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An Equilibrium Dialysis Study of the Interaction of Proteins with Local Anesthetics

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AN EQUILIBRIUM DIALYSIS STUDY OF THE INTERACTION OF PROTEINS WITH LOCAL ANESTHETICS

by

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LIFE

Vincent John Sawinski was born in Chicago, Illinois on March 28, 1925. In June, 1943, he was graduated from Weber High School, Chicago, Illinois. From Loyola University, he received the degree of Bachelor of Science in February, 1948 and the degree of Master of Arts in February, 1950.

In September, 1949, the writer joined the faculty of Loyola University in the Departments of Chemistry, Physiology, and Pharmacology at the School of Dentistry under the chairmanship of Dr. Gustav W. Rapp.

The author resumed advanced study in the Department of Biochemistry of the Graduate School of Loyola University and attended classes both at the Stritch School of Medicine and at the Lake Shore Campus. He began his research studies at the Stritch School of Medicine under the direction of Dr. Hugh J. McDonald.

Since April, 1957, the candidate has served as an official abstractor for Chemical Abstracts in the section on Biological Chemistry and occasionally in other sections.

With Dr. Hugh J. McDonald, chairman of the Department of Biochemistry at the Stritch School of Medicine, the candidate is co-author of the article, "A Colorimetric Reaction for Tolbutamide," published in Texas Reports on Biology and Medicine, volume 16, page 479, 1958.

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INTRODUCTION

The Importance of Blood Serum Protein-Small Ion Interaction Studies in Biological Sciences.

From the standpoint of biology, blood serum proteins have many functions in the circulatory system. One of their most interesting properties is the role that they play as transporting agents for chloride ion (1), iodide ion (1), mercuric ion (2), steroids (3,4), urea (5), and zinc ion (6), and many other physiological and non-physiological entities.

Importance of interactions of various drugs with blood serum proteins has been analyzed to the greatest extent in connection with pharmacological problems. Notable work in this direction has been done by Storm van Leeuwen (7), Bennhold (8), Davis (9), and Goldstein (10). A number of aspects of these interactions have been studied. To begin with, there have been problems connected with the effective concentration of a drug in the presence of serum proteins. Secondly, the possible role of protein complexes in transporting substances from one part of the body to another has been sought. Finally, the influence of protein binding on the excretion and general persistence of small molecules and

ions in tissues has been widely considered.

Presumably, the biological activity of a drug is dependent, at least in many cases, on uncomplexed drug molecules. Evidently, as more biological processes begin to be viewed on a molecular level, the value of interaction studies will be extended.

Chemical Aspects of Protein Binding of Small Ions.

The aims of a chemical study of the interactions of proteins with relatively smaller ions comprises the following:

1) Determination of the number of interacting molecules that are held by a given protein molecule under specified environmental conditions.

2) Determination of the maximum number of sites on a protein molecule available to a given interacting species.

3) Thermodynamic consideration of the strength of the bond between the protein and any given interacting species.

4) A quantitative statement as to the effect of the environment on the energy of combination.

5) Quantitative information on the structural characteristics of the interacting molecules which favor combination with the protein.

6) Quantitative elucidation of the molecular and configurational nature of the site on the protein at which a given species is bound.

Summary of Procedures.

There are many methods which are available for studying protein interactions. They are conveniently divided into two main groups, namely, the process of studying protein interactions by changes in the smaller interacting molecules and the process which studies changes in the behavior of the protein. The first category includes the following: solubility, reduction in thermodynamic activity (equilibrium dialysis, ultrafiltration, ultracentrifugation, distribution between phases, electromotive force measurements), migration in an electric field, polarographic reduction, diffusion, changes in spectra, and biological activity. Methods which are dependent upon changes in the properties of proteins include: changes in spectra (titration curves, shifts in iso-ionic pH), optical measurements (spectrophotometry, refractometry, light scattering, optical rotation), osmotic pressure, sedimentation, electrophoresis, precipitation, viscosity, surface tension, magnetic properties, and biological activity.

Of the above methods which would most closely resemble the state of affairs in a living system without itself being a living system, the equilibrium dialysis procedure was believed to be most appropriate. Hence, this method was picked to study the interactions of local anesthetics and blood serum proteins.

Usefulness of the method for demonstrating protein interaction with salts was first shown about 50 years ago by Osborne (11) and later by Northrop and Kunitz (12,13,14) in studies of metal-protein complexes. Many recent investigations of other protein interactions have also adopted this technique (15,16). A fairly recent application of equilibrium dialysis to the study of organic ions was that of Klotz, Walker, and Pivan (17) who measured the extent of binding of sulfonate anions from methyl orange and azosulfathiazole by bovine serum albumin. Other significant work with organic ions includes that by Teresi and Luck (18) and the investigations of Klotz, Gelewitz, and Urquhart (19). A list of other chemical moieties which have been shown to interact with serum albumin is shown in Table I.

Technique of Equilibrium Dialysis.

As mentioned previously, this technique is dependent on a reduction in the thermodynamic activity of the interacting substance. The apparatus, in its simplest form, consists of two compartments separated by a membrane. This dialysis membrane is prepared so as to allow free penetration of small interacting molecules only. First, a protein solution is placed in one compartment and a solution of relatively smaller molecules in the other. At equilibrium, the total number of small molecules per unit volume in the protein compartment

TABLE I

LITERATURE SUMMARY OF INTERACTIONS BETWEEN SERUM ALBUMIN AND VARIOUS CHEMICAL ENTITIES.

Chemical Entity	Literature Reference
Adenine	Klotz (20)
Adenosine	Klotz (20)
Adenylic Acid	Klotz (20)
Alkaloids	Beutner (21)
Aromatic Carboxy Acids	Teresi (18)
Barbiturates	Goldbaum (22)
Calcium	Klotz (23)
Copper	Klotz (24)
Dodecyl Sulfate	Putnam (25)
Estrogens	Boettiger (3)
Fatty Acids	Boyer (26)
Penicillins	Tompsett (27)
Phenol Red	Grollman (28)
Sulfonamides	van Dyke (29)
Sulfonated Azo Dyes	Klotz (30)

will be greater than the total number of small molecules per unit volume in the original small molecular compartment. This is a result of an interaction occurring between the protein and the small molecules. The binding data are assembled into appropriate graphic representations and the equations of multiple equilibria are applied to calculate the degree of binding of the small molecules with the protein molecules. If no interaction should occur between the small molecules and the protein, the concentration of the small molecule in the one compartment with the protein should be approximately equal to the concentration of the small molecule in the other compartment without the protein, at equilibrium, within limits of experimental error. In this latter case, application of the equations for multiple equilibria is not possible since the degree of binding of the small molecules with the protein molecules is negligible.

Drugs Used in This Study.

The drugs used in the investigation belong to a class known as local anesthetics. Local anesthetics are drugs which in sufficient concentration are capable of blocking nerve conduction along both sensory and motor fibers. When carefully controlled concentration is used, it is possible to produce loss of sensation without motor paralysis because sensory fibers are always affected before motor fibers. Most local anesthetics, when used properly, can produce anesthesia in

a certain definite portion of the body without producing a systemic effect. Some of the anesthetic agents exert not only a local action but also a central one. The synthetic agents, however, have slight effect on the central nervous system. These synthetic agents are the type of drugs which were chosen for this study.

The particular drugs which were considered are:

1) Procaine·HCl (Novocaine·HCl). It is 2'-diethyl-aminoethyl-4-aminobenzoate hydrochloride.

2) Butethamine·HCl (Monocaine·HCl) (31,32). It is 2'isobutylaminoethyl-4-aminobenzoate hydrochloride.

3) Lidocaine·HCl (Xylocaine·HCl) (33,34). It is ∝-diethylamino-2,6-aceto-xylidide hydrochloride.

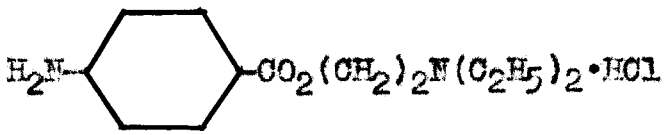
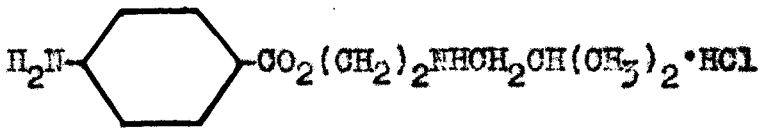
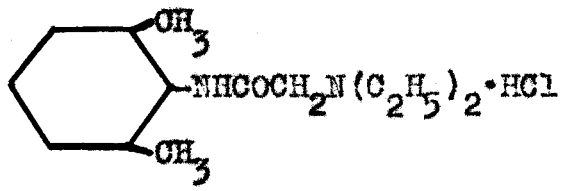
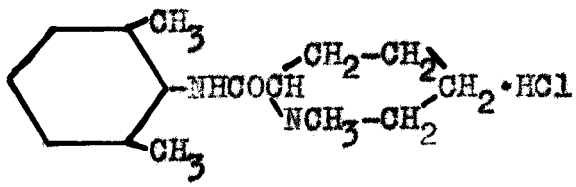
4) Mepivacaine·HCl (Carbocaine·HCl (35,36,37,38). It is d,1-N-methyl-pipecolic acid-2,6-dimethylanilide hydrochloride.

The chemical formulas of these compounds are shown in Table II.

The most common group of local anesthetics is composed of complex nitrogen containing compounds. Their structures all conform to a general configuration and three groups and linkages are recognized in their molecules: 1) An aromatic nucleus; 2) a nitrogen atom or nitrogen containing heterocyclic nucleus; and 3) an intervening side chain which

TABLE II

LOCAL ANESTHETIC AGENTS USED IN THE STUDY

Generic Name and (Proprietary Name)	Chemical Formula
1) Procaine•HCl (Novocaine•HCl)	
2) Butethamine•HCl (Monocaine•HCl)	
3) Lidocaine•HCl (Xylocaine•HCl)	
4) Mepivacaine•HCl (Carbocaine•HCl)	

separates these two. One or two atoms of nitrogen, usually in the form of an amino group, are always present in the molecule. The amino groups, which are usually tertiary or secondary, confer basic properties to these derivatives. The majority of local anesthetics of importance are esters or amides. Of the local anesthetics used in this study, procaine and butethamine are esters, while lidocaine and mepivacaine are amides.

Proteins Used in This Study.

In regard to the stable protein complexes, much attention has been directed toward interactions between native proteins and small organic anions, since many small molecules of biological importance normally occur as charged species in aqueous media. The nature of these interactions varies from the extreme specificity of serological reactions to the comparative non-specificity of serum albumin with regard to structure (39,40), size, and isomerism of the small organic anion. Serum albumin is an outstanding protein in its ability to bind anions in a very general, non-specific way(41). There are several other proteins, such as beta lactoglobulin, etc, which approach the binding affinity of serum albumin, but they are not as interactive, and the great majority of proteins will not form complexes non-specifically with organic anions (42,43).

Interactions of proteins with small neutral molecules have been less extensively investigated but combinations with the small non-electrolytes have been observed (44). Pasynskii and Chernyak (45) have described combinations of small neutral molecules and the serum globulins and albumin. Also, it has been known for some time that cholesterol, some of its esters, and related steroids form complexes with serum albumin and globulins (46).

Investigations of cationic interactions with proteins have been relatively few as compared with the extensive studies done with organic anions. Recent work indicates that interactions with cations show some similarities as well as some major differences to interactions with anions. Quantitative comparisons of binding ability by the equilibrium dialysis method show, however, that among ions of equal size, organic cationic dyes are bound much less than organic anionic dyes by plasma albumin (19). On the other hand, Glassman (47) has observed that long chain detergents form a stoichiometric complex with plasma albumin, in which the number of detergent molecules corresponds closely to the number of carboxyl groups on the protein.

Various organic compounds, as has been pointed out previously, are effective local anesthetic agents and their usefulness in the treatment of patients has been demonstrated.

On the other hand, certain limitations to the effectiveness of the drugs have resulted from unfavorable side effects which develop in some patients following increasing concentrations of the drugs. If these side effects, which may become quite serious in some patients and result in necessary withdrawal of the drugs, could be eliminated, then the usefulness of the drugs could be greatly extended. As has been stated, the plasma proteins, by interacting with small organic ions and molecules, render them biologically inactive, thus decreasing the concentration of free drug and in turn reducing the toxic effects. Also, the interaction between local anesthetics and the plasma proteins would aid in the transport of the compounds from one part of the body to another as well as reducing the rate of excretion of the compounds from the animal body. This investigation was undertaken in an effort to determine the extent of interaction between local anesthetics and two readily obtainable purified proteins, namely, human serum albumin and human serum gamma globulin.

The Purpose of This Study.

In the light of the previous discussions of this introduction, the purpose of this study on the interaction of proteins with local anesthetics was to determine the extent, if any, of the binding of certain human serum proteins with a select group of local anesthetic agents. Another aspect of

this investigation was an attempt to correlate the extent of the interactions with some of the properties of local anesthetics in reference to their approach to an ideal anesthetic. The chief properties of an ideal local anesthetic will be discussed later, but let it be said here that one characteristic of an ideal local anesthetic agent is that it be devoid of any allergy producing characteristics. Since allergies may be due to an antibody - antigen reaction, it may well be that the binding of a local anesthetic by a serum protein leads to the production of a foreign protein which in turn initiates antibody formation against this foreign protein; hence, the result is an allergy reaction.

CHAPTER II

HISTORICAL BACKGROUND

The introduction of cocaine as a local anesthetic into the arsenal of therapeutics is one of the great landmarks in this field. Wöhler, the well-known chemist, whose pupil Niemann in 1859 isolated cocaine, wrote in 1860: "The compound has a bitter taste and affects the nerves of the tongue in a peculiar way, for a short while making the site of application numb and almost devoid of any sensation." However, this observation was not utilized until the Austrian ophthalmologist, Karl Koller, in 1884 established the local anesthetic properties of cocaine on the eye. In the same year cocaine was used in dentistry by Hall (48) and within a year for all kinds of surgery, as reported by Halsted (49) in 1885. But a number of side effects of the drug were noticed, such as, intoxication on overdosage with confusion and hallucinations, or in other cases convulsions, respiratory and circulatory failure, the risk of abuse, and deterioration of the compound when being sterialized. Within the first 5 years, 175 cases of intoxication, including 10 fatalities were reported. It was these developments, then, that actualized the search for new substances which would be

equal or superior to cocaine in their anesthetic properties but with less incidence of complications.

One contribution was the combination of cocaine with adrenaline, which prolonged the anesthetic effect, and thus implied a decrease of the amount injected. Another contribution was the introduction of a new series of anesthetics, chemically related to p-amino-benzoic acid, starting with procaine (novocaine), introduced by Einhorn in 1905, and followed by benzocaine, pantocaine, tutocaine, butyn, monocaine (32,50), metycaine, and others, just to mention a few of those mostly used.

In 1935, two Swedish chemists, Erdtman and Löfgren, while synthesizing indole derivatives noticed an isomer of the alkaloid gramine (donaxin) to have an anesthetic effect, when tested on the tongue. In 1937, fourteen substances belonging to this series of anilides were reported. Their anesthetic properties were established by U. S. von Euler. Continued research with these compounds led to the synthesis of xylocaine in 1946 followed by its introduction into clinical work (33) in 1948.

Carbocaine is a local anesthetic agent described by Eckenstam, Egnér, and Pettersson (37) in 1956. Carbocaine belongs to a group of cyclic acids with pipecolic acid as its acid component. The nitrogen in the piperidine ring

is methylated and its aromatic component consists of a bond with the dimethyl anilide.

By analgesia is generally meant insensibility to pain, whereas other sensations as heat and cold, itching, tingling, vibratory, and kinesthetic sensations are generally not included in the term. Pain is usually conceived as a sensation produced 1) peripherally by a stimulus acting on the minute sensory fibers which is 2) conducted along special pathways, larger and thicker fibers, to the spinal cord and 3) to a reception and perception center, localized in the superficial layer of a well defined region of the brain, the precentral gyrus. Accordingly, the sensation of pain can be interrupted at any part of this system. General analgesia interrupts pain sensations at the center [3] , whereas local analgesia produces painlessness by acting either at the periphery [1] or [2] , the latter by blocking a main nerve, as in spinal anesthesia, where the large nerves leaving the spinal canal are blocked, that is, are made incapable of conducting pain and other sensory sensations.

The common local anesthetics are organic bases. Since they are not readily soluble in water and since their solutions are not stable, the local anesthetics in practical anesthesiology are used in the form of salts, for the most part as hydrochlorides. It has been advocated by Gros in

1910 that the anesthetic effect is produced only by the anesthetic bases and by Trevan and Boock in 1924 and Gardner and Semb (51) in 1935 that the potency of an anesthetic is directly proportional to the amount of free base present.

The exact knowledge of the efficiency of local anesthetics under clinical conditions is, contrary to what would be expected, very slight. Only scattered information obtained by different types of laboratory experiments is usually available. Most local anesthetics have been investigated with the aid of several methods and a multitude of figures are to be found in the literature. It is of primary importance to realize that data obtained with each of these methods can be directly compared only with data obtained under identical experimental conditions, and cannot without great care be related to results arrived at by other means, as has often been done. Even with the same method different workers do not always obtain precisely identical results (52,53,54). Potency values arrived at in animal experiments cannot be directly assumed to be valid for man even if experience shows most anesthetics to display the same relative effect in different mammalian species. For final conclusions, experiments must be carried out on human subjects. Unfortunately, there are few methods suitable for this use. At best, only a general idea of the clinical value of a particular drug can be obtained with the available methods.

The ideal local anesthetic should possess certain fundamental pharmacologic characteristics including 1) local anesthetic properties which make it useful for all types of regional analgesia, whether this be infiltration, topical application, or injection about a nerve; 2) selectivity of action, that is, the action should be confined principally to nerve tissue; 3) low toxicity, particularly in concentrations used clinically; 4) complete reversibility of action, so that after its effects disappear, normal function of the nerve or nerves returns without any sequelae; 5) the drug should not cause any local pain during or after the injection; 6) the time required for the onset of analgesia (latency) should be reasonably short; 7) the duration of analgesia should be sufficiently long; and 8) it should not produce idiosyncratic reaction. In addition it should meet the following physicochemical requirements: 1) it should be sufficiently soluble in saline and water to be easily preparable; 2) it should be amenable to sterilization without decomposition; 3) it should be compatible with vasoconstrictive drugs; 4) when placed in solution, the drug should be stable and its action unimpaired by light, air, or minor variations in pH.

The ideal local anesthetic agent would have all of these properties to the optimal degree, but, unfortunately, such a drug has not yet been made available. Although a particular

drug may not meet these requirements of the ideal agent, it may still be useful clinically if it provides certain definite advantages. Thus, under certain circumstances (for example, postoperative pain) one may willingly compromise on rapid onset of analgesia if the drug is capable of producing a prolonged block.

CHAPTER III

THEORETICAL TREATMENT OF SYSTEMS EXHIBITING MULTIPLE EQUILIBRIA

The earliest attempts at investigating protein complexes indicated that more than one interacting species could be bound on a given protein molecule and, as a result of this, mathematical treatments involving multiple equilibria were developed to treat the resultant data (55). Development of the pertinent equations was quickened by workers not so much interested in protein interactions but in adsorption and electrostatic processes (56,57).

If the assumption is made that all binding sites on a protein molecule that are capable of interacting have identical affinities for the drug molecules and that the affinity of any group is unaffected by the combination of drug molecules with other groups, several related equations can be derived from the law of mass action (58).

For n sites of interaction of X with protein P, the equilibria involved are

$$P + X = PX,$$

$$PX + X = PX_2,$$

. . . . . .

$$PX_{i-1} + X = PX_i, \qquad [1]$$

. . . . . .

$$PX_{n-1} + X = PX_n.$$

In terms of equilibrium constants, the following relationships are obtained according to the law of mass action:

$$\frac{(PX)}{(P)\ (X)} = k_1, \qquad [2]$$

$$\frac{(PX_2)}{(PX)\ (X)} = k_2, \qquad [3]$$

. . . . . .

$$\frac{(PX_i)}{(PX_{i-1})\ (X)} = k_i, \qquad [4]$$

. . . . . .

$$\frac{(PX_n)}{(PX_{n-1})\ (X)} = k_n. \qquad [5]$$

From these equations it follows that

$$(PX) = k_1(P)\ (X), \qquad [6]$$

$$(PX_2) = k_2(PX)\ (X) = k_1k_2(P)\ (X)^2, \qquad [7]$$

. . . . . .

$$(PX_i) = k_i(PX_{i-1})\ (X) = (k_1k_2 \cdots k_i)\ (P)\ (X)^i, \qquad [8]$$

. . . . . .

$$(PX_n) = k_n(PX_{n-1})\ (X) = (k_1k_2 \cdots k_i \cdots k_n)\ (P)\ (X)^n. \qquad [9]$$

If each protein site is uninfluenced by a neighboring site and if each site has the same intrinsic affinity for X, then the equilibrium constants $k_1 \cdots k_i \cdots k_n$ are not independent

but bear certain definite relationships to each other. For the first complex PX, there are n possible forms of PX, depending upon the particular site on P to which X is attached. If the possible forms of PX are distinguished by the notation $_1PX$, $_2PX$. . . $_iPX$. . . $_nPX$, and if the intrinsic affinity of each site for X is identical, then the equilibrium constant, k, for the association

$$P + X = {}_1PX \qquad [10]$$

is the same as that for

$$P + X = {}_2PX, \qquad [11]$$

and so on.

