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" summarizes the importance of drug-plasma protein interactions.
The Goldstein publication contributed to an increased awareness and stimulated many researchers to investigate the protein drug binding phenomenon. Since 1949, many publications describing the binding of a variety of drugs by plasma proteins have appeared. Among the drug examined were anticoagulants, hypnotics, parasympatholytics, anti-infectives, CNS stimulants, cardiovascular agents, etc. (Meyer and Guttman, 1968).

Weiner et al (1950) showed that several human protein fractions had different affinities for binding dicumarol. They found that beta and gamma globulins bound 20% of the drug while alpha globulin and albumin bound 50 and 99% respectively. The low free plasma level, slow rate of metabolism, slow rate of transformation, and low elimination rate of dicumarol were attributed to extensive plasma protein binding.

Mark et al (1951) found that procaine amide was primarily localized in various tissues, i.e. kidney, liver, spleen, etc. To estimate the extent of drug localization, it was necessary to determine the amount of procaine amide bound by plasma proteins. They found that 15% of the drug was bound to non-diffusible constituents of plasma.

A report by Tonnesen (1956) stated that the slow elimination of atropine in man might be due to interaction with plasma protein. Employing ultrafiltration and ultracentrifugation techniques, he found that after the administration of a therapeutic dose of atropine, 50% was absorbed onto the plasma proteins. Tonnesen concluded that the plasma proteins served as a carrier by which atropine is transported to various organs. He also intimated that this binding may slow the elimination of atropine in man.
In vivo results reported by Paul et al (1960) showed that 30% of the non-ionized nitrofurans (nitrofurazone) were bound to plasma proteins, 50 - 90% of the anionic compounds (nitrofuran), while the cationic drugs (furalradone) were not bound to any appreciable extent. The authors failed to establish any connection between the protein binding of these anti-infectives and their systemic effects. The relative binding power of the protein for the drug was not established nor was the possibility of displacement within the drug-protein complex. It is the consensus that most drug-protein complexes are therapeutically inactive.

In 1960-1961, Anton, using equilibrium dialysis, confirmed that in vitro protein bound sulfonamides were devoid of antibacterial activity and that the binding activity varies greatly between mammalian species. He also presented in vivo evidence that the distribution of sulfonamide in the rat could be modified by competitive interference with its binding to plasma protein by other drugs, i.e. sulfinpyrazone, ethyl bis-coumacetate, phenylbutazone, and iophenoxic acid. The total number of binding sites per molecule of human serum albumin for a sulfonamide was determined by Clausen (1966). Clausen also reported that other plasma proteins (prealbumin, glycoproteins, and alpha 2-macroglobulin) possessed secondary binding properties.

Eichman et al (1962) demonstrated that xanthine molecules (caffeine, theophylline, theobromine, etc.) formed drug-albumin complexes. Their results indicated that the extent of the binding was partially dependent upon the hydrogen ion concentration of the system, and the acid strength of the drug.
The inactivation of penicillins F, G, K, and X by human and rabbit serum protein interaction was revealed by Eagle (1947). Keen (1965) found that the binding of these antibacterials showed considerable variation between oxen, goats, horses, sheep, and pigs. According to Keen, these differences may be accounted for by the average plasma concentrations of albumin, differences in affinity for the drug molecule, and ionic constitution of the different plasmas. Temperature variation has been characterized as another factor operant in the binding of penicillins, calcium, and anionic dyes. In 1966, Keen reported that sulphamethoxypridazine, phenozmethyl penicillin, and phenol red could be displaced from serum albumin by several anionic drugs. This displacement by agents such as phenylbutazone and salicylate showed specificity. Furthermore, Keen determined the number of binding sites for phenoxy methyl penicillin and benzylpenicillin to bovine serum albumin.

In 1954, Goldbaum and Smith investigated the in vitro binding characteristics of several barbituric acids to bovine serum albumin using ultrafiltration. Among the barbiturates studied were thiopental, seconal, pentobarbital, phenobarbital and barbital. Their results showed that gradually increasing the length of the substituted alkyl side chain from two to six carbons increased the percent of the drug bound. Hence, those drugs having the shorter duration of pharmacological action had the greatest fraction bound. Variation of pH, albumin concentration, and drug concentrations produced:

1) maximum drug-albumin complex at approximately pH 7.8;
2) an increase in bound drug with increased albumin concentration which plateaued near 4%;

3) maximal and minimal barbiturate-albumin interaction at drug concentrations of $25 \times 10^{-4}$ and $1 \times 10^{-3}$ M respectively.

They demonstrated that bound barbiturates could be partially displaced from the drug-albumin complex by addition of other barbiturates and dissimilar organic anions. In vivo distribution and in vitro tissue homogenate studies in the rabbit generally indicated the highest binding of the barbiturates occurred in plasma liver heart kidney lung brain muscle red cells. Finally, they estimated the number of binding sites on bovine serum albumin for the barbiturates.

The work by Goldbaum and Smith provided evidence that barbiturate binding is "related to pH", drug concentration, and protein availability. Also, binding in vitro seems to be related in part to the distribution of drugs in vivo.

Employing dosages approximately five to ten times that used therapeutically, Lous (1954) examined the fate of three barbituric acids in man. Using three to four normal subjects per drug, his work indicated that the highest plasma concentration of barbital (B), allypropymal (A), and phenobarbital (P), occurred between four to eight hours, three to nine hours, and twelve to eighteen hours, respectively. After administration, the drugs were eliminated from plasma following an exponential curve with total urine excretion for B, A, and P being 75-95%, 9-18%, and 13-37% respectively. The renal clearance was shown to be "independent of plasma concentration but dependent upon urinary flow". Lous found, by ultrafiltration experiments, that the percent of barbiturate bound to
plasma was 95% (88-104) for barbital, 50% (33-71) for phenobarbital, and 60% (54-65) for allypropymal.

Also, in 1954, Taylor et al investigated the binding of thio-pental by rabbit plasma in vivo and in vitro. These data indicated that the amount of drug bound in vitro closely resembled the amount bound in vivo. They also demonstrated that as the concentration of the thio-pental increased, the percentage of the thio-pental bound decreased, resulting in an enhancement of the sleeping time duration.

