AN EQUILIBRIUM DIALYSIS STUDY OF CERTAIN HISTAMINE-PROTEIN-ANTIHISTAMINE INTERACTIONS

by

BLANCHARD LEROY MICKEL

B. S., Washburn Municipal University, 1949

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1951

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INTRODUCTION

Although histamine was prepared by synthesis (1) in 1907, it was not until 1910 that the physiological activity of this substance was recognized (2). Since that time, an abundant literature has accumulated regarding this simple imidazole derivative, its physiological effects, and presence in body tissues.

The physiological action of histamine, upon experimental administration or release by allergies and other injurious reactions, has been summarized by Sir Henry Dale (3). Nearly all plain muscle is said to be stimulated by histamine. The intensity of this action is not uniformly distributed, but the plain muscle coats of the bronchioles seem especially responsive. Histamine is said to bring about the asthmatic reaction of the allergies as a result of its bronchiole-constrictive properties (3). The urticarial reactions of the allergies are said to be a consequence of its capillary-dilatant action (3).

To effect symptomatic relief in allergy, the attention of therapeutic chemists was drawn to substances exhibiting a specific antagonism toward histamine. Of the many compounds reported in the literature as exhibiting such antihistiminic activity, Antergan, or N,N-Dimethyl-N'-phenyl-N'-benzylethylenediamine, was the first therapeutic agent commonly employed.

A multitude of compounds similar to Antergan have been used more recently. Although these various antihistiminics

differ in physiological activity, they have certain structural features that are common to all. Bovet (4) notes that in the first place, each possesses one or more strongly saturated tertiary or quarternary amines groups. Secondly, this amino group is found in the β position, and attached to an aliphatic chain. Thirdly, another amino group or an oxygen atom forms a linkage between a strongly electropositive nucleus and the basic side chain.

Most evaluations of antihistiminic potency have been of a highly empirical nature and generally involve the reaction of a particular animal tissue in solutions of varying histamine-antihistamine concentration. With regard to a mechanism of reaction, Wells (5) stated:

> The histamine antagonists fail to meet the requirements of certain of the well-known types of antagonism, such as "physiological" or "chemical" antagonism, and we have thus assumed that their mechanism of action is what has been termed as "specific" antagonism, a more descriptive name for which is "competitive inhibition". The implications of such a mechanism of antagonism are that both the active drug and its antagonist compete for the same site of attachment in a tissue, the antagonist combining with this site without eliciting a response of the tissue. In more picturesque terms, the antagonist may be considered as acting the part of the "dog in the manger".

In view of this theory of competitive binding, it was thought that a study of the possibilities of such interaction would prove worthwhile. For this purpose, a representative protein having general binding tendencies was selected and the separate reactions of this protein with histamine, and with each of several antihistamines were observed. To render binding

tendencies comparable, conditions of pH and temperature were maintained constant throughout the work. The method of study was quantitative in that both the extent of drug-protein interaction and the accompanying free energy changes could be calculated. By determining the extent of the drug-protein interaction and the accompanying free energy change coincident with any complex formation, it was hoped that the mechanism of the histamine-protein-antihistamine reaction could be better understood.

METHOD OF STUDY

Derivation of Equations

One of the simplest and most straightforward methods of protein binding study is that developed by Irving M. Klotz of Northwestern University (6). Derived from the law of mass action, this method lends itself well to the accumulation of thermodynamic data and requires a minimum of complex apparatus and procedures. A full derivation of the final equations needed in using this technique will be presented in this thesis.

The binding by protein material of ions or molecules (hereafter referred to as drug molecules) is considered to be reversible and to occur in a stepwise manner. The successive binding reactions and equilibrium expressions for each may be represented as

(1) $\quad Pr + M \rightleftharpoons PrM \qquad k_1 = \dfrac{(PrM)}{(Pr)\,(M)}$

$$PrM + M \rightleftharpoons PrM_2 \qquad k_2 = \frac{(PrM_2)}{(PrM)\,(M)}$$

$$PrM_2 + M \rightleftharpoons PrM_3 \qquad k_3 = \frac{(PrM_3)}{(PrM_2)\,(M)}$$

. . . .

$$PrM_{i-1} + M \rightleftharpoons PrM_i \qquad k_i = \frac{(PrM_i)}{(PrM_{i-1})(M)}$$

. . . .

$$PrM_{m-1} + M \rightleftharpoons PrM_m \qquad k_m = \frac{(PrM_m)}{(PrM_{m-1})\,(M)}$$

In the preceding, Pr represents the protein, M the binding drug, and PrM_i the protein-drug complex in which i drug molecules are bound to each protein molecule. The subscript letter m designates the maximum number of drug molecules capable of being bound by the given protein molecule. Brackets in the equilibrium expressions indicate the activity of the substances enclosed therein.

The quantity r, defined as the total moles of bound drug per total moles of protein, may be expressed by the equation

$$(2) \qquad r = \frac{(PrM) + 2(PrM_2) + 3(PrM_3) \;.\;.\;. + m(PrM_m)}{(Pr) + (PrM) + (PrM_2) \;.\;.\;. + (PrM_m)}.$$

The concentration of bound drug, (M), in the numerator of the preceding must be expressed in terms of the protein-drug complex molecules. This is equal to the concentration of each distinct complex molecule multiplied by the number of potential individual drug molecules bound in each complex.

The products of the successive equilibrium constants may be shown to have the general form

$$(3) \qquad k_1k_2k_3 \cdot \cdot k_m = \frac{(PrM_m)}{(Pr)\,(M)^m}.$$

A substitution for the activities of the complex in equation 2 brings about the elimination of these terms.

$$(4)\quad r = \frac{k_1(Pr)(M)+2k_1k_2(Pr)(M)^2+3k_1k_2k_3(Pr)(M)^3\ldots+mk_1k_2\ldots k_m(Pr)(M)^m}{(Pr)+k_1(Pr)(M)+k_1k_2(Pr)(M)^2 \quad \cdots +k_1k_2 \cdots k_m(Pr)(M)^m}$$

Dividing out the (Pr) terms from the numerator and denominator, and factoring (M) in the numerator of equation (4) yields

$$(5)\quad r = \frac{(M)\ k_1 + 2k_1k_2(M) + 3k_1k_2k_3(M)^2 \quad \cdots +mk_1k_2 \cdots k_m(M)^{m-1}}{1 + k_1(M) + k_1k_2(M)^2 \quad \cdots +k_1k_2 \cdots k_m(M)^m}$$

The numerator of equation 5 is seen to be the product of (M) times the first derivative of the denominator (f) with respect to (M).

$$(6)\quad \frac{df}{d(M)} = k_1 + 2k_1k_2(M) \quad \cdots +mk_1k_2 \cdots k_m(M)^{m-1} = \text{numerator.}$$

$$(7)\quad \frac{(M)\ df}{d(M)} = (M)\left[k_1 + 2k_1k_2(M) \quad \cdots +mk_1k_2 \cdots k_m(M)^{m-1}\right] = (M)f',$$

where f' is the first derivative of the denominator with respect to (M). Therefore

$$(8)\quad r = (M)f'/f\ .$$

McLean and Hastings (7) also approached protein complex formation from the standpoint of reversible binding. Studying the interaction of calcium ion with serum albumin, they considered the protein molecule to be a group of independent binding sites opposite in sign and charge to that of the ion bound. They set up an equilibrium expression for the dissociation process of the following form

$$(9)\quad K = \frac{(M)\ (Pr)}{(MPr)}\ ,\ \text{where}$$

(Pr) represents the concentration of free protein binding sites rather than that of the free protein molecule. Similarly (MPr) expresses the concentration of complex bonds formed rather than that of moles of complex. McLean and Hastings assume that, in addition to the nature of the reactants, the only other factor regulating such binding is of a statistical character. Such a treatment demands that all bonds be considered equivalent, regardless of the order of binding, and exclusive of any steric or intra-molecular electrostatic effects.