Hence,

$$k = \frac{(_1PX)}{(P)\ \ (X)} = \frac{(_2PX)}{(P)\ \ (X)} = \ldots = \frac{(_nPX)}{(P)\ (X)} . \qquad [12]$$

But

$$(PX) = (_1PX) + (_2PX) + \ldots + (_nPX), \qquad [13]$$

so that

$$k = \frac{(PX)}{(P)\ \ (X)} = \frac{(_1PX) + (_2PX) + \ldots + (_nPX)}{(P)\ \ (X)} \qquad [14]$$

$$= k + k + \ldots + k = nk.$$

For the complex PX_2, there are $\frac{n(n-1)}{2}$ possible forms depending on the particular combination of two sites on P to which the two X's are attached. This is obtained from the general algebraic relationship for the number of possible

combinations of n sites taken m at a time.

$$ {}_nC_m = \frac{n!}{m!\ (n-m)!} \cdot \qquad [15] $$

Here m = 2, so that

$$ {}_nC_2 = \frac{n!}{2!\ (n-2)!} = \frac{n(n-1)}{2} \cdot \qquad [16] $$

The different complexes of type PX_2 may be denoted by the symbols ${}_{1,2}PX_2$, ${}_{1,3}PX_2$, $\cdots$ ${}_{j,1}PX_2$, where the subscripts at the left indicate the sites on P at which the two X's are attached. But

$$ ({}_1PX) = ({}_2PX) = \cdots = ({}_nPX), \qquad [17] $$

since the values of k, (P), and (X), respectively, are identical. An analogous set of equilibrium expressions can be written for the complexes involving ${}_{j,1}PX_2$, from which it is a simple matter to prove that

$$ ({}_{1,2}PX_2) = ({}_{1,3}PX_2) = \cdots = ({}_{j,1}PX_2) \qquad [18] $$

if it is assumed that the intrinsic affinity constant, k, is the same for any single site taking up one X. Therefore,

$$ k_2 = \frac{(PX_2)}{(PX)\ \ (X)} $$

$$ = \frac{\left[({}_{1,2}PX_2) + \cdots + ({}_{j,1}PX_2) + \cdots \right]}{\left[({}_1PX) + \cdots ({}_iPX) + \cdots \right] (X)} $$

$$= \frac{\frac{n(n-1)}{2} \; (_{1,2}PX_2)}{n \; (_1PX) \quad (X)}$$

$$= \frac{n-1}{2} k. \qquad [19]$$

For the complex PX_1,

$$k_1 = \frac{(PX_1)}{(PX_{1-1}) \; (X)} \; . \qquad [4]$$

If n binding sites are taken i at a time,

$$_nC_i = \frac{n!}{i! \; (n-i)!} \; . \qquad [20]$$

For PX_{i-1},

$$_nC_{i-1} = \frac{n!}{(i-1)! \; [n-(i-1)]!} \; . \qquad [21]$$

Therefore,

$$k_i = \frac{\frac{n!}{i!(n-i)!}}{\frac{n!}{(i-1)!(n-i+1)!}} k$$

or

$$k_i = \frac{n-i+1}{i} k \qquad [22]$$

The factor $\frac{n-i+1}{i}$ can also be derived from statistics so that it is frequently referred to as the statistical factor.

A convenient method of measuring the extent of combina-

tion of small molecule X with the protein is the quantity

$$r = \frac{\text{Moles bound X}}{\text{Moles total protein}} \qquad [23]$$

Since the number of moles of bound X equals the number of moles of PX plus twice the number of moles of PX_2, etc. we may write

$$r = \frac{(PX) + 2(PX_2) + \cdots + i(PX_i) + \cdots + n(PX_n)}{(P) + (PX) + (PX_2) + \cdots + (PX_i) + \cdots + (PX_n)} \qquad [24]$$

Also,

$$r = \frac{k_1(P)(X) + 2k_1k_2(P)(X)^2 + \cdots + i(k_1k_2 \cdots k_i)(P)(X)^i + \cdots}{(P) + k_1(P)(X) + \cdots + (k_1k_2 \cdots k_i)(P)(X)^i + \cdots}$$

or

$$r = \frac{k_1(X) + 2k_1k_2(X)^2 + \cdots + i(k_1k_2 \cdots k_i)(X)^i + \cdots + n(k_1k_2 \cdots k_n)(X)^n}{1 + k_1(X) + k_1k_2(X)^2 + \cdots + (k_1k_2 \cdots k_i)(X)^i + \cdots + (k_1k_2 \cdots k_n)(X)^n} \qquad [25]$$

But $k_1 = nk$,

$$k_2 = \frac{n-1}{2}k, \qquad [19]$$

$$\cdots\cdots\cdots$$

$$k_i = \frac{n-i+1}{i}k, \qquad [22]$$

$$\cdots\cdots\cdots$$

$$k_n = \frac{n-n+1}{n}k = \frac{1}{n}k. \qquad [26]$$

Therefore,

$$r = \frac{nk(X) + \frac{2n(n-1)}{2!}k^2(X)^2 + \cdots + i\frac{n(n-1)\cdots(n-i+1)}{i!}k^i(X)^i + \cdots + n\frac{n!}{n!}k^n(X)^n}{1 + nk(X) + \frac{n(n-1)}{2!}k^2(X)^2 + \cdots + \frac{n(n-1)\cdots(n-i+1)}{i!}k^i(X^i) + \cdots + \frac{n!}{n!}k^n(X)^n} \cdot \quad [27]$$

But the denominator is

$$\left[1 + k(X)\right]^n \quad [28]$$

Also, the numerator is

$$= (X)\frac{\partial}{\partial(X)}(\text{denominator}) = (X)\frac{\partial}{\partial(X)}\left[1 + k(X)\right]^n \quad [29]$$

$$= (X)nk\left[1 + k(X)\right]^{n-1} .$$

Hence,

$$r = \frac{(X)nk\left[1 + k(X)\right]^{n-1}}{\left[1 + k(X)\right]^n} \quad [30]$$

$$r = \frac{nk(X)}{1 + k(X)} \cdot \quad [31]$$

Rearranging, we have

$$\frac{1}{r} = \frac{1}{nk}\frac{1}{(X)} + \frac{1}{n} \quad [32]$$

or

$$\frac{r}{(X)} = km - kr. \qquad [33]$$

Either of these latter two equations, namely, [32] and [33], provides a linear relationship which is useful in the evaluation of n. For example, if equation [32] is used and 1/r is plotted as the ordinate versus 1/(X) as the abscissa, the intercept on the ordinate is 1/n. Using equation [33], if r/(X) is plotted as the ordinate versus r as the abscissa, the intercept on the abscissa is n.

CHAPTER IV

EQUILIBRIUM DIALYSIS METHODS AND MATERIALS

Experimental Procedure of Equilibrium Dialysis.

The equilibrium dialysis method was chosen as a means of investigating the extent of interaction between the local anesthetics and the two purified proteins, human serum albumin and human serum gamma globulin since it appeared to be the most reliable of the many methods available. In order to determine the extent of binding, it was necessary to have some analytical method available by which the concentration of the free, unbound local anesthetic in the protein free compartment could be measured at the time that equilibrium had been attained.

In practice, there are two inherent sources of error in the equilibrium dialysis method. First, there may be some asymmetry in the distribution of the small molecule, if it is ionic, as a consequence of the Donnan membrane effect. Suitable corrections can be made for the Donnan effect, or an inocuous electrolyte can be added to reduce it to negligible proportions. Also, investigation may be undertaken at a pH where the protein exhibits its isoelectric point and the asymmetric distribution of the small ions due to the Donnan

membrane effect is eliminated. Secondly, there may also be some adsorption of the small molecule to the membrane but this can be corrected by setting up control dialyses in which the protein is absent and measuring the depletion of the small molecule from the solution. The effect of osmotic pressure is negligible with the low concentration of protein used in these experiments since this physical property is dependent on the number of particles present, which in this case are relatively few due to the rather large molecular weights of most proteins.

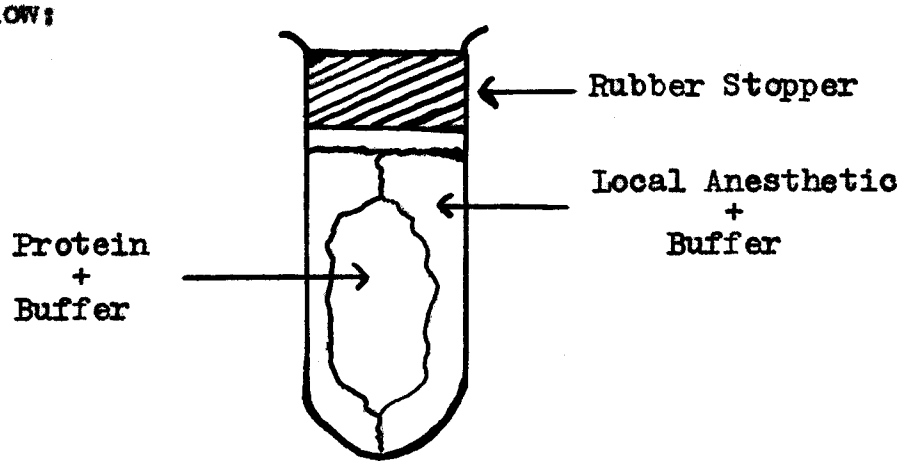
Experiments using the method of equilibrium dialysis were devised to produce quantitative data of the interaction of proteins with the relatively small local anesthetic molecules. Fifty ml. samples of a buffered solution of a local anesthetic at several known concentrations were pipetted into 200 mm. X 38 mm. Pyrex test tubes. A dialysis bag was prepared containing 50 ml. of the protein solution in the same buffer as the local anesthetic, the bag being tied at each end. Then the bag was immersed in the buffered local anesthetic solution previously placed in the Pyrex test tubes and the tubes tightly stoppered with clean, dry rubber stoppers. A control dialysis assembly was prepared to accompany each sample unit to eliminate any errors due to adsorption of the local anesthetic on the cellulose tubing. The control

assemblies differed from the experimental assemblies only in that they contained 50 ml. of buffer inside the dialysis sack rather than the 50 ml. of buffered protein solution. All of the dialysis systems were allowed to equilibrate at a constant temperature of 4°C. This temperature was used because lower temperatures inhibit protein hydrolysis and denaturation. Also, many investigations have demonstrated that temperature changes do not greatly affect the extent of binding of ions (59). After equilibrium was reached, as determined by using a series of identical vessels and removing and analyzing samples at varying intervals to ascertain the time necessary for the concentration of local anesthetic outside the bag to show no further change in concentration, an aliquot of the solution outside the bags was removed and analyzed for unbound local anesthetic.

To determine if there was significant adsorption of the local anesthetic to the cellulose tubing used in the dialysis assemblies, an analysis was made of the local anesthetic solution used in constructing the dialysis assembly. One-half of this concentration would represent the concentration of local anesthetic which would be outside the dialysis bag of the control tubes at equilibrium if there were no adsorption taking place. The control tubes consisted of a 50 ml. aliquot of buffer solution contained in a dialysis bag and suspended

in 50 ml. of the local anesthetic solution. The results of this analysis showed that there was no significance in adsorption of any of the small molecules to the dialysis tubing.

An illustration of a typical dialysis assembly showing the test tube with the cellophane dialysis bag in it is shown below:



The term "unbound drug" rather than the term "free drug" is used in these discussions in opposition to the term "bound drug" to designate that portion of the particular local anesthetic studied which does not complex with protein. The term "free drug" is employed in two utterly unrelated contexts in the scientific literature. First, it is meant to distinguish a drug in its original state from the same drug after conjugation (e.g. "free sulfonamide" as opposed to conjugated sulfonamide). Second, it is used to connote unbound drug as opposed to protein-bound drug. While this latter sense would conform to general usage in physical chemistry, the

former sense is well established in pharmacology and related endeavors. In order to be consistent, correct, and, at the same time less easily misunderstood, it was believed that the term "unbound drug" rather than the term "free drug" would be more appropriate in this investigation.

Materials for Equilibrium Dialysis.

After quantitative analytical methods were developed to determine the unbound local anesthetic concentrations, equilibrium dialysis experiments were performed to determine the extent of binding by the two purified human serum proteins, namely, albumin and gamma globulin. They were chosen because the ability of blood serum proteins to bind organic molecules and the physiological consequences of such interactions have been realized for many years (60). Also, it is known that of the various protein fractions of blood serum, albumin shows the most significant degree of binding with organic molecules and gamma globulin is usually associated with antibody - antigen interactions (61).

1) Crystallized Human Serum Albumin: This was a crystalline product obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, lot 5685 (100% pure by electrophoresis). The molecular weight of the human serum albumin was taken as 7.0×10^4.

2) Human Serum Gamma Globulin: This was Fraction II from

human serum obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, lot 2355 (96.3% pure by electrophoresis).

3) Dialysis Tubing: The dialysis tubing was obtained from the Visking Company, Chicago, Illinois. It has an inflated diameter of 36 mm. The tubing is a seamless product made of regenerated cellulose by the viscose process. Except for traces of water, glycerin, and sulfur, the tubing is pure cellulose. It is permeable to water and will permit the passage of low molecular weight compounds in aqueous solution while retaining higher molecular weight materials such as proteins. Performance of the biuret test on the solution outside the dialysis bags showed that none of the human serum albumin or human serum gamma globulin escaped from the dialysis sacks made of the cellulose tubing. Since the manufacturer treated the material with a humectant to prevent brittleness, the tubing was exhaustively dialyzed in distilled water prior to all equilibrium dialysis studies in order to remove the material which was probably glycerin.

4) Buffers: The phosphate buffers used in the equilibrium dialysis experiments were prepared from analytical reagent grade chemicals. The concentration of the buffers,

M/15, was selected so as to be large enough to render the Donnan correction negligible for the protein concentrations used in the equilibrium dialysis experiments (62). The reagents that were used were Baker's Analyzed Potassium Dihydrogen Phosphate, KH_2PO_4, and Baker's Analyzed Disodium Hydrogen Phosphate, $Na_2HPO_4 \cdot 12H_2O$.

5) Local Anesthetics: The four pure crystalline local anesthetic agents used in the study were:

   a) Novocaine Hydrochloride, obtained from Mallinckrodt Chemical Works, St. Louis, Missouri, lot 7172,JTN.

   b) Monocaine Hydrochloride, obtained from Novocol Chemical Manufacturing Company, Brooklyn, New York, lot H15.

   c) Xylocaine Hydrochloride, obtained from Astra Pharmaceutical Products, Worcester, Massachusetts, lot 467.

   d) Carbocaine Hydrochloride, obtained from Cook-Waite Laboratories, New York, New York, lot R-013-TF.

General Procedure for Data Acquisition.

In order to collect the necessary data for this investigation, the following general procedure was followed for each experiment:

1) Buffered solutions of various concentrations of the local anesthetic were prepared so as to encompass as closely as possible a 100-fold to a 1000-fold concentration range of the drug.

2) Two sets of duplicate dialysis assemblies were set up for each concentration of the local anesthetic used.
   a) One set of duplicates had blank dialysis assemblies containing buffered drug solutions outside the dialysis bags and plain buffer solutions inside the dialysis bags. This set acted as a control.
   b) The other set of duplicates had dialysis assemblies containing buffered drug solutions outside the dailysis bags and buffered protein solutions inside the dialysis bags.
3) After equilibrium had been established, the concentrations of the local anesthetic of both sets of dialysis assemblies were determined by the appropriate quantitative methods and the average values of the first set were used to indicate the concentrations of the drugs before dialysis and the average values of the second set were used to indicate the concentrations of the drugs after equilibrium had been reached. Use of the first set of dialysis assemblies eliminated any error in the experiments which might be caused by adsorption of the drugs to the dialysis bags. The concentration of the proteins of the second set was low enough to obviate any Donnan effect which might occur but at the same time high

enough so that, at the highest concentrations of the drugs used, the ratios of the concentrations of the proteins to the concentrations of the drugs approached the ratios of these entities when found in the living organism upon injection of the drugs.

4) From the appropriate mathematical analysis of the data, the results of the experiments were graphed in order to determine the number of statistical binding sites, n, of the drugs on the proteins, if any binding does indeed occur.

   a) One method of determining n is to plot 1/r as ordinate versus $\frac{1}{(X)}$ as abscissa, where r is the ratio of bound drug molecules to total protein molecules, and (X) is the concentration of unbound drug molecules at equilibrium. The intercept on the ordinate of the extrapolated theoretical straight line obtained from the points is 1/n, the reciprocal of the number of binding sites for the drug on the protein.

   b) Another method of determining n is to plot $\frac{r}{(X)}$ as ordinate versus r as abscissa. The intercept on the abscissa of the extrapolated theoretical straight line obtained from the graph then is n, the theoretical number of binding sites on the protein for the drug.

Spectrophotometric Method for Procaine and Butethamine Analysis.

The method selected for the determination of procaine and butethamine was a modification of the method of Zollner (63) for the colorimetric determination of diazotizable amines. Essentially, the procedure consists of diazotizing the free amino group of procaine or butethamine with nitrous acid followed by coupling of the diazotization product with thymol to yield an orange colored product in alkaline solution.

Reagents for Procaine and Butethamine Determination.

1) 0.1N HCl - The HCl solution was prepared from DuPont's Hydrochloric Acid Reagent (36.5 - 38.0% by weight) dissolved in distilled water.
2) 0.5% $NaNO_2$ - This solution was prepared fresh daily from Baker's Analyzed Sodium Nitrite (crystal) dissolved in distilled water.
3) 0.5% Thymol in 95% Ethanol - A 0.5% Thymol solution in Ethyl Alcohol was prepared fresh daily from Baker and Adams, U.S.P., Thymol (crystal) dissolved in 95% Ethyl Alcohol.
4) 5% NaOH - This reagent was prepared by dissolving Baker's Analyzed Reagent Sodium Hydroxide pellets in distilled water.

Experimental Procedure for Procaine and Butethamine Determination.

An aliquot of the aqueous solution containing 0.1 mg.

to 1.0 mg. procaine·HCl or butethamine·HCl was pipetted into a 100 ml. volumetric flask. In some cases where the volume of the aliquot was relatively large, the solution was acidified, as a preliminary step, with a few drops of concentrated hydrochloric acid to destroy the buffer capacity of the buffer in the experimental anesthetic solution. Next, 2 ml. of 0.1 N HCl was added and the flask was cooled in water. Then 1 ml. of 0.5% sodium nitrite was added and the solution was allowed to stand for 3 minutes. Next, 0.4 ml. of 0.5% alcoholic thymol solution and 4 ml. of 5% NaOH were added. After mixing, the volume was brought up to 100 ml. with distilled water and mixed thoroughly. The optical density of 10 ml. of the orange solution was measured in duplicate with a Coleman Universal Spectrophotometer at 465 mμ within one hour.

A blank solution containing all of the reagents except procaine or butethamine diluted to 100 ml. with distilled water was prepared for each set of determinations at the same time as the colored solutions containing the procaine or butethamine, and this blank solution was used to standardize the spectrophotometer.

Preparation of a Standard Curve for Procaine and Butethamine.

In order to determine the range over which procaine or butethamine follows the Beer-Lambert law, it was necessary to prepare a standard curve. The standard curve was prepared by

making up various concentrations of procaine·HCl or butethamine·HCl and running them through the colorimetric procedure. The standard curve was found to be linear for aliquots of procaine or butethamine solutions containing about 0.1 mg. to about 1.0 mg. Beyond this amount of procaine·HCl or butethamine·HCl in the final colored solution the standard curve deviated from Beer's law and in subsequent equilibrium dialysis experiments, the necessary volumes of procaine·HCl or butethamine·HCl solutions were used in order to work within the range over which Beer's law was applicable.

Data for the standard curves are given in Table III for procaine·HCl and in Table IV for butethamine·HCl. The standard curve for procaine·HCl is reproduced in Figure 1 and the standard curve for butethamine·HCl is reproduced in Figure 2.

Spectrophotometric Method for Lidocaine and Mepivacaine Analysis.