Waddell and Butler (1957) delved deeper into the distribution and excretion of phenobarbital in dog, mice and man, stressing the effects of pH alteration upon the parameters studied. In contrast to the results obtained by Goldbaum and Smith, these authors presented in vitro phenobarbital binding data obtained by dialysis which indicated that the percentage of the drug bound was only slightly affected by pH within the normal physiological range found during acidosis and alkalosis. Furthermore, they stated that "in a 4 gm. per 100 ml. solution of albumin, the proportion of phenobarbital bound is almost independent of the phenobarbital concentration over the range of 20 to 100 mg. per liter". They also stated that binding by "human serum albumin did not differ significantly from bovine albumin". In vivo experiments in dog, mice and man revealed that after precipitation of acidosis, induced by CO₂ inhalation, and alkalosis, by hyperventilation or intravenous infusion of NaHCO₃, plasma phenobarbital concentrations fell as blood pH decreased. Experiments measuring the effects of pH on phenobarbital-induced anesthesia in mice indicated that alkalosis lightens phenobarbital anesthesia by
decreasing drug concentration in the brain. Thus, tissue/plasma concentration varies reciprocally with blood pH. Finally, Waddell and Butler presented data showing that the renal clearance of phenobarbital was similar in man and dog; "The clearance of phenobarbital increases with increasing urine flow in acid or alkaline urine, but at any given rate of flow the clearance is much higher in alkaline than in acid urine."

The work cited here indicates that the pharmacological action of many drugs is closely related to plasma protein binding and binding site availability. A number of these papers have reported that drug binding to these proteins, whether albumin, globulins, glycoproteins, etc., is a function of pH, drug or protein concentration. Duration of action and metabolic fate are apparently influenced by these phenomena. The investigations involving phenobarbital by Goldman and Smith, Lous, Waddell and Butler provide evidence showing that the drug is primarily bound by serum albumin. However, the effects of variation in drug concentration, serum albumin concentration, and pH upon phenobarbital binding are in some instances contradictory. Also, binding site availability on human serum albumin for phenobarbital has not as yet been established.

Inconsistencies in previous reports on phenobarbital binding by serum albumin suggested the need for clarification in this area. The ratio of moles of drug bound per mole of albumin calculated by Goldbaum and Smith was determined, using a value of $14.5 \times 10^{-5} \text{M}$ for their bovine albumin concentration. On recalculation, it appears that this value should have actually been $14.7 \times 10^{-5} \text{M}$. The larger value could have resulted in a lower binding site estimation. Waddell and Butler stated
that the binding of phenobarbital to human serum albumin was affected only to a slight degree by pH and that it was nearly independent of pH, whereas Goldbaum and Smith state that phenobarbital binding to albumin is related to pH. Waddell and Butler reported that alkalosis induced in mice increased the median anesthetic dose of phenobarbital. They also observed a decrease in phenobarbital plasma concentrations after inducing acidosis in the dog. These findings do not appear to be in concert with their in vitro data; although Waddell and Butler indicated that they performed some experiments involving the binding of phenobarbital to human serum albumin, they failed to present any data documenting their findings. The only direct data concerning the binding of phenobarbital to human serum proteins were reported by Lous. Lous estimated that in vivo binding of the drug concentration found in human serum ranged between 33-71%. However, the upper and lower values were obtained in a single individual. He did not determine the percent of protein in the serum nor the blood pH of his samples. Therefore, research involving these problems entailed a confirmation and clarification of the characteristics exhibited by human serum albumin binding of phenobarbital sodium when drug concentration, albumin concentration and pH were varied. A second phase included the determination of the number of binding sites per molecule of albumin available to the drug. These data were further examined in regard to implications as to pharmacological effect in humans.
CHAPTER II
MATERIALS AND METHODS

In this study, experiments were designed to determine the percentage of drug bound in vitro to human serum albumin (HSA) while varying specific physical and chemical conditions. However, all experiments were conducted in an in vitro system approximating the normal in situ HSA environment as closely as possible.

The percentage of drug bound to HSA was determined by a modification of the equilibrium dialysis procedure of Anton (1960). Equilibrium dialysis confines the protein component within a bag formed from a semi-permeable membrane. This membrane allows unbound drug molecules to freely diffuse into the solution surrounding the bag until equilibrium is attained. When unbound drug concentrations on both sides of the membrane become equal, any drug increment in the protein compartment is presumed to represent drug bound to protein. The concentration and percent of drug bound is determined as follows:

1) \[ \text{Concentration of bound barbiturate} = \frac{\text{Concentration of barbiturate in the bag}}{\text{Concentration of barbiturate in dialysate}} \]

2) \[ \% \text{ bound barbiturate} = \frac{\text{Concentration of bound barbiturate}}{\text{Concentration of barbiturate in bag}} \times 100 \]

The technique of equilibrium dialysis provided an uncomplicated method for the study of protein binding. It also allowed the convenience of an overnight binding reaction and easily controlled temperatures.

Phenobarbital was assayed by the ultraviolet, spectrophotometric method of Goldbaum (1952), and Goldbaum and Smith (1954). This procedure
combines precision, specificity, simplicity and sensitivity for the measurement of microgram quantities of phenobarbital in saline, blood, serum, and urine solutions. To minimize experimental variance due to pipetting errors, determination of data from standard curves, and other extraneous variables, each experimental point was established on the basis of three separate determinations. Each such determination was conducted in duplicate. The average and standard error of the mean for each set of phenobarbital values was calculated. Protein concentrations were determined by the procedure of Gornall et al (1948).

Preliminary Experiments

Pilot experiments were conducted to assure that no interference in binding or assay resulted from the cellulose dialysis tubing. In system one, 0.001 M phenobarbital Na in Sorensen's M/15 phosphate buffer solution was placed in the dialysis tube (outer phase) while the dialysis bag (inner phase) contained only the buffer. In a second system, the phases were reversed. A buffer solution containing phenobarbital equal in amount to that in the tube of system one was placed in the bag. This bag was then put into a dialysis tube containing only buffer solution. After 16 hours dialysis, the buffer solutions in the tubes and bags of both systems were analyzed for phenobarbital concentration.

Additional experiments estimated the minimal amount of time necessary to attain system equilibrium. The above procedure was repeated; however, a three percent human serum albumin-buffer solution was placed into the dialysis bags. The dual systems were then dialyzed and assayed after eight, twelve and sixteen hours. Minimal equilibrium time was
determined to be twelve hours.

Previous work by Goldbaum and Smith (1954) using ultrafiltration indicated that equilibrium values for barbiturate binding to bovine albumin was unaffected by temperatures ranging from 4°C - 40°C. Experiments were conducted at 37.5°C. and at room temperature, confirming the above results. Thus, for convenience, equilibrium dialyses were carried out overnight through a period of 16 to 17 hours at room temperature.

A final set of preliminary experiments was conducted to confirm the sensitivity of the assay and the percentage of phenobarbital Na recovered. The partition coefficient was determined as described by Martin (1962) and employed to calculate the theoretical extraction and percentage recovery of the drug.