The concentration of free binding sites (Pr) and of complex bonds formed (MPr) may be related to the concentration of free protein and bound protein respectively, since

(10) $(MPr) = (M_{bound}) = (M_{total}) - (M_{free})$, and

(11) $(Pr) = \frac{\text{maximum moles M bound (m)}}{\text{mole Protein}}$ (total moles Protein)-(MPr).

Substituting these values into equation 9 gives

(12) $$K = \frac{(M_{free})\left[mPr_{total} - (M_{bound})\right]}{(M_{bound})} ,$$

which may be rearranged to express the moles of bound drug per total moles of protein, previously defined as r.

(13) $$r = \frac{(M_{bound})}{(Pr_{total})} = \frac{m(M_{free})}{K + (M_{free})} .$$

At this point, it is interesting to note that equation 13 is of the same form as that of the Langmuir adsorption isotherm equation

(13a) $$y = \frac{aP}{1 + bP} .$$

Here, y is the amount of a gas adsorbed per unit area or mass of adsorbent, a and b are constants, and P is pressure.

Klotz (6) has set equation 13 equal to equation 8 and removed (M_{free}) from the numerator of each, obtaining

(14) $$f'/f = \frac{m}{K + (M)}, \text{ or}$$

(15) $$df/d(M) = \frac{mf}{K + (M)}.$$

Solution of equation 15, a simple differential equation, yields

(16) $$\ln f = m[\ln K + (M)] + \text{constant}.$$

Since quantities are equal whose logarithms are equal

(17) $$f = [K + (M)]^m = [1 + (M)/K]^m + \text{constant}.$$

If the denominator of equation 5, or f, is set equal to f of equation 17, it is seen that

(18) $$1 + k_1(M) + k_1k_2(M)^2 \cdot \cdot + k_1k_2 \cdot \cdot k_m(M)^m = [1 + (M)/K]^m + \text{constant}.$$

For the purpose of a simplified exposition, m is arbitrarily assigned the value of four. Using this value of m, equation 14 is successively differentiated with respect to (M).

(19a) $$d/d(M)\left[1+k_1(M)+k_1k_2(M)^2+k_1k_2k_3(M)^3+k_1k_2k_3k_4(M)^4\right] = d/d(M)\left[1+(M)/K\right]^4$$

$$= k_1 + 2k_1k_2(M)+3k_1k_2k_3(M)^2+4k_1k_2k_3k_4(M)^3 = 4/K\left[1 + (M)/K\right]^3$$

(19b) $$d^2/d(M)^2 = 2k_1k_2+6k_1k_2k_3(M)+12k_1k_2k_3k_4(M)^2 = 3\cdot4/K^2\left[1+(M)/K\right]^2$$

(19c) $$d^3/d(M)^3 = 6k_1k_2k_3 + 24k_1k_2k_3k_4(M) = 2\cdot3\cdot4/K^3\left[1 + (M)/K\right]$$

(19d) $$d^4/d(M)^4 = 24k_1k_2k_3k_4 = 1\cdot2\cdot3\cdot4/K^4.$$

The generalized form of the last derivative is seen to be

(20) $$m!(k_1k_2k_3\cdots k_m) = m!/K^m ,$$

and is applicable to the product of the successive equilibrium constants regardless of the size of m.

Equation 20 is next consecutively substituted back through the series of successive derivatives. By this procedure, (M) terms are cancelled, and the products of the k values are obtained in terms of K alone.

(21a) $$k_1k_2k_3 = 4!/3!K^3 = 4/K^3$$

(21b) $$k_1k_2 = 6/K^2$$

(21c) $$k_1 = 4/K$$

Solving for individual k values in terms of the k product values from the preceding equation series yields

(22a) $$k_2 = k_1k_2/k_1 = 3/2K$$

(22b) $$k_3 = k_1k_2k_3/k_1k_2 = 2/3K$$

(22c) $$k_4 = k_1k_2k_3k_4/k_1k_2k_3 = 1/4K.$$

The general form for these individual equilibrium constants is seen to be

(23) $$k_i = \frac{m - (i - 1)}{iK} .$$

Here m represents the maximum moles of bound substance per mole of protein, K is the McLean-Hastings constant, and i is the order of consecutive binding. The term k_i may be defined as the equilibrium constant for the reversible binding of the ith mole of drug by protein which has already bound (i - 1) moles of that drug per mole of protein.

Evaluation of Constants

To use equation 23 in the calculation of successive equilibrium constants, K and n must first be evaluated. This may be accomplished by a suitable treatment of equation 13. In the derivation of equation 13, all binding sites were considered to be of the same nature. Electrostatic effects of bound groups upon entering groups were assumed to be negligible. The extent of binding was taken to depend only upon the concentration of reactants (in accordance with the law of mass action) and the intrinsic binding tendencies of these reactants. If the assumptions made in the derivation of equation 13 are justified, a graph of the moles bound per mole of protein versus the equilibrium concentration of the free drug should produce a straight line. Taking the reciprocal of equation 13 and rearranging gives

$$(24) \qquad 1/r = \frac{(K) + (M)}{n(M)} = \frac{K}{n}\frac{1}{(M)} + \frac{1}{n} \ .$$

A plot of $1/r$ against $1(M)$ should produce a straight line. From this equation, K/n is seen to be the slope, and $1/n$ the intercept on the vertical axis. At a value of $1/(M)$ equal to zero, the concentration of M is seen to be infinite. At infinite M concentration, binding would be expected to reach its maximum. By graphical extrapolation to the vertical axis, $1/n$ may be determined from which follows the value of n, or maximum bound entities per protein molecule.

The slope of the binding curve is then calculated. By equating K/n to the slope, n being known, the intrinsic disso-

ciation constant, K, may be evaluated.

By the Method of Least Squares. Points plotted from experimental data may follow a random linear distribution not lending itself well to an accurate extrapolation. Under such circumstances, recourse to the method of least squares for a determination of constants may be advantageous. Details of procedure will not be discussed at this point, but the method involves the setting up of normal equations, from the data, for the two constants. Solution of these simultaneous equations yields the most accurate values of 1/m and of K/m from the available data.

Thermodynamic Calculations

Standard Free Energy Change. By substitution of K and m values into equation 23, the value for any successive binding equilibrium constant may be calculated. The equilibrium constant for any reversible reaction may be related to the standard free energy change for that reaction. The free energy of any substance may be defined as

$$F_A = F_A^o + RT \ln (A) , \tag{25}$$

where F_A is the total free energy of substance A, F_A^o its free energy in the standard state, and (A) its activity. The gas constant is represented by R, and T is the absolute temperature. When one mole of A reacts with one mole of B to form a mole, respectively, of products C and D, the free energy change for the reaction is

$$\Delta F = (F_C + F_D) - (F_A + F_B) , \tag{26}$$

which by substitution of equation 25 for each reactant and product may be rewritten as

$$\Delta F = \Delta F^{o} + RT \ln \frac{(C)(D)}{(A)(B)} . \quad (27)$$

The thermodynamic condition for equilibrium is that the free energy change for the system shall be zero. Therefore

$$0 = \Delta F^{o} + RT \ln \frac{(C)(D)}{(A)(B)} , \text{ and} \quad (28)$$

$$\Delta F^{o} = -RT \ln \frac{(C)(D)}{(A)(B)} . \quad (29)$$

Since the standard free energy change for a given reaction at any one temperature is constant, the activity product ratio must also be constant, this quantity therefore being defined as the thermodynamic equilibrium constant, or

$$\Delta F^{o} = -RT \ln k_{eq} . \quad (30)$$

Here ΔF^{o} is the standard free energy change for the reaction in which reactants start and form products all of unit activity. If R is set equal to 1.987 calories per degree per mone, and T is the absolute temperature at which the reaction occurred, then ΔF^{o} is in units of calories per mole. The thermodynamic equilibrium constant must be in terms of reactant and product activities. At the low concentrations employed in this binding study, the use of concentration to represent activity is common practice (8).