Various organic bases are known to react with ammonium reineckate reagent in aqueous solution producing precipitates which can be isolated, dissolved in acetone, and determined speotrophotometrically by measuring the optical density at 530 mμ. It has also been found by Ortenblad (64) that under controlled conditions lidocaine is readily and quantitatively precipitated by ammonium reineckate so that it too may be accurately estimated in the same manner. It was a modification

this method that was chosen for determining the concentration of lidocaine·HCl and mepivacaine·HCl in the equilibrium dialysis experiments.

Reagents for Lidocaine and Mepivacaine Determination.

1) HCl - 6N HCl was prepared from DuPont's Hydrochloric Acid Reagent (36.5 - 38.0% by weight) dissolved in distilled water.
2) Ammonium Reineckate - The saturated solution was freshly prepared on the day of use. It was made by shaking 1.5 grams of the salt with 100 ml. of distilled water at room temperature for 15 minutes and then filtering. The salt was obtained from Matheson, Coleman, and Bell Company, lot AX 1365.
3) Acetone - Baker's Acetone, N.F., was used without purification as the solvent for the colored precipitate.

Experimental Procedure for Lidocaine and Mepivacaine Determination.

A volume of solution containing between 1.0 and 150 mg. of lidocaine·HCl or mepivacaine·HCl is acidified with HCl to pH 2 or less, and 5 ml. of a saturated aqueous solution of ammonium reineckate is then slowly added with vigorous stirring (for every 10 ml. of the original solution). The mixture is then allowed to stand for one hour after which

time the precipitate is collected by suction on a small filter and washed with two portions of distilled water (2 ml. per portion). When drained, the precipitate is dissolved in acetone while still on the filter and the resulting violet colored solution is transferred to a 10 ml. volumetric flask or a 100 ml. volumetric flask depending upon the amount of lidocaine·HCl or mepivacaine·HCl which was present in the original solution. The solution is diluted with acetone and the optical density is measured in duplicate with a Coleman Universal Spectrophotometer at 530 mμ within four hours by transferring 10 ml. of the colored solution to a colorimeter tube. The spectrophotometer is standardized at the same time by simultaneously preparing a blank solution using the above procedure for distilled water.

Preparation of a Standard Curve for Lidocaine and Mepivacaine.

The range over which lidocaine·HCl or mepivacaine·HCl follows the Beer-Lambert law was determined by preparing a standard curve. This was done by making up various concentrations of lidocaine·HCl or mepivacaine·HCl and carrying out the procedure previously described. The standard curve was found to be linear for solutions containing about 15 to 150 mg. lidocaine·HCl or mepivacaine·HCl when the final acetone solution had a volume of 10 ml. In subsequent equilibrium

dialysis experiments, the necessary volumes of lidocaine·HCl or mepivacaine·HCl solutions were used in order to yield colored solutions within the range over which Beer's law was applicable.

Data for the standard curves are given in Table V for lidocaine·HCl and in Table VI for mepivacaine·HCl. The standard curve for lidocaine·HCl is reproduced in Figure 3 and the standard curve for mepivacaine·HCl is reproduced in Figure 4.

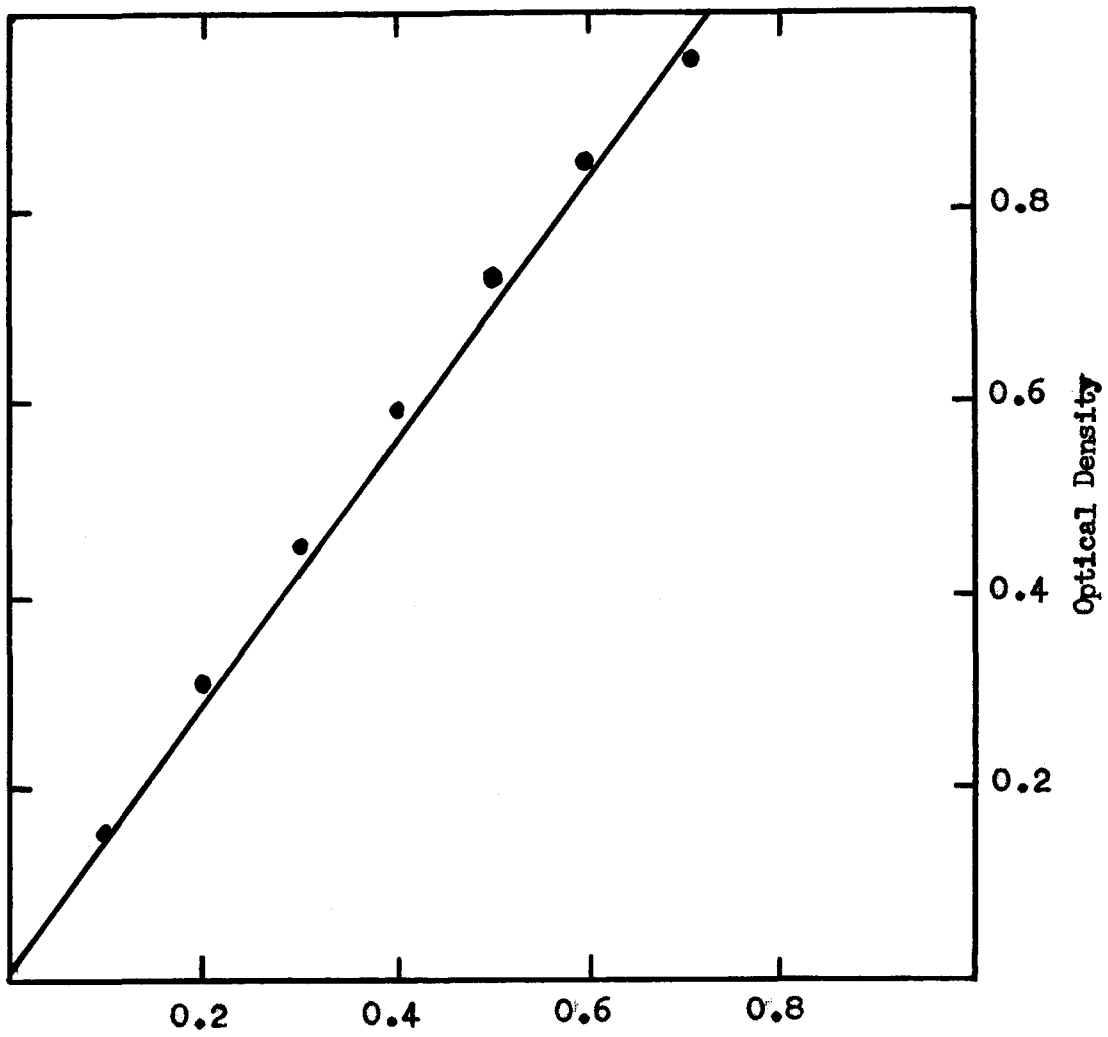
TABLE III

STANDARD CURVE DATA FOR PROCAINE·HCl

Milligrams of Procaine·HCl per 100 ml. Colored Solution	Optical Density (Average)
0.1	0.159
0.2	0.314
0.3	0.460
0.4	0.600
0.5	0.737
0.6	0.853
0.7	0.962
0.8	1.060
0.9	1.150
1.0	1.230

FIGURE 1

STANDARD CURVE FOR PROCAINE·HCl



Milligrams of Procaine·HCl per 100 ml. Colored Solution

Graph of the Data of Table III

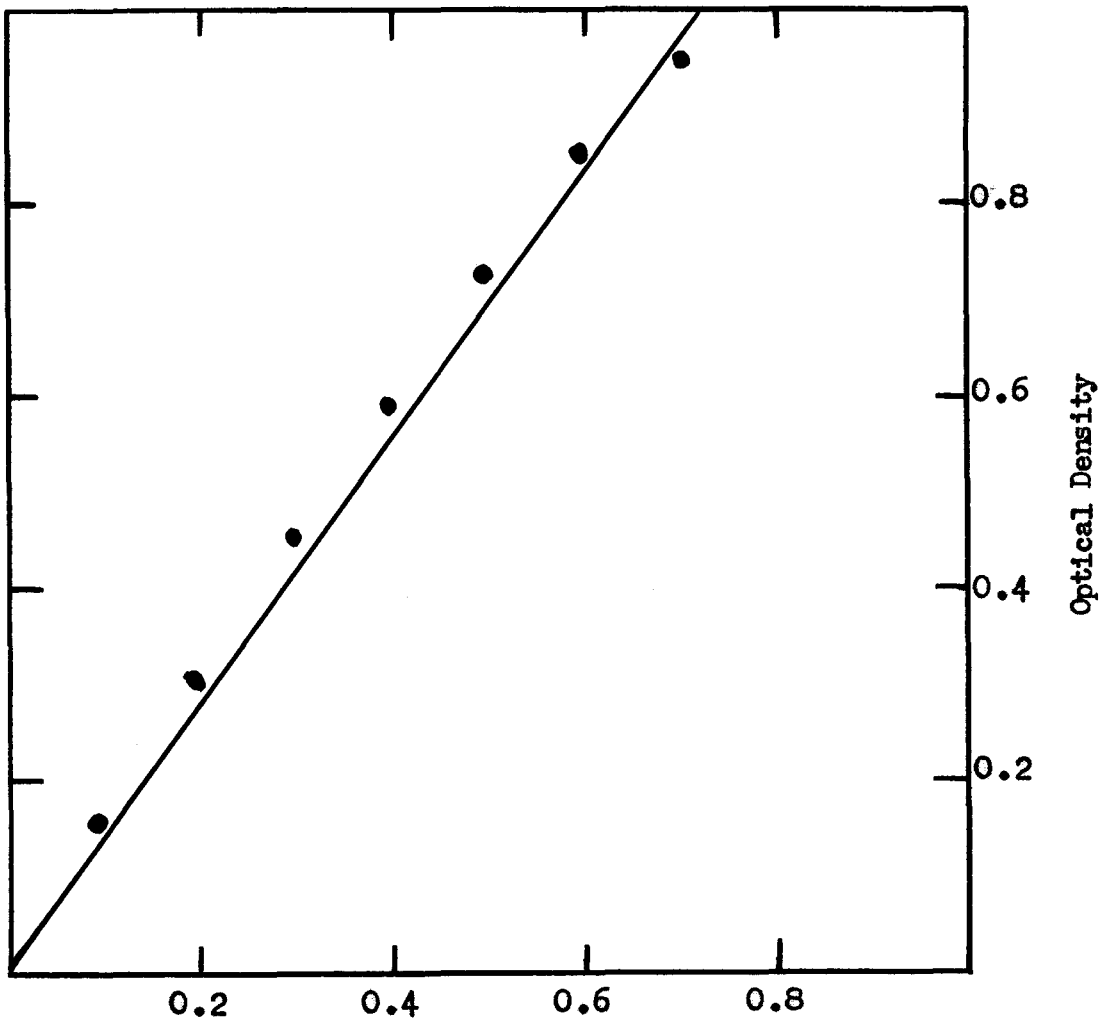
TABLE IV

STANDARD CURVE DATA FOR BUTETHAMINE·HCl

Milligrams of Butethamine·HCl per 100 ml. Colored Solution	Optical Density (Average)
0.1	0.158
0.2	0.303
0.3	0.455
0.4	0.592
0.5	0.727
0.6	0.847
0.7	0.951
0.8	1.05
0.9	1.14
1.0	1.22

FIGURE 2

STANDARD CURVE FOR BUTETHAMINE·HCl



Milligrams of Butethamine·HCl per 100 ml. Colored Solution

Graph of the Data of Table IV

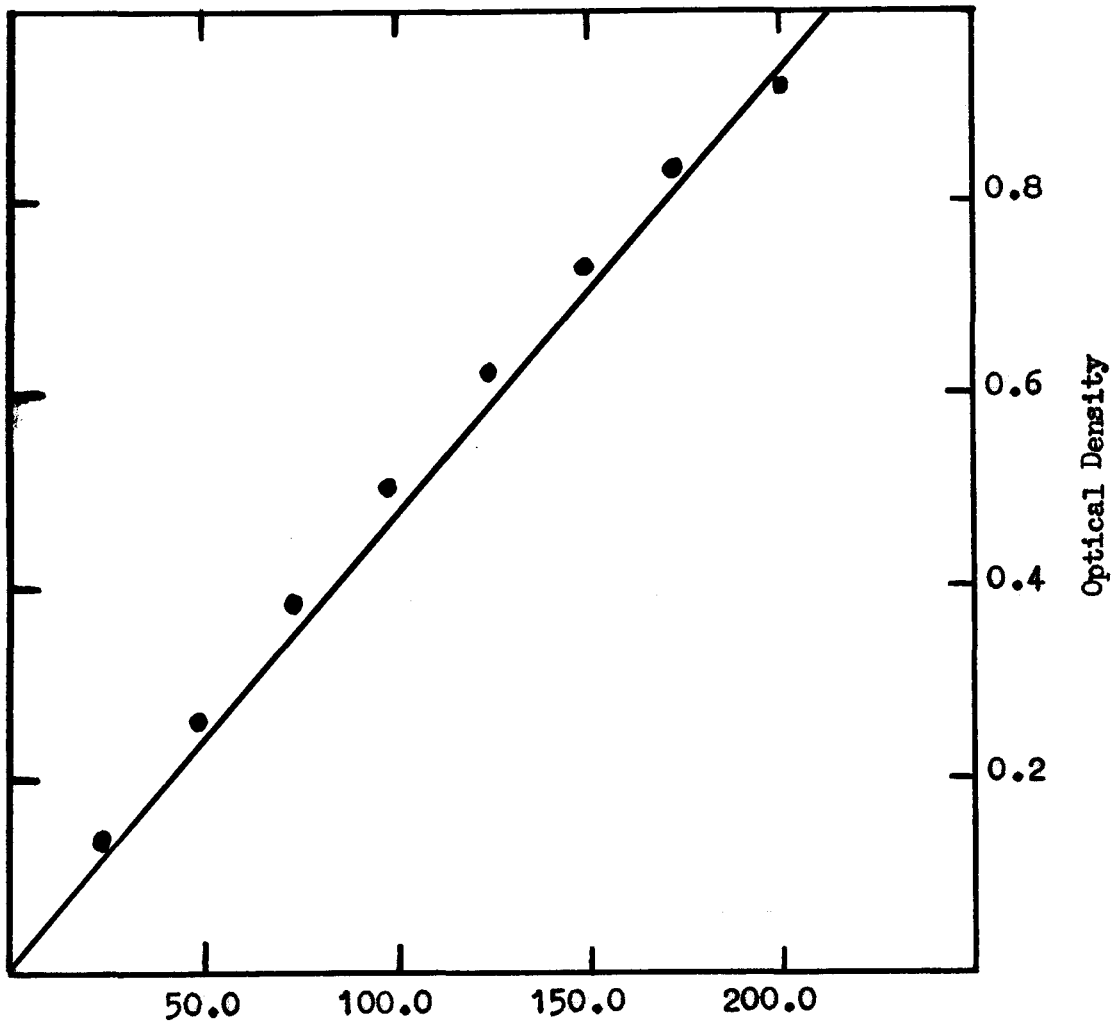
TABLE V

STANDARD CURVE DATA FOR LIDOCAINE·HCl

Milligrams of Lidocaine·HCl per 100 ml. Acetone Solution	Optical Density (Average)
25.0	0.133
50.0	0.258
75.0	0.382
100.0	0.503
125.0	0.621
150.0	0.730
175.0	0.830
200.0	0.922

FIGURE 3

STANDARD CURVE FOR LIDOCAINE·HCl



Graph of the Data of Table V

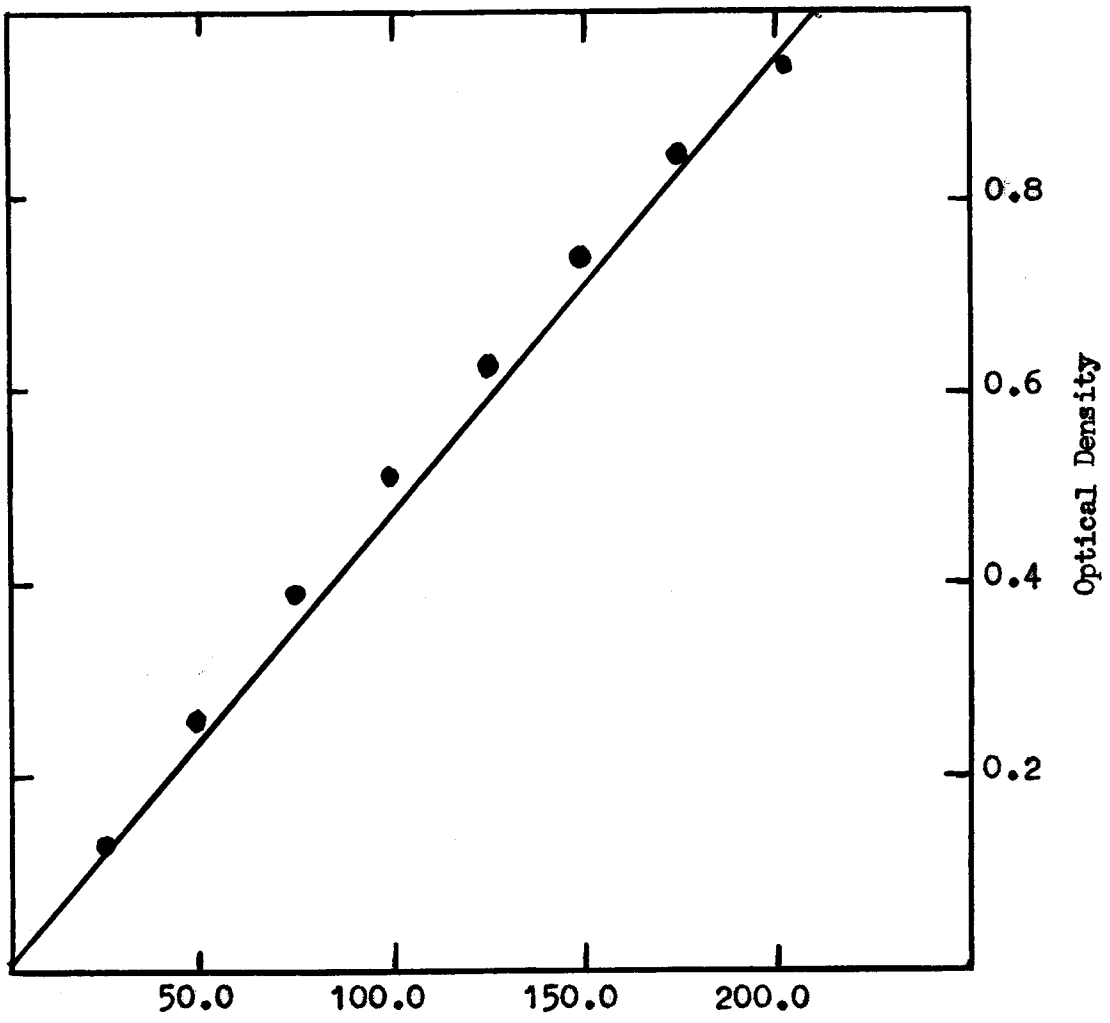
TABLE VI

STANDARD CURVE DATA FOR MEPIVACAINE·HCl

Milligrams of Mepivacaine·HCl per 100 ml. Acetone Solution	Optical Density (Average)
25.0	0.129
50.0	0.260
75.0	0.389
100.0	0.513
125.0	0.633
150.0	0.742
175.0	0.848
200.0	0.950

FIGURE 4

STANDARD CURVE FOR MEPIVACAINE·HCl



Graph of the Data of Table VI

CHAPTER V

THE EFFECT OF CONCENTRATION OF SOME LOCAL ANESTHETICS ON HUMAN SERUM ALBUMIN INTERACTION AT pH 7.0

This part of the investigation into the binding ability of human serum albumin with procaine, butethamine, lidocaine, and mepivacaine was a study of the effect that the concentration of a local anesthetic has on the binding capacity of the protein. For each anesthetic agent, in turn, a series of concentration levels of the anesthetic was prepared between about 0.2% and 0.002% in M/15 phosphate buffer of pH 7.0. Duplicate 50 ml. aliquots of these solutions were placed in the dialysis assembly test tubes outside of the dialysis bags. Within the dialysis membranes were placed 50 ml. aliquots of 0.2% human serum albumin also in M/15 phosphate buffer of pH 7.0. Information on the moisture content of human serum albumin that was necessary in the preparation of the protein solutions is given in Appendix I. As described previously, duplicate blank assemblies were prepared in an identical manner with the exception that inside the dialysis bags were placed 50 ml. aliquots of phosphate buffer without the protein. A double knot of the membrane itself was used to close off the bag at each end. The upper concentration levels of the anes-

thetics were chosen so that the system would approximate as closely as possible the ratio of local anesthetic to serum albumin that prevails in the human body when these agents are used for injection for regional analgesia. In other words, since the approximate average concentration of albumin in human serum is about 4% and approximately 2% solutions of local anesthetics are usually used for anesthesia, the ratio of human serum albumin concentration to local anesthetic concentration is 2:1. Similarly, since 0.2% concentrations of human serum albumin were used in the experimental dialysis systems, and since a concentration of 0.2% of local anesthetic outside the dialysis bag would result in a concentration of approximately 0.1% both outside the dialysis bag and within the dialysis bag at equilibrium, the ratio of human serum albumin concentration to local anesthetic concentration at equilibrium would approximate a 2:1 ratio of human serum albumin concentration to local anesthetic concentration. A comparison of the per cent concentrations and the corresponding molarities, used in the dialysis experiments, of the various anesthetics for the study is given in Appendix II.

