

THE EVALUATION OF THERMODYNAMIC FUNCTIONS OF
THE HISTAMINE-HEPARIN INTERACTIONS
BY EQUILIBRIUM DIALYSIS

by

WAYNE BURL ROSE

B. S., Adams State College, 1955
M. Ed., Adams State College, 1957

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY
MANHATTAN, KANSAS

1961

LD
2668
T4
1961
R68
c. 2
Document

TABLE OF CONTENTS

INTRODUCTION	1
HISTORICALLY	1
LITERATURE REVIEW	4
EQUILIBRIUM DIALYSIS	7
DEFINITION OF SYMBOLS	10
DERIVATION OF EQUATION	11
HISTAMINE TO HEPARIN BINDING.	16
HISTAMINE ANALYSIS.	17
MECHANICAL APPARATUS	21
THERMODYNAMIC DATA	21
DISCUSSION OF RESULTS	22
EXTENSIONS	37
ACKNOWLEDGMENTS	38
LITERATURE CITED	39
APPENDIX	41

INTRODUCTION

There frequently appear references to heparin in the literature of histamine. These sources indicate that these two substances are found in the body tissue. The references go farther in showing that histamine and heparin are found within the same body units viz. The mast cells. These mast cells are a rich source of both histamine and heparin. When an allergic reaction occurs within a portion of a living body, histamine and heparin appear in the blood of that area. During anaphylactic shock when histamine is released into the lymph and blood, it is not long until heparin also appears in these fluids. The simultaneous appearance of these compounds has led to the question of whether or not they might be in combination. It is the purpose of this paper to measure the amount of interaction by some experimental means. The problem is whether this appearance is coincidence, or if these two powerful chemicals form some type of complex or coordination compound.

HISTORICALLY

Kehrer (1908) demonstrated the strong stimulating agent ergotamine on plain muscle tissue; the same year Windaus prepared histamine by enzymatic decarboxylation of histidine (22). Dale observed this demonstration by Kehrer, and became interested in the agent that could cause such a powerful reaction. He isolated this compound from ergot, and, from the enzymatic decarboxylation of histidine. Dale (1910) identified both of these compounds as being histamine. A discrepancy arose when it was observed that the compound obtained from the histidine reaction would cause a rise in the blood pressure of rabbits.

Histamine has always been known to cause a decrease in blood pressure. This obstacle was removed when histamine was found to raise the blood pressure of rabbits under certain conditions.

Ehrlich (1878) first observed the interaction of heparin with certain basic dyes (20). He noted that certain cells would stain with toluidine blue, while cells adjacent to them would not. He reported this differential staining, and observed that these cells seemed to cluster around small blood vessels. It was because of this location that he called these cells, "mast cells" meaning "well fed."

McLean (20) in 1925 isolated heparin from dog liver in the form of a phosphotide. Heparin received its name from its discovery in the liver, and heparin actually means "in the liver." Howell recognized its high anti-coagulant character. Jorpes (1942) found that there was a direct relationship between the amount of heparin that could be extracted from tissue and the number of mast cells present (10).

About this time there was disagreement in the literature concerning the homogeneity and probable structure of heparin. However, workers did agree that heparin of different animals exhibited different anticoagulability. It was not until 1950 that Jorpes (11) and Wolform (29) found through independent research that the potency of heparin seems to be due to amine sulfate linkage ($-\text{NHOSO}_2^-$).

Histamine, 4-ethylamineimidazole is a weak organic base.

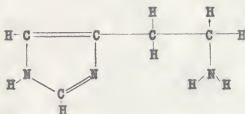


Figure 1

Histamine shows pK values of 6.14 and 9.87 for two of the amine groups at 25°C. A titration curve typical of a weak dibasic amine is obtained in titrating histamine. (Plate I)

Heparin appears to have a polysaccharide structure, containing two specific ring structures within a unit, glucosamine and glucuronic acid.

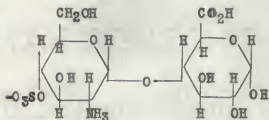


Figure 2

Wolfrom (29) reports the following structure, as one of the possible heparin structures.

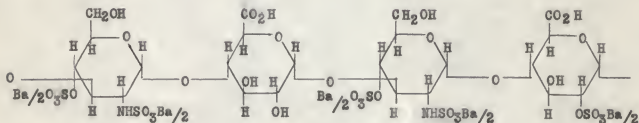


Figure 3

For this unit the molecular weight is 1415.274, and it has the following formula ($C_{24} H_{33} O_{35} N_2 S_5 Ba_{2\frac{1}{2}}$). The optical rotation of the sodium salt of heparin is reported in this paper to be $(\alpha)^{24}_D$ 16.5 degrees in chloroform.

Upon titration of heparin with KOH a typical acid base titration curve results. The end point appears at pH of 7.5 (Plate I).

LITERATURE REVIEW

Histamine is known to be present in the blood during anaphylactic shock, and it is present in high concentration in areas of the skin which exhibit allergy reactions. Anaphylactic shock can be induced by the injection of histamine into a test animal, or by an injection of a foreign substance to which the test animal had previously been sensitized. In his book Rocha e Silva (22), summarizes the literature on histamine. He draws attention to the fact that histamine has been found in the lymph of the thoracic duct during anaphylactic shock, accompanied by the simultaneous appearance of anticoagulability of the blood.

Jaques and Waters (8) found heparin in dog's blood during anaphylactic shock; but not in normal dogs blood. They also determined that this heparin came from the liver. White and Woodard (26) report that heparin is found in the lymph of the thoracic duct following shock in dogs. Other interesting observations have been made. Dragstedt, Wells and Rocha e Silva (4) have shown that suppression of histamine release in rabbit blood can be accomplished with heparin. However, the amount needed for this is far above the concentration needed to prevent coagulation. Macht (16) further summarizes work done on lower animals. Pigeons, guinea pigs, rabbits and rats can be protected from severe anaphylactic shock by previously injecting them with heparin. Gerendas, Caefko, and Udvardy (6) have postulated a chain mechanism by which histamine will cause a decrease in the activity of heparin on blood coagulation time. Haley and Stodarsky (7) found that the concentration of histamine needed to cause this antiheparin activity is a toxic amount.

Cass, Head, Riley, Stroud and West (5) reported both heparin and histamine present in mast cell tumors of a dog in relatively large quantities. These mast cells appear in all vertebrates apparently originating around small blood vessels in connective tissue. Riley and West (21) report that these mast cells occur in quite different tissues among different species of animals that have been investigated. They have observed a high correlation between the abundance of mast cells in tissue; and the amount of histamine that can be extracted. Eaton (19) and Fawcett (5) have caused histamine release by : (1) disruption of the mast cells with distilled water; (2) histamine liberators; and (3) simple chemical compounds. A mixture of low polymers of p-methoxy N-methyl-phenylethyl amine, called 48/80, is one of these histamine liberators.