HSA-Phenobarbital Binding Experiments

To ascertain a picture of HSA binding capacity and estimate the number of binding sites per molecule of albumin available to phenobarbital, the effect of drug concentration variation upon binding was studied. Concentrations ranging from that considered therapeutic to those causing toxic reactions were investigated as follows:

a) $12.5 \times 10^{-5}$M  

b) $25 \times 10^{-5}$M  

c) $5 \times 10^{-4}$M  

d) $1 \times 10^{-3}$M  

e) $2 \times 10^{-3}$M  

f) $4 \times 10^{-3}$M  

g) $8 \times 10^{-3}$M

The $12.5 \times 10^{-5}$M concentration was employed in all other experiments. This concentration was assumed to be therapeutically equivalent to that
found in a normal 70 Kg. individual after administration of 150 mg. of phenobarbital.

The normal range of arterial blood pH is 7.35 - 7.45, "any pH below 7.35 or above 7.45 is generally defined acidosis and alkalosis respectively", (Woodbury, 1965). Many acute and chronic disease states clinically reveal an acid-base disturbance; therefore effects of pH variation (6.6 - 7.8) upon binding were investigated. A pH of 7.4 was maintained in all other experiments.

The effects of varying concentrations of HSA (1 to 6%) on binding were also examined since there are clinical conditions in which HSA variations from normal may occur. For all other experiments, 3.0% HSA was utilized.

Finally, the number of binding sites per molecule of human serum albumin was estimated using the procedure described by Karush (1950).

System Components and Procedure

Unless specified, all solutions were made up using glass distilled water and employed at room temperature. All compounds were weighed on an analytical balance to the nearest 0.1 mg.

Dialysis Bags (Scientific Products #D1615-2)

The dialysis bags were prepared from high purity, seamless, regenerated cellulose tubing (150 mm. in length and 28 mm. in width). The tubing was rinsed in glass distilled water, then washed for 15 minutes in 90°C. glass distilled water, and finally rerinsed three times. Washing was done to eliminate any substances in or adhering to the cellulose which might interfere with the analytical procedure. The tubing was

12.
double knotted and trimmed at the distal end while the proximal portion was tied securely to the end of a glass tube with blanched nylon thread. The dialysis bags measured approximately 4.5 cm. from the end of the glass tube to the proximal end of the knot. All bags were tested for leaks by submerging the bags in water and blowing through the proximal end of the glass tube, absence of bubbles being indicative of positive closure.

Dialyzer Container

The dialyzer container consisted of a Pyrex (Corning #9820) 25x100 mm. culture tube, Bacti-Capall (Scientific Products #T-1395-4) and a one hole neoprene stopper (18x24 mm), through which was placed Pyrex glass tubing (6 mm I.D. x 55 mm lg.). The glass tube was fire polished at one end and blunted to form a lip at the distal end. The proximal end of the glass tube then provided a porthole used to admit the HSA into the dialysis bag while the distal lip provided a ledge to which the bag was secured.

The Dialysis System Assembly Procedure (Figure 1)

1. A dialysis bag was tied to the glass tube of the neoprene stopper and allowed to dry.

2. Ten ml. of the appropriate phenobarbital Na/buffer solution concentration was pipetted into a culture tube and the base of the tube inserted into a Bacti-cap which provided a standup platform.

3. Three ml. of the appropriate HSA/buffer concentration was pipetted into the dialysis bag through the glass tube insertion port.
4. The neoprene-dialysis bag assembly was then inserted into the culture tube. The glass tubing at the proximal end of the stopper assembly was depressed until the HSA solution meniscus in the bag was at a level equal to that of the culture tube phenobarbital Na solution.

5. A Bacti-cap was placed over the head of the entire assembly, sealing in the contents. The contents were dialyzed for 16 hours at room temperature in a metabolic shaker at 80 strokes/min. using a 1-1/2 inch stroke.

6. At the end of this period, the contents of both the dialysis bag and dialysis tube were analyzed for phenobarbital and protein concentration.

**Human Serum Albumin Solution (Calbiochem-Pentex Lot #17, 21, 23)**

Electrophoretically pure crystalline human albumin was dissolved in the appropriate M/15 buffer and refrigerated. The HSA solutions were used within 24 hours.

**Phenobarbital Sodium (Mallinckrodt Chemical Works Lot #DET)**

The powder was dissolved in the phosphate buffer at the concentrations indicated on page 11. The drug was solubilized in HSA solution at a concentration of 0.001 M when determining system equilibrium.

**Borate Buffer**

74.554 gms. of KCl, reagent grade, and 61.844 gms. of boric acid, reagent grade, diluted to one liter with water. It was then stored at room temperature for 24 hours and filtered.
Sorensen's M/15 Phosphate Buffer (Hepler, 1960)

Sorensen's M/15 Phosphate Buffer was prepared by mixing the appropriate quantities from stock solutions of:

1. M/15 Potassium Acid Phosphate (KH$_2$PO$_4$), 9.08 gm. qs. to 1 liter.
2. M/15 Disodium Phosphate (Na$_2$HPO$_4$), 9.47 gm. qs. to 1 liter.

Sodium Hydroxide Solutions:

Sodium hydroxide solutions were prepared by diluting a commercially available 10N Analytical Reagent Grade Sodium Hydroxide solution with glass distilled water. 1 N sodium hydroxide was used for washing the chloroform extracting solvent. Approximately 0.36 N was used in the phenobarbital analysis. The alkalinity of this solution was adjusted to a pH of 10.4 by adding NaOH and borate buffer in a 2:1 ratio as required.

Chloroform

Reagent grade chloroform was washed with 1 N sodium hydroxide followed by two rinsings with glass distilled water. For every liter of solvent, 100 ml. of wash solution was used. Only the volume necessary for daily use was washed because the chloroform tends to decompose upon standing.

Biuret Reagent (Harleco Lot #70101)

The reagent was prepared in accordance with the procedure described by Gornall et al. The preparation consisted of a modified alkaline copper tartrate stabilized with potassium iodide.
(For a detailed description of the procedures quoted, see the Appendix).
CHAPTER III
RESULTS

Prior to commencing any experiments on the binding of phenobarbital, we attempted to validate the analytical procedure for barbiturates as described in 1952 by Goldbaum (see Appendix). The analytical procedure involved an initial series of four extractions of phenobarbital by CHCl₃ from Sorensen's Buffer following addition of HCl. Following this step, the pooled CHCl₃ extract was re-extracted by NaOH. Extraction of phenobarbital in this second phase was virtually complete. Using this procedure, the phenobarbital recovery was found to be approximately 92-93%. A theoretical recovery of 93.8% was calculated for phenobarbital. Thus the theoretical and actual recovery of the barbiturate were comparable.