After the dialysis assemblies were prepared, they were placed in a refrigerator at 4° Centigrade and allowed to reach equilibrium. The time necessary for the dialysis process to

reach equilibrium was found to be between three and four days so that each set of dialysis experiments was allowed to equilibrate at least four days before chemical analyses of the anesthetic solutions outside of the dialysis bags were carried out according to the procedures discussed in Chapter IV.

A comparison of the pH value before and after equilibrium dialysis showed that no change in pH occurred during the course of the experiment. The blank assemblies were used to indicate the exact initial concentrations of the local anesthetics since twice the concentration of the local anesthetic outside of the dialysis bag in a blank set-up at equilibrium would be equal to the initial concentration of the local anesthetic outside of the dialysis bag for the experimental assembly. Any errors caused by evaporation of the solutions during dialysis and interaction of the local anesthetics with the dialysis membrane were eliminated by this procedure. As already mentioned previously, the Donnan effect produced by the relatively large charged protein molecules was held in abeyance by the relatively high concentrations of buffer that were used, thereby reducing the Donnan effect to the point where it may be considered negligible.

Results for each of the human serum albumin-local anesthetic equilibrium dialysis systems at pH 7.0 are shown in the accompanying Tables VII through X. The first column in

each table shows the initial anesthetic concentration that was obtained by doubling the concentration of the blank system at equilibrium. The second column gives the unbound anesthetic concentration, (X), at equilibrium from analysis of the experimental dialysis assemblies. The other columns depict the calculated values of r, 1/(X), 1/r, and r/(X) from the data of the first and second columns and the fact that the concentration of bound local anesthetic is equal to the algebraic difference between the concentration in the first column and twice the concentration of the second column.

The concentration ranges over which interaction between human serum albumin and the local anesthetic agents was found differed to some extent for each of the agents studied, as seen in the tables. Graphical representations of the ranges are shown in Figures 5 through 8 where appropriate plots were made in accordance with the linear equation [33] that was obtained in the theoretical treatment of systems exhibiting multiple equilibria in Chapter III. The theoretical interpretation of these data and graphs is not clear since detailed information of the molecular structure of the protein has not been fully established.

The four anesthetics appear to fall into two distinct classes with regard to their ability to interact with the protein. These classes seem to parallel the two kinds of

internal chemical linkage found in these agents, namely, the ester linkage in the case of procaine and butethamine and the amide linkage in the case of lidocaine and mepivacaine. In any event, the ability of the protein to bind these agents is not exactly the same although the data bring out the fact that the statistical number of binding sites on the protein for the anesthetic agent is about two in all cases. It may well be that the number of binding sites on the protein in the case of these agents is governed by their approximately equal size and the concentration range of binding depends upon their charge distribution in reference to the charge distribution of the protein at this particular pH value for, certainly, the hydrogen ion concentration does influence the net electrical charge on or near the surface of the protein molecule as a reflection of the number of positively and negatively charged groups found within the molecular structure of the protein molecule.

TABLE VII

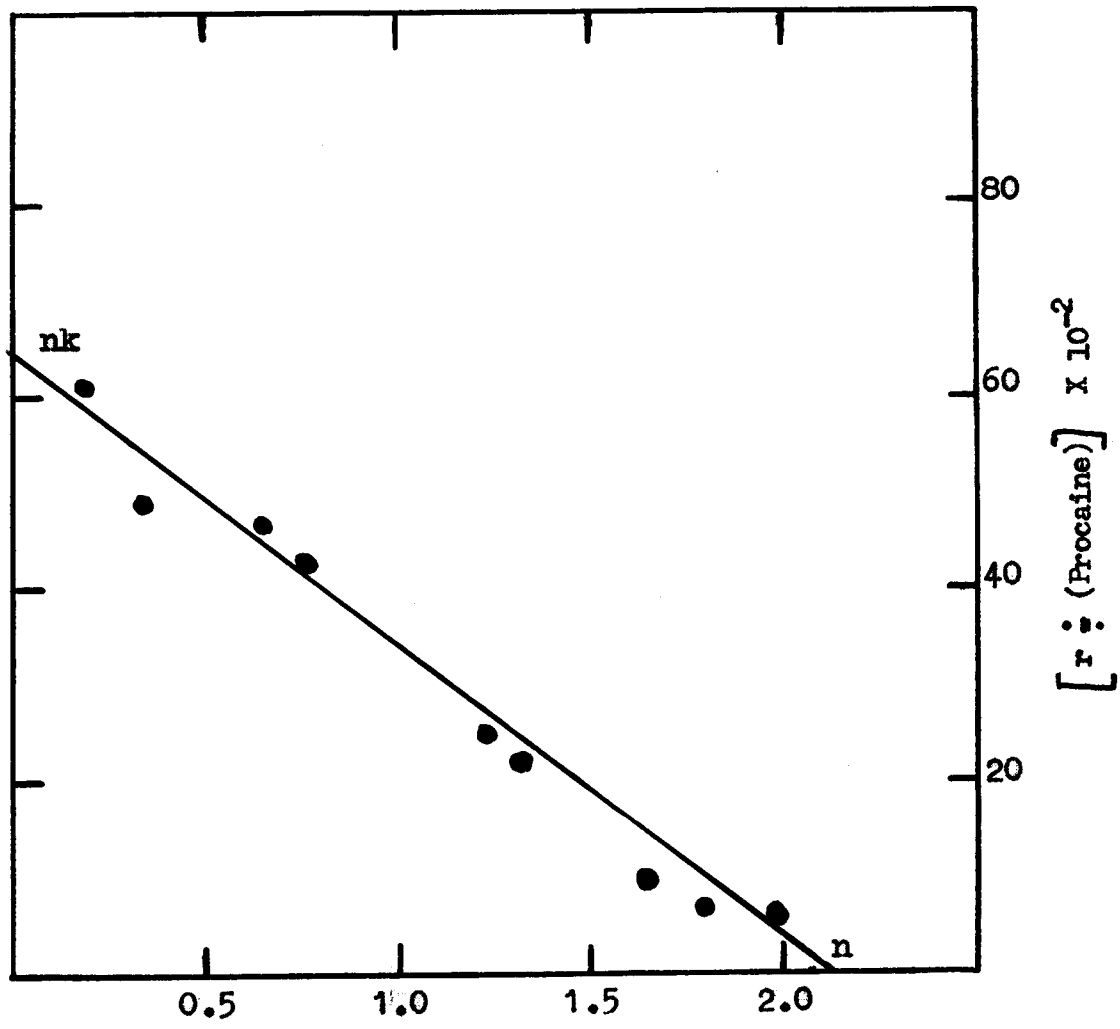
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN--PROCAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Procaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Procaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Procaine·HCl / Moles Total Albumin. r
6.716×10^{-3}	3.330×10^{-3}	1.96
5.092×10^{-3}	2.503×10^{-3}	1.82
3.398×10^{-3}	1.670×10^{-3}	1.67
1.238×10^{-3}	6.00×10^{-4}	1.33
1.036×10^{-3}	5.00×10^{-4}	1.25
3.82×10^{-4}	1.80×10^{-4}	0.77
2.99×10^{-4}	1.40×10^{-4}	0.66
1.53×10^{-4}	7.15×10^{-5}	0.35
7.20×10^{-5}	3.30×10^{-5}	0.20
1/(X)	1/r	r/(X)
3.00×10^{2}	0.51	5.88×10^{2}
4.00×10^{2}	0.55	7.28×10^{2}
5.99×10^{2}	0.60	1.00×10^{3}
1.67×10^{3}	0.75	2.22×10^{3}
2.00×10^{3}	0.80	2.50×10^{3}
5.56×10^{3}	1.30	4.24×10^{3}
7.14×10^{3}	1.52	4.75×10^{3}
1.40×10^{4}	2.86	4.90×10^{3}
3.03×10^{4}	5.00	6.06×10^{3}

FIGURE 5

BINDING OF PROCAINE WITH HUMAN SERUM ALBUMIN AT pH 7.0



r = Moles Bound Procaine ÷ Moles Total Albumin

Plot of Equation [33]

Graph of the Data of Table VII

TABLE VIII

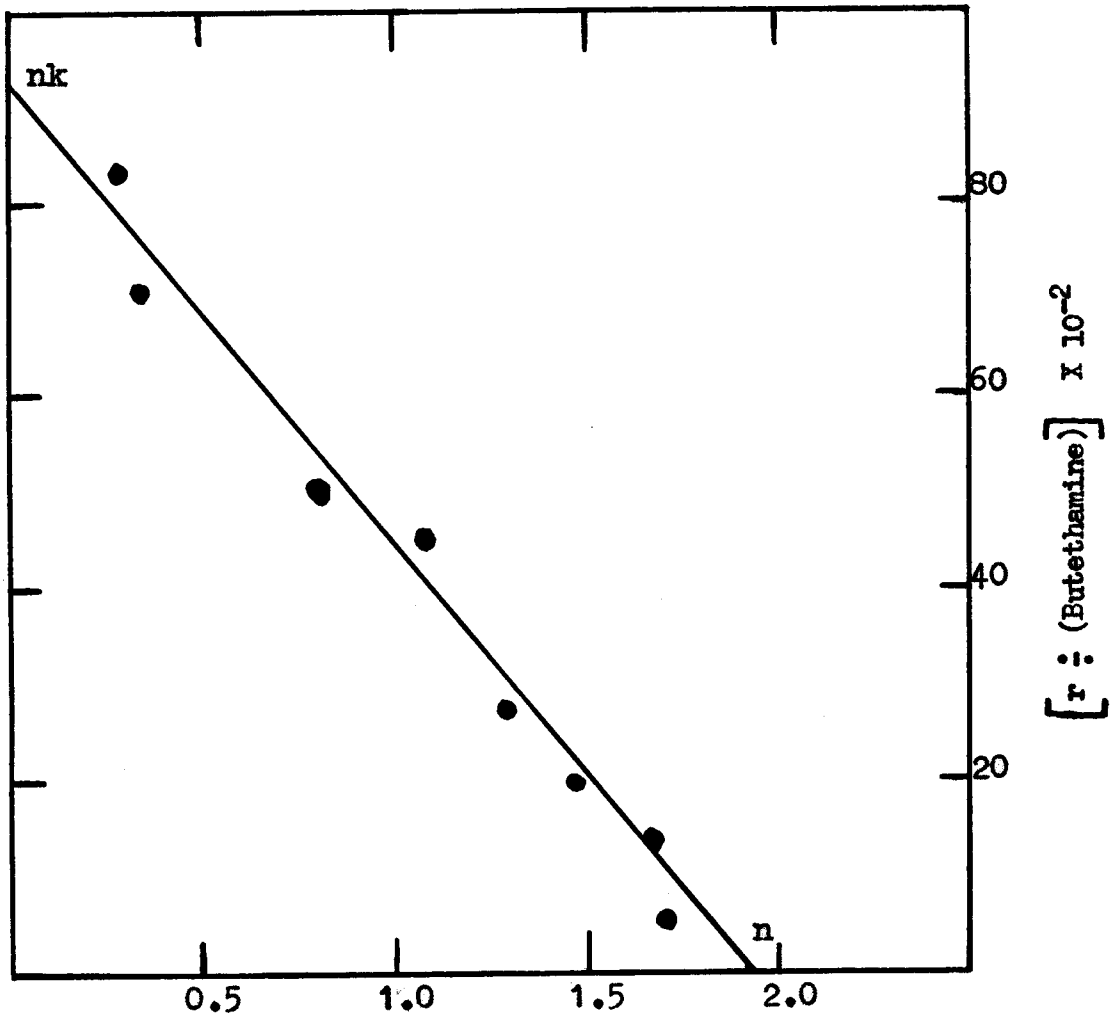
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—BUTETHAMINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Butethamine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Butethamine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Butethamine·HCl / Moles Total Albumin r
7.250×10^{-3}	3.602×10^{-3}	1.72
2.587×10^{-3}	1.270×10^{-3}	1.66
1.522×10^{-3}	7.40×10^{-4}	1.47
9.97×10^{-4}	4.80×10^{-4}	1.30
5.07×10^{-4}	2.38×10^{-4}	1.08
3.43×10^{-4}	1.60×10^{-4}	0.80
1.06×10^{-4}	4.82×10^{-5}	0.34
7.54×10^{-5}	3.37×10^{-5}	0.28
1/(X)	1/r	r/(X)
2.78×10^{2}	0.57	4.87×10^{2}
7.87×10^{2}	0.60	1.31×10^{3}
1.35×10^{3}	0.68	1.98×10^{3}
2.08×10^{3}	0.77	2.70×10^{3}
4.20×10^{3}	0.93	4.54×10^{3}
6.25×10^{3}	1.25	5.00×10^{3}
2.07×10^{4}	2.94	7.04×10^{3}
2.97×10^{4}	3.57	8.32×10^{3}

FIGURE 6

BINDING OF BUTETHAMINE WITH HUMAN SERUM ALBUMIN AT pH 7.0



r = Moles Bound Butethamine ÷ Moles Total Albumin

Plot of Equation [33]

Graph of the Data of Table VIII

TABLE IX

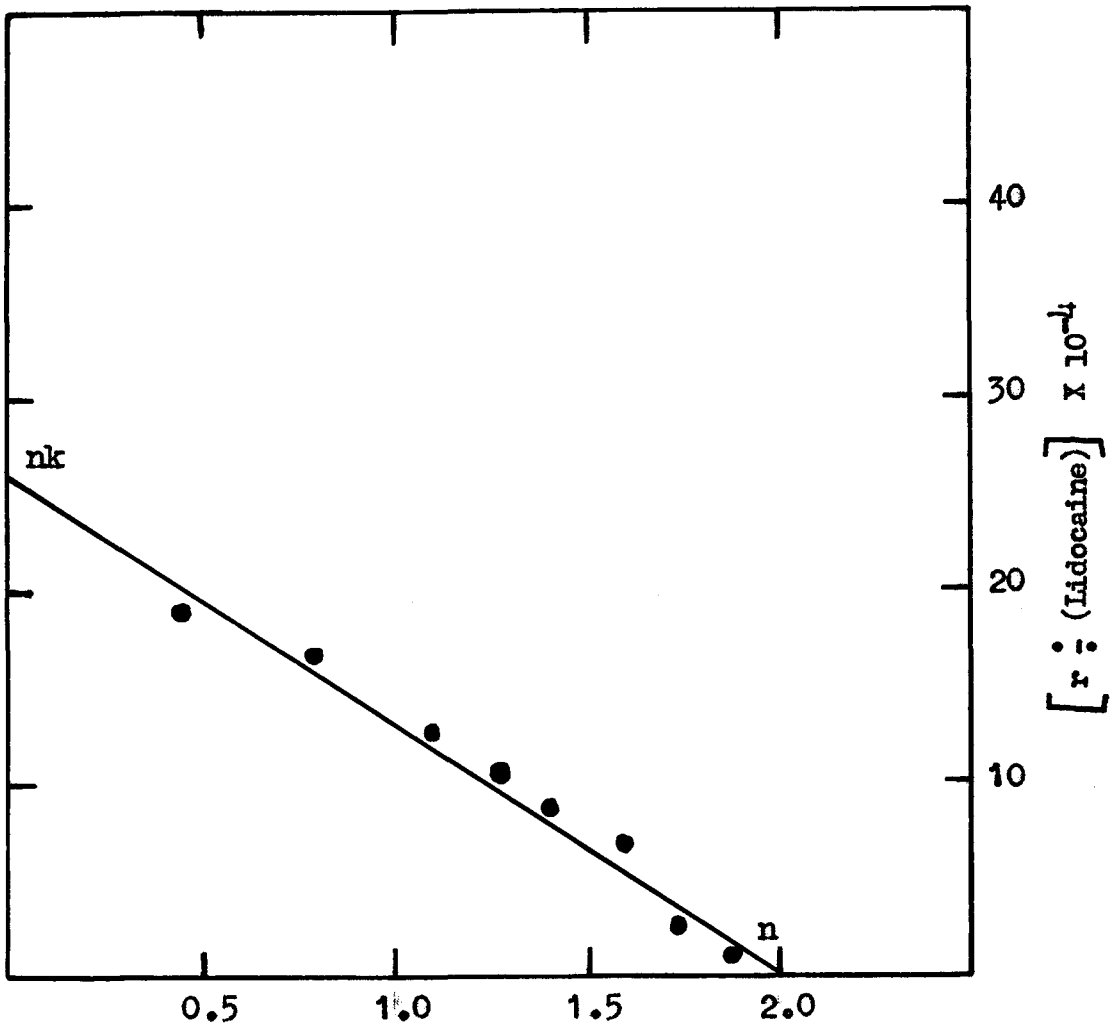
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—LIDOCAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer, M/15.

Initial Lidocaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Lidocaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Lidocaine·HCl / Moles Total Albumin. r
3.94×10^{-4}	1.70×10^{-4}	1.90
1.71×10^{-4}	6.04×10^{-5}	1.76
9.28×10^{-5}	2.34×10^{-5}	1.62
7.32×10^{-5}	1.63×10^{-5}	1.42
6.30×10^{-5}	1.29×10^{-5}	1.30
4.94×10^{-5}	8.85×10^{-6}	1.11
3.28×10^{-5}	4.83×10^{-6}	0.81
1.81×10^{-5}	2.42×10^{-6}	0.46
1.03×10^{-5}	1.82×10^{-6}	0.23
1/(X)	1/r	r/(X)
5.88×10^{3}	0.53	1.12×10^{4}
1.66×10^{4}	0.57	2.92×10^{4}
4.27×10^{4}	0.62	6.92×10^{4}
6.13×10^{4}	0.70	8.70×10^{4}
7.75×10^{4}	0.77	1.01×10^{5}
1.13×10^{5}	0.90	1.25×10^{5}
2.07×10^{5}	1.23	1.68×10^{5}
4.13×10^{5}	2.17	1.90×10^{5}
5.49×10^{5}	4.35	1.26×10^{6}

FIGURE 7

BINDING OF LIDOCAINE WITH HUMAN SERUM ALBUMIN AT pH 7.0



r = Moles Bound Lidocaine ÷ Moles Total Albumin

Plot of Equation [33]