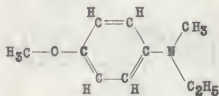


Figure 4

Histamine-heparin relationship appears to be associated with the metabolism of animals. Histamine is apparently not released in the body, except as heparin is found in the body fluids.

Sanyal and West (23) and Amann and Werle (1) have found that a heparin histamine complex is formed in a water solution from which it can be extracted by either acetone or ethanol. By careful analysis of the precipitate from the acetone water mixture, they have determined the ratio at which heparin and histamine appear to combine. They also found that the complex formation is definitely pH dependent. Above a pH of 7 there are lesser amounts of histamine removed upon precipitation of the complex. Sanyal (23) feels that the complex

contains the same ratio of heparin and histamine as do the mast cells within the body.

Amann and Werle (1) and Werle and Amann (24,25) found the heparin to histamine ratio to be 5 to 1 by weight. In their work they report that at a pH of 5 a complex definitely forms. In neutral or basic solutions little or no complex is found. The optimum pH was 2.8 for the formation of the heparin histamine complex. The experimental methods, these researchers used, were equilibrium dialysis, paper chromatography, and precipitation methods. Werle (24,25) further studied the heparin toluidine blue interaction using paper chromatography. Here also he found that the equilibrium was pH dependent. In neutral or basic solutions, little or no complex formed; but a definite complex forms if the system is made acid. A competition was observed to exist between toluidine blue and histamine. If an excess of one were added to a complex, the other may be completely removed.

Wolfson (27,28,29) and Jorpes (10,11) studied the chemical properties of heparin in an attempt to establish the mechanism by which it lengthens the coagulation time of blood, and to determine its structure more exactly. Jorpes (10) had previously proposed that the high molecular weight of heparin might be the reason for its anticoagulant activity; although, other high molecular weight polysaccharides do not appear to have this anticoagulant property. Wolfson (29) and Jorpes (10,11) by careful analytical analysis have shown the activity of heparin will decrease upon slowly warming solutions of heparin dissolved in 0.04 M acid. The only apparent change during this process was the release of sulfuric acid and the accompanying appearance of a free amine group. This reduction of activity with the loss of a small per cent of the sulfate groups may explain other discrepancies; one of which is the reaction of toluidine blue with heparin. Using heparin from one species of animal, MacIntosh (17) was able to

develop a technique of quantitative heparin analysis. The interaction of heparin and toluidine blue causes a color change of the dye. The color change is from deep blue to purple. MacIntosh made use of this phenomenon by observing the absorbance change in a constant concentration of toluidine blue with varying concentrations of heparin. For a given source of heparin this absorbance change is linear in relation to heparin potency. Succeeding workers have found that this relationship does not hold for heparins that have been obtained from other sources.

The literature indicates at least two conclusions about histamine liberations: (1) first whenever histamine appears in the lymph fluid of the body, heparin also appears; and second histamine cannot be released without heparin soon appearing in the blood. The ratio of the heparin to histamine in the mast cells (2) has been determined. The complexes of heparin and histamine have been observed (3) and the ratio of their combination calculated. The next step would seem to indicate the necessity for determining the thermodynamic quantities of this interaction in aqueous solutions.

Heparin having a molecular weight of 12,000 to 20,000 will not permeate a dialysis bag. Within a few hours the smaller histamine molecule will, by dialysis, establish equilibrium concentration in this system. This allows the use of equilibrium dialysis as an experimental technique for studying such interactions. With this information thermodynamic constants can be readily calculated.

EQUILIBRIUM DIALYSIS

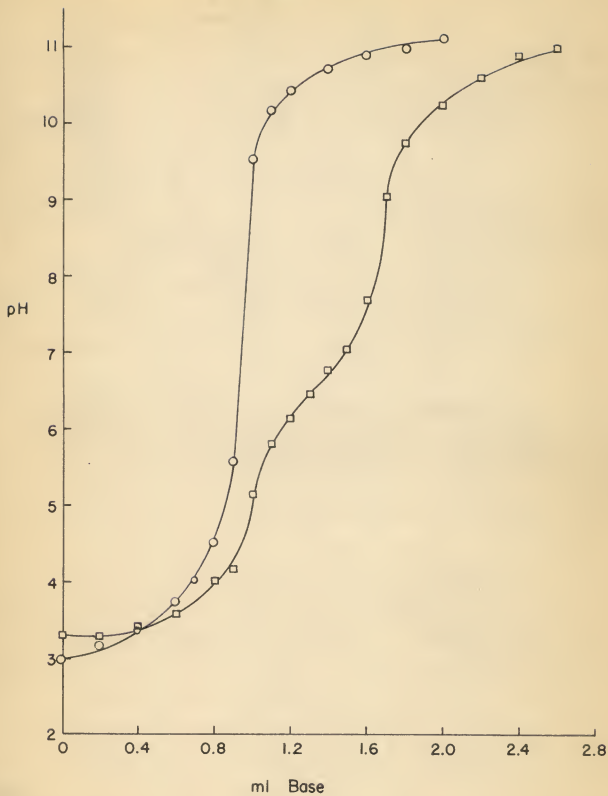
In this technique the container was divided into two sections by a semi-permeable membrane. One section contained the large heparin molecules; the other contained the histamine. The heparin molecule will not permeate the membrane,

2000-2001

1000-1000

1000-1000

PLATE I



while the smaller histamine molecule moves through freely. If the heparin combines with some of the histamine; there will be a decrease histamine concentration in the heparin free portion. This difference is a relative measure of the bound histamine to the heparin.

In this technique errors of at least two types may occur; namely (1) the Donnan effect and (2) the interaction of histamine with the dialysis membrane. The Donnan effect is the unequal distribution of charges and is negligible if a strong electrolyte is used. This second error may be eliminated if a blank cell is treated in an identical manner to the heparin sample. This blank cell contains no heparin within the dialysis bag, only the buffered solution.

To evaluate the data obtained from equilibrium dialysis, relationships are needed between the equilibrium constant and the measurable quantities of the system. Koltz (12) in his discussion of techniques of analysis; he derived an equation for what he called the "intrinsic association constant" of interacting species. This constant is related to experimentally measurable quantities in an actual dialysis. The derivation of the expression connecting these measurable quantities assumes, first that the overall binding reaction occurs in a stepwise manner and second that there exists no interaction between binding sites.