Employing a semi-log plot, Figure 2 illustrates the effects of varied barbiturate concentrations upon the percentage of phenobarbital bound by human serum albumin (HSA). The amount of drug bound increased while the percentage bound diminished as phenobarbital concentrations increased. Thus, at a concentration considered therapeutically effective, 1.25 x 10⁻⁵ M, a high fraction of the drug was bound. The percent of phenobarbital bound at 0.5 - 8 x 10⁻³ M, concentrations which would appear to be toxic or lethal were significantly less bound than at 12.5 x 10⁻⁵ M (Table 1).
FIG. 2
EFFECT OF PHENOBARBITAL CONCENTRATION VARIATION ON PHENOBARBITAL BINDING BY 3% HSA

EFFECT OF PHENOBARBITAL CONCENTRATION VARIATION ON PHENOBARBITAL BINDING BY 3% HSA
TABLE I

Effect of Varying Phenobarbital Na Concentration on Percentage of Drug Bound by HSA at pH 7.4

<table>
<thead>
<tr>
<th>Drug Concentration (10^{-5} M)</th>
<th>% Drug Bound ±S.E.*</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>53.0 ±0.58</td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>56.9 ±0.54</td>
<td>0.01</td>
</tr>
<tr>
<td>50.0</td>
<td>46.8 ±0.40</td>
<td>0.001</td>
</tr>
<tr>
<td>100.0</td>
<td>43.6 ±0.66</td>
<td>0.001</td>
</tr>
<tr>
<td>200.0</td>
<td>39.4 ±0.70</td>
<td>0.001</td>
</tr>
<tr>
<td>400.0</td>
<td>37.4 ±1.01</td>
<td>0.001</td>
</tr>
<tr>
<td>800.0</td>
<td>27.7 ±1.58</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* S.E. represents the standard error of the mean percentage of drug bound

The changes in phenobarbital binding by human serum albumin (HSA) binding due to pH alterations are depicted in Figure 3. The fraction of barbiturate bound declined as the hydrogen ion concentration was increased. The sharpest decrease in binding occurred between pH 7.2 and 7.4. According to Woodbury (1965), any pH above 7.45 or below 7.35 indicates alkalosis and acidosis respectively. Table II indicates that the amount of drug bound was significantly less at pH's representing acidosis than at normal physiological pH. Above pH 7.4, the percentage of drug bound began to plateau. However, at pH's representing alkalosis, a slight increase in phenobarbital binding occurred which became significant at pH 7.8. In the pH range investigated, the drug binding affinity of the protein appeared to decrease as the pH was lowered.
FIG. 3
EFFECT OF pH VARIATION ON 3% HSA BINDING
OF 12.5 X 10^{-5} M PHENOBARBITAL Na

% 12.5 X 10^{-5} M PHENOBARBITAL BOUND

\begin{align*}
\text{pH} & \quad 6.6 \quad 6.8 \quad 7.0 \quad 7.2 \quad 7.4 \quad 7.6 \quad 7.8 \\
\% \text{ Bound} & \quad 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 60 \quad 70
\end{align*}
### TABLE II

Effect of pH Alteration on 3% HSA Binding of 12.5 x 10^{-5} M Phenobarbital Na

<table>
<thead>
<tr>
<th>pH</th>
<th>% Drug Bound ±S.E.</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>53.0 ±0.76</td>
<td>-</td>
</tr>
<tr>
<td>Acidosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>38.6 ±0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>7.0</td>
<td>34.3 ±0.70</td>
<td>0.001</td>
</tr>
<tr>
<td>Alkalosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>53.2 ±0.30</td>
<td>N.S.</td>
</tr>
<tr>
<td>7.8</td>
<td>57.8 ±0.24</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Changes in the percentage of phenobarbital bound at various concentrations of human serum albumin are portrayed in Figure 4. A progressive increase in the portion of the drug bound occurred as the concentration of HSA was increased. The sharpest rise in binding occurred between two and three percent HSA after which percentage of drug bound began to plateau. Little difference in binding was observed between three and four percent protein concentration. When considering the moles of drug bound per mole of albumin, the fraction of phenobarbital bound was generally reduced as albumin concentrations increased (Table III).
FIG. 4
EFFECT OF HSA CONCENTRATION VARIATION ON
BINDING OF 12.5 X 10^-5M PHENOBARBITAL Na

% 12.5 X 10^-5M PHENOBARBITAL BOUND

% HSA

0 10 20 30 40 50 60 70

1 2 3 4 5 6
TABLE III

Effect of Varying HSA Concentration on Binding of 12.5 x 10^{-5} M Phenobarbital at pH 7.4

<table>
<thead>
<tr>
<th>% HSA Concentration</th>
<th>% Drug Bound ± S.E.</th>
<th>Moles Drug Bound x 10^{-5}</th>
<th>Conc. HSA Moles (10^{-5})</th>
<th>Fraction Drug Bound/Mole HSA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.8 ±0.82</td>
<td>2.72</td>
<td>14.5</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>32.1 ±0.74</td>
<td>4.01</td>
<td>29.0</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>53.0 ±0.58</td>
<td>6.62</td>
<td>43.5</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>58.8 ±0.27</td>
<td>7.35</td>
<td>58.0</td>
<td>0.13</td>
</tr>
<tr>
<td>6</td>
<td>63.5 ±0.59</td>
<td>7.94</td>
<td>86.8</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Assuming a molecular weight of 69,000 for HSA

The reduction in the fraction of phenobarbital bound per mole of albumin can be attributed to a dilution of the drug in the albumin. As the albumin concentration was increased, the number of binding sites was increased while the barbiturate concentration remained constant.