Graph of the Data of Table IX

TABLE X

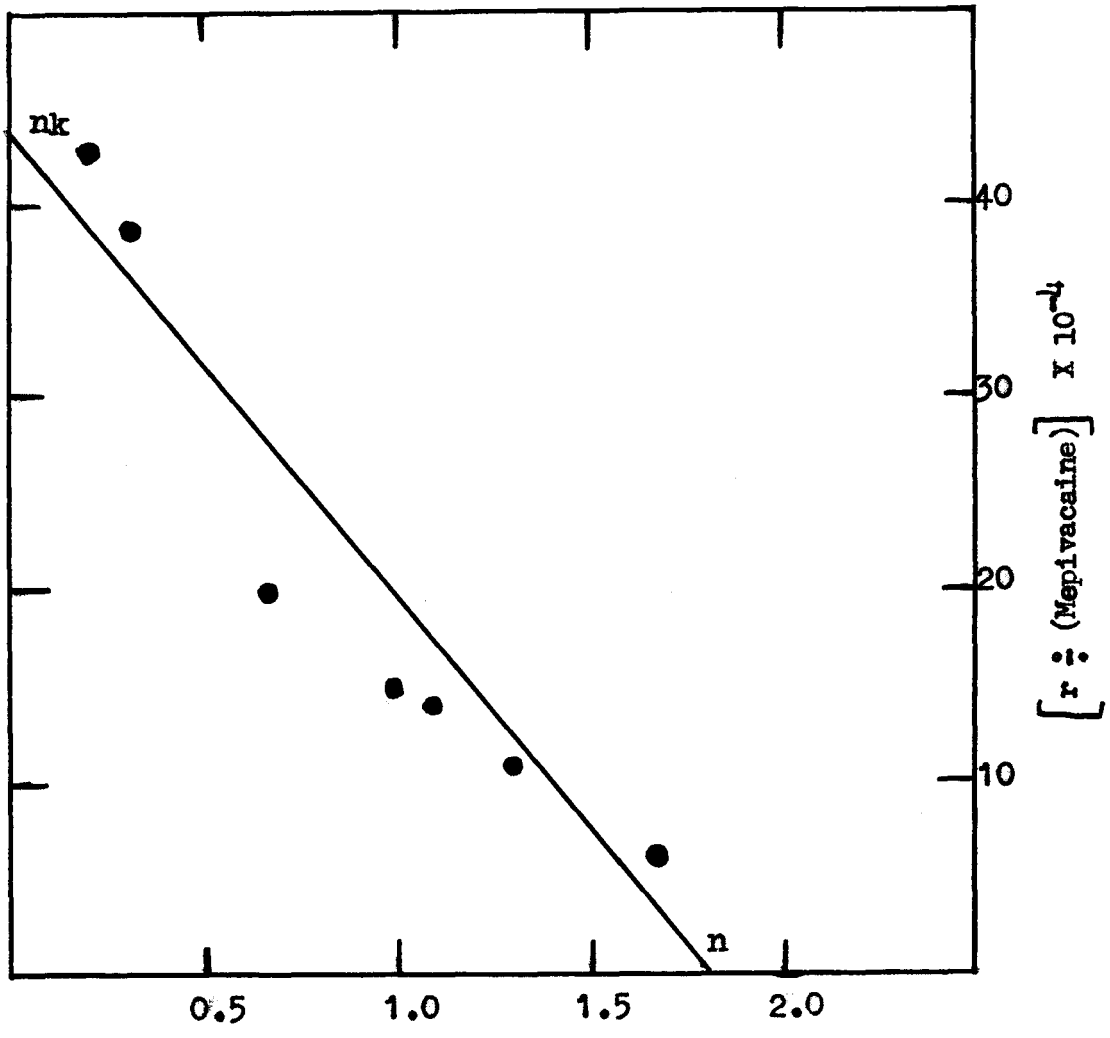
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—MEPIVACAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Mepivacaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Mepivacaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Mepivacaine·HCl / Moles Total Albumin. r
1.09×10^{-4}	3.00×10^{-5}	1.70
6.30×10^{-5}	1.26×10^{-5}	1.32
4.70×10^{-5}	7.65×10^{-6}	1.11
4.34×10^{-5}	7.40×10^{-6}	1.00
2.66×10^{-5}	3.55×10^{-6}	0.68
1.08×10^{-5}	8.25×10^{-7}	0.32
7.32×10^{-6}	5.15×10^{-7}	0.22
1/(X)	1/r	r/(X)
3.33×10^{4}	0.59	5.66×10^{4}
7.94×10^{4}	0.76	1.05×10^{5}
1.31×10^{5}	0.90	1.35×10^{5}
1.35×10^{5}	1.00	1.45×10^{5}
2.82×10^{5}	1.47	1.92×10^{5}
1.21×10^{6}	3.13	3.87×10^{5}
1.94×10^{6}	4.55	4.27×10^{5}

FIGURE 8

BINDING OF MEPIVACAINE WITH HUMAN SERUM ALBUMIN AT pH 7.0



r = Moles Bound Mepivacaine ÷ Moles Total Albumin

Plot of Equation [33]

Graph of the Data of Table X

CHAPTER VI

THE EFFECT OF CHANGES IN pH ON HUMAN SERUM ALBUMIN BINDING OF LOCAL ANESTHETICS

The experimental procedure comprising this phase of the study on the interaction of human serum albumin with the local anesthetics procaine, butethamine, lidocaine, and mepivacaine was an investigation into the effect that the pH value of a solution of a local anesthetic has on the binding ability of the protein at several concentrations of each of the local anesthetics. As in Chapter V, a series of concentration levels of each anesthetic was prepared between approximately 0.2% and 0.002% in M/15 phosphate buffer. Two different pH values of buffer were used, namely, pH 6.8 and pH 7.2. Information on the binding of each anesthetic agent for a series of the same concentration levels at pH 7.0 was already discussed in Chapter V. Also, as before, duplicate 50 ml. aliquots of the anesthetic solutions were placed in the large test tubes used for dialysis and within the dialysis bags were placed 50 ml. aliquots of 0.2% human serum albumin also in M/15 phosphate buffer at the corresponding pH values. Duplicate blank equilibrium dialysis set-ups were prepared for the experiments at pH values 6.8 and 7.2 as they were for the experiments at pH 7.0. After

preparation of the dialysis tubes, the assemblies were placed in a refrigerator at 4° Centigrade and allowed to equilibrate four days before spectrophotometric analyses of the anesthetic solutions outside of the dialysis bags were carried out according to the usual procedures. Measurements of the pH values of the tubes before and after equilibrium dialysis showed no change in pH of the tubes during the course of the experiment.

Results for each of the human serum albumin—local anesthetic equilibrium dialysis systems at pH 6.8 and at pH 7.2 are tabulated in Tables XI through XVIII. The first and second columns list the initial anesthetic concentrations outside of the dialysis bags and the unbound anesthetic concentrations outside of the dialysis bags at equilibrium, respectively. The remaining four columns list the calculated quantities from the information given in the first two columns. Tables XIX through XXII indicate the results of fitting straight lines according to statistical procedures to the data given in Tables VII through XVIII using the straight line equations

$$\frac{r}{(X)} = - kr + kn \qquad [33]$$

and

$$\frac{1}{r} = \frac{1}{kn} \cdot \frac{1}{(X)} + \frac{1}{n} \qquad [32]$$

that were derived previously in Chapter III. The data for equation [33] and the fitted straight lines are plotted in Figures 9 through 12 for each of the local anesthetics used at the three pH values 6.8, 7.0, and 7.2. The curves for all three pH values for each anesthetic, in turn, are shown on one graph for the sake of facilitating comparison of the binding data at these three pH values. A short treatment of the mathematics of fitting straight lines to linear data is presented in Appendix IV.

The data and the graphed results show that each of the four local anesthetics exhibit variation of the extent of their binding with human serum albumin in accordance with variation in pH. However, in the pH range chosen for the study, the approximate statistical number of binding sites of each of the local anesthetics for the protein remains in the neighborhood of two. Again, the theoretical interpretation of this information coupled with the fact that each anesthetic has an upper concentration level above which saturation of the protein with local anesthetic molecules is as complete as possible (percentage of binding of anesthetic is a minimum) seems to justify the hypothesis that the extent of binding of a particular local anesthetic agent with human serum albumin is some function of the ionic charges on both the anesthetic and the protein and the

number of binding sites on the protein for the anesthetic is some function of the structural size of the local anesthetic with respect to the structure of the binding sites on the protein. Whether or not a direct relationship exists between the ionic charges of both the anesthetic and the protein and the extent of binding on the one hand, and whether or not a direct relationship exists between the molecular size of the local anesthetic and the number of binding sites on the other hand, cannot be decided from the information and manipulation of data attempted in this work, but it is hoped that this study will lead to a better understanding of the interaction that occurs between human serum albumin and local anesthetic agents. Also, from the results gathered here it cannot be conjectured that all other known local anesthetic agents will behave similarly under these same conditions. More detailed information concerning the molecular structure of human serum albumin must be attained before this will become possible.

In recent years there has arisen the firm conviction that not only the chemical but also the physical properties of proteins, or at least many of them, can be given a rational explanation in terms of the amino acid composition, if this is sufficiently well-known and can be adequately interpreted. However, one of the most disheartening features of the amino

acid analyses of proteins at the present time is that the results have little meaning. To a limited extent they are useful for assessing the nutritional values of proteins but they do not explain at all true biological functions, that is, why one protein is an enzyme, another a hormone, another a structural protein, another a toxin, and so on. However, in spite of this pessimistic view, it is probably true that many properties of proteins, including the binding properties one aspect of which is the basis of this study, will become explicable in terms of the amino acid composition. It is clear, though, that knowledge of the order of the amino acids in the peptide chains, the orientation of groups in the folded chains of the native proteins, and the effect of the juxtaposition of a group, or groups, on the properties of any single type of side chain must be obtained before such an interpretation becomes possible.

Although certain chemical groups are known to be responsible for some of the simpler properties of proteins, for example, the distribution and concentration of cationic and anionic groups determine the isoelectric point and electrophoretic mobility, there is still little or no evidence to correlate the properties of a protein with its constituent groups. Isolated experiments on a few proteins have revealed that certain groups are essential for biological activity to

be maximal. For example, phosphokinases, transaminase, succinic dehydrogenase, and many other enzymes require the intactness of their sulfhydryl groups; insulin requires disulfide and phenoxyl groups, and the lactogenic hormone of the pituitary requires the presence of free amino groups.

Therefore, although physical and biological properties of proteins are not simple functions of their constituent groups, it is obvious that these groups must be the fundamental determinants of these properties. The relationships between composition and function will be more clearly defined when the following factors are established:

1) The influence of various side chains, both individually and collectively, upon the properties of any one side chain.

2) The modification of function induced by

a) movement of groups alone a peptide chain and

b) the folding of peptide chains.

3) What groups, individually and collectively, are essential for a particular function.

Such relationships may become clear when the intermediate peptides from the initial dipeptide and upwards become available for study. From a review of the rapid progress that has occurred in protein analysis in the last few years, it seems most probable that the development of this field of study

will not be long delayed.

A glance at Tables XIX through XXII will reveal that the order of magnitude of the intrinsic affinity constants, k, of human serum albumin for procaine and butethamine is 10^3, whereas for lidocaine and mepivacaine the order of magnitude of k is 10^5. This result indicates that the affinity of human serum albumin for either procaine or butethamine is weaker than the corresponding affinity of human serum albumin for either lidocaine or mepivacaine. Therefore, the interpretation may be made that this may be one of the reasons why each of these pairs of local anesthetics, namely, procaine and butethamine on the one hand and lidocaine and mepivacaine on the other hand, have different concentration ranges over which changes in binding from maximal binding to minimal binding were found in these experiments. Perhaps, local anesthetic agents that interact more strongly with human serum albumin behave differently in certain respects, whether chemical or physiological, than local anesthetic agents that interact less strongly with human serum albumin.

TABLE XI

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—PROCAINE HYDROCHLORIDE SYSTEM AT pH 6.8

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4 °C, pH 6.8, Ionic Strength 0.13, Phosphate Buffer M/15.

Initial Procaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Procaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Procaine·HCl / Moles Total Albumin. r
4.520×10^{-3}	2.240×10^{-3}	1.68
1.640×10^{-3}	8.00×10^{-4}	1.40
8.76×10^{-4}	4.23×10^{-4}	1.07
6.13×10^{-4}	2.93×10^{-4}	0.95
4.63×10^{-4}	2.20×10^{-4}	0.80
3.44×10^{-4}	1.63×10^{-4}	0.63
2.00×10^{-4}	9.38×10^{-5}	0.43
9.24×10^{-5}	4.32×10^{-5}	0.21
1/(X)	1/r	r/(X)
4.46×10^{2}	0.60	7.50×10^{2}
1.25×10^{3}	0.71	1.75×10^{3}
2.36×10^{3}	0.93	2.53×10^{3}
3.41×10^{3}	1.05	3.24×10^{3}
4.55×10^{3}	1.25	3.63×10^{3}
6.13×10^{3}	1.59	3.87×10^{3}
1.07×10^{4}	2.32	4.58×10^{3}
2.06×10^{4}	4.76	4.86×10^{3}

TABLE XII

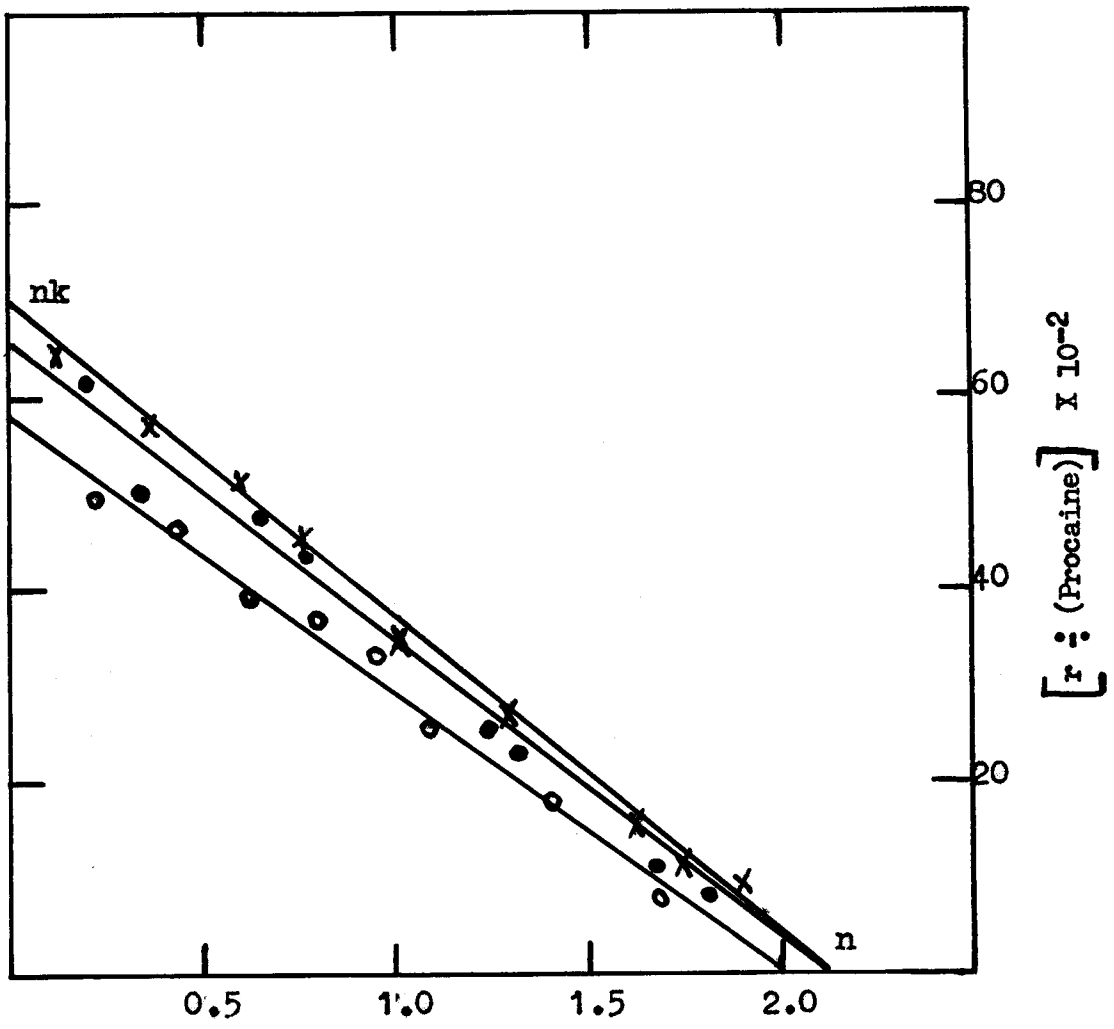
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—PROCAINE HYDROCHLORIDE SYSTEM AT pH 7.2

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.2, Ionic Strength 0.16, Phosphate Buffer M/15.

Initial Procaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Procaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Procaine·HCl / Moles Total Albumin. r
4.622×10^{-3}	2.284×10^{-3}	1.90
3.384×10^{-3}	1.667×10^{-3}	1.75
2.263×10^{-3}	1.108×10^{-3}	1.64
9.99×10^{-4}	4.81×10^{-4}	1.28
6.23×10^{-4}	2.97×10^{-4}	1.02
3.60×10^{-4}	1.69×10^{-4}	0.76
3.27×10^{-4}	1.55×10^{-4}	0.60
1.38×10^{-4}	6.37×10^{-5}	0.36
4.09×10^{-5}	1.88×10^{-5}	0.12
1/(X)	1/r	r/(X)
4.39×10^{2}	0.53	8.32×10^{2}
5.98×10^{2}	0.57	1.05×10^{3}
9.00×10^{2}	0.61	1.48×10^{3}
2.08×10^{3}	0.78	2.66×10^{3}
2.96×10^{3}	0.98	3.44×10^{3}
5.92×10^{3}	1.32	4.50×10^{3}
6.45×10^{3}	1.67	5.17×10^{3}
1.57×10^{4}	2.78	5.65×10^{3}
5.32×10^{4}	8.33	6.40×10^{3}

FIGURE 9

COMPARISON OF BINDING OF PROCAINE WITH HUMAN SERUM ALBUMIN AT pH 6.8, pH 7.0, AND pH 7.2



r = Moles Bound Procaine ÷ Moles Total Albumin

Plot of Equation [33] at
a) pH 6.8 — open circles
b) pH 7.0 — dots
c) pH 7.2 — X's

Graphs of the Data of Tables VII, XI, and XII

TABLE XIII

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—BUTETHAMINE HYDROCHLORIDE SYSTEM AT pH 6.8

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 6.8, Ionic Strength 0.13, Phosphate Buffer M/15.

Initial Butethamine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Butethamine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Butethamine·HCl / Moles Total Albumin. r
4.322×10^{-3}	2.172×10^{-3}	1.64
1.884×10^{-3}	9.20×10^{-4}	1.50
1.340×10^{-3}	6.70×10^{-4}	1.34
9.47×10^{-4}	4.57×10^{-4}	1.16
5.55×10^{-4}	2.64×10^{-4}	0.96
4.49×10^{-4}	2.13×10^{-4}	0.85
3.33×10^{-4}	1.57×10^{-4}	0.70
1.49×10^{-4}	6.91×10^{-5}	0.38
1/(X)	1/r	r/(X)
4.61×10^{2}	0.61	7.55×10^{2}
1.09×10^{3}	0.67	1.63×10^{3}
1.49×10^{3}	0.75	2.00×10^{3}
2.19×10^{3}	0.86	2.55×10^{3}
3.79×10^{3}	1.04	3.64×10^{3}
4.69×10^{3}	1.18	4.00×10^{3}
6.37×10^{3}	1.43	4.47×10^{3}
1.45×10^{4}	2.63	5.50×10^{3}

TABLE XIV

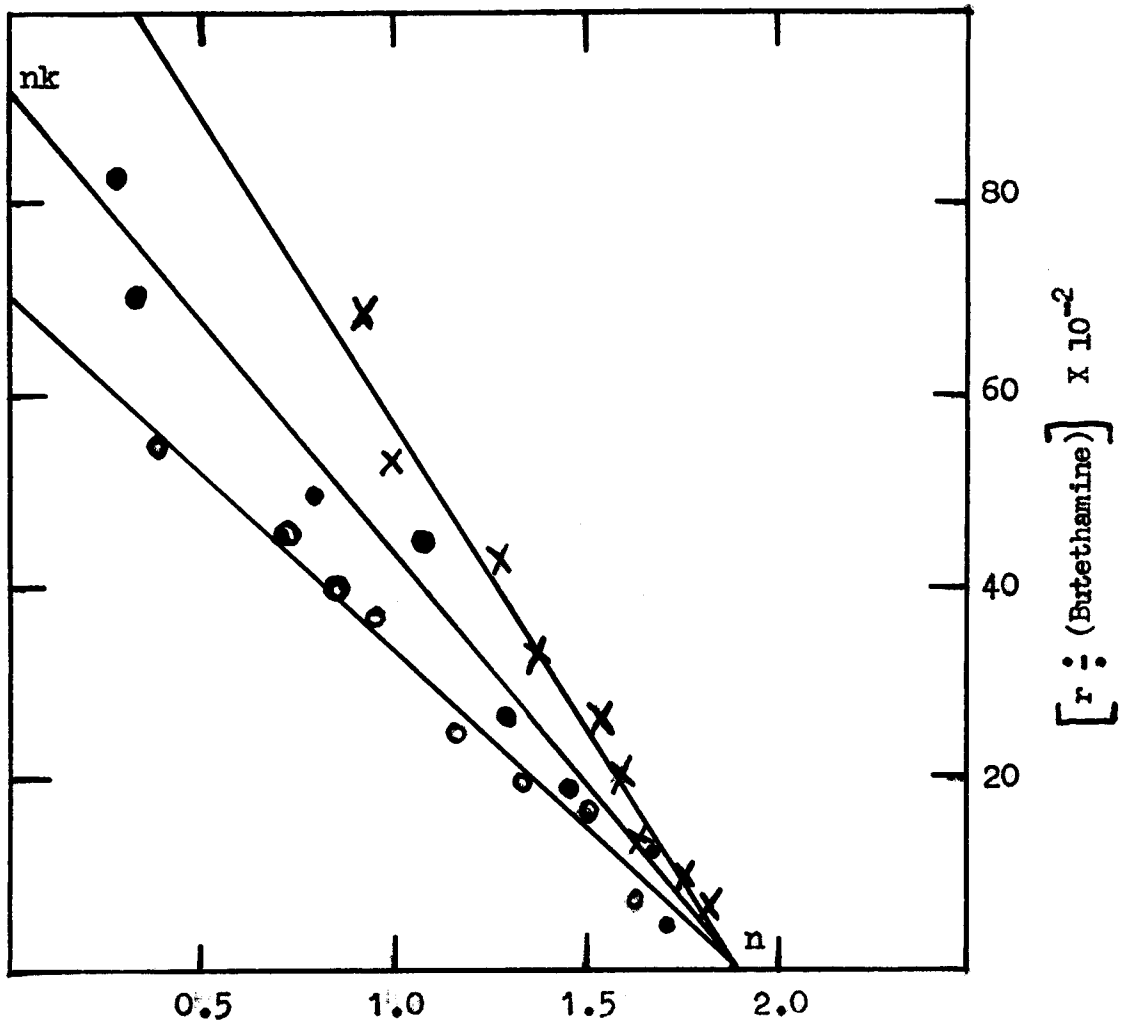
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—BUTETHAMINE HYDROCHLORIDE SYSTEM AT pH 7.2

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.2, Ionic Strength 0.16, Phosphate Buffer, M/15.