DEFINITIONS OF SYMBOLS

- (---) : Brackets indicate concentration in moles per liter.
- ligand : Molecules of histamine (11).
- (11)_t : Total concentration of ligands in the system.
- (11)_f : Concentration of free ligand in a system.
- (11)_b : Ligands bound.

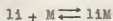
\bar{n}	:	Maximum number of ligands bound per molecule of heparin.
r	:	Experimentally determined amount of ligands per molecule of heparin
$k_1, k_2, k_3 \dots$:	Stepwise formation constants of complexing reaction.
K	:	Intrinsic equilibrium constant for the formation reaction between the ligand and heparin.
$K_n^- = k_1 k_2 k_3 \dots$:	$K_n^- = k_1$
M	:	Concentration of central molecule (in this paper, heparin)
M_t	:	Total concentration of central molecule.
A	:	Absorbance of solution.
C	:	Concentration in moles per liter.

DERIVATION OF EQUATION

Each binding site of the heparin molecule is considered to be independent of the other sites. Further it is assumed that the intrinsic association constant is the same for each binding site. The association constant for the j th site is $k_j = (l_{jM})/(l_1)(l_{j-1M})$. The molecule l_{jM} contains j ligands bound to each M , this molecule has j times the chances of losing a ligand as does the molecule with only one ligand bound. Then its intrinsic association constant will be lowered by a factor of $1/j$. The molecule with $(j-1)$ bound ligands will have $\bar{n}-(j-1)$ sites upon coordination. Its chances of reaction with a species (l_1) before a molecule with only one available site is $\bar{n}-j+1$ and the intrinsic association constant is raised by this factor. So then the relationship between k_j and K is

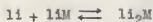
$$k_j = (\bar{n}-j+1) K/j \dots\dots\dots (1)$$

Consider the equilibria involved in the following reactions.



$$k_1 = \frac{(liM)}{(li)_f (M)}$$

$$(liM) = k_1 (li)_f (M) \dots\dots\dots (2)$$

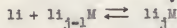


$$k_2 = \frac{(li_2M)}{(li)_f (liM)}$$

$$(li_2M) = k_2 (li)_f (liM)$$

$$(li_2M) = k_1 k_2 (li)_f^2 (M) \dots\dots\dots (3)$$

⋮
⋮
⋮

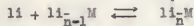


$$k_j = \frac{(li_jM)}{(li)_f (li_{j-1}M)}$$

$$(li_jM) = k_j (li)_f (li_{j-1}M)$$

$$(li_jM) = k_1 k_2 \dots k_j (li)_f^j (M) \dots\dots\dots (4)$$

⋮



$$k_n = \frac{(li_nM)}{(li)_f (li_{n-1}M)}$$

$$(1i_{\bar{n}M}) = k_{\bar{n}}^{-1} (1i)_f (1i_{\bar{n}-1}M)$$

$$(1i_M) = k_1 k_2 \dots k_j \dots k_{\bar{n}}^{-1} (1i)_f^{\bar{n}} (M) \dots \dots \dots (5)$$

From the definition of r on page 11

$$r = \frac{\text{moles histamine bound to heparin}}{\text{moles of heparin present}}$$

here the moles of histamine bound may be represented in the following way.

$$(1i_b) = (1iM) + 2(1i_2M) + \dots + j(1i_jM) + \dots + \bar{n}(1i_{\bar{n}M}) \dots (6)$$

Substituting into equation (6) the quantities from the equilibrium constant equations (2), (3), (4), and (5) the following relation is obtained.

$$(1i_b) = k_1 (1i)_f (M) + 2k_1 k_2 (1i)_f^2 (M) + \dots + j k_1 k_2 \dots k_j (1i)_f^j (M) + \dots (6')$$

$$\bar{n} k_1 k_2 \dots k_j \dots k_{\bar{n}}^{-1} (1i)_f^{\bar{n}} (M).$$

Factoring out the quantity (M) and substituting in for each association constant, its value in terms of the intrinsic association constant as shown in equation (1):

$$(1i_b) = (M) (\bar{n}K (1i)_f + \frac{2\bar{n}(\bar{n}-1)}{2!} K^2 (1i)_f^2 + \dots + \frac{j\bar{n}(\bar{n}-1)(\bar{n}-2)}{j!} \dots$$

$$\frac{(\bar{n}-j-1) K^j (1i)_f^j}{\bar{n}!} + \frac{\bar{n}\bar{n}!}{\bar{n}!} K^{\bar{n}} (1i)_f^{\bar{n}}). \dots \dots \dots (7)$$

The same operation may be carried out on the denominator.

$$(M_t) = (M) + (1iM) + (1i_2M) + \dots + (1i_jM) + \dots + (1i_{\bar{n}M}).$$

Making the substitution for the complexed heparin and factoring out the common term (M)

$$(M_t) = (M) (1 + k_1 (11))_f + k_1 k_2 (11)_f^2 + k_1 k_2 \dots k_j (11)_f^j + \dots + k_1 k_2 \dots k_j \dots k_n (11)_f^n$$

Again substituting in for $k_j = \left(\frac{\bar{n}-j+1}{j} \right) K$

$$(M_t) = (M) \left(1 + \bar{n}K (11) + \frac{\bar{n}(\bar{n}-1)}{2!} K^2 (11)^2 + \dots + \frac{\bar{n}(\bar{n}-1)(\bar{n}-2)}{3!} K^3 (11)^3 + \dots + \frac{(\bar{n}-j-1)}{j!} K^j (11)^j + \dots + \frac{\bar{n}!}{\bar{n}!} K^{\bar{n}} (11)^{\bar{n}} \right)$$

From the binomial theorem, the denominator may be reduced to $(1 + K (11))^{\bar{n}}$.