By substitution of the previously determined binding equilibrium constants into equation 30, standard free energy changes for each successive step in such binding may be calculated.

Entropy and Enthalpy Change. By determining the free energy

change of a given reaction at several different temperatures, a graph of ΔF as ordinate against T as abscissa may be constructed. The free energy change of any process may be related directly to the enthalpy and entropy changes of that process by the equation

(31) $$\Delta F = \Delta H - T\Delta S ,$$

where ΔH and ΔS are the enthalpy and entropy changes, respectively, and T is the absolute temperature. A familiar form of the Gibbs-Helmholtz equation is

(32) $$\Delta F = \Delta H + T(\partial \Delta F / \partial T) ,$$ from which it follows

(33) $$-(\partial \Delta F / \partial T) = \Delta S .$$

Equation 33 is seen to also represent the slope of the temperature free energy curve at any one temperature. Therefore, the entropy change at that temperature may be directly determined. Substitution into equation 31 permits calculation of the corresponding heat change.

EXPERIMENTAL

Technique

To apply the equations as developed in the preceding section, data regarding two variable factors must be obtained. The first of these variables is the concentration of unbound drug, (M), in equilibrium with that drug which is bound by the protein. The second is the amount of this drug bound per mole of protein, or r, at the equilibrium concentration referred to above. Such data was obtained experimentally by the method of equilibrium dialysis, as developed by Klotz et al (9). A description of the typical

experimental procedure is given below.

Drug Solutions. A series of seven solutions, each having different concentrations of the drug to be studied was prepared in buffer solvent. The series was prepared by successive dilution with buffer of a stock solution, also in buffer, whose concentration was high compared to that of the series. A representative group of seven such solutions ranged in concentration from 4×10^{-5} to 10×10^{-5} moles per liter, the concentration of each solution decreasing by increments of 1×10^{-5} moles per liter. The stock solution, from which the series was prepared, was of 40×10^{-5} molar concentration.

The necessity for the use of low solute concentrations is apparent in view of the preceding thermodynamic derivations. Furthermore, mono-molecular dispersion, essential in cases involving spectrophotometric analysis, is more probable in highly dilute solutions

Dialysis Cells. Twenty milliliters of each solution were pipetted into separate clean, dry, twenty-five by two hundred millimeter Pyrex test-tubes. Into each of these tubes was placed a small cellophane dialysis sack, knotted at both ends, and containing exactly 10 or exactly 20 ml of the protein solution to be studied. For every protein containing dialysis cell a corresponding blank cell was prepared, identical in all respects to the former except that it contained buffer, rather than protein solution, in the cellophane sack. The dialysis cells were closed with clean, dry, corks or rubber stoppers, placed vertically in a shaker (Plate I), and allowed to come to concentration equi-

EXPLANATION OF PLATE I

Fig. 1. Dialysis cells in rack.

Fig. 2. Complete shaker assembly.

PLATE I

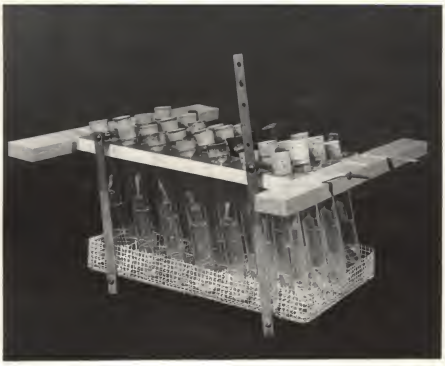


Fig. 1.

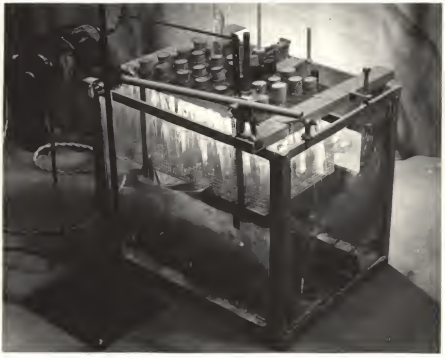


Fig. 2.

librium. A temperature of 4° C. to 5° C. was maintained in the cold room in which the cells were equilibrated.

The purpose of the blank cell was to compensate for any interaction between the drug and cellophane. If equal areas of cellophane were immersed in each pair of cells, concentration changes between the two cells resulting from membrane-drug interaction would be cancelled. Differences in final concentration of unbound drug between the two cells would then, presumably, be due, exclusively, to binding by the protein molecules.

Protein Solutions. The protein solutions, made up in the buffer solvent, had been equilibrated with buffer, prior to use, by a continuous dialysis procedure. Protein concentration of a typical binding determination was 0.15 percent by weight. This low concentration is desirable in consideration of Donnan membrane effects.

Equilibration. Time for concentration equilibration at 4° to 5° in the shaker varied from twenty-four to seventy-two hours. The condition of equilibrium was determined by analysing at intervals for an unchanging concentration outside the dialysis sack of the cell containing the most concentrated drug solution. When this concentration was observed to decrease no further with time, the system was assumed to be in equilibrium.

Concentrations Measured. Upon equilibration, the concentration of free drug outside the protein containing dialysis sack was determined and the determination repeated for each of the blank cells. The difference in equilibrium drug concentration between any protein containing cell and its corresponding blank

cell should have occurred only as a consequence of drug-binding by the protein. (Experimentation revealed the operation of certain factors which rendered the preceding statement only true in theory. An empirical correction for these complicating factors is described in a later section.) The difference in concentration between each pair of solutions, after suitable corrections, was reduced therefore to the number of moles of drug in the total volume of the protein cell (including the initial volume within the dialysis sack) removed from solution by protein complex formation. The number of moles of drug removed was then divided by the moles of protein present in the dialysis sack, the quotient being the number of moles of bound drug per mole of protein. This is represented by r in equation 24.

The unbound drug concentration inside the protein sack was taken to be equal to that determined, spectrophotometrically, outside the dialysis membrane. This was the free drug concentration at which bound and unbound drug were in equilibrium with one another. The reciprocal of this equilibrium concentration, or $1/(M)$, was plotted as abscissa against the reciprocal of r as ordinate. This plot was extrapolated, as explained previously, or a least squares solution taken, and the constants K and n evaluated.

Reversibility of Binding. It is seen, from the section regarding the derivation of fundamental equations, that only reversible binding may be properly treated by the method of equilibrium dialysis. Therefore, upon completion of a binding de-

termination, several protein containing dialysis sacks were removed from the original cells, and each was immersed in 20 ml of buffer solution. The procedure was repeated for each of the original corresponding blank dialysis sacks. If the binding was reversible, the drug molecules should have diffused out through the membrane and into the surrounding buffer solution. The new equilibrium thus established, and the relative protein and blank cell concentrations, should have been in proportion to the equilibrium values observed originally.

Drug Concentration Determination

Optical Absorption and Concentration. A spectrophotometric method of analysis was applied throughout this work. Justification for such an analytical technique rests upon certain fundamental rules of absorption behavior.

The general law of optical absorption may be represented, mathematically, as

(34) $$-dI = I\mu\, dx$$

where -dI expresses the decrease in original intensity, I, of a light beam as that beam passes through an increasing length, dx, of absorbing material. The proportionality constant relating the variables at any one wavelength is μ, and is referred to as the absorption coefficient.

If equation 34 is re-arranged and integrated within limits, as shown below, an important relation is obtained.

(35) $$\int_{I_o}^{I} dI/I = -\mu \int_{0}^{x} dx \qquad \text{is equal to}$$

(36) $\ln I/I_0 = -\mu x$ which in turn is equal to

(37) $I = I_0 e^{-\mu x}$.

Equation 37 is the common expression of Lambert's Law, and relates the intensity of light, I, of original intensity I_0 to the length of absorption medium, x, through which it has passed.