Initial Butethamine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Butethamine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Butethamine·HCl / Moles Total Albumin. r
5.465×10^{-3}	2.706×10^{-3}	1.84
3.570×10^{-3}	1.760×10^{-3}	1.76
2.547×10^{-3}	1.250×10^{-3}	1.65
1.608×10^{-3}	7.81×10^{-4}	1.60
1.228×10^{-3}	5.92×10^{-4}	1.55
8.50×10^{-4}	4.05×10^{-4}	1.37
6.39×10^{-4}	3.01×10^{-4}	1.28
4.06×10^{-4}	1.89×10^{-4}	1.00
3.45×10^{-4}	1.59×10^{-4}	0.92
1/(X)	1/r	r/(X)
3.69×10^{2}	0.54	6.80×10^{2}
5.68×10^{2}	0.57	1.00×10^{3}
8.00×10^{2}	0.60	1.32×10^{3}
1.28×10^{3}	0.63	2.05×10^{3}
1.69×10^{3}	0.65	2.62×10^{3}
2.47×10^{3}	0.73	3.38×10^{3}
3.32×10^{3}	0.78	4.24×10^{3}
5.29×10^{3}	1.00	5.30×10^{3}
6.29×10^{3}	1.09	6.85×10^{3}

FIGURE 10

COMPARISON OF BINDING OF BUTETHAMINE WITH HUMAN SERUM ALBUMIN AT pH 6.8, pH 7.0, AND pH 7.2



r = Moles Bound Butethamine ÷ Moles Total Albumin

Plot of Equation [33] at
a) pH 6.8 -- Open circles
b) pH 7.0 — dots
c) pH 7.2 — X's

Graphs of the Data of Tables VIII, XIII, and XIV

TABLE XV

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—LIDOCAINE HYDROCHLORIDE SYSTEM AT pH 6.8

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 6.8, Ionic Strength 0.13, Phosphate Buffer M/15.

Initial Lidocaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Lidocaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Lidocaine·HCl / Moles Total Albumin. r
3.12×10^{-4}	1.30×10^{-4}	1.82
1.45×10^{-4}	4.97×10^{-5}	1.60
9.53×10^{-5}	2.72×10^{-5}	1.43
7.39×10^{-5}	1.78×10^{-5}	1.34
5.80×10^{-5}	1.27×10^{-5}	1.14
4.61×10^{-5}	8.77×10^{-6}	1.00
2.83×10^{-5}	4.85×10^{-6}	0.65
1.83×10^{-5}	2.56×10^{-6}	0.46
1/(X)	1/r	r/(X)
7.69×10^{3}	0.55	1.40×10^{4}
2.01×10^{4}	0.63	3.22×10^{4}
3.68×10^{4}	0.70	5.25×10^{4}
5.62×10^{4}	0.75	7.53×10^{4}
7.87×10^{4}	0.88	9.00×10^{4}
1.14×10^{5}	1.00	1.14×10^{5}
2.06×10^{5}	1.54	1.34×10^{5}
3.91×10^{5}	2.17	1.80×10^{5}

TABLE XVI

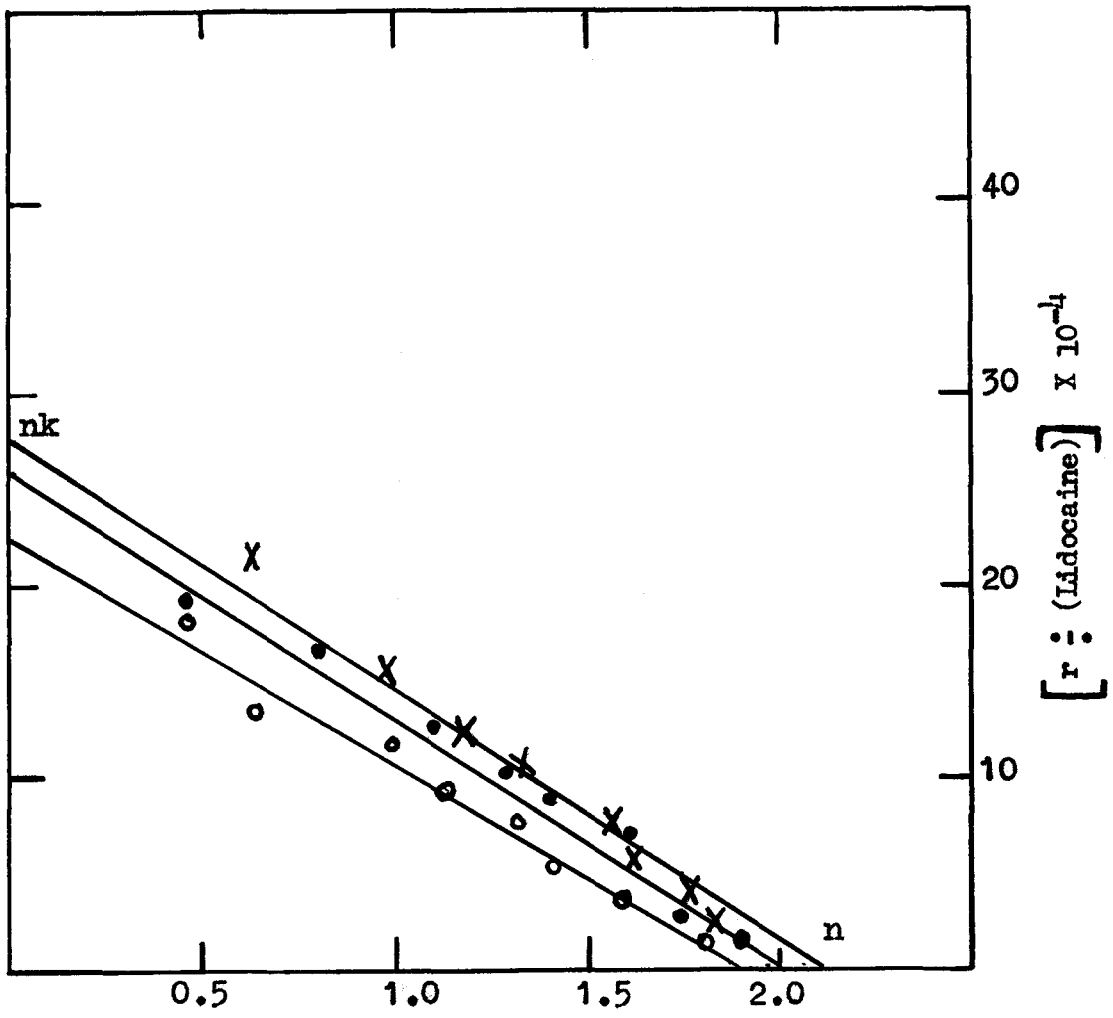
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—LIDOCAINE HYDROCHLORIDE SYSTEM AT pH 7.2

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.2, Ionic Strength 0.16, Phosphate Buffer M/15.

Initial Lidocaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Lidocaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Lidocaine·HCl / Moles Total Albumin. r
2.01×10^{-4}	7.40×10^{-5}	1.85
1.40×10^{-4}	4.47×10^{-5}	1.78
1.07×10^{-4}	3.00×10^{-5}	1.64
8.56×10^{-5}	2.05×10^{-5}	1.56
6.44×10^{-5}	1.29×10^{-5}	1.35
5.38×10^{-5}	9.75×10^{-6}	1.20
4.12×10^{-5}	6.61×10^{-6}	0.98
2.43×10^{-5}	3.02×10^{-6}	0.64
1/(X)	1/r	r/(X)
1.35×10^{4}	0.54	2.50×10^{4}
2.24×10^{4}	0.56	3.98×10^{4}
3.33×10^{4}	0.61	5.46×10^{4}
4.88×10^{4}	0.64	7.60×10^{4}
7.75×10^{4}	0.75	1.05×10^{5}
1.03×10^{5}	0.83	1.23×10^{5}
1.51×10^{5}	1.02	1.54×10^{5}
3.31×10^{5}	1.56	2.12×10^{5}

FIGURE 11

COMPARISON OF BINDING OF LIDOCAINE WITH HUMAN SERUM ALBUMIN AT pH 6.8, pH 7.0, AND pH 7.2



r = Moles Bound Lidocaine ÷ Moles Total Albumin

Plot of Equation [33] at
a) pH 6.8 --open circles
b) pH 7.0 --dots
c) pH 7.2 --X's

Graphs of the Data of Tables IX, XV, and XVI

TABLE XVII

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN--MEPIVACAINE HYDROCHLORIDE SYSTEM AT pH 6.8

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 6.8, Ionic Strength 0.13, Phosphate Buffer M/15.

Initial Mepivacaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Mepivacaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Mepivacaine.HCl / Moles Total Albumin. r
2.64×10^{-4}	1.08×10^{-4}	1.67
1.14×10^{-4}	3.48×10^{-5}	1.54
7.24×10^{-5}	1.69×10^{-5}	1.35
5.83×10^{-5}	1.20×10^{-5}	1.20
4.58×10^{-5}	8.29×10^{-6}	1.02
3.37×10^{-5}	5.00×10^{-6}	0.83
1.42×10^{-5}	1.64×10^{-6}	0.38
1/(X)	1/r	r/(X)
9.26×10^{3}	0.60	1.55×10^{4}
2.87×10^{4}	0.65	4.43×10^{4}
5.92×10^{4}	0.74	8.00×10^{4}
8.33×10^{4}	0.83	1.00×10^{5}
1.21×10^{5}	0.98	1.23×10^{5}
2.00×10^{5}	1.20	1.66×10^{5}
6.10×10^{5}	2.63	2.32×10^{5}

TABLE XVIII

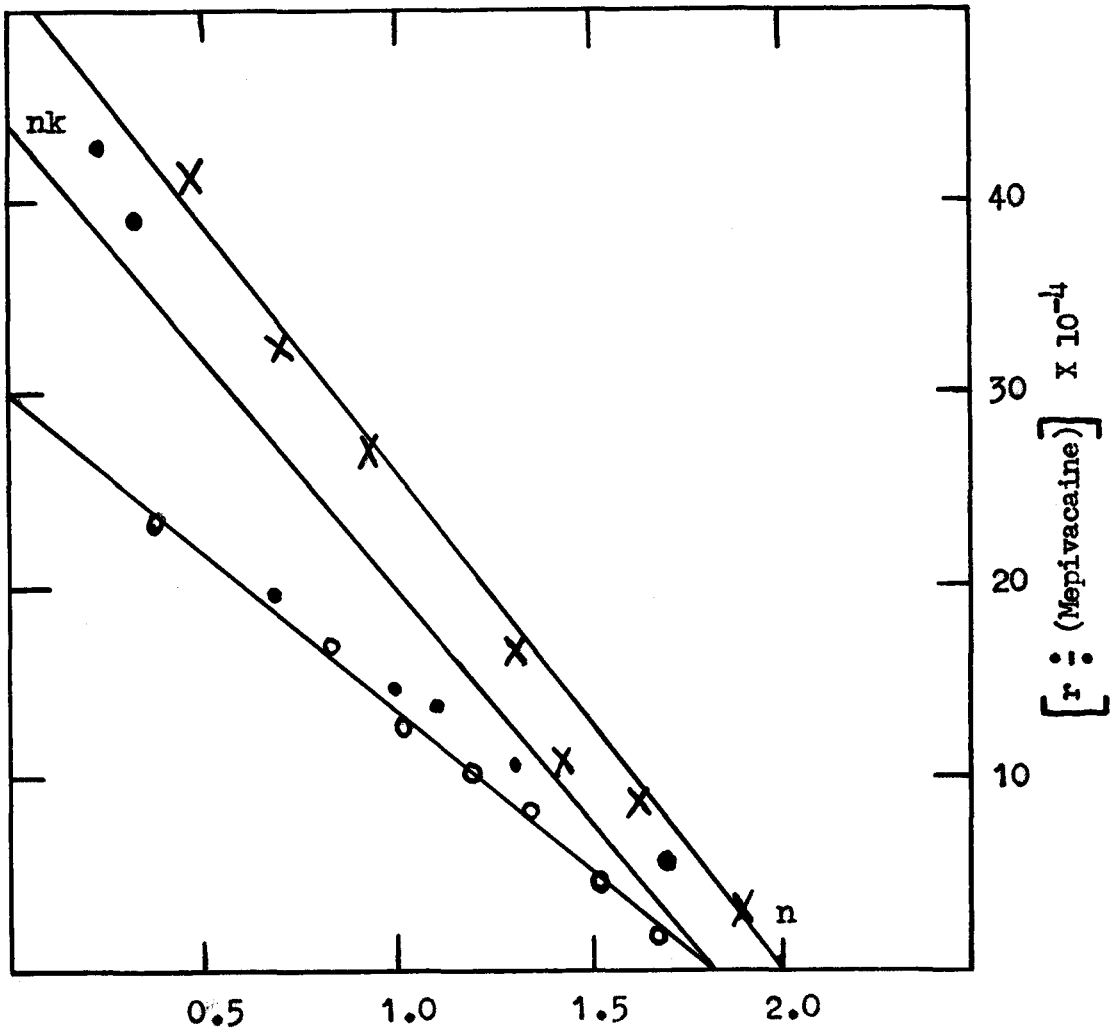
EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM ALBUMIN—MEPIVACAINE HYDROCHLORIDE SYSTEM AT pH 7.2

Experimental Conditions: Human Serum Albumin Concentration 0.2%, Temperature 4°C, pH 7.2, Ionic Strength 0.16, Phosphate Buffer M/15.

Initial Mepivacaine·HCl Concentration Outside of Dialysis Bag. (Molarity)	Unbound Mepivacaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium. (Molarity)	Moles Bound Mepivacaine·HCl / Moles Total Albumin. r
1.81×10^{-4}	6.33×10^{-5}	1.90
8.45×10^{-5}	1.91×10^{-5}	1.62
6.73×10^{-5}	1.32×10^{-5}	1.43
5.34×10^{-5}	7.94×10^{-6}	1.31
3.38×10^{-5}	3.44×10^{-6}	0.94
2.44×10^{-5}	2.17×10^{-6}	0.70
1.54×10^{-5}	1.11×10^{-6}	0.46
1/(X)	1/r	r/(X)
1.84×10^{4}	0.53	3.00×10^{4}
5.24×10^{4}	0.62	8.47×10^{4}
7.50×10^{4}	0.70	1.08×10^{5}
1.26×10^{5}	0.76	1.65×10^{5}
2.91×10^{5}	1.06	2.73×10^{5}
4.61×10^{5}	1.43	3.22×10^{5}
9.01×10^{5}	2.17	4.15×10^{5}

FIGURE 12

COMPARISON OF BINDING OF MEPIVACAINE WITH HUMAN SERUM ALBUMIN AT pH 6.8, pH 7.0 AND pH 7.2



r = Moles Bound Mepivacaine ÷ Moles Total Albumin

Plot of Equation [33] at
a) pH 6.8 — open circles
b) pH 7.0 — dots
c) pH 7.2 — X's

Graphs of the Data of Tables X, XVII, and XVIII

TABLE XIX

FITTING STRAIGHT LINES TO EQUILIBRIUM DIALYSIS DATA OF PROCAINE HYDROCHLORIDE AND BUTETHAMINE HYDROCHLORIDE

The equation, derived previously in Chapter III,

$$\frac{r}{(X)} = - kr + kn, \qquad [33]$$

when compared with the equation of a straight line,

$$y = mx + b,$$

yields the following:

$$y = \frac{r}{(X)}, \quad x = r, \quad m = - k, \quad b = kn.$$

Procaine Hydrochloride

pH	6.8	7.0	7.2
m	-2.88×10^3	-3.13×10^3	-3.27×10^3
b	5.73×10^3	6.49×10^3	6.89×10^3
k	2.88×10^3	3.13×10^3	3.27×10^3
n	2.0	2.1	2.1

Butethamine Hydrochloride

pH	6.8	7.0	7.2
m	-3.74×10^3	-4.86×10^3	-6.31×10^3
b	7.05×10^3	9.19×10^3	12.15×10^3
k	3.74×10^3	4.86×10^3	6.31×10^3
n	1.9	1.9	1.9

TABLE XX

FITTING STRAIGHT LINES TO EQUILIBRIUM DIALYSIS DATA OF PROCAINE HYDROCHLORIDE AND BUTETHAMINE HYDROCHLORIDE

The equation, derived previously in Chapter III,

$$\frac{1}{r} = \frac{1}{kn} \cdot \frac{1}{(X)} + \frac{1}{n}, \qquad [32]$$

when compared with the equation of a straight line,

$$y = mx + b,$$

yields the following:

$$y = \frac{1}{r}, \quad x = \frac{1}{(X)}, \quad m = \frac{1}{kn}, \quad b = \frac{1}{n}.$$

Procaine Hydrochloride

pH	6.8	7.0	7.2
m	1.70×10^{-4}	1.52×10^{-4}	1.47×10^{-4}
b	0.51	0.50	0.51
k	2.94×10^{3}	3.29×10^{3}	3.40×10^{3}
n	2.0	2.0	2.0

Butethamine Hydrochloride

pH	6.8	7.0	7.2
m	1.44×10^{-4}	1.06×10^{-4}	9.10×10^{-5}
b	0.52	0.55	0.51
k	3.65×10^{3}	5.05×10^{3}	5.78×10^{3}
n	1.9	1.8	1.9

TABLE XXI

FITTING STRAIGHT LINES TO EQUILIBRIUM DIALYSIS DATA OF LIDOCAINE HYDROCHLORIDE AND MEPIVACAINE HYDROCHLORIDE

The equation, derived previously in Chapter III,

$$\frac{r}{(X)} = - kr + kn, \qquad [33]$$

when compared with the equation of a straight line,

$$y = mx + b,$$

yields the following:

$$y = \frac{r}{(X)}, \quad x = r, \quad m = - k, \quad b = kn.$$

Lidocaine Hydrochloride

pH	6.8	7.0	7.2
m	-1.18×10^5	-1.26×10^5	-1.32×10^5
b	2.26×10^5	2.60×10^5	2.77×10^5
k	1.18×10^5	1.26×10^5	1.32×10^5
n	1.9	2.0	2.1

Mepivacaine Hydrochloride

pH	6.8	7.0	7.2
m	-1.66×10^5	-2.52×10^5	-2.42×10^5
b	2.98×10^5	4.36×10^5	5.10×10^5
k	1.66×10^5	2.52×10^5	2.42×10^5
n	1.8	1.8	2.0

TABLE XXII

FITTING STRAIGHT LINES TO EQUILIBRIUM DIALYSIS DATA OF LIDOCAINE HYDROCHLORIDE AND MEPIVACAINE HYDROCHLORIDE

The equation, derived previously in Chapter III,

$$\frac{1}{r} = \frac{1}{kn} \cdot \frac{1}{(X)} + \frac{1}{n}, \qquad [32]$$

when compared with the equation of a straight line,

$$y = mx + b,$$

yields the following:

$$y = \frac{1}{r}, \quad x = \frac{1}{(X)}, \quad m = \frac{1}{kn}, \quad b = \frac{1}{n}.$$

Lidocaine Hydrochloride

pH	6.8	7.0	7.2
m	4.86×10^{-6}	4.03×10^{-6}	3.25×10^{-6}
b	0.50	0.46	0.50
k	1.03×10^{5}	1.20×10^{5}	1.50×10^{5}
n	2.0	2.1	2.0

Mepivacaine Hydrochloride

pH	6.8	7.0	7.2
m	2.92×10^{-6}	2.02×10^{-6}	1.90×10^{-6}
b	0.57	0.63	0.47
k	1.90×10^{5}	2.97×10^{5}	2.50×10^{5}
n	1.8	1.8	2.1

CHAPTER VII

THE EFFECT OF CONCENTRATION OF SOME LOCAL ANESTHETICS ON HUMAN SERUM GAMMA GLOBULIN INTERACTION AT pH 7.0

An inquiry into the behavior of procaine, butethamine, lidocaine, and mepivacaine in the presence of buffered human serum gamma globulin solutions was made in this portion of the study. As in the previous two phases, a series of different concentrations of each of the local anesthetics was prepared between about 0.2% and 0.002% levels in M/15 phosphate buffer at pH 7.0. Two sets of 50 ml. aliquots of each of these solutions were placed in the dialysis assemblies outside of the dialysis bags. Also, 50 ml. aliquots of 0.1% human serum gamma globulin in the same buffer were placed in the dialysis bags. The moisture content of human serum gamma globulin for solution preparation purposes is reported in Appendix V. Again, as in previous dialysis set-ups, duplicate blank assemblies were prepared in an identical manner with the exception that within the dialysis bags were placed 50 ml. aliquots of phosphate buffer without the protein. The upper concentration levels of the local anesthetic agents were chosen so that the system would contain a ratio of local anesthetic to serum gamma globulin similar to the

ratio that prevails in the human body when these agents are employed for injection to produce local analgesia. Therefore, since the approximate average concentration of gamma globulin in human serum is about 2% and approximately 2% solutions of local anesthetics are usually used for anesthesia, the ratio of human serum globulin concentration to local anesthetic concentration is 1:1. Similarly, since 0.1% concentrations of human serum gamma globulin were used in the experimental dialysis systems, and since a concentration of 0.2% of local anesthetic outside the dialysis bag would result in a concentration of approximately 0.1% both outside the dialysis bag and within the dialysis bag at equilibrium, the ratio of human serum gamma globulin concentration to local anesthetic concentration at equilibrium would approximate a 1:1 ratio of protein concentration to local anesthetic concentration.