To estimate the number of binding sites per molecule of albumin, the moles of drug bound per mole of albumin (r) was determined for several concentrations of the drug (see Appendix). These ratios were then related to the molar concentration of free drug present (c) by making a lineal plot of r/c vs. r. To minimize experimental error which might have resulted due to techniques employed and to improve the accuracy of binding site estimation, the percentages of drug bound used to calculate r were determined from Figure 2, as indicated in Table IV. The semi-log plot allowed the determination of values from a straight line. The molecular weight for human serum albumin was assumed to be 69,000 (Phelps and Putnam, 1960). The ionic strength of the phosphate buffer at pH 7.4 was calculated at 0.17 arbitrary units (Martin, 1962).
TABLE IV

Values Utilized for Characterization of Phenobarbital Binding Sites

<table>
<thead>
<tr>
<th>Initial Drug Conc. (10^{-5} M)</th>
<th>% Drug Bound</th>
<th>Final Free Drug Conc. (c) (10^{-5} M)</th>
<th>Amount Drug Bound (10^{-5} M)</th>
<th>Moles Bound Drug/mole HSA (r)</th>
<th>r/c x 10^3</th>
<th>r/c x 10^3 (f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>53.7</td>
<td>5.8</td>
<td>6.7</td>
<td>0.15</td>
<td>2.59</td>
<td>1.44</td>
</tr>
<tr>
<td>25</td>
<td>50.3</td>
<td>12.4</td>
<td>12.6</td>
<td>0.29</td>
<td>2.33</td>
<td>1.35</td>
</tr>
<tr>
<td>50</td>
<td>46.9</td>
<td>26.6</td>
<td>23.4</td>
<td>0.54</td>
<td>2.03</td>
<td>1.15</td>
</tr>
<tr>
<td>100</td>
<td>43.2</td>
<td>56.8</td>
<td>43.2</td>
<td>0.99</td>
<td>1.74</td>
<td>1.04</td>
</tr>
<tr>
<td>200</td>
<td>39.9</td>
<td>120.0</td>
<td>79.8</td>
<td>1.83</td>
<td>1.52</td>
<td>0.91</td>
</tr>
<tr>
<td>400</td>
<td>36.8</td>
<td>253</td>
<td>147.0</td>
<td>3.38</td>
<td>1.34</td>
<td>0.87</td>
</tr>
<tr>
<td>800</td>
<td>33.3</td>
<td>534</td>
<td>266.0</td>
<td>6.11</td>
<td>1.14</td>
<td>0.85</td>
</tr>
</tbody>
</table>
concentration of human serum albumin was $43.5 \times 10^{-5}$ M (3%).

Figure 5 presents the graphical estimation of phenobarbital binding sites per molecule of albumin. According to Karush (1950), Scatchard (1949, 1950), and others, homogeneity of binding sites produces a straight line plot. When the line is extrapolated to the ordinate and abscissa, the intercepts equal $nK$ and $n$ respectively (see Appendix). If the $r/c$ vs. $r$ relationship produces a curved line, two alternatives are present. The deviation from the straight line may be due to electrostatic factors or the binding sites are heterogeneous.

The plot of these data produced a curved line, Figure 5. Therefore, we replotted the data using the electrostatic interaction correction factor. The formula for electrostatic interaction is $r/c (f)$ where $f$ equals $e^{2w(Zp+r)}$ (Keen, 1966). The factor $e$ is the log of the natural logarithm and $w$ is the electrostatic parameter taken as 0.025, extrapolated from Tanford et al (1955). According to Goldstein (1949), the isoelectric point of albumin is 4.9 and carries a net negative charge at pH 7.4. Consequently, the charge on the protein ($Zp$) is taken as -12 (McMenamy, 1968). A plot of $r/c (f)$ vs. $r$, Table IV, produced a curved line, Figure 6.
FIG. 5

GRAPHICAL ESTIMATION OF PHENOBARBITAL BINDING SITES PER MOLECULE OF HSA

- - Plot of Points Uncorrected for Electrostatic Interaction
- - - Plot of Calculated Points
\[ \frac{n_1 K_1}{1 + K_1 c} \times \frac{n_2 K_2}{1 + K_2 c} \]
where \( r/c = \frac{n_1 K_1}{1 + K_1 c} \times \frac{n_2 K_2}{1 + K_2 c} \)

▲ ▲ Extrapolation of \( n_1 \) and \( n_1 K_1 \)

— — Extrapolation of \( n_2, \xi n \) and \( \xi n K \)

\( r \) represents the moles of drug bound per mole of albumin, and \( c \) equals the final free drug concentration (10^{-5} M). \( n \) is the number of binding sites, and \( K \) is the binding site association constant.
**FIG 6**

**PLOT OF PHENOBARBITAL BINDING VALUES ASSUMING ELECTROSTATIC INTERACTION**

- The plot shows a curve for $r/c$ vs. $r$ without the electrostatic correction factor ($f$) as depicted in Figure 5.
- The curve obtained using the electrostatic correction factor.

$r$ represents the moles of drug bound per mole of albumin, and $c$ equals the final free drug concentration ($10^{-5}$ M).
Since we failed to demonstrate the presence of homogeneous binding sites, an assumption of heterogeneous binding sites for phenobarbital was made (see Appendix). Resolution of the binding curve (Figure 5) indicated two components corresponding to sites, \( n_1 \) and \( n_2 \). Table V shows the values obtained for the albumin molecules binding sites and their association constants for phenobarbital.

### TABLE V

<table>
<thead>
<tr>
<th>Sites</th>
<th>( n )</th>
<th>( nK )</th>
<th>( K )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary binding sites</td>
<td>0.95</td>
<td>950</td>
<td>1000</td>
</tr>
<tr>
<td>Secondary binding sites</td>
<td>14.05</td>
<td>1750</td>
<td>124</td>
</tr>
<tr>
<td>Total binding sites</td>
<td>15</td>
<td>2700</td>
<td></td>
</tr>
</tbody>
</table>

The relationship between free and bound drug concentration for these groups of sites is expressed by an equation calculated by Karush (1950)

\[
\frac{r}{c} = \frac{n_1 K_1}{1 + K_1 c} + \frac{n_2 K_2}{1 + K_2 c}
\]

When the above values were substituted in the equation, the broken line in Figure 5 was plotted. This curve approximates that obtained with the experimental data. Extrapolation of both curves estimates the total number of binding sites to be approximately 15. The data suggest a primary group of sites with a greater affinity for phenobarbital and a
secondary more numerous group with a lesser affinity as indicated by the differences in the group association constants.
CHAPTER IV

DISCUSSION

The response of living systems to chemical stimuli such as drugs is often widely varied between and within the species and frequently within an individual organism. "Most of the variation attending the use of drugs, especially in therapeutics, lies in the wide ranges of physical, biochemical, and pathological conditions that confront the drug when it is administered to a living organism" (Condouris, 1965). Plasma-protein binding of drugs, particularly by serum albumin, demonstrates a notable involvement in drug-response variation. Drug binding by albumin can alter therapeutic effectiveness, delay metabolic transformation or excretion, and participate in drug interaction. The slow release of a bound drug may prolong drug blood levels, resulting in sustained therapy, or the bound complex may act as a protective carrier system by which a drug can be transported to or removed from its site of action. (Tonnesen, 1956; Anton, 1960; Keen, 1966; Meyer and Guttman, 1968; Goldstein, 1949.) The binding of drugs by albumin is related to environmental pH, albumin concentration, and drug concentration (Goldstein, 1949; Goldbaum and Smith, 1954). Consequently, variations in the physical, biochemical, or pathological condition of an organism which change pH or albumin concentrations can influence drug binding and alter therapeutic effectiveness.