At any given wavelength, the absorption coefficient may be shown to be

(38) $\mu = k_\lambda C$,

where C is the concentration of absorbing material, and k_λ is the proportionality constant for a given absorbent at a particular absorbed wavelength. Substituting this value of μ into equation 37 yields the conventional formula for the law of Beers',

(39) $I = I_0 e^{-k_\lambda Cx}$.

Putting equation (39) in logarithmic form, and changing to base ten gives

(40) $2.303 \log I_0/I = k_\lambda Cx$.

The optical density, D, of an absorbing medium is defined as

(41) $D = \log I_0/I$.

Substituting D into equation (40) and re-arranging yields

(42) $D = k_\lambda Cx/2.303$ which may be simplified to

(43) $D = k'_\lambda Cx$.

It is seen that at constant wavelength and light path, the optical density of an absorbing substance is in direct proportion to the concentration of that substance present. Therefore, a plot of optical density against concentration, holding light path and wavelength constant, would be expected to yield a straight line. This graph could then be used in the analysis of

solutions of unknown concentration. By determining the optical density of such an unknown solution, concentration could readily be obtained by interpolation from the curve prepared previously for that substance.

The Instrument and Its Use. The Beckman Model DU Spectrophotometer, used throughout this work, is an instrument in which the readings are made directly in terms of optical density. Both its design and operation are explained fully in literature prepared by the manufacturer. For this reason, no detailed description of instrumental procedure will be attempted in this thesis. Instead, only the major steps in its application to this work will be outlined.

The first step, prior to analysis, was construction of an absorption curve for each of the colorless drugs studied. Optical densities were measured from a wavelength of 4000 Å to about 2200 Å, the ultra-violet limit of the instrument for the solute concentrations employed. These absorption curves are shown in Fig. 1.

Secondly, a wavelength providing considerable density change per unit change in concentration was selected. At this wavelength, density measurements were made upon a series of drug solutions of known but varying concentration. This range covered the range of sample concentrations to be analysed. From the data, a graph of optical density plotted against concentration was drawn. Examples are shown in Fig. 2.

Thirdly, the instrument was used in subsequent quantitative analyses of each drug studied. From the optical density of a

given sample, as indicated by the Beckman, concentration of that sample was readily determined by interpolation of its concentration-density curve.

Errors in Ultra-Violet Analysis. Absorption analysis in the ultra-violet region is complicated by a number of factors not encountered when analysing at visible wavelengths. These are concerned with the buffer, cellophane plasticizer, and the protein itself.

Since the phosphate buffer salts present in all solutions exhibit optical absorption beginning at a wavelength of about 3400 Å (Fig. 1), several precautions regarding the buffer were necessitated when analysing below this wavelength. One precaution taken was to equilibrate the protein with buffer in a continuous dialyser for two to three days prior to use. This was necessary in order that no buffer binding by protein should occur and be analysed as drug-binding instead. Another precaution taken was to equilibrate the cellophane dialysis tubing with the buffer in order that buffer concentration would not change as a result of cellophane-buffer interaction. This interaction was studied by immersing a strip of the cellophane in buffer for several days, then analysing photometrically for any change in buffer concentration. Though no change was observed, it is possible that certain substances, possibly plasticizers, dissolved out of the cellophane might have obscured this effect. A third and important consideration regarding the buffer was to obtain a representative optical blank to cancel buffer salt and water absorption when analysing with the spectrophotometer.

Since all drug and protein solutions were made up in the same buffer, it was essential that the buffer salt concentration in the optical blank be the same as that in the samples analysed. Concentration by evaporation of the buffer was observed to occur in the siphon tube leading to the storage carboy. Therefore, a portion of the buffer was drawn off and discarded before collecting solution to be used as a blank.

When the cellophane sacking alone was immersed in distilled water for several days, optical density of the solvent was observed to increase. This most likely indicated the presence of plasticizer dissolved out of the cellophane. The absorption of this substance appeared just beyond the visible range at a wavelength near 3800 $\mathring{A}$, and increased steadily to the ultra-violet limit of the instrument, as shown in Fig. 1. To minimize this error which was effective throughout the entire range of optical analysis, it was necessary to remove as much of this absorbing material as possible. This was accomplished by two methods. First, the cellophane was soaked several days prior to use in buffer solution. Several times during this soaking period, the liquid was poured off the cellophane and replaced with fresh buffer. The plasticizer was dissolved out, and the cellophane equilibrated with buffer in one operation. The second method involved continuous extraction with distilled water. A large commercial reagent bottle, filled with distilled water, was inverted and supported by a ringstand such that the mouth of the bottle was just below the top of a liter beaker containing the cellophane. A small glass funnel was placed, stem up, at the

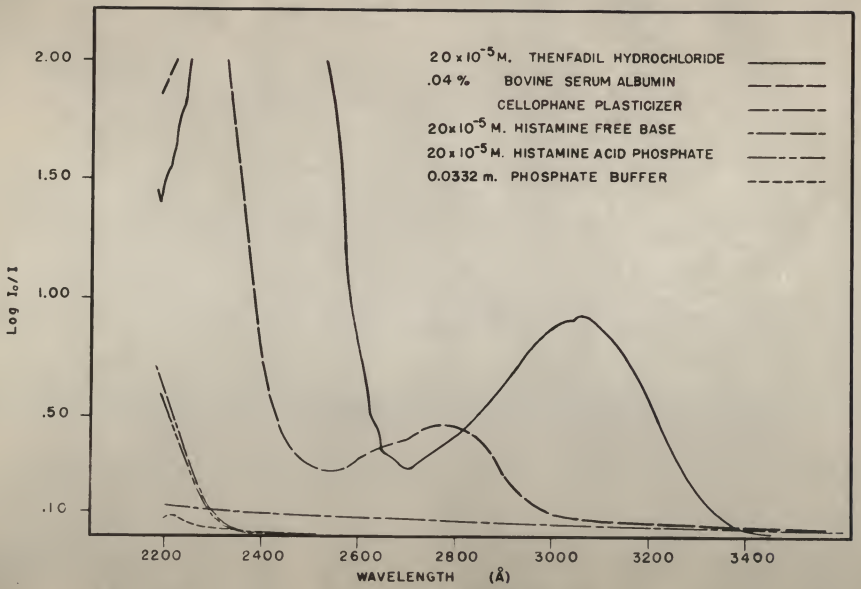


Fig. 1. Absorption curves of substances studied.

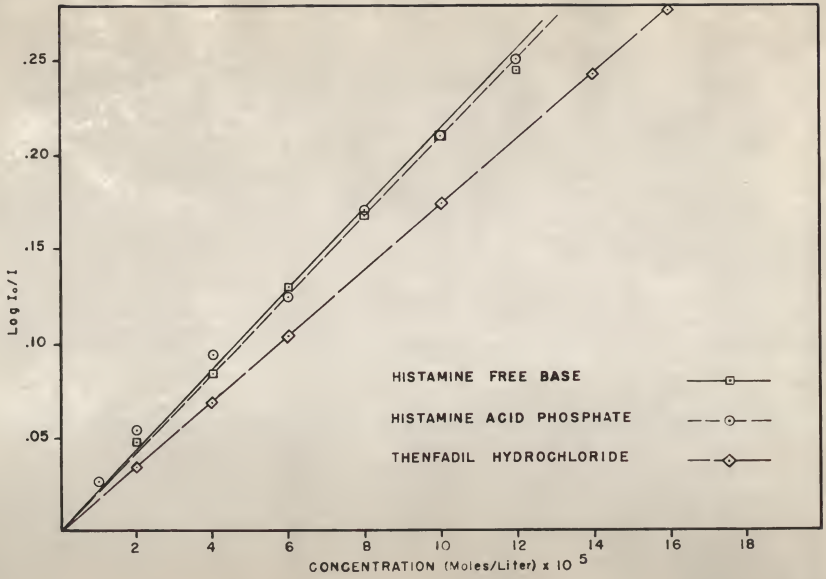


Fig. 2. Validity of Beer's Law for solutions studied.

bottom of the beaker and attached at the stem to a rubber siphon hose. Flow through this siphon was regulated by means of a screw clamp. As water containing extracted plasticizer was siphoned off below the cellophane in the beaker, fresh water bubbled in at the same rate from the bottle at the top. After extraction, the cellophane was equilibrated with buffer as described previously.