After preparation, the dialysis assemblies were placed in a refrigerator at 4° Centigrade until equilibrium had been established, that is, at least four days. Then chemical analyses of the anesthetic solutions outside of the dialysis bags were completed according to the procedures discussed in Chapter IV. Investigation of the pH value before and after the dialysis showed that no change in pH occurred during the course of the experiment as was true in the previous experiments with serum albumin. The blank assemblies were employed to

indicate the exact initial concentrations of the local anesthetics since double the concentration of the local anesthetic outside of the dialysis bag in a blank assembly at equilibrium would be equal to the initial concentration of the local anesthetic outside of the dialysis bag for the experimental assembly.

Information for each of the human serum gamma globulin—local anesthetic equilibrium dialysis systems at pH 7.0 is given in the subsequent Tables XXIII through XXVI. The data of the first column shows each of the concentrations for each of the local anesthetic solutions as soon as they were prepared but before the solutions were placed in the dialysis assemblies. The second column gives the results that were obtained for both the blank and the experimental dialyses at equilibrium since these results were exactly the same. One column was used to depict these values in order to eliminate unnecessary duplication. Therefore, the equilibrium dialysis experiments with the protein within the dialysis bags behaved in a fashion similar to that of the blank experiments which contained no protein inside the dialysis membrane bags. In other words, this phenomenon could only be interpreted to mean that binding of the local anesthetic agents used in this study with human serum gamma globulin did not occur or was too

small in magnitude to be capable of measurement by the analytical methods used in this investigation. Hence, gamma globulin, at least human serum gamma globulin, apparently does not enter into protein--small molecule interactions as opposed to human serum albumin, which does enter into this combination.

In order to account for the apparent differences in the behavior of human serum albumin, studied in Chapters V and VI, and human serum gamma globulin toward the four local anesthetic agents used in this work, it probably is necessary to look for the explanation in the two main classes of protein interactions, namely, specific and nonspecific interactions. Specificity is characteristic of the vast majority of enzymatic and physiological reactions, but one example, serological specificity, will suffice to illustrate the general principle. Fairly detailed molecular pictures have been developed to explain this specificity. In general, the lock-and-key concepts have been elaborated in detail to include modern information on atomic sizes and interatomic distances. Thus, the specific interaction of an antibody, a specific gamma globulin, with an antigen is attributed to the presence on the gamma globulin surface of an invaginated region into which the antigen can fit well, but toward which other ions or molecules can approach only with difficulty owing to steric

repulsions or to insufficient interaction energy because of looseness. It is perhaps unnecessary to carry the look-and-key analogy to the extent of picturing an actual invagination, but the postulate of a very specific configuration on the antibody surface which is complementary to the antigen structure is certainly the best molecular interpretation available at present. At the other extreme are nonspecific protein interactions, such as with albumin, in which specificity is essentially nonexistent.

Serum albumin is remarkable for its ability to interact with a wide variety of small molecules. The most striking feature of this interaction or binding, however, is the lack of any reasonably marked specificity. Changes in the structures of the bound molecules do have some effects on the strength of binding. These changes in affinity are not, however, as large as one would expect to find if the surface of the serum albumin molecule were covered with a mosaic of rigid binding sites having a reasonably high degree of complementarity to the necessarily limited number of types of substances which such a mosaic might potentially bind. In order to account for this the concept of a configurational adaptability has been provisionally proposed in the protein field according to which the serum albumin molecule is considered to possess a degree of internal flexibility. As

a result, the surface conformation can be changed so as to establish a measure of complementarity with the surface of almost any molecule that approaches it, and there is no need to deal with a limited number of fixed potential binding sites, hence, the use of the term statistical binding sites in connection with this investigation. A strong point in support of this concept is the fact that serum albumin does possess a high degree of internal flexibility as compared with other proteins, which also show a far smaller affinity for small molecules. In a sense, the serum albumin molecule behaves like a lump of putty onto which one can stick an unlimited number of different shapes. This postulate of configurational adaptability is important because it shows that complementarity need not imply specificity. Furthermore, it raises the possibility that, in some instances, the specific complementary structure may be developed only when the substrate is present.

To summarize then, there must exist on the serum albumin protein a number of sites, each associated with several side-chains, which to a varying extent can assume a large number of configurations in equilibrium with each other and of approximately equal energy. In the presence of the proper small molecule or ion that configuration is stabilized which by virtue of its structural relation to the molecule or ion

permits the various portions of the molecule or ion to interact with appropriate groups of the protein. Therefore, in aqueous solutions of serum albumin, there are a large number of configurations of approximately equal energy and in thermodynamic equilibrium with each other. With proteins such as gamma globulin it is not the unavailability of the loci which prevents binding, but rather the rigid configuration of the sites. How far the concept of configurational adaptability can be pressed to explain the unavailability of phenolic or sulfhydryl groups, especially in gamma globulin, is uncertain. In ionic binding, electrostatic forces are primarily involved though aided by van der Waals' interactions. However, covalent bonds are usually formed in the estimation of masked groups in proteins with the usual chemical reagents. Also, the charged groups are generally available to titration or other means of analysis.

TABLE XXIII

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM GAMMA GLOBULIN—PROCAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Gamma Globulin Concentration 0.1%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Procaine·HCl Concentration Outside of Dialysis Bag on Preparation. (Molarity)	Unbound Procaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium for Blank and Experimental Assemblies. (Molarity)
7.12×10^{-2}	3.55×10^{-2}
3.61×10^{-2}	1.79×10^{-2}
7.32×10^{-3}	3.66×10^{-3}
5.72×10^{-3}	2.82×10^{-3}
3.57×10^{-3}	1.76×10^{-3}
2.85×10^{-3}	1.42×10^{-3}
1.86×10^{-3}	9.02×10^{-4}
7.04×10^{-4}	3.44×10^{-4}
3.56×10^{-4}	1.74×10^{-4}
2.86×10^{-4}	1.38×10^{-4}
2.21×10^{-4}	1.07×10^{-4}
7.32×10^{-5}	3.41×10^{-5}

TABLE XXIV

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM GAMMA GLOBULIN—BUTETHAMINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Gamma Globulin Concentration 0.1%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Butethamine·HCl Concentration Outside of Dialysis Bag on Preparation. (Molarity)	Unbound Butethamine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium for Blank and Experimental Assemblies. (Molarity)
7.21×10^{-2}	3.56×10^{-2}
3.61×10^{-2}	1.79×10^{-2}
7.30×10^{-3}	3.60×10^{-3}
5.80×10^{-3}	2.82×10^{-3}
3.57×10^{-3}	1.75×10^{-3}
2.80×10^{-3}	1.38×10^{-3}
1.78×10^{-3}	8.52×10^{-4}
6.96×10^{-4}	3.49×10^{-4}
3.50×10^{-4}	1.71×10^{-4}
2.82×10^{-4}	1.43×10^{-4}
2.16×10^{-4}	1.08×10^{-4}
7.00×10^{-5}	3.52×10^{-5}

TABLE XXV

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM GAMMA GLOBULIN—LIDOCAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Gamma Globulin Concentration 0.1%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Lidocaine·HCl Concentration Outside of Dialysis Bag on Preparation. (Molarity)	Unbound Lidocaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium for Blank and Experimental Assemblies. (Molarity)
7.30×10^{-2}	3.55×10^{-2}
3.62×10^{-2}	1.80×10^{-2}
7.35×10^{-3}	3.62×10^{-3}
5.80×10^{-3}	2.80×10^{-3}
3.57×10^{-3}	1.73×10^{-3}
2.85×10^{-3}	1.42×10^{-3}
1.78×10^{-3}	8.60×10^{-4}
7.16×10^{-4}	3.30×10^{-4}
3.50×10^{-4}	1.66×10^{-4}
2.90×10^{-4}	1.41×10^{-4}
2.20×10^{-4}	1.05×10^{-4}
7.08×10^{-5}	3.44×10^{-5}

TABLE XXVI

EQUILIBRIUM DIALYSIS STUDY ON THE HUMAN SERUM GAMMA GLOBULIN—MEPIVACAINE HYDROCHLORIDE SYSTEM AT pH 7.0

Experimental Conditions: Human Serum Gamma Globulin Concentration 0.1%, Temperature 4°C, pH 7.0, Ionic Strength 0.15, Phosphate Buffer M/15.

Initial Mepivacaine·HCl Concentration Outside of Dialysis Bag on Preparation. (Molarity)	Unbound Mepivacaine·HCl Concentration, (X), Outside of Dialysis Bag at Equilibrium for Blank and Experimental Assemblies. (Molarity)
7.00×10^{-2}	3.46×10^{-2}
3.45×10^{-2}	1.68×10^{-2}
6.98×10^{-3}	3.45×10^{-3}
5.62×10^{-3}	2.75×10^{-3}
3.43×10^{-3}	1.64×10^{-3}
2.75×10^{-3}	1.40×10^{-3}
1.65×10^{-3}	8.62×10^{-4}
7.07×10^{-4}	3.32×10^{-4}
3.54×10^{-4}	1.65×10^{-4}
2.81×10^{-4}	1.36×10^{-4}
2.05×10^{-4}	1.00×10^{-4}
7.00×10^{-5}	3.47×10^{-5}

CHAPTER VIII

CONCLUSIONS FROM THIS STUDY AND DISCUSSION OF THE RESULTS IN RELATION TO BIOLOGICAL SIGNIFICANCE

From the data of Chapters V, VI, and VII and the resulting mathematical calculations from these data, two main conclusions can be reached. Firstly, the binding of the chosen local anesthetics with human serum albumin has certain earmarks of similarity in that all of these agents appear to bring out the fact that the number of binding sites of these particular molecules on the albumin molecules is in the neighborhood of two. Secondly, the interaction of these local anesthetics with human serum gamma globulin is, for practical purposes, negligible, in accordance with the behavior of many organic anions and cations in reference to their binding inability with gamma globulin.

Besides these main conclusions the experimental results bear out the fact that the local anesthetics studied fall into two distinct groups in their interaction ability with human serum albumin. These two groups parallel their molecular structural make up in reference to the fact that procaine and butethamine both contain the ester type linkage whereas lidocaine and mepivacaine both contain the amide type

linkage. The first two, namely procaine and butethamine, exhibit minimal binding at higher molar concentrations than the last two, namely, lidocaine and mepivacaine. Exactly what bearing these facts have on the sum total of the properties of each of these agents cannot be decided on the basis of these observations. However, it may be alluded that the concentration range of each of these agents between the occurrence of minimum and maximum interaction may be related to the most common, albeit the most efficient, concentration levels at which these agents are used for the purposes of injection for regional analgesia.

Besides what has already been stated in defense of the method of equilibrium dialysis that was used to carry out this investigation, the results just mentioned show that the technique is eminently suited for quantitative studies, especially when an interaction is to be explored through a range of drug concentrations. In this way, nearly complete saturation of a protein with a given drug can often be achieved and information of prime physical and chemical importance derived. It is one of the few methods of choice in this type of endeavor and it is both quantitative in its results and thermodynamically sound in theory, for it is a measure of the change in the thermodynamic activity of the reactant (that is, local anesthetic) species. Thermo-

dynamic activity, used here in its usual physicochemical sense, is a definite function of the concentration of a given active moiety, in this case the unbound local anesthetic, and is obtained by determining the algebraic product of the activity coefficient and the concentration. However, the method of equilibrium dialysis does not yield accurate information about the status of an interaction in the blood serum in vivo. Even if whole serum containing the substance under study is dialyzed, physiological conditions will no longer be present because of the dilution of the aqueous phase. Should the drug-protein interaction be reversible, dissociation may occur and neither the amount bound per mole of protein nor the fraction of the total amount of drug bound will necessarily reflect the state of affairs before equilibrium was disturbed.

As a word of explanation for the choice of human versus other sources of serum proteins, such as bovine or equine sources, the following comment is offered. It has been established in many instances that most drugs that exhibit binding to proteins are bound to about the same extent by human, bovine, and horse serum proteins, a fortunate coincidence since these are the common sources of albumin for experimental work. In general, however, the complete unpredictability of species differences and the variation of

relative binding power with different drugs dictate caution in transferring interaction data from one species to another.

It should be emphasized that the most fundamental aspect of the interactions of drugs with serum proteins is that these diverse combinations must be regarded as model systems for elucidating the nature of the primary interaction of each drug with its protein receptor in the body. In some instances the affinity for serum proteins seems to parallel the primary potency of a drug. Davis and Wood (65) showed that this is true in the sulfonamide series and another parallel situation has been indicated in the competition of certain substances for kidney tubular excretion and for binding to albumin. This, however, is not always the case and, certainly, not the general rule. For example, even among the sulfonamides, acetylation abolishes antibacterial action without affecting protein binding. Also, penicillins K and X, in the absence of albumin, are both more potent antibacterials than G, although the order for affinity or binding for albumin is K>G>X. Again, in the barbiturate series, interaction with protein cannot be correlated with either therapeutic potency or susceptibility to metabolic destruction in the human body. In fact, simple parallelisms are not to be expected. Only as the nature of the forces that are responsible for interaction specificity are explained and an insight into the precise nature of the

protein surface is achieved, shall the understanding of the binding phenomena be advanced, for it must be remembered that although no specificity exists between the two interacting molecular species, they have a selective affinity for each other.

A practical consequence of binding or interaction studies is that some drugs or other substances may be combined with local anesthetics and other materials to potentiate their action. For example, it is known that potassium salts enhance the analgesic effect of procaine. However, infiltration of tissues with solutions containing potassium usually causes pain, edema, and redness in the post-anesthetic period. Protins, also, are known to potentiate local analgesic action. Most commonly, gliadin, human plasma, and serum albumin, when added to procaine, are known to prolong its effect. The same is true for other local anesthetic agents. The increase in duration, however, is not remarkable from a clinical standpoint and usually the benefits derived are not worth the effort in the preparation of the solutions. These facts, then, at the present time, have a theoretical value or significance at best.

The behavior of human serum albumin toward local anesthetic as determined in this investigation is explainable by the theory that amino acid residues with -OH groups, on the

protein, interact preferentially with $-COO^-$ side chains. This internal binding or interaction demands that an organic cation must have a much higher interaction to be bound at a $-COO^-$ site than an organic anion requires to be bound at an $\equiv NH^+$ site. Among molecules of similar size and structure, anions are therefore bound much more strongly than cations; in this study, the cations are local anesthetics when prepared in solution as the hydrochlorides.

The results that were obtained in this investigation with human serum gamma globulin bear out the concept that this protein fraction of blood is, more than likely, not linked with the production of foreign proteins. Rather, as is well-known, the gamma globulins are primarily concerned with antibody production. The severity of an allergic reaction to a particular local anesthetic is possibly a measure of the ability of an organism to synthesize antibodies versus the foreign proteins that are produced when human serum albumin complexes with local anesthetics. Ordinarily, antibody formation is thought of only as a method of protection, while the harmful effects that can be produced by the presence of circulating antibodies is overlooked. As a matter of fact, antibody formation is a reaction of the body against foreign high molecular substances, having its counterpart in the body's reaction against low molecular weight substances, which is

termed detoxication. It should be borne in mind that both detoxication and antibody responses are the result of special conditions, and may be harmful as well as beneficial to the organism.

Since there is no difference in the number of binding sites but only in the degree of binding at these sites, for the local anesthetics used in this study with human serum albumin at the pH values investigated, namely, 6.8, 7.0, and 7.2, it may probably be safe to say that near the normal body pH values, the hydrogen ion concentration does not materially influence the statistical binding sites of the protein for local anesthetics. If this conjecture is correct, probably some levity in the preparation of anesthetic solutions for injection use, with reference to the pH of the solutions, is permitted. It might be noted, however, that the ability of local anesthetics to block nerve impulses of the sciatic nerve in decapitated frogs decreases as the pH is lowered appreciably, that is, as the degree of ionization increases. Therefore, the ability of local anesthetics to block nerve impulses may not bear a direct relationship with the binding of these agents. However, a study of the binding of local anesthetics at pH values farther removed than a few tenths of a pH unit from neutral or pH 7.0 would introduce another unphysiological factor, besides those already present,

into the artificial, but yet justified on theoretical grounds, conditions under which this project was carried out.

Alkalinization of solutions of local anesthetic drugs has been recommended, although sometimes unsuccessfully, to enhance the local anesthetic effects, but the procedure is of doubtful value. Although the free base and not the hydrochloride salt is the agent which penetrates into the cell and causes the physiological change attributed to a local anesthetic, regardless of the pH that a solution may have before injection, it becomes adjusted to the pH of the tissues after injection by virtue of the buffer action of the electrolytes in the tissue fluids. Most anesthetic solutions that are used clinically are acid in reaction because they are prepared from the salts and their pH value is raised to that of the tissues upon injection. Likewise, the pH of alkaline solutions is lowered to that of the tissues upon injection with the result that the effects of previous alkalinization are nullified.

One fundamental question which any investigation into the binding of proteins with drugs raises is whether or not and under what conditions the common interactions of drugs with proteins in the serum are capable of giving rise to antigenicity, and if so, under what conditions. There is no particular evidence in the literature or in the present

investigation to suggest a strict parallelism between affinity for serum proteins and allergic potentiality, but this does not bear upon the question. Obviously, interactions of a drug with a serum protein or proteins are not a sufficient condition for allergy, because, for example, all penicillin treated patients would become sensitive to this drug since it does exhibit protein binding. Whether interaction of the kind carried out in this work is a necessary condition remains to be proved.

From thermodynamic considerations and the theory of multiple equilibria discussed in Chapter III the following approximate calculations may be made. First of all, from equation [22] of the theory of multiple equilibria and the values of the intrinsic affinity constants, k, at pH 7.0 for example, for the anesthetic agents studied, the values of the equilibrium constants, k_1, are approximately 6.3×10^3, 9.7×10^3, 2.5×10^5, and 5.0×10^5 for procaine, butethamine, lidocaine, and mepivacaine, respectively. Next, respective values for the equilibrium constants, k_2, are approximately 1.6×10^3, 2.4×10^3, 6.3×10^4, and 1.3×10^5. Then, according to the theory of thermodynamics, the free energies of binding, or more commonly, the binding energies, at 4° Centigrade for the equilibria corresponding to these constants are approximately -4.85, -5.10, -6.89,

and -7.27 respectively, for k_1, and approximately -4.10, -4.34, -6.12, and -6.50 respectively, for k_2, in kilocalories per mole, calculated from the equation

$$\Delta F^{\circ} = -RT \ln K.$$

Here ΔF° is the standard free energy of binding, R is the gas law constant, T is the absolute temperature, and K is the appropriate equilibrium constant, k_1 or k_2. From the results it can be seen that the binding energy values are larger in absolute magnitude for the interaction of the first local anesthetic molecule with human serum albumin in the reaction

$$P + X = PX$$

than for the interaction of the second local anesthetic molecule with human serum albumin in the reaction

$$PX + X = PX_2,$$

where P is the protein and X is the local anesthetic agent.

These results are valid if, in the first place, the number of actual binding sites on the human serum albumin molecule is two for the local anesthetics procaine, butethamine, lidocaine, and mepivacaine. Also, the formulation given above is valid only if the interaction of the first local anesthetic ion does not influence the binding of a second ion except to the extent that there is one less site to which the second ion can attach itself. Ions that are successively bound do frequently interact. This means

that at times electrostatic interaction is considerable and can greatly outweigh the statistical effect. There can likewise be van der Waals' interaction between organic ions; this, no doubt, occurs in the interaction of local anesthetic agents with human serum albumin. A discussion of this type of interaction in reference to this study will be made now.