Factoring out a (11) in the numerator it is found the numerator =

$$(11) \frac{d}{d(11)} (1 + K (11))^{\bar{n}} = (11) \bar{n}K(1 + K(11))^{\bar{n}-1}. \text{ The value for } r \text{ may be}$$

represented in the following way.

$$r = \frac{(11) \bar{n}K(1 + K(11))^{\bar{n}-1}}{(1 + K(11))^{\bar{n}}} = \frac{\bar{n}K (11)}{(1 + K (11))} \dots \dots \dots (8)$$

Rearranging this equation, it becomes a linear equation in $1/r$ and $1/(11)$.

$$r = \frac{\bar{n}K (11)}{1 + K (11)} \dots \dots \dots (8)$$

Multiplying both sides by $(1 + K(11))$

$$r(1 + K(11)) = \bar{n}K (11)$$

and then dividing both sides by r and $\bar{n}K$ (11)

$$\frac{1}{\bar{n}K} \frac{1}{(11)} + \frac{1}{\bar{n}} = \frac{1}{r} \dots\dots\dots (9)$$

This is the equation derived by Klotz in his work. Starting with equation (8) again one may obtain the equation, commonly called the Scatchard equation.

$$r = \frac{\bar{n}K (11)}{1 + K (11)} \dots\dots\dots (8)$$

multiplying both sides by $(1 + K (11))$ and carrying out the expansion,

$$r + rK (11) = \bar{n}K (11)$$

dividing both sides by (11)

$$\frac{r}{(11)} + rK = \bar{n}K$$

subtracting rK from both sides gives the Scatchard equation

$$\frac{r}{(11)} + \bar{n}K - rK \dots\dots\dots (10)$$

The approximate standard free energy, enthalpy, and entropy quantities were calculated by means of the following equations. Equations (9) and (10) may be used graphically to determine the association constant and the maximum number of histamine molecules per molecule of heparin. In equation (9) the Klotz equation $1/r$ is graphed with respect to $1/(11)$. The slope of the graph is $1/\bar{n}K$, and the intercept is $1/\bar{n}$. The Scatchard equation is graphed with $r/(11)$ plotted against r . The slope of the line is $-K$, and the intercept is $\bar{n}K$. The method of least squares may be used to evaluate the best straight

line through the group of points graphed in either equation. The approximate standard free energy, enthalpy, and entropy quantities were calculated by means of the following equations.

$$\Delta F = -RT \ln K$$

$$\Delta H = \left(\frac{\partial \Delta F / T}{\partial (1/T)} \right)_p$$

$$\Delta S = -\left(\frac{\partial \Delta F}{\partial T} \right)_p$$

HISTAMINE TO HEPARIN BINDING

Studies of heparin binding to histamine were carried out in a water bath at pH values of 4.40, 6.83, and 8.99. The temperatures were 0°C and 25°C. Equilibrium was achieved by placing dialysis bags containing the heparin solutions in 18 x 25 mm. test tubes containing histamine solutions. These test tubes were then mounted into the constant temperature bath. The 25° bath was maintained constant by a bimetal regulator in connection with a Fisher-Serfass relay. This system kept the temperature within 0.1 of 25°. The 0° bath was controlled with a similar bimetal regulator, but to maintain this low temperature a refrigeration unit had to be substituted for the heating element. The refrigeration unit was controlled by a modified Fisher-Serfass relay. This modification consists simply of putting the relay in series with the coil of a 1950 Ford car solenoid. The relay plug-in was rewired so that it would cause the current to flow from one side of the plug-in to the other. Here the relay acts as an on-off switch for the six volts D. C. used to activate the solenoid. The modification was required because the heavy compressor motor continued to burn off the points of the Fisher-Serfass relay (graph 3). The pH was maintained constant with phosphate buffer in all cases.

HISTAMINE ANALYSIS

An analytical method for the quantitative determination of histamine was developed by Koessler and Hanke (13). The method used here is a modification developed by Mickel (18) and by Lyons (14,15), of the Koessler and Hanke procedure and is as follows.

A stock solution of sulfanilic acid is made by dissolving 0.047 moles of sulfanilic acid in nine milliliters of concentrated (37%) hydrochloric acid and diluting to 100 milliliters with distilled water. A five per cent solution of sodium nitrite is made by dissolving 5 grams of the salt in distilled water, and diluted to 100 milliliters. A 20% solution of sodium carbonate is made by dissolving two hundred grams of sodium carbonate in distilled water and diluting to one liter.

To prepare the coupling reagent, all solutions must be at 0° C. Two ml. of the cooled sulfanilic acid solution is placed in a fifty ml. volumetric flask. Six ml. of the sodium nitrite is slowly added with swirling, and allowed to equilibrate for five minutes at 0° C. An excess of six ml. of sodium nitrite solution is added. This solution is thoroughly mixed and allowed to stand in an ice bath for an additional five minutes. The solution is then diluted to 50 ml. with distilled water and allowed to temperature equilibrate for fifteen minutes before being used.

To make the azo dye of histamine, 5 ml. of the sodium carbonate solution is pipetted into a 50 ml. erlenmeyer flask. Into this is pipetted 2 ml. of the histamine solution to be tested. Two ml. of the diazo reagent are added, and the solution mixed thoroughly. The absorbance of the resulting solution is determined by the use of a Beckmann spectrophotometer. Using the sodium carbonate in the blank cell, and the azo dye solution in the other cell, the

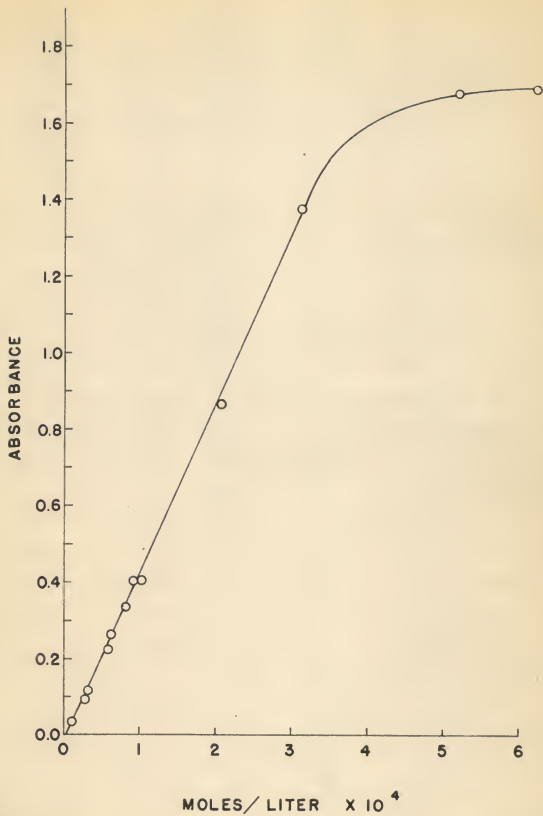
absorbance measurement is made at a wave length of 500 millimicrons. This procedure is repeated again and again, until a maximum absorbance is observed for the azo dye. This repetition is necessary because the dye formed is somewhat unstable. The dye in forming, blooms slowly from a pale yellow to a deep orange. The maximum absorbance has been used in establishing the relationship of absorbance to concentration. The relationship is linear within the range of concentrations used. (See Plate 2)

A second method which allows all solutions to be measured at relatively the same time, has been used with good success. In the technique described, the time needed to make twenty absorbance readings was about two and one-half hours. This time delay will see a change in the diazo reagent. A decided decrease in absorbance is observed in solutions whose absorbance should remain constant. In almost all cases where the time needed to take readings extended over two hours, an apparent change in concentration took place. The second technique, then, requires standard solutions of histamine to be made up of about the same concentration as the unknown solutions. This can be easily done by diluting the original histamine solution to the appropriate concentrations. The diazo reagent is made up as previously described. Five ml. of the sodium carbonate solution are pipetted into a series of 50 ml. erlenmeyer flasks. Two ml. of the histamine solution to be measured are introduced into each flask. Two ml. of the diazo reagent are added to each flask and thoroughly shaken. All flasks are tightly stoppered and allowed to stand twelve hours. After the azo dye goes through the maximum as previously described, it fades slowly to what appears to be a relatively stable color. It has been demonstrated that this final dye color formed also plots a linear graph of concentration against absorbance. Other methods of analysis of histamine have been developed. Included in these is the procedure by Lyons and Andrews (15), which utilizes p-bromo aniline as the diazo coupling agent.