A further complicating factor of protein leakage appeared in the ultra-violet region. Since even extremely dilute bovine serum albumin exhibits pronounced and extensive absorption areas in the ultra-violet, as shown in Fig. 1, any leakage produced errors of magnitude rendering such readings valueless. Though leakage occurred infrequently it was immediately apparent by density readings far larger than reasonable.

Empirical Correction. Even after the elaborate precautions described in the preceding paragraphs, binding determinations analysed at wavelengths approaching 2200 Å exhibited a behavior not completely understood. Through some buffer-membrane interaction or membrane effect, the equilibrium optical densities of the blank drug solutions were observed to be higher than might be expected if the original solutions were diluted by the volume of buffer contained in the dialysis sack. Therefore, in later work, at the beginning of each binding determination, duplicate blank cells containing buffer within and without the dialysis sack were prepared. As suspected the optical densities of these buffer-membrane-buffer blank solutions were observed to increase with time above the density of stock buffer solution. The

average of these buffer blank density increases, when deducted from the density readings of the drug-containing blank cells, reduced the equilibrium optical densities of the latter to within reasonable limits.

A blank similar to that used in the binding determination, and identical to the membrane-buffer blank with the exception that the dialysis sack contained the same protein solution was set up. Optical density of the outer solution in this membrane-protein blank was also observed to increase with time. In binding determinations, the measured optical densities of the drug-protein cells were, therefore, also corrected by the average of this deviation corresponding to the time for drug-protein equilibration. In the following table, the increases with time of the membrane-buffer and membrane-protein blank optical densities for duplicate cells, analysed at 2235 Å are shown.

Table 1. Variation of empirical density corrections with time.

Hours	Membrane-protein blanks			Membrane-buffer blanks		
	Cell 1	Cell 2	Average	Cell 1	Cell 2	Average
21	0.018	0.016	0.017	0.057	0.044	0.051
36	0.018	0.018	0.018	0.066	0.043	0.055
62	0.024	0.023	0.024	0.068	0.047	0.058

This table shows that the membrane-protein blank density increases are much less than those of the membrane-buffer blanks. This would suggest protein binding of a substance extracted from

from the cellophane, in spite of the painstaking extraction procedures to which the latter was subjected.

At wavelengths approaching the visible range, correction values were observed to diminish significantly, indicating that the error was produced by a substance absorbing primarily at the shorter wavelengths. Both the buffer and cellophane plasticizer exhibit such increased absorbance at these shorter wavelengths, as shown in Fig. 1.

Protein Concentration Determination

Protein concentration determination and correction for water content was effected in one operation. The amount necessary to produce the desired approximate protein concentration was weighed out and put into solution in the buffer. After dialysing in buffer and diluting to the proper volume for the chosen approximate concentration, a 20 ml sample was pipetted into a clean, dry, weighing dish. Both the dish and its lid had been weighed prior to filling with the protein solution. The sample was then dried at 80° to 90°, to avoid boiling and possible loss at higher temperatures, for twenty-four to forty-eight hours. After this preliminary drying, the sample was placed in a second oven and maintained at 110° for another twenty-four hours. According to Mellor, the second heating should have removed water of hydration from the buffer salts. Other investigators (9) have stated that such treatment is sufficient to properly dry the protein.

Upon completion of the drying procedure, the lid was

placed on the dish, to prevent uptake of moisture from the air, and weighed after cooling. The weight of anhydrous buffer salt in 20 ml of solution was subtracted from the weight of the dry residue, and the weight remaining was taken to be that of protein present in 20 ml of solution.

Materials

Buffer. In equilibrium dialysis study, solutions of both protein and dialysate must be made up in a buffer solvent. Such a buffer solvent serves not only as a stabilizer of pH, but as an equalizer of ionic strengths on the two sides of a semi-permeable membrane separating a diffusible and a non-diffusible electrolyte. This is necessary in order to reduce the Donnan membrane equilibrium effect to a minimum. From Donnan theory it may be shown that, in the simplest system involving equal volumes of electrolyte on the two sides of a semi-permeable membrane,

(44) $$x = (C_d)^2 / [(C_n) + 2(C_d)] .$$

Here, x represents the concentration of a diffusible electrolyte which has passed through a semi-permeable membrane and into a region of non-diffusible electrolyte of concentration (C_n). The quantity (C_d) expresses the initial concentration of the diffusing electrolyte. From equation (44) it is seen that to permit maximum transfer of electrolyte through the membrane, (C_n) should be low relative to (C_d). From equation (44), it would appear that in equilibrium dialysis studies, drug ion concentration should be held high relative to that of the protein

in order that near equal drug ion concentrations should exist on both sides of the membrane.

The Donnan relation in equilibrium dialysis is far more complex than that expressed by the simple preceding equation. Furthermore, the ratio of drug to protein is limited by the sensitivity of the analytical procedure. Drug-protein concentration ratios, if too high, preclude the accurate determination of slight complex formation. Nevertheless, it has been found that by holding buffer concentration high relative to that of the protein, thus rendering the protein ion concentration low relative to the overall ion concentration, dilute drug solutions may be employed with only slight Donnan inhibition to drug migration.

Karush and Sonenburg (10) observed that with protein concentrations from 0.05 percent to 0.50 percent, phosphate buffer of 0.025 moles per liter was sufficient to render any Donnan correction negligible. Klotz and Urquhart (11) used phosphate buffer of ionic strength 0.132 with 0.2 percent bovine serum albumin. The major portion of this work was done using $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 of reagent grade, as sold by Baker and Adamson. Equi-molal amounts of the two phosphate salts were weighed out such that the total molality of the solution prepared was 0.0332. The ionic strength was calculated to be 0.20 from the formula

$$\mu = (1/2) \sum cv^2 \quad , \tag{45}$$

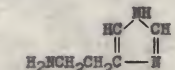
where μ is ionic strength, c is the stoichiometric concentration of each ion in molalities, and v is the valence of the ion.

The di-sodium phosphate was dried to constant weight prior

to use, while the mono-hydrate mono-sodium salt was weighed directly from the reagent bottle. This latter hydrated salt was observed to lose water of hydration when placed in a calcium chloride desiccator. Though the mono-hydrate is described as being stable below 100°, drying in an oven at reduced temperatures was avoided in fear of a possible dehydration and consequent error in weight. Buffer solution was made up with distilled water in twenty liter quantities, and stored for use in a pyrex carboy. The pH of all batches so prepared was 6.8, as indicated by a Leeds and Northrup pH meter.

Phosphate buffer was chosen for this work since its competition with organic ions for binding sites has been shown to be slight(12).

Histamine Free Base. Histamine, known variously as β-imidazolyl-4-ethylamine, 4-imidazoleethylamine, β-aminoethyl glyoxaline, and ergamine, has the structure



The molecular weight of histamine is 111.15. Its melting point is 86° in a sealed capillary.

In this work, the colorless crystalline drug was used as obtained from General Biochemicals, Incorporated.

Histamine Acid Phosphate. This crystalline diphosphate salt, having a corrected melting point of 132° to 133°, and a molecular weight of 307.18, was furnished through the courtsey of the Eli Lilly Company. Data regarding its purity have not

been received, as yet, from the manufacturer, but decomposition of the solutions of this salt, believed to be bacterial in nature, was observed. When several histamine phosphate solutions, in buffer, stood for three days at room temperature, they were observed to become colloidal in nature. Another solution, prepared at the same time but kept in a cold room at 4° to 5° remained clear.