As two atoms approach each other there is a weak attraction which is primarily electrostatic in nature. This is known as the van der Waals' attraction force and varies as the sixth power of the distance between the atoms. The energy of such a "bond" is about 1000 calories per mole and is of importance in maintaining the crystal structure of many compounds. At shorter distances of separation a powerful van der Waals' repulsive force becomes effective which is similar in nature and magnitude to the forces of chemical bond formation and is due to the overlapping of the electronic distribution of the respective atoms. In a crystal these van der Waals' attractive and repulsive forces are balanced at a certain distance of approach of the atoms. By means of X-ray diffraction studies, it is possible to measure the distance separating atoms in a crystal and, accordingly, to assign a so-called van der Waals' radius to the atom. This distance represents the closest approach that can be made to an atom without chemical bond formation. Those

radii are useful in constructing molecular models and a chemical bond results when the repulsive forces are overcome and two atoms approach closer than the sum of their van der Waals' radii. There are several forms which this interaction between the two atoms can assume and which leads to the formation of different types of chemical bonds. The ionic bond involves a discrete transfer of electrons from one atom to the other, whereas a covalent bond results when two electrons are shared by the two atoms. Various hybrids of the ionic and covalent bonds are possible.

In any postulated structure of a protein which is to be used for fully explaining interaction phenomena the bond distances and bond angles must have accepted values. The values to be regarded as acceptable are those derived from X-ray studies of small molecules, such as amino acids and small peptides, and the chance of any large deviations from the average values is negligible. The most important feature is that the six atoms of the peptide group (-C-CO-NH-C-) invariably lie in a plane, or very nearly so. This is attributed to resonance, which is also responsible for the relatively short (HN-CO) bond. The structure retains freedom of motion in spite of the planarity of the peptide bond, rotation being possible about the two single bonds attached to each carbon atom. Apart from the covalent bonds, which

are usually obtained from amino acid sequence studies, the most important links in structures of proteins are the hydrogen bonds, such as (NH • • • OC) or (OH • • • OC). From results of investigations obtained thus far, virtually all the NH, CO, OH or similar groups which the structure contains are somehow linked up in hydrogen bonds; but the particular groups paired to one another cannot be predicted. The stereochemical conditions which a hydrogen bond must satisfy are not so restrictive as those governing covalent bonds, but the bond distance and bond angle must fall nevertheless within certain limits. Finally there are the van der Waals' contacts between neighboring atoms. These are not directional bonds nor are the permissible distances very precisely determined. However, a structure cannot have van der Waals' "bonds" which are unacceptably short. All in all, the conditions imposed by stereochemistry are very severe, and the number of configurations allowed by them for a structure is often very small. This does not necessarily mean that all the allowed configurations can be simply discovered. These considerations and the complete amino acid sequence of human serum albumin and most other proteins have not yet been completely worked out so that an all inclusive explanation of the binding sites and binding phenomena of human serum albumin cannot be given at this time.

A comparison of the binding energies of the hydrogen bonds, (NH • • • OC) and (OH • • • OC) of proteins, which are in the vicinity of 3.5 and 6.0 kilocalories per mole, respectively, with the approximate 5 kilocalories per mole free energy of interactions of local anesthetics and human serum albumin shows that the ability of some local anesthetic agents, at least, to bind with protein lies between these two types of hydrogen bonding. The approximate value of 5.0-7.0 kilocalories per mole obtained in this study probably means that the competition of local anesthetic agents for protein sites is greater between the hydrogen bonding (OH • • • OC) than between (NH • • • OC), everything else being equal. This must be the case since much less energy is liberated in the formation of the (NH • • • OC) hydrogen bonding than in the formation of the (OH • • • OC) hydrogen bonding. In any case, the local anesthetic agents probably combine at the sites, on the protein, which are left over from (NH • • • OC) and (OH • • • OC) hydrogen bonding, pH, temperature, ionic strength, concentration, and other conditions altering this competition one way or another depending upon the yet unknown configuration of the protein under any set conditions.

As a recapitulation it may be desirable to review the chemical aspects of protein binding of small ions with

reference to the binding of local anesthetic agents with human serum albumin. These aspects were listed in Chapter I.

1) Determination of the number of interacting molecules held by a human serum albumin molecule under specified environmental conditions resulted in a range between zero and two molecules of local anesthetic per molecule of protein depending on the concentration of the local anesthetic.

2) Determination of the maximum number of sites on a human serum albumin molecule available to a local anesthetic interacting species resulted in an approximate statistical value of two.

3) Thermodynamic considerations of the strength of the bond between the protein and any given local anesthetic used in this study gave a value in the neighborhood of 5000-7000 calories per mole as the binding energy of a local anesthetic with human serum albumin.

4) The effect of pH on the extent of binding of human serum albumin with local anesthetics was discussed in Chapter VI and showed that the pH of a solution of a local anesthetic and the protein affects the extent of binding at a particular concentration of local anesthetic, but the pH does not affect the maximum number of binding sites.

5) Concerning the structural characteristics of the interacting molecules which favor combination with human

serum albumin in this study, it may be said that the interacting local anesthetic agents belong to two main classes depending upon whether they are ester type local anesthetics or amide type local anesthetics. The amide type local anesthetics seem to be bound more strongly.

6) The molecular and configurational nature of the site on the protein at which a given local anesthetic agent is bound will be intimately dependent upon the yet to be complete elucidation of the details of protein structures in general and human serum albumin in particular.

It must be realized at this point that the prediction and analysis of small molecule-protein interactions under physiological conditions is a long way from the study of these same chemicals in relatively simple, artificial systems. However, the determination of the nature of such interactions under artificial conditions is sure to serve as a guide to the behavior of living systems.

In order to consider the pharmacological overtones into which this investigation has encroached, it may be best to begin by looking into the role that local anesthetic agents play with respect to the interference in the conduction of nerve impulses. In fact, the field of drug action on axonal conduction is limited to blocking drugs known as local anesthetics. Both the chemical mechanisms responsible for the

observed electrical phenomena in the conduction of nerve impulses along nerve axons and the chemical mechanisms of drug action on axonal conduction are not completely understood. However, the view has no emerged that the electrical events in a nerve fiber are governed by the differential permeability of its surface membrane to sodium and potassium ions and that these permeabilities themselves depend upon the electric field across the surface. The interaction of these two factors leads, at a certain critical threshold level, to excitation, that is, to a regenerative release of electrical energy from the axon membrane and the propagation of this change along the fiber in the form of a brief, all-or-none, electrical impulse called the spike or action potential.

The nerve cell wall is normally positively charged on the outside and negatively charged on the inside. This means that there is a tendency for electrically charged particles to move across the membrane in response to this charge or electrical potential. The only charged particles of consequence in this connection are ions in aqueous solution. In fact, for the purpose of discussing the principal features of the nerve signal, the structural picture can be reduced to that of a long cylindrical tube with a surface membrane which separates two aqueous solutions of equal osmolarity but of different chemical composition. In the external

medium, more than 90% of the osmotic balance is made up of sodium and chloride ions while inside the cell, these ions account for less than 10% of the solutes, potassium taking the place of sodium, and various organic anions, presumably synthesized within the cell itself, taking the place of chloride. Therefore, the concentration of sodium ions is about ten times higher outside of the cell than inside and the concentration of potassium ions is about 30 times higher inside the cell than outside. Thus, there is a potential difference across the cell membrane of some 60 to 90 millivolts while there are no detectable potential differences within the interior of the normal resting cell.

The structure of the cell membrane is such that it is relatively impermeable to the large hydrated sodium ions and an active metabolic mechanism dependent on oxidative processes literally "pumps" sodium ions out of the cells. According to present views of the ionic maintenance mechanisms of the resting cell, it is the cellular metabolism that is responsible for the upkeep of ionic concentration differences between the cell and its surroundings. This is accomplished by synthesizing large organic anions which cannot diffuse through the membrane and by providing the energy for an active ion-exchange mechanism, expelling sodium ions and accumulating potassium ions, possibly one

for one. The cell membrane has a generally extremely low ionic permeability so that even in the complete absence of pumping action it would take many hours before the sodium/potassium concentration gradients ran down. The ionic permeability of the resting membrane, apart from being small, is also differentially very selective, that to potassium being much higher than to sodium, so that the potential difference across the resting membrane is approximately that of a potassium concentration cell. Although it does not quite reach this level, it would do so only if the sodium permeability were negligible. Be this as it may, as in most instances of specific cellular utilization of metabolic energy yields, there is no theory at present to explain how the driving reaction of the sodium pump is geared to the express purpose of expelling sodium ions and "piling up" potassium ions.

A suitable stimulus brings about a momentary reversal of the electric potential across the nerve cell membrane and this is what is measured as the nerve impulse. Why the electric potential is reversed instead of merely destroyed is not thoroughly understood. It is due, in part at least, to a marked and specific alteration of the ionic permeability of the nerve membrane to sodium ions. These ions rush through the membrane into the cell momentarily (for about one milli-

second or less) and the excess of cations thus created inside the cell forces potassium ions outward to neutralize the potential difference. During the recovery process sodium ions are pumped to the outside of the cell and potassium ions move inside, and the normal state of polarization is thus restored. It should be noted that the electrical return of the system during the impulse is not brought about by a reversal of the ionic movement but by the leakage of an equivalent quantity of potassium ions. Both sodium and potassium are moving downhill during the impulse, but the quantities are so small that no detectable change of the internal concentration would occur during one impulse, and there is plenty of time for the pumping process to restore the chemical situation in resting periods later on.

The reversal of potential across the membrane at the site of the nerve impulse causes a sudden flow of ions through the adjacent axonal membrane. This flow of ions is sufficient to trigger a depolarization with a resulting action potential in this area, and by repetition of this process the impulse is propagated without decrement along the axon resulting in conduction or propagation of the nerve impulse.

The flow of ions initiated by the approaching nerve impulse is believed to cause the release of acetylcholine from an inactive precursor. The released acetylcholine

triggers a receptor protein in such a way as to cause depolarization of the membrane. Then acetylcholine is destroyed by acetylcholinesterase and resynthesized later by choline acetylase for storage. Since many local anesthetics contain chemical structures resembling acetylcholine, it is possible and most probable that they compete with the enzyme or enzymes governing this chain reaction and thereby interrupt the propagation of the nerve impulse.

If it is conjectured, in the light of the results of this study that local anesthetic agents interact with human serum albumin under specified conditions, that the receptor protein, which acetylcholine triggers in the propagation of the nerve impulse, is an albumin-like protein, it is possible that the blocking of the nerve impulse by a local anesthetic is brought about by the binding of the local anesthetic compound with the receptor protein, in competition with acetylcholine. In this way, then, it may well be that local anesthetics accomplish the antiacetylcholinesterase activity usually attributed to them. In other words, the blocking of acetylcholine action by local anesthetics may be carried out by inactivating the receptor protein by means of binding or interaction.

The statistical effect of this binding or interaction, as determined by this study, can be appreciated by comparing

the situation facing a local anesthetic molecule, X, as it approaches a human serum albumin molecule, P, with the situation when it approaches the complex, PX. In the latter case, the number of binding sites open to the local anesthetic molecule, X, is only one, whereas two binding sites were available to the local anesthetic molecule, X, approaching the human serum albumin molecule, P. Therefore, the chances of a successful interaction in the case of X approaching PX are only one-half that of X approaching P, and thus the probability of forming the second complex, PX_2, is less than that of forming PX. Another statistical effect working against the formation of PX_2, as compared to PX, arises even after the complexes have been formed. In the case of PX_2, there are two positions at which a single X molecule may be removed, since the first X molecule as well as the second molecule may come off. Therefore, the chance of PX_2 dissociating a single X molecule is twice as much as that of the complex, PX, losing a single X molecule. Therefore, again a probability factor is present which tends to reduce the concentration of the complex, PX_2. Then, from the thermodynamic standpoint discussed previously, k_2, will be reduced if the concentration of PX_2 is reduced and ΔF_2° corresponding to k_2 will not be as negative as it would have been if the probability factor were not operative. In addition to the

statistical effect, there is the other factor, discussed previously, which tends to reduce the relative stability of the complex, PX_2, as compared to the complex, PX. Since ions are being bound, charged units and, furthermore, species of like charge are being dealt with. Therefore, it is to be expected that the first bound local anesthetic ion would exert an electrostatic repulsion toward an approaching second ion. Again, the tendency of the second local anesthetic ion to be bound by PX would be reduced, and hence the strength of the bond decreased.

Modern concepts concerning the structure of proteins emphasize regularity in the pattern of occurrence of amino acid residues within the proteins. More simply, the fact that the fundamental peptide chains are all derived from peptide bond formation through the alpha carboxyl and alpha amino groups insures a strict spacing of the emergent residues along the peptide chain. This spacing between two peptide bonds is, therefore, a very regular distance called the identity distance and is equal to about 3.6 Angstroms. It is especially interesting, in view of the "two site binding results" obtained in this investigation of the interaction of local anesthetic agents with human serum albumin, that studies throughout the past decade relating various types of pharmacologic activity to intramolecular distances between

groupings in various series of drugs have revealed repeatedly the identity distance or some whole number multiple of the identity distance as the distance most readily correlated with optimum activity. Usually this multiple of the identity distance is an even-numbered multiple and for many nerve blocking agents this distance is about 7.2 Angstroms or about twice the identity distance. Therefore, if the multiple sites which bind a given drug with a protein are in reality the emergent amino acid residues of a protein, it is not surprising that the distances on the protein involved with drug activity follow the multiplicity rule with respect to the identity distance. In the present case, since the approximate length of the acetylcholine molecule is about eight Angstroms and the approximate length of the local anesthetic agents is in this same vicinity, there may be some connection between the "drug activity" of both acetylcholine itself and the local anesthetic agents which may take its place on the receptor protein, and the fact that their lengths are a little greater than twice the identity distance.

Recently (66,67), experiments have been performed which seem to show that the so-called receptor protein that is believed to be involved in the nerve impulse transmission mechanism has been isolated from an extract of electric tissue of Electrophorus electricus (electric eel). First,

there is a striking parallel between the binding of curare and related compounds to this protein in solution and their affinity for the receptor as measured by their effects on the electrical activity of the isolated electrophax. This interaction was studied by equilibrium dialysis under controlled conditions of pH and ionic strength. Also, testing of the binding to the protein of a whole series of choline derivatives showed that acetylcholine was bound most strongly. Interaction of the protein with procaine and other local anesthetics has also been found which was strikingly parallel to the graded action of the compounds on conducting membranes of both nerve and electroplax as well as on synapses of electroplax. These observations strongly support the assumption that the isolated protein is indeed the receptor. The protein has been purified to a considerable extent, to essentially one component in electrophoretic studies, and refinements in the isolation are still being continued. However, thus far, the complete identity and structure of the protein, as is true in the case of most proteins of biological importance, has not been completely worked out. Therefore, whether or not the receptor protein is partially or completely identical with the human serum albumin used in this investigation cannot be decided at this time.

In conclusion, therefore, it may be said that the

interaction of local anesthetics with human serum albumin may have biological significance from two viewpoints. First, the binding of local anesthetics with the protein may have one or more effects on the injection concentration, in the immediate neighborhood of a nerve, of the local anesthetic that is necessary for initiating proper and lasting analgesia. Second, the interaction may be important in the chemical and physical phenomena which lead, in the final analysis, to the local analgesia or nerve block by interfering with the normal train of events necessary for the conduction of a nerve impulse. Clarification of this possible dual role of local anesthetic binding with human serum albumin must await more complete knowledge of the chemical and physical three-dimensional picture of this protein.

Also, the failure of human serum gamma globulin to interact with the local anesthetic agents of this study cannot be explained until the actual chemical structure of this protein, likewise, has been determined and elucidated.

The present concept of a protein molecule is undoubtedly a gross oversimplification. Structural models cannot indicate the nature of the wide differences between different proteins such as are found in hormones, enzymes, blood proteins, and viruses. Some of the behavior of proteins may reside in localized complex surface patterns or three-dimensional

templates. This aspect of protein structure is one that is least known at the present time.

APPENDIX I

DETERMINATION OF HUMAN SERUM ALBUMIN MOISTURE CONTENT (Drying to Constant Weight at 105° Centigrade)

Sample Number	1	2
Weight of Weighing Bottle and Cover	19.04333g.	18.62314g.
Weight of Undried Albumin + Weighing Bottle and Cover	19.29370g.	18.90460g.
Weight of Undried Albumin	0.25037g.	0.28146g.
Weight of Dry Albumin + Weighing Bottle and Cover	19.28161g.	18.89182g.
Weight of Dry Albumin	0.23828g.	0.26868g.
Weight of Water Lost	0.01209g.	0.01278g.
Percentage Water Content	4.83%	4.54%
Average Percentage Water Content of Human Serum Albumin	4.69%	

APPENDIX II

A COMPARISON OF THE PERCENTAGE CONCENTRATIONS AND MOLAR CONCENTRATIONS OF SOME OF THE LOCAL ANESTHETIC SOLUTIONS USED IN THE EXPERIMENTS

1) Procaine·HCl

a) 0.2% = 7.32×10^{-3} M

b) 0.1% = 3.66×10^{-3} M

c) 0.05% = 1.83×10^{-3} M

d) 0.02% = 7.32×10^{-4} M

e) 0.01% = 3.66×10^{-4} M

f) 0.002% = 7.32×10^{-5} M

2) Butethamine·HCl

a) 0.2% = 7.32×10^{-3} M

b) 0.1% = 3.66×10^{-3} M

c) 0.05% = 1.83×10^{-3} M

d) 0.02% = 7.32×10^{-4} M

e) 0.01% = 3.66×10^{-4} M

f) 0.002% = 7.32×10^{-5} M

3) Lidocaine·HCl

a) 0.2% = 7.38×10^{-3} M

b) 0.1% = 3.69×10^{-3} M

c) 0.05% = 1.85×10^{-3} M

d) 0.02% = 7.38×10^{-4} M

e) 0.01% = 3.69×10^{-4} M

f) 0.002% = 7.38×10^{-5} M

4) Mepivacaine·HCl

a) 0.2% = 7.07×10^{-3} M

b) 0.1% = 3.54×10^{-3} M

c) 0.05% = 1.77×10^{-3} M

d) 0.02% = 7.07×10^{-4} M

e) 0.01% = 3.54×10^{-4} M

f) 0.002% = 7.07×10^{-5} M

APPENDIX III

PHOSPHATE BUFFER SOLUTIONS USED IN EQUILIBRIUM DIALYSIS EXPERIMENTS

pH	Molarity	Ionic Strength
6.8	M/15	0.133
7.0	M/15	0.148
7.2	M/15	0.162

These buffers were prepared by mixing volumes of solutions of M/15 secondary sodium phosphate (Na_2HPO_4) and M/15 primary potassium phosphate (KH_2PO_4), in proper proportion, and checking the pH of the resulting solutions with a pH meter.

APPENDIX IV

FITTING A STRAIGHT LINE TO LINEAR DATA

All straight lines in a plane are represented by rectangular coordinates by the formula:

$$y = mx + b.$$

This formula is completely characterized when the constants m and b have been determined. One method that is used in the determination of the constants m and b is the method of least squares. In this method, the sum of the squares of deviations of given values from the mean is minimized through the methods of the calculus to yield values for m and b such that their variability about the mean is weighed with respect to both their number and their degree of individual variation. The resulting formulas for m and b are

$$m = \frac{n\Sigma xy - \Sigma x \Sigma y}{n\Sigma x^2 - (\Sigma x)^2}$$

$$b = \frac{\Sigma x^2 \Sigma y - \Sigma x \Sigma xy}{n\Sigma x^2 - (\Sigma x)^2}$$

where n = number of paired data values,

Σx = sum of all x,

Σy = sum of all y,

Σx^2 = sum of the squares of all x values,

$(\Sigma x)^2$ = square of the sum of all x values,

and Σxy = sum of the products of all corresponding pairs of x and y values.

APPENDIX V

DETERMINATION OF HUMAN SERUM GAMMA GLOBULIN MOISTURE CONTENT (Drying to Constant Weight at 105° Centigrade)

Sample Number	1	2
Weight of Weighing Bottle and Cover	18.39172g.	17.90194g.
Weight of Undried Globulin + Weighing Bottle and Cover	18.48514g.	18.00326g.
Weight of Undried Globulin	0.09342g.	0.10132g.
Weight of Dry Protein + Weighing Bottle and Cover	18.47710g.	17.99467g.
Weight of Dry Protein	0.08538g.	0.09273g.
Weight of Water Lost	0.00804g.	0.00859g.
Percentage Water Content	8.61%	8.48%
Average Percentage Water Content of Human Serum Gamma Globulin	8.55%	

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Approval Sheet

The dissertation submitted by Vincent John Sawinski has been read and approved by a board of five members of the Graduate School faculty of Loyola University.

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 dissertation is now given final approval with reference to content, form, and mechanical accuracy.

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

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Date

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Signature of Adviser