EXPLANATION OF PLATE II

Absorbance curve of histamine azo dye at maximum absorbance,
graphed against concentration of histamine.

PLATE II



MECHANICAL APPARATUS

The shaker unit was so devised that the test tubes would be held beneath the water level, and was agitated by a rocking-jerking motion. This was done by a motor attached either through reduction gears or a series of pulleys to a rocker arm. This rocker arm holds the test tubes in place in the bath. To obtain the jerking motion a steel plate with a horizontal slot was attached to the vertical rod of the rocker arm. The driving rod was forced to slide back and forth in this slot. At each end of the slot, the driving rod would hit and cause the dialysis bags to agitate the solution, thus achieving and maintaining the equilibrium.

THERMODYNAMIC DATA

In the calculation of this data from Scatchard's equation an assumption was made as to what constitutes a basic heparin unit. The model chosen to represent one heparin molecule contains five sulfate groups and it is further assumed that each of the five groups will react with histamine. (Figure 3) This number is used in the Scatchard equation for the maximum number of histamine molecules that will be bound to each heparin unit. The equation is

$$\frac{r}{(11)} = 5K - rK \dots\dots\dots (10)$$

In the evaluation of the data obtained from equilibrium dialysis, the mean association constant was taken to be the true constant for that particular temperature and pH.

Table 5. Thermodynamic values from equilibrium dialysis

pH	Temperature	$\Delta F \left(\frac{\text{Kcal}}{\text{Mole}} \right)$	$\Delta H \left(\frac{\text{Kcal}}{\text{Mole}} \right)$	$\Delta S \frac{\text{cal}}{\text{deg mole}}$
4.40	0°	-5.23	0.616	21.43
	25°	-5.77		
6.83	0°	-4.40	-0.824	13.08
	25°	-4.72		
8.99	0°	-3.59	-7.021	-12.56
	25°	-3.28		

DISCUSSION OF RESULTS

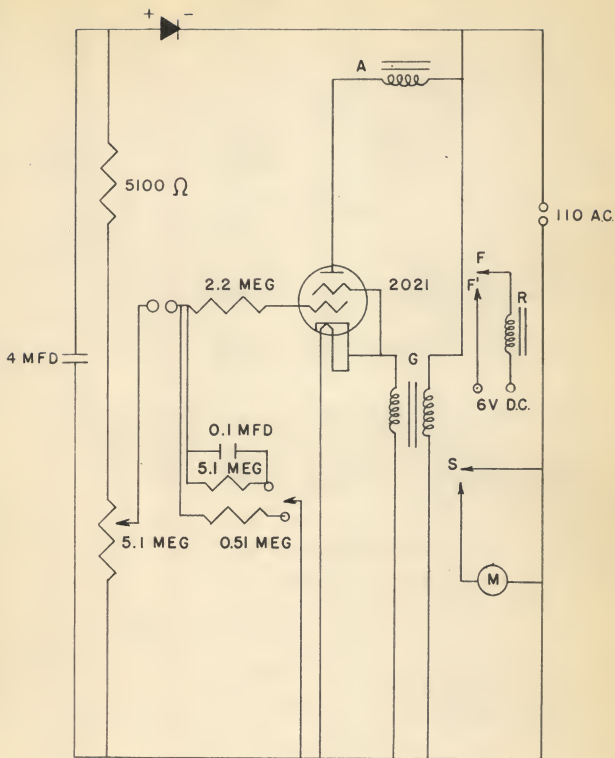
It can be seen from the thermodynamic data that a definite binding occurred between heparin and histamine. This is in agreement with the conclusions of Amann (1), Sanyal (23), and Werle (24,25). The free energy values indicate that when the two molecules are in a system together there is a definite association. From the examination of equilibrium of the heparin-histamine system once it is established, it remains stable. This is quite dissimilar to the binding of the histamine-protein complex as reported by Lyons (14). Lyons found that histamine would form a complex with protein; although it would again be released into the system after an initial interaction. Apparently the heparin to histamine bond is stronger, or of a different type. Andrews and Lyons found that by using a metal ion, namely copper, that the complex of histamine and protein was more stable (2).

The free energy values are of the order of hydrogen bonding. These free energy quantities agree with the hypothesis that the complex formed is a coordination compound. It is proposed here that the protinated histamine coordinates

EXPLANATION OF PLATE III

- A - Coil of Fisher-Serfass relay
- F, F' - Fisher-Serfass relay points
- G - Fisher-Serfass transformer
- M - Refrigeration motor
- R - Solenoid coil
- S - Solenoid points

PLATE III



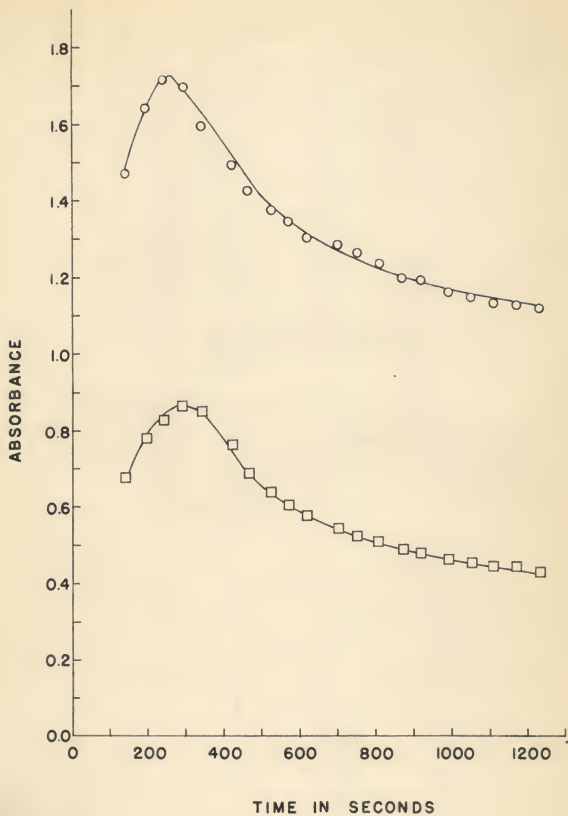
EXPLANATION OF PLATE IV

Azo dye absorbance change with regard to time,
and concentration of histamine.