The optical absorption of solutions stored in the cold room for less than a week was observed to decrease, indicating some chemical or physical change. A table showing the change in optical density of an initially 20 x 10^{-5} molar histamine acid phosphate solution is included below.

Table 2. Change in optical density of a histamine acid phosphate solution.

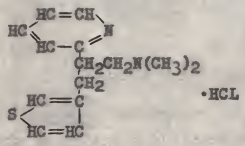
Hours from first reading	Optical density
0	0.415
24	.405
56	.385
97	*.397

* Possibly indicates new absorption by decompositinn product.

In later work with histamine acid phosphate, solutions were prepared and dialysis cells loaded at temperatures not exceeding 5°. This was accomplished either in a cold room at this temperature, or with the assistance of a constant temperature bath maintained at 0.5°. Only freshly prepared solutions of either

the acid phosphate or free base were studied. Changes in solutions of the free base were not as apparent photometrically as changes in the acid phosphate.

Thenfadil Hydrochloride. A member of the pyribenzamine family of antihistiminics, N, N-Dimethyl-N'-3-thenyl-N'- pyridylethylene-diamine Hydrochloride has the following structure:



The molecular weight of this compound is 297.85. According to correspondence with its manufacturers, the corrected melting point is 168°-170° C., and its purity, as used, exceeds 99 percent.

The antihistiminic activity of Thenfadil is said to be about one and one-half times as great as that of its benzyl analog, Pyribenzamine (13), which in turn is twice as potent as Antergan (14), the first commonly applied antihistamine. Extending this scale of comparison, Antergan has been demonstrated to be approximately three times as active as Benadryl, the first purely anithistiminic drug to be synthesized in this country (14)

The Protein. Bovine serum albumin was the protein employed in this work. This was the crystalline form recently made available commercially by Armour and Company. The bovine serum albumin molecule is said to have the form of an ellipsoid of revolution with a major axis of 150 Å and a minor axis of 38 Å.

Its molecular weight is generally taken to be 69,000 (15), and its iso-electric point to be 4.88. The preparation of the albumin for use and correction for water content has been discussed in a preceding section.

The majority of equilibrium dialysis binding studies reported in the literature have involved the interaction of various dyes, drugs, and detergents with bovine serum albumin. By virtue of these studies, bovine serum albumin has very nearly achieved the status of a reference protein. Its ability to combine with a variety of ions and molecules is said to be a result of its great configurational adaptability (16). Karush (16) expertly phrases this concept and its significance in the following manner:

> ...there exist a number of sites on the protein, each associated probably 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 an organic anion, for example, that configuration is stabilized which, by virtue of its structural relation to the anion, permits the various portions of this anion to interact with appropriate groups of the protein. This is manifested by the formation of a complex.

Through the use of bovine serum albumin, a new insight into the nature of protein complex formation in general has been obtained.

Dialysis Sacks. Visking cellophane tubing of 18/32 inches inflated diameter was found to be best adapted to this work. According to correspondence with the manufacturer, this tubing was 0.00095 inches thick, and of pore size such that it was impermeable to serum albumin. With regard to ionic diffusion through this tubing, the manufacturer stated that, in general,

negative ions diffuse more readily than do positive ions. This is said to be a result of the binding of cations by the cellophane, which acquires a negative charge when immersed in water. The extended time for equilibration necessary in this work as compared to the times of equilibration required in anion binding studies reported by other investigators (11, 16) may be explained by this membrane inhibition to cationic migration.

DISCUSSION

The Data

Binding of Histamine Acid Phosphate. Two separate duplicate binding determinations involving a total of twenty-eight individual protein containing dialysis cells were made on this substance.

In the first duplicate determination, protein of only .04 percent by weight was bound, and the equilibrium optical densities of the drug blank cells were noted to be higher than would be anticipated for the amount of drug diffusion into the volume of the dialysis sacks. Both acid phosphate determinations were made prior to the use of the membrane-protein and membrane-buffer blands. These early determinations demonstrated the need for such correcting devices. Therefore, the equilibrium optical densities of the drug blank cells were arbitrarily reduced to that which drug diffusion into the membrane should have produced. From the concentration differences between these arbitrarily corrected blank cells and the uncorrected protein cells, the

amounts of bound acid phosphate per mole of protein were calculated for the four duplicate cells whose initial drug solutions were most concentrated.

In the second duplicate determination, 0.122 percent by weight of protein was equilibrated with fresh drug solutions of the same initial concentrations as those of the first run. Because of the increased amounts of bound drug, with increased protein concentration, membrane-buffer and membrane-protein errors were decreased relative to the amount of binding. However, these errors were still apparent. Optical density readings, in this second determination with the exception of the first two, were used in calculations as taken from the spectrophotometer.

As shown in Table 3, and as might be expected from treatment of the data, the extent of binding is observed to be less in the first than in the second determination at comparable equilibrium concentrations.

In order that the best values of the constants might be obtained from the available data, a least squares solution was taken. The normal equations for this solution are given in a later section and values of the constants so obtained are shown in Table 5. A plot of the data, using the intercept and slope calculated by the method of least squares, is shown in Fig. 3.

Successive equilibrium constants and the corresponding standard free energy changes, as calculated from equations 23 and 30, respectively, are shown in Table 6.

The primary value of the acid phosphate binding determination

rests in its contribution to the improvement of the technique as applied in later studies, and for this reason it is included in this thesis. Perhaps of secondary importance are the calculated free energy changes and maximum available binding sites on the protein molecule. These are considered significant only in comparison to those values as calculated for histamine free base. The comparatively large amounts and high energy change of acid phosphate binding would indicate an ion-dipole or ion-ion interaction (17) with the protein molecule.

Histamine Free Base. The first determination of free base binding involved the use of seven pairs of duplicate cells with drug concentrations ranging from 2×10^{-5} to 14×10^{-5} moles per liter. A second duplicate determination was conducted using solutions of 10, 12, and 14×10^{-5} molar concentration. Membrane-buffer and membrane-protein blanks were set up for these runs, and all equilibrium densities were corrected by the amounts corresponding to the time of equilibration, as indicated in Table 1. These corrected concentration values varied proportionally with the amount of bound drug and the initial drug concentration. Calculated binding values between the first and second duplicate-determinations were in substantial agreement with one another, as shown in Table 4. Constants were evaluated by the method of least squares (Table 5), and the graph through the experimental points constructed therefrom (Fig. 4). Although these plotted points follow a more random distribution than those for the acid phosphate, this is believed to be indicative of experimental error only. It is felt that this determination

is free from any large systematic error which would shift the points, as well as the value of the constants, in one and only one direction.

Successive equilibrium constants and corresponding free energy changes are given in Table 7.

Thenfadil Hydrochloride. One of the more active antihistiminics, Thenfadil Hydrochloride showed only inconsequential and unsystematic binding by serum albumin (Table 8). From blank cells prepared with the run, small membrane-buffer and membrane-protein corrections were applied in this determination. A similar duplicate determination, made several months prior to the improvement of the technique, indicated this same lack of binding by the protein.

Least Squares Evaluation of Constants. When presenting calculations based upon a least squares solution, it is proper to show the normal equations used in such a solution.

Before calculating successive equilibrium constants and free energy changes for the acid phosphate and free base binding systems, it was first necessary to evaluate the constants n and K. These appear in the simple linear equation 24. By standard least squares procedure, the normal equation in K/n may be shown to be

$$(45) \qquad \left[\Sigma(1/M)^2\right] K/n + \left[\Sigma(1/M)\right] 1/n = \left[\Sigma(1/M)(1/r)\right] ,$$

while the normal equation for 1/n is

$$(46) \qquad \left[\Sigma(1/M)\right] K/n + 1/n = \left[\Sigma(1/r)\right] .$$

Upon substitution of the summed data values, as indicated in equations 45 and 46, for the points plotted in Fig. 3 and Fig. 4,

Table 3. Binding of histamine acid phosphate by bovine serum albumin at pH 6.8.