In 1954, Lous estimated the percentage of phenobarbital bound in vivo by serum proteins. He employed three normal human subjects and repeated the experiment in one of the subjects to obtain four values.
Each subject received 750 mg. of the barbiturate orally. Using ultrafiltration, he found that serum proteins bound phenobarbital at a level of 33 - 71% (50 ±8%). Unfortunately, Lous did not report the blood pH or serum protein content for any of the subjects. Nevertheless, the calculated average of 50% was fairly close to results we reported. Our data indicated that approximately 43 - 47% of a comparable in vitro concentration, between $5 \times 10^{-4}$ and $1 \times 10^{-3}$ M, was bound to albumin alone. Thus, there appears to be some relationship between in vivo and in vitro phenobarbital binding. It has been reported that minor secondary binding and sometimes primary binding occurs with other serum proteins such as prealbumin, alpha and gamma globulins, and glycoproteins (Goldstein, 1949; Clausen, 1966; Meyer and Guttman, 1968). However, albumin is considered the primary binding entity for most drugs including phenobarbital.

Barbiturates in sedative or hypnotic doses act principally at the level of the thalamus and the ascending reticular formation, interfering with impulse transmission to the cortex (Cutting, 1967). These drugs are capable of depressing many biological functions and are considered unspecific in their effects. Although little is known about their mechanism of action, barbiturates are only partially ionized at biological pH's. Available information indicates that the free acid (undissociated) form is responsible for the depressant effects. (Hardman et al, 1959; Sharpless, 1966). "Moreover, the drugs cross the cellular membrane only in the form of undissociated molecules" (Maynert, 1965).

Examination of our pH data indicates a sharp increase in phenobarbital bound as the pH is increased, 35% at pH 7.2 to 53% at 7.6.
As the hydrogen ion content decreases, the fraction of the barbiturate bound substantially increases and the amount of drug available to produce an effect is diminished.

Waddell and Butler reported a 6% difference in the fraction of phenobarbital bound by albumin in vitro between pH 7.2 and 7.6. They stated that "binding is affected only to a small degree by pH", and that "the binding is nearly independent of pH". Nevertheless, their in vivo experiments in mice indicate a 20% increase in the median anesthetic dose of phenobarbital after NaHCO₃ induced alkalosis. Waddell and Butler also observed a decrease in plasma phenobarbital concentration when acidosis was induced by CO₂ in dogs. Assuming that the drugs cross cellular membranes only in the form of undissociated molecules and that the bound form is inactive (Goldstein, 1949; Maynert, 1965), the in vivo and in vitro results and conclusions of Waddell and Butler appear to be inconsistent. On the other hand, our results and those of Goldbaum and Smith correlate nicely with the in vivo data of Waddell and Butler. The information reported above indicates that phenobarbital binding as well as drug response are apparently pH dependent.

The binding of an anionic drug molecule by albumin can be ascribed to the availability of the free imidazole, ε-amino, and guanido groups of the basic amino acids - histidine, lysine, and arginine, respectively.

The dissociation constants for phenobarbital and the amino acids are represented by their pKa values. The pKa for phenobarbital, a weak acid, is 7.4 (Martin, 1962). The pKa's for the imidazole group
of histadine, the ε-amino group of lysine, and the guanido group of arginine are 5.6 - 7.0, 9.4 - 10.6, and 11.6 - 12.6 (White, Handler, and Smith, 1964). The acidic and basic dissociation constants may be calculated by substitution in the following equations:

\[ pKa = pH + \log \frac{\text{non-ionized acid}}{\text{ionized acid}} \]  \hspace{1em} (1)

\[ pKa = pH + \log \frac{\text{ionized base}}{\text{non-ionized base}} \]  \hspace{1em} (2)

Examination of the pKa, pH and the equation involved provides a basis for determining the extent of drug or amino acid ionization.

As the hydrogen ion concentration in a solution containing phenobarbital is decreased, the amount of the drug ionized is increased (equation 1). Thus, the anionic form of the phenobarbital molecule becomes more available for binding as the pH is increased from 6.6 - 7.8. In contrast, this alteration of pH results in a positive charge on the free groups of the basic amino acids (equation 2). The presence of increasing amounts of the anionic and cationic charged molecules in the solution provides for increases in the drug-protein binding. Since the imidazole group has the lowest pKa, it is likely that this is the group primarily responsible for the binding of phenobarbital to human serum albumin.

Like Goldbaum and Smith, many researchers have reported a reversal of the fraction of drug bound above pH 7.8. This decrease in association above pH 7.8 is rationalized by Eichman et al (1962). They assume: 1) the occurrence of a specific interaction between a
"proteinated site" on the albumin and the drug, or 2) a change in the configuration of the binding sites "resulting from the neutralization of a proteinated group on the protein". Either or both may result in a disruption of the drug-protein complex. They point out that *ε*-amino groups dissociate in the pH range where binding reversal transpires, and that "it is quite possible that neutralization of such groups is related to the marked decrease in the interaction".

Our pH data and that of Goldbaum and Smith suggest that change in the blood hydrogen ion content may affect the dosage at which phenobarbital and other barbiturates are therapeutically effective. Binding differences over a normal pH of 7.35 - 7.45 amount to approximately 4.5% in vitro. Disease states evoking disturbances in acid-base metabolism leading to acidosis or alkalosis such as renal disorders, respiratory disorders (tuberculosis, emphysema), types of adrenocorticism, or diabetes mellitus, provoke significant pH alterations. Although barbiturates may often be contraindicated in these conditions, sedative and hypnotic doses of phenobarbital are administered to relieve insomnia and anxiety prior to knowledge of their etiology. Undissociated phenobarbital availability appears to be a factor in drug dose-response relationship. Consequently, any disease states evoking pH changes would contribute to the problems of drug dosage variability between and within patients.