○ - 3.32×10^{-4} Molar

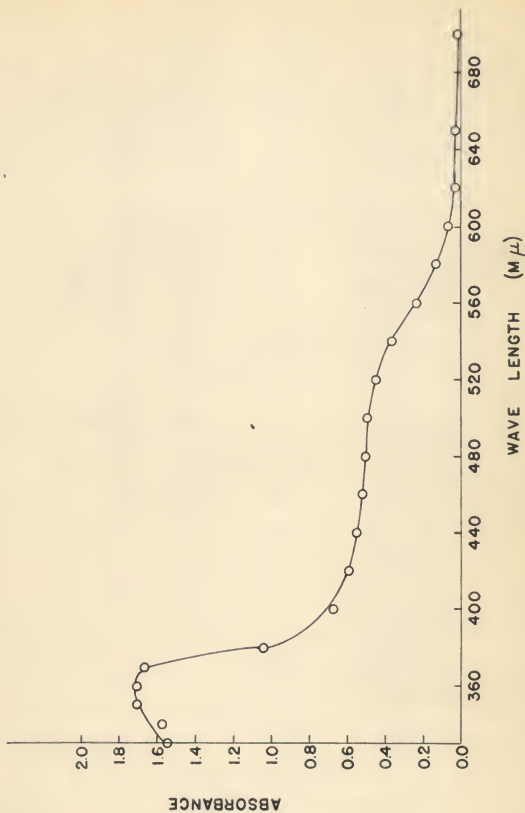
□ - 1.67×10^{-4} Molar

PLATE IV



EXPLANATION OF PLATE V

Visible and ultraviolet spectrum of the
histamine azo dye.

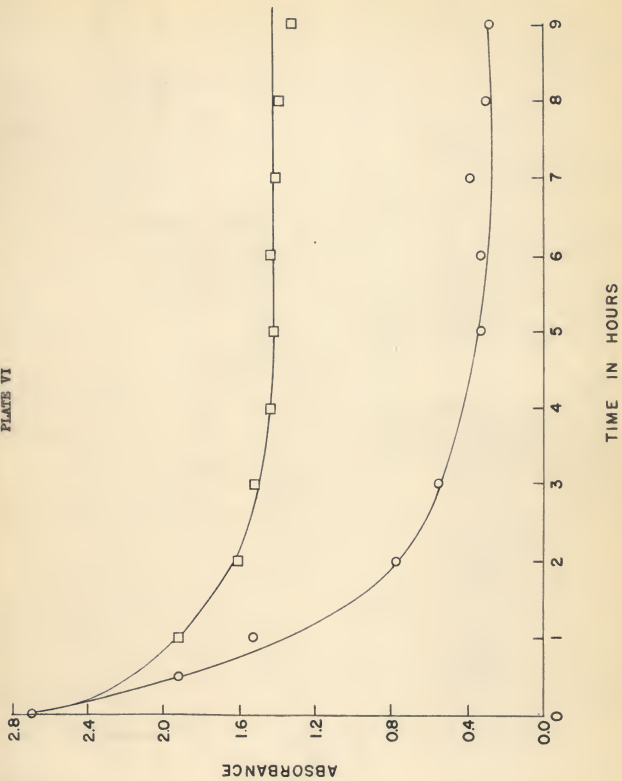


EXPLANATION OF PLATE VI

Equilibrium attainment of histamine with
heparin at a pH of 4.40 0° C.

○ - Absorbance of cells containing buffer

□ - Absorbance of cells containing heparin



with the negative sulfate groups of the heparin anion. The electron deficient histamine acts as a Lewis acid, and the electron rich sulfate sites as the Lewis base.

It is observed that the ΔS values are negative. This negative entropy may be explained in two ways; (1) the heparin molecule may be associated in some way with water or buffer molecules at the lower temperature and when the temperature is raised they become less associated; (2) the heparin molecule may be folded at the lower temperature, and when the temperature is raised it unfolds. This makes the site more available for binding.

The explanation of the pH dependence of the complex may have been predicted from a study of the structure of the two compounds involved. Recalling the discussion of this coordination compound and the postulate of protonated histamine reacts with the sulfate groups. It would seem feasible that the more protonated histamine present, the more successful collisions would occur between the two reacting species. These free energies indicate histamine binding to just one site. If this was not true a leveling off of the free energy would be shown between a pH of 6.83 and pH of 4.40.

In the process of histamine analysis an unexplained phenomenon occurred. Weighed amounts of histamine were dissolved into solution and these varied concentrations were used to make the azo dye. The different absorbance readings of the resulting dye were measured, and the concentration to absorbance was found to be linear with concentration. These histamine preparations were allowed to stand under normal conditions for a few hours and when new azo dye solutions were then made of these samples, the concentration to absorbance graph was no longer linear. There occurred in all cases a definite decrease in histamine present. Several approaches have been investigated to find a possible answer;

(1) increasing the area of glass in contact with the histamine solution; (2) using test tubes rinsed with dilute HCl, compared to test tubes not rinsed with the acid; (3) dialysis against the soap used in washing the equipment; (4) allowing equal molar concentrations to stand under different thermal and lighting conditions. Of these four procedures only the fourth gave any indication of a difference in concentration. Here samples were allowed to stand at room temperature in the sunlight. After a period of twelve hours, a definite difference in concentration was observed. Of two sets of histamine samples, the one left at room temperature, had a discernible decrease in concentration compared to the samples of histamine at 0°C .

Consideration of the instability of the azo dye of histamine has led to another short investigation. The reaction of sulfanilic acid yields a colored solution under the conditions given for making the azo dye. The color formed is not stable. It blooms to a peak absorbance and then apparently fades asymptotically to a stable color. (Plate 4). A short study was made of this instability to determine the order of the reaction. The method used was to observe the change in absorbance with respect to time for several concentrations. The assumption was made that the dye concentration is directly related to the absorbance of the solution, and that absorbance of the dye measured the dye concentration in all calculations. The order of the reaction changes from an initial zero order to fractional orders as the reaction proceeds. Plate 4 gives evidence that the color does not fade as rapidly as previously suspected.