Observed average equil. optical density.		Corrected average equil. optical density.		Average equil. conc. ($M./l. \times 10^5$)		$M. \times 10^5$ bound in pr. cell	$M. \times 10^5$ protein in cell	M. bound mole pr. (r)
Prot. cell	Blank cell	Prot. cell	Blank cell	Prot. cell (M)	Blank cell			
First duplicate determination								
0.041	0.119	0.041	0.063	2.05	3.15	0.044	0.0116	3.79
.042	.142	.042	.084	2.10	4.20	.084	.0116	7.24
.052	.155	.052	.103	2.55	5.05	.100	.0116	8.62
.066*	.168	.066	.128	3.25	6.25	.120	.0116	10.34
Second duplicate determination								
0.017*	0.037*	0.005	0.025	0.25	1.25	0.030	0.0177	1.69
.031	.055	.013	.037	.57	1.77	.036	.0177	2.04
.013	.067			.57	2.97	.072	.0177	4.07
.025	.097			1.15	4.45	.099	.0177	5.59
.036	.126			1.65	5.75	.123	.0177	6.95
.045*	.152			2.05	6.95	.147	.0177	8.31
.058*	.200			2.65	9.25	.198	.0177	11.19

*single cell readings.

Table 4. Binding of histamine free base by bovine serum albumin at pH 6.8.

Observed average equil. optical density.		Corrected average equil. optical density.		Average equil. conc. $\times 10^5$		M. $\times 10^5$ bound in pr. cell	M. $\times 10^5$ protein in cell	M. bound mole pr. (r)
Prot. cell	Blank cell	Prot. cell	Blank cell	Prot. cell (M)	Blank cell			
first duplicate determination								
0.029	0.070	0.009	0.014	0.45	0.65	0.006	0.0177	0.34
.030	.097	.010	.041	.50	1.95	.044	.0177	2.49
*.037	*.135	.017	.079	.80	3.75	.089	.0177	5.03
.047	.165	.027	.109	1.30	5.15	.116	.0177	6.55
.059	.186	.039	.130	1.85	6.15	.129	.0177	7.29
.068	.238	.048	.182	2.30	8.60	.189	.0177	10.68
second duplicate determination								
0.038	0.170	0.018	0.104	0.85	4.90	0.122	0.0177	6.89
.058	*.224	.038	.168	1.80	7.90	.183	.0177	10.34
.074	.257	.054	.201	2.60	9.50	.207	.0177	11.69

*single cell readings

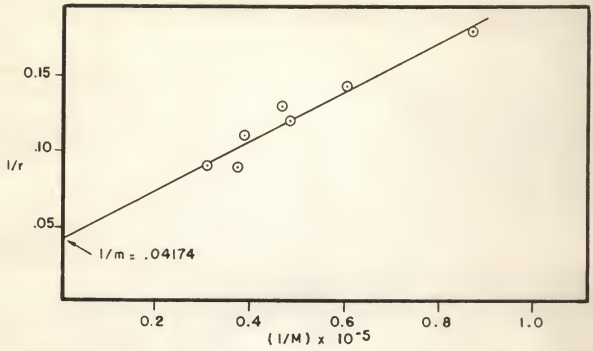


Fig. 3. Binding curve of histamine acid phosphate.

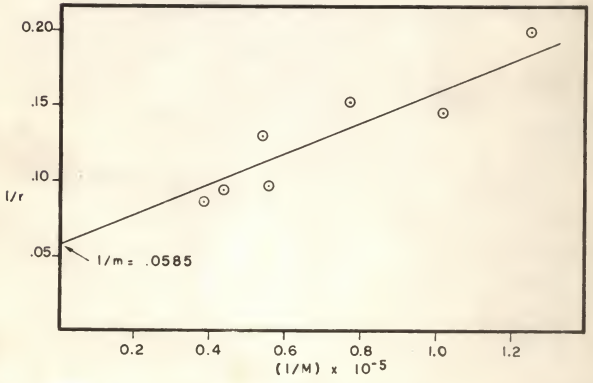


Fig. 4. Binding curve of histamine free base.

solution of these simultaneous equations yields the most accurate values for the constants from the available data. These values, so calculated, are shown in Table 5.

Table 5. Values of constants as calculated by method of least squares.

Constant	Histamine acid phosphate	Histamine free base
1/m	0.04174	0.0585
m	23.96	17.1
K/m	1.62×10^{-4}	9.83×10^{-3}
K	3.88×10^{-5}	1.68×10^{-1}

Interpretation of Data

First Interpretation. A typical active antihistamine was not bound by the protein. Therefore, if the protein molecule studied contained representative "active centers" as referred to by Wells (5), then the mechanism of the antihistamine reaction is not one of competitive adsorption.

Second Interpretation. Assuming that the bovine serum albumin molecule does NOT contain these "active centers", then the anti-histamine and histamine binding of other proteins, possibly even certain tissues, should be studied. A comparison of the extent and energy of such binding would tend to strengthen or detract from the theory of competitive binding action.

Third Interpretation. Assuming the mechanism to be one of

Table 6. Free energy changes for successive binding by histamine acid phosphate.

i (binding order)	$k_i \times 10^{-4}$	$-\Delta F^\circ$ (Kcal./mole)
1	61.85	7.37
2	29.64	6.96
3	18.90	6.71
4	13.53	6.53
5	10.31	6.38
6	8.26	6.25
7	6.63	6.13
8	5.48	6.03
9	4.58	5.93
10	3.87	5.84
11	3.28	5.75
12	2.79	5.66
13	2.38	5.57
14	2.03	5.48
15	1.72	5.39
16	1.45	5.29
17	1.21	5.19
18	1.00	5.09
19	0.81	4.97
20	.64	4.84
21	.49	4.69
22	.35	4.51
23	.22	4.25
24	.11	3.87

Table 7. Free energy changes for successive binding by histamine free base.

i (binding order)	k_i	$-\Delta F^\circ$ (Kcal./mole)
1	101.10	2.55
2	47.62	2.13
3	29.76	1.88
4	20.83	1.68
5	15.48	1.51
6	11.90	1.37
7	9.35	1.24
8	7.44	1.11
9	5.95	.99
10	4.76	.86
11	3.79	.74
12	2.98	.60
13	2.29	.46
14	1.70	.29
15	1.19	.10

Table 8. Absence of significant binding by Thenfadil Hydrochloride.

Observed equil. optical density		Corrected equil. optical density		Equil. conc. ($M./l. \times 10^5$)		Prot. cell Δconc. ($M./l. \times 10^5$)
prot. cell	blank cell	prot. cell	blank cell	prot. cell	blank cell	
0.026	0.027	0.022	0.021	1.30	1.25	+0.05
0.027	0.030	0.024	0.024	1.35	1.40	-0.05
0.052	0.059	0.048	0.053	2.80	3.08	-0.28
0.069	0.059	0.065	0.053	3.75	3.08	+0.67
0.073	0.075	0.069	0.069	4.00	4.00	0.00
0.089	0.076	0.085	0.070	4.90	4.05	+0.85
0.118	0.120	0.114	0.114	6.60	6.60	0.00
0.119	0.121	0.115	0.115	6.65	6.65	0.00
0.158	0.164	0.154	0.158	8.90	9.10	-0.25
0.161	0.163	0.157	0.157	9.08	9.08	0.00
0.185	0.188	0.181	0.182	10.40	10.45	-0.05
0.186	0.190	0.182	0.184	10.45	10.55	-0.10
0.228	0.233	0.224	0.227	12.80	13.05	-0.25
0.235	0.236	0.231	0.230	13.30	13.20	+0.10

competitive inhibition, then the lack of binding by a typical antihistamine indicates the absence of the "active centers" in the serum albumin molecule.