The concentration of albumin in serum between individuals is highly varied. Altman and Dittmer (1961), using reports published in the 1950's, compiled a table showing a range of 2.0 - 4.5 g/100 ml. Other sources suggest an average concentration of 3.5 g/100 ml. The consensus
places the average HSA concentration between three and four percent. Little
difference in binding was observed (5 percent) between HSA concentrations
of 3 to 4 percent. A sharp decrease in the amount of drug bound was ob-
served below 3 percent. Our work supports the findings of Goldbaum and
Smith. We all observed that 3 percent albumin bound approximately 43% of
the phenobarbital. Differences between human serum albumin and bovine
serum albumin seem small and many drugs are bound to approximately the
same extent by both. "In general, however, the complete unpredictability
of species differences and variation of relative binding power with
different drugs dictate caution in transferring interaction data between
species" (Goldstein, 1949; Tanford, 1955). The results obtained appear
to indicate that HSA expresses its greatest influence upon the drug binding
at concentrations below 3 percent. More subtle binding changes occur
above 4 percent. Conditions such as dehydration, renal disease, liver
disease, and malnutrition, lead to HSA alterations. Changes in albumin
concentration could be a participating factor in the regulation of pheno-
barbital dose-response by controlling the number of albumin molecules with
available binding sites.

Given a specific pH and HSA concentration, increasing pheno-
barbital concentrations provide a larger quantity of undissociated drug.
Consequently, toxicity results when a sufficient amount of the free acid
becomes available. As the phenobarbital concentration increased, the
moles of drug bound per mole HSA ratio increased proportionately until
concentrations approximating toxic levels were attained. At $100 \times 10^{-5} \text{M}$,
this proportionality began to decrease. Although the percentage of drug
bound at $800 \times 10^{-5}$ M was low, the ratio indicates that approximately 40 times more drug was bound per molecule of HSA at this concentration than at $12.5 \times 10^{-5}$ M (Table IV). It would appear that the presence of phenobarbital may affect the affinity of the protein for the barbiturate. The decrease in proportionality may be attributed to binding site saturation and/or a distortion of binding site configuration due to saturation.

When the environment of the albumin is altered, the response of the organism to phenobarbital can become quickly magnified. Higher concentrations of the drug not only provide more of the free acid form but can cause depressed respiration leading to respiratory acidosis. This decrease in pH would result in a further magnification of the phenobarbital response.

Karush together with Goldbaum and Smith have stressed the importance of the number of binding sites per molecule of protein ($n$) in the drug-protein complex. It was estimated from these results that human serum albumin has a total $n$ of 15 for phenobarbital. There was a primary class of sites ($n_1 = 0.95$) and a more numerous secondary class ($n_2 = 14.05$). Goldbaum and Smith determined that bovine serum albumin (BSA) had 22 $n$. The BSA sites consisted of a primary group with a smaller $n_1$ (0.3) and a larger secondary $n_2$ (21.7). Moreover, the $n_1$ association constant was extremely high when compared with ours (287,000 vs. 1000). A comparison between secondary association constants showed some agreement, 90 vs. 124. Thus, BSA not only had more binding sites per molecule albumin than HSA but the overall affinity for binding phenobarbital was stronger.

Given constant physiological and biochemical conditions, it is
tempting to attribute species differences in phenobarbital response to the number and character of the binding sites found on serum albumin. However, binding data such as reported in this dissertation have not become available for albumin of species other than bovine.

In conclusion, a comparison between these data and results obtained by Goldbaum and Smith, Taylor et al (1954), Waddell and Butler, and Lous, provides a correlation between in vitro and in vivo binding of phenobarbital by serum albumin. The drug-protein complex is a function of the number of binding sites available. But the extent of phenobarbital binding is dependent upon pH and related to the serum albumin concentration and the drug concentration. Any disease states altering these parameters alter phenobarbital binding by HSA and may produce variations in the drug dose-response relationship.

**SUMMARY**

1. Phenobarbital binding is related to hydrogen ion concentration, being maximal at about pH 7.8. The percent of drug bound sharply decreased below pH 7.35.

2. At a given concentration of phenobarbital, the fraction of drug bound approaches maximum with increasing albumin concentration. However, the fraction of bound drug per mole HSA diminishes.

3. As the phenobarbital concentration increases, the fraction bound decreases; however, more drug is bound per molecule of HSA.

4. A total number of 15 binding sites per molecule of HSA is estimated for phenobarbital. Two classes of sites were determined, a primary group with high affinity and a secondary group with much less affinity.
5. The percentage of phenobarbital bound by HSA in vitro was found to be similar to previously published results obtained in vivo.

6. Alterations in physical, and chemical conditions, are related to the binding of phenobarbital by HSA. In the opinion of this author, alterations in these parameters which cause changes in phenobarbital binding can contribute to drug dose-response variations.
Phenobarbital Determination: Method of Goldbaum (1952)

This procedure has its basis in the fact that barbiturates have
one absorption band in strong alkali and another in solutions between 10.2
and 10.6 showing characteristics of two different resonance forms. The
optical density difference between the two forms is greatest at 260 μm
"for alkaline solutions containing 2-30 mcg. of barbiturates/ml." These
differences follow Beers Law and are used to quantitatively measure
phenobarbital concentrations. From a survey of the literature, it is
concluded that ultraviolet, spectrophotometric techniques are the most
sensitive for quantitative and qualitative identification of barbiturates.
The Goldbaum method provides a highly specific identification for barbi-
turates. It also allows for differentiation between many of the barbi-
turates.

Reagents: Borate buffer, 0.36 N sodium hydroxide, washed
chloroform, glass distilled water, and the
appropriate M/15 Sorensen's pH buffer.

Decant solutions from dialysis bag and tube into separate test
tubes. Pipette a portion of each solution into separate volumetric flasks
and dilute to a concentration approximating 50 mcg./ml. Place 5 ml. of
each diluted solution into separate, dry, 125 ml. polypropylene-stoppered
separatory funnels. Acidify each solution with 3 drops of 6 N HCl. The
pH should be between 1-2 using pH paper.

Add 15 ml. of chloroform to each funnel and shake vigorously
for 1 minute. Drain the chloroform layer through a #1 Whatman filter paper
containing approximately 10 gms. of Na$_2$SO$_4$ into a dry separatory funnel. Repeat the extraction with aliquots of 15, 10 and 10 ml. of chloroform. Collect the filtered extracts in the second separatory funnel. Finally, rinse the filter with 10 ml. of chloroform.

Pipette 10 ml. of 0.36 N NaOH into the separatory funnel containing the chloroform extracts and shake for 10 min. on a mechanical shaker to extract the phenobarbital into the NaOH layer. After shaking, drain and discard the chloroform layer and collect the aqueous alkaline layer in a 12 ml. centrifuge tube. Centrifuge for 2-3 min. at 2000 rpm to precipitate any chloroform.