EXPLANATION OF PLATE VII

Interaction of histamine with heparin

- - pH 6.85 25° C
- - pH 6.83 0° C
- - pH 4.40 25° C
- ◆ - pH 4.40 0° C



EXPLANATION OF PLATE VIII

Interaction of histamine with heparin.

- - pH 8.99 25° C
- - pH 8.99 0° C



EXTENSIONS

Numerous extensions may be made from this problem. The first might be a further study of the heparin interaction with histamine using a different experimental technique. This could be done in several ways. Amann (1), Senyal (23), and Werle (24,25) have already made studies of this complex, using paper chromatography, precipitation techniques, and equilibrium dialysis. Since heparin does have a reported optical activity, there is a possibility of studying the heparin-histamine complex with polarimetry. A second extension would be to study the heparin-imidazole complex formation and the histidine-heparin complex formation. These two compounds follow naturally the study of histamine. Imidazole is the basic ring structure of histamine; and histidine is an amino acid with very similar structure. Histamine is derived from histidine by decarboxylation. Potentiometric titrations have possibilities for this investigation. Both histamine and heparin have titration curves. (Plate I) The information from such titrations could give information as to the type of complex that is being formed between histamine and heparin. Examination of the titration curves of histamine indicate that a sharp increase in complex formation may occur at pH's just less than each end point.

An exploration of the apparent disappearance of histamine from its solution should be carried out. This problem could be probed in the following ways. Investigate the effect of sunlight and artificial light including ultra violet, visible, and infra red; the effect of heat; the effect of pH on the rate of histamine disappearance.

ACKNOWLEDGMENTS

The author wishes to express his deep gratitude to Dr. A. C. Andrews for his guidance and infinite patience, without which this work could not have been completed.

Sincere acknowledgment is expressed to the National Institute of Health, Public Health Service, grant G-3920 for the support of this work. Appreciation is shown for the help and advice of Ivan C. Smith and Joseph S. Cantrell, and to the other associates of the author for their generous encouragement.

LITERATURE CITED

- (1) Amann, R., and E. Werle. "Formation of Complexes by Heparin with Histamine and Other di and Polyamines." *Klin. Wochschr.* 34:207-9 (1956).
- (2) Andrews, A. C. and T. D. Lyons. "Binding of Histamine and Antihistamine to Bovine Serum Albumin by Mediation with Cu (II)." *Science.* 126:561-562 No. 3273 (1957).
- (3) Cass, R., K. W. Head, J. F. Riley, S. W. Stroud and G. B. West. "Heparin and Histamine in Mast Cell Tumours." *J. of Physiology.* 125:47 (1954).
- (4) Dragstedt, C. A., J.A. Wells and M. R. e Silva. "Inhibitory Effect of Heparin on Histamine by Trypsin, Antigen, and Protease." *Proc. Soc. Exptl. Biol. Med.* 51:191-2 (1942).
- (5) Fawcett, K. W. "Release of Histamine by Mast Cells." *J. of Exp. Med.* 100:217 (1954).
- (6) Gerendas, M., I. Csefko and M. D. F. Udvardy. "Histamine Heparin Thrombin Chain Mechanism." *Nature.* 162:257-8 (1948).
- (7) Haley, T. J. and F. Stolarsky. "Effect of Histamine and Antihistamines on Coagulation of Normal and Heparinized Rabbit Plasma." *Proc. Soc. Exptl. Biol. Med.* 76:299-301 (1951).
- (8) Jaques, L. B., and E. T. Waters. "The Identity and Origin of the Anticoagulant of Anaphylactic Shock in the Dog." *J. of Physiology.* 99:454-66. (1941).
- (9) Jensen, R., O. Snellmann, and B. Sylven. "The Inhomogeneity of Commercial Heparin Preparations from a Physico Chemical Point of View." *J. Biol. Chem.* 39:497-592 (1919).
- (10) Jorpes, E. "The Chemistry of Heparin." *Biochem. J.* 36:203-13 (1942).
- (11) Jorpes, E., H. Bostrom, and V. Matt. "The Linkage of the Amino Group in Heparin." *J. of Biol. Chem.* 183:607 (1950).
- (12) Klotz, I. M. "Protein Interactions." *The Proteins*, Edited by N. Neurath Vol. 1., Part B., pp. 727-747, Academic Press Inc., (1955).
- (13) Koessler, K. K., and M. T. Hanks. "Studies of Proteinogenous Amines." *J. Biol. Chem.* 39:497-592 (1919).
- (14) Lyons, D. T., Jr., "Possible Mechanisms of Histamine-Human Gamma Globin Interactions." *Masters Thesis, Kansas State College* (1955).

- (15) Lyons, T. D. and A. C. Andrews. "A Spectrophotometric Method for the Determination of Histamine." *Trans. Kans. Acad. Sci.* 58:435-438 (1955).
- (16) Macht, D. I. "Experimental Studies of Heparin and its Influence on Toxicity of Digitaloids, Congo Red, Cobra Venom, and Other Drugs." *Ann. Internal. Med.* 18:772-81 (1943).
- (17) MacIntoch, F. C. "A Colorimetric Method for the Standardization of Heparin Preparations." *Biochem. J.* 35:776-82 (1941).
- (18) Mickel, B. L. Master's Thesis, Kansas State College, (1951).
- (19) Paton, W. D. M. "Histamine Release by Compounds of Simple Chemical Structure." *Pharmacological Review.* 9:269 (1957).
- (20) Riley, J. F. "The Mast Cells." *Endeavor.* 19:4-8 (1960).
- (21) Riley, J. F. and F. B. West. "The Presence of Histamine in Tissue Mast Cells." *J. of Physiology.* 120:528 (1953).
- (22) Rocha e Silva, M. "Histamine, Its Role in Anaphylaxis and Allergy." Charles C. Thomas, Publisher, Springfield, Ill. USA.
- (23) Sanyal, K. K. and G. B. West. "Binding of Histamine in Mammalian Tissues." *Nature.* 178:1293 (1956).
- (24) Werle, E. and R. Amann. "Physiology of Mast Cells and Carriers of Heparin and Histamine." *Klin. Wochschr.* 34:624-30 (1956).
- (25) Werle, E., and R. Amann. "Reaction of Histamine and Heparin." *Naturwissenschaften.* 42:583 (1955).
- (26) White, R. P. and P. H. Woodard. "Heparin Content of Thoracic Duct Lymph Following Shock in Dogs." *Am. J. Physiol.* 188:189-192 (1957).
- (27) Wolfrom, M. L., D. I. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean. *J. Am. Chem. Soc.* 65:2077 (1943).
- (28) Wolfrom, M. L. and W. H. McNeely. "Relation Between the Structure of Heparin and Its Anticoagulant Activity." *J. Am. Chem. Soc.* 67:748-53 (1945).
- (29) Wolfrom, M. L., R. Montgomery, J. V. Karabinos, and P. Rathgeb. "The Structure of Heparin." *J. Am. Chem. Soc.* 72:5796-7 (1950).