Histamine was shown to be bound by the bovine serum albumin molecule. This is a fairly loose bond as seen from its low free energy of formation. Therefore, it seems reasonable to suspect that, under normal conditions, histamine is tied up in an inactive form by this serum molecule. In consideration of the looseness of this bond it also appears reasonable that, under certain pathologic conditions, only a small amount of energy is necessary to displace histamine from its inactive position on albumin and other protein molecules. Histamine so liberated could then be transferred to pre-sensitized "active centers."

Evidence supporting this theory of histamine inactivation is found in the work of Benda and Urquia (18) who noted that the subcutaneous injection of human or animal blood serum increased the histamine resistance of guinea pigs. This action is thought, by these investigators, to be due to the binding of histamine by this serum. Dragstedt and Mead (19) report the rapid disappearance from the blood stream of histamine injected into the anaesthetized dog.

Proposed Extensions

A continuation of the histamine-serum albumin interaction study is considered with regard to the nature of binding sites on both the protein and the histamine molecule. Changes in binding behavior with blocking of certain protein and drug groups could be related to the nature of the complex bonds normally formed. Changes in binding behavior with change in pH would provide an interesting study.

The kinetics of the binding reaction could be studied quite simply and treated statistically by the method of least squares. By conducting the binding determination at several temperatures, and calculating free energy values at each, both the enthalpy and entropy changes of binding could be evaluated. This, together with kinetic data, would aid, further, in an understanding of the mechanism of this reaction.

With regard to the technique of equilibrium dialysis, it is felt that elimination of the dialysis sack would greatly reduce experimental and systematic errors appearing in equilibrium analyses. Toward this end, a potentiometric method of analysis might be adapted, removing the need for a separate protein compartment in the binding cells.

Obviously, the technique is not limited to binding by protein material alone. Multiple binding by any material could be studied, granted that the molecular or particle concentration of the binding substance was capable of being determined.

Similarly, binding by surfaces such as those of a contact catalyst, an asphalt substrate, a solid adsorbent such as silica or charcoal, etc., could be studied by this technique. The validity of thermodynamic treatment of such a highly organized binding system is uncertain at this time, but it is felt that free energy values calculated for binding per unit area of adsorbents would at least be of comparative value.

SUMMARY

In order to better understand the mechanism of the histamine-protein-antihistamine reaction, the binding of histamine and a typical antihistamine by a reference protein was studied. The method of equilibrium dialysis was applied to obtain the necessary data. A spectrophotometric analytical technique was used. All constants, necessary in subsequent calculations, were evaluated by the method of least squares.

Both histamine acid phosphate and histamine free base were observed to be bound by the protein. A maximum of twenty-four acid phosphate cations was calculated to be bound per mole of protein; maximum binding of free base molecules was concluded to be seventeen per molecule of protein. Tables showing the calculated free energies of successive binding were constructed. Free energies of binding for the first acid phosphate and free base molecules were calculated

to be 7.37 and 2.55 Kcal./mole, respectively.

A typical active antihistamine, Thenfadil Hydrochloride, was observed to be inconsequentially bound by the protein.

The data was interpreted in terms of whether the protein did or did not contain the "active centers" of histamine response. If bovine serum albumin did contain these centers it was suggested, in view of the data, that the antihistamine reaction was not one of competitive adsorption. If the protein did NOT contain such centers, then the mechanism of reaction was not elucidated. However, an interpretation of the data suggested a possible histamine inactivation by the plasma proteins. Such inactivation would explain certain biological phenomena reported in the literature.

ACKNOWLEDGMENT

Deepest appreciation is expressed to Dr. A. C. Andrews, major instructor, for his interest, instruction, enthusiastic support, and encouragement during the course of this study; to Dr. R. E. Silker, Head of the Department of Chemistry, for making available the facilities of the department; to Dr. R. K. Burkhard for the use of materials and literature while this work was in progress; to Dr. C. H. Whitnah for the use of his calculator and other apparatus; to Dr. C. W. Colver and Mr. C. C. Blood for services in the obtaining of reagents and apparatus; to Mr. E. B. Beetch and Mr. W. G. Stanley for assistance in the building of equipment and in photographic procedures, and to the Eli Lilly Company, the Sterling-Winthrop Research Institute, Merck and Company, the Upjohn Company, and American Cyanimid for drugs donated to this research.

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AN EQUILIBRIUM DIALYSIS STUDY OF CERTAIN HISTAMINE-PROTEIN-ANTIHISTAMINE INTERACTIONS

by

BLANCHARD LEROY MICKEL

B. S., Washburn Municipal University, 1949

AN ABSTRACT OF

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1951

The asthmatic and other reactions of the allergies are reported in the literature to be caused by histamine. To effect symptomatic relief in allergy, a series of structurally similar compounds exhibiting an antagonism for histamine have been prepared by therapeutic chemists. The mechanism of such antihistaminic action is not clear. One mechanism has been suggested to be that of competitive binding for certain sites on the affected tissues. This work, therefore, was devoted to an investigation of the separate reactions of histamine and a typical antihistamine with bovine serum albumin as a representative protein.

The method of binding study used was that of equilibrium dialysis as developed by Klotz. He considers most binding of ions or molecules by protein material to be reversible, and to occur in a stepwise fashion. This extent of binding is said to be regulated by the nature of the reactants and mass action principles alone. A full derivation of the final equation used in calculating successive binding equilibrium constants was presented in this thesis. From a knowledge of these successive equilibrium constants, the corresponding free energies of binding may be calculated by standard thermodynamic procedures. The method permits not only a determination of the extent of binding, but a calculation of the accompanying free energy changes as well.

The experimental procedure involved an equilibration of protein solution, contained in a dialysis sack, with a drug solution of known initial concentration. For each protein con-

taining cell, a corresponding blank cell was prepared identical to the first except that it contained only solvent in the dialysis sack. The difference in equilibrium drug concentrations between cells in a given pair was taken to be the amount of drug bound by the protein. Additional cell pairs were set up with each pair containing drug solutions of different initial concentration. The amounts of drug-protein interaction were related to the equilibrium concentrations of free drug, from which followed the calculation of equilibrium constants and free energy changes. All binding determinations were conducted in duplicate, then repeated in duplicate when binding was observed to occur.

A spectrophotometric analytical technique was applied to determine the various drug concentrations. Because of the ultra-violet range of analysis necessitated, certain errors not appearing upon absorption analysis in the visible region were encountered. These involved a solvent-membrane and a protein-membrane interaction. A correction for these effects was developed and applied in later binding determinations.

Binding of both histamine acid phosphate and histamine free base by bovine serum albumin was observed to occur. A maximum of twenty-four acid phosphate cations and seventeen free base molecules were calculated to be bound per mole of protein. The free energy change for binding of the first mole of acid phosphate was found to be 7.37 Kcal., while that for the free base was only 2.55 Kcal. per mole. The greater extent and free energy change of acid phosphate binding is believed to be a

consequence of the pronounced electrostatic nature of this interaction. A typical active antihistamine, Thenfadil Hydrochloride, appeared to be inconsequentially bound by the protein.

Two interpretations of the data in terms of protein character were presented. First, assuming that bovine serum albumin contains sites similar to those in sensitive tissues at which the histamine response is initiated, the lack of binding by a typical antihistamine suggests that the mechanism of antihistaminic action may not be one of competitive binding. Secondly, assuming that the protein does not contain these histamine reactive sites, then the observed binding of histamine by bovine serum albumin could indicate a mechanism of histamine inactivation. Furthermore, the low energies of binding calculated suggests that only a slight pathological reaction such as that of an allergy, would be necessary to dislodge the loosely bound histamine from its inactive position, upon which it would be free to seek a reactive tissue. Certain evidence in the literature substantiates this idea of the antihistaminic action of serum albumin itself.