Transfer approximately 3 ml. of the alkaline extract to matched quartz cuvette and determine the optical density at 260 m$_\mu$. in a Beckman DU spectrophotometer against a water blank. Pipette 4 ml. of the alkaline extract into a tube containing 2 ml. of the Borate buffer. Pour 3 ml. of this solution into a clean cuvette and determine the optical density at 260 m$_\mu$.

The appropriate Sorensen's buffer is carried through the same procedure as a reagent blank.

The optical density of the alkaline-borate solution is corrected for dilution by multiplying by 1.5, and then it is subtracted from the optical density of the alkaline extract. The difference is employed to determine the phenobarbital concentration after appropriate dilution corrections.

A standard curve is plotted of the optical density differences obtained from several known concentrations of phenobarbital Na extracted
using the above procedure. The experimental phenobarbital concentrations were determined from the standard curve.
Protein Determination

The method described by Gornall et al (1948) is based on the formation of a violet color when copper sulfate in a strongly alkaline solution reacts with proteins or polypeptides having peptide or amide linkages.

Reagents: Biuret reagent of Gornall et al contains 1.5 gm. cupric sulfate (CuSO$_4$.5H$_2$O), 6.0 gm. potassium tartrate (NaKC$_4$H$_6$.4H$_2$O) dissolved in approximately 500 ml. of distilled water to which is added 300 ml. of 10% NaOH. qs. to 1 liter.

The dialysate, the HSA solutions, and the appropriate Sorensen's Buffer (reagent blank) are diluted 1:10 with water and one ml. of each is pipetted into separate test tubes. 4 ml. of the reagent is pipetted into each tube and the mixture allowed to stand for 30 minutes at room temperature. The optical densities of the solutions were determined in a Beckman DU with one cm. cells at 540 mu using a blank of glass distilled water.

Human serum albumin standards were used as controls and various concentrations were employed to plot standard curves from which protein concentrations were determined.
Partition Coefficient (Pc) and Per Cent Theoretical Extraction

The Pc of phenobarbital Na and its extraction was determined using the method described by Martin, (1962).

The Pc is based upon the distribution of a solute between immiscible solvents. When a substance is added to the immiscible solvents in a quantity insufficient to saturate the solutions, it becomes distributed between the two solvents in a definite ratio. If $C_1$ and $C_2$ are the equilibrium concentrations of the substance in solvent 1 and solvent 2, then the equilibrium constant ($k$) is

$$
Pc = \frac{\text{concentration of Phenobarbital in Sorensen's Buffer (C_1)}}{\text{concentration of Phenobarbital in CHCl_3 (C_2)}}
$$

Once the Pc is determined, it is employed in the following formulas to calculate the theoretical extraction and percent recovery.

1. $W_1 = W \frac{k V_1}{k V_1 + V_2}$, \hspace{1cm} $W_2 = W_1 \left( \frac{k V_1}{k V_1 + V_2} \right)^2$

2. $X$ mg./cc. theoretically extracted = $W_1 + W_2$

3. Percent theoretical recovery = $\frac{X}{\text{concentration in original solvent}} \times 100$

Where $W$ = theoretical number of milligrams of solute extracted repeatedly from $V_1$ ml. of one solvent (Sorensen's buffer) with successive portions of $V_2$ ml. of a second immiscible solvent (CHCl_3), $W_1$ = amount of solute left in original solvent after the first extraction, and $W_2$ = the amount of solvent remaining in the original solvent after repeating the extraction procedure using $V_2$ number of ml. of solvent.

The Pc procedure was as follows:

1. Dilute 500 mg. of phenobarbital Na to 100 ml. with Sorensen's pH 7.4
buffer \((C_1)\). Pipette 20 ml. of this solution into a separatory funnel and add 20 ml. washed \(\text{CHCl}_3\) (pipette). Add 3 drops of 6 N HCl and shake for 1 minute. Pipette 10 ml. of the \(\text{CHCl}_3\) layer into a tared beaker. Evaporate \(\text{CHCl}_3\) to dryness and weigh beaker to determine the phenobarbital residue gravimetrically \((C_2)\).

2. a) Dilute 500 mg. of phenobarbital Na to 100 ml. with Sorensen's buffer. Pipette 40 ml. of this solution into a separatory funnel and add 40 ml. \(\text{CHCl}_3\) (pipette). Add 3 drops of 6 N HCl and shake one minute. Remove 10 ml. \(\text{CHCl}_3\) and repeat step #1, making the dilution correction. This serves as a control \(\text{CHCl}_3\) extraction of phenobarbital and confirms step #1.

b) Using a pipette, remove 20 ml. of the \(\text{CHCl}_3\) layer remaining in the separatory funnel from (a) and place into a second separatory funnel. Pipette 20 ml. 0.36 N NaOH into the funnel. Shake the contents on a mechanical shaker for 10 minutes. Pipette 10 ml. of the \(\text{CHCl}_3\) layer into a tared beaker and repeat (a).

c) Subtract (b) from (a) to determine the amount of phenobarbital extracted by NaOH from \(\text{CHCl}_3\).

The partition coefficient for

\[
\frac{\text{Concentration of Phenobarbital in Sorensen's Buffer}}{\text{Concentration of Phenobarbital in CHCl}_3}
\]

was calculated to be 1.226.
Estimation of Protein Binding Sites - Method of Karush (1950)

The mathematical analysis of binding data has been thoroughly discussed by Scatchard (1949), Scatchard et al (1950), Karush and Sonenberg (1949), Karush (1950), Eichman et al (1962), and Keen (1966). These investigators estimated the number of binding sites per molecule of albumin for various drugs 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 linear 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:

\[ \frac{r}{c} = nK - nr \]

Should the r/c versus 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 $r/c (f)$ versus $r$, when $f$ equals $e^{2w(Zp+r)}$. "$e$" is the natural log, "$w$" the Debye-Huckle parameter, and $Zp$ is the net charge on the protein. The relationship between the concentrations of bound and free drugs are represented by the formula (Keen, 1966):

$$r/c (f) = nK - rK$$

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, $n$ 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:

$$r/c = \frac{n_1 K_1}{1 + K_1 c} + \frac{n_2 K_2}{1 + K_2 c}$$

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. This plot should produce a curve similar to that originally drawn and extrapolated (Keen, 1966).

The estimation of the number and character of phenobarbital binding sites per molecule of human serum albumin was determined in the manner described.
CHAPTER VI

BIBLIOGRAPHY


The thesis submitted by Ronald K. Browne has been read and approved by the director of the thesis. Furthermore, the final copies have been examined by the director of the thesis 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 and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

[Signature]

Date: 23 Jan 69