APPENDIX

Sample Calculations:

The following sample calculations are for the two temperatures reported in this paper. One set of data for each temperature have been calculated from the following experimental values:

a) absorbance of histamine sample dialyzed against heparin = 0.383

b) absorbance of histamine sample dialyzed against buffer = 1.200

In the following equations A represents absorbance, and c represents concentration of histamine in moles per liter. By the use of least squares the data graphed on Plate 2 yielded the following relationship. (A reference to least squares may be found in J. W. Mellor's "Higher Mathematics of Chemistry and Physics." p. 326-330). $A = 4.270 \times 10^5 c - 0.04$.

The following calculation is for the concentration of histamine in a .

$$(0.383) = 4.27 \times 10^5 c_h - 0.04$$

$$4.27 \times 10^5 c_h = 0.383 - 0.04 = 0.387$$

$$c_h = 9.06 \times 10^{-6} \text{ moles per liter}$$

where c_h is the molar concentration of the free histamine.

Since only 25 milliliters was the total volume of the solution, and so the number of moles present in the solution was

$$(9.06 \times 10^{-6} \text{ moles/liter})(2.5 \times 10^{-2} \text{ liters}) = 2.26 \times 10^{-6} \text{ mole}$$

Similarly for the standard solution a value of

$$c_s = 7.05 \times 10^{-6} \text{ moles histamine was obtained.}$$

The difference in these two quantities, the number of moles of histamine bound to the heparin within the dialysis bag, is

$$7.05 \times 10^{-6} - 2.26 \times 10^{-6} = 4.79 \times 10^{-6} \text{ moles.}$$

The concentration of the heparin solution was 2.46×10^{-5} moles per liter.

The volume of solution within the dialysis bag was 10 milliliters; therefore the total concentration of heparin present was 2.46×10^{-5} moles. Scatchard's equation from page 9 is

$$r/(1i)_f = rK - rK \dots\dots\dots (7)$$

Assuming the value for n to be 5, and substituting in the experimentally determined values the association constant is obtained as follows:

$$2.76 \times 10^4 = (5)K - (.194)K$$

$$2.76 \times 10^4 = K(5 - 0.194) = K(4.806)$$

$$K = 2.76 \times 10^4 / 4.806 = 5.74 \times 10^3$$

Repeating the same procedure for 25°C. , the calculation association constant is

$$K = 8.03 \times 10^3$$

Using the above data, the following thermodynamic quantities are calculated:

$$\Delta F = -RT(2.303) \log K$$

$$\Delta F_{00} = -(1.36 \times 10^3)(\log 5.74 \times 10^3) = 4.70 \text{ Kcal/Mole}$$

$$\Delta F_{250} = -(1.36 \times 10^3)(\log 8.03 \times 10^3) = 5.32 \text{ Kcal/Mole}$$

$$\Delta S = -(\partial \Delta F / \partial T)_p = -(-5320 - 4700)/25 = 24.8 \text{ cal/deg mole}$$

$$\Delta H = (\partial \Delta F / \partial 1/T)_p = \frac{5320}{298} - \frac{-4700}{273}$$

$$\Delta H = 51000/25 = 2040 \text{ cal/mole.}$$

Table 1. Titration data of histamine dihydrochloride, and heparin using KOH.

ml base	pH Histamine Solution	pH Heparin Solution
0.0	3.28	2.99
0.1	3.15	3.06
0.2	3.28	3.13
0.3	3.38	3.23
0.4	3.38	3.36
0.5	3.49	3.51
0.6	3.58	3.73
0.7	3.72	4.03
0.8	3.97	4.50
0.9	4.30	5.57
1.0	5.12	9.51
1.1	5.78	10.16
1.2	6.14	10.57
1.3	6.44	10.70
1.4	6.77	10.80
1.5	7.02	10.87
1.6	7.66	10.91
1.7	9.02	10.98
1.8	9.72	11.03
1.9	10.00	11.08
2.0	10.20	
2.1	10.38	
2.2	10.57	
2.3	10.73	
2.4	10.88	
2.5	10.93	
2.6	10.93	
2.7	-----	
2.8	11.07	
2.9	11.12	
3.0	11.16	

Table 2. Absorbance of the azo dye of histamine related to time and concentration of histamine

Time after making azo dye (sec.)	:	Histamine concentration moles/liter		
		:	absorbance readings	:
	:	5.55×10^{-4}	3.33×10^{-4}	1.66×10^{-4}
140		2.23	1.47	0.678
195		2.43	1.64	0.780
240		2.58	1.713	0.825
290		2.78	1.898	0.865
340		2.45	1.592	0.850
420		2.45	1.492	0.760
465		2.35	1.425	0.690
525		2.30	1.377	0.640
570		2.28	1.547	0.605
620		2.27	1.303	0.573
700		2.31	1.287	0.545
750		2.26	1.262	0.525
805		2.17	1.233	0.510
870		2.27	1.303	0.490
920		2.30	1.190	0.479
990		2.22	1.161	0.465
1050		2.13	1.147	0.455
1110		2.13	1.130	0.447
1170		2.17	1.127	0.443
1230		2.17	1.127	0.443
1800		2.13	1.090	0.367
2400		2.15	1.082	0.345
3000		2.08	1.07	0.337
39600		1.999	1.007	0.315

Table 3. Time study of heparin-histamine binding. pH 4.40 at 0° C.

Time (hours) blank	Absorbance of sample	Molar concentra- tion X 10 ⁴
0	2.50	5.74
1	1.658	5.13
2	1.400	4.33
4	1.233	3.82
5	1.208	3.74
6	1.226	3.79
7	1.200	3.71
8	1.180	2.71
9	1.132	3.50
heparin solutions		
0	2.50	5.74
1	1.327	4.24
2	0.768	2.74
3	0.548	1.77
4	0.477	----
5	0.327	1.06
6	0.325	1.05
7	0.383	1.24
8	0.293	0.95
9	0.277	0.90

Table 4. Effect of pH and temperature on the binding of heparin to histamine.

pH	Temperature deg. C.	Concentration in moles		
		Histamine $\times 10^6$	Heparin $\times 10^5$	Histamine bound $\times 10^7$
4.40	0	4.44	2.46	30.60
		9.18	1.01	39.00
		9.61	1.50	40.00
		7.76	1.83	54.20
	25	2.60	2.15	14.10
		3.69	1.08	19.10
		10.36	1.28	69.10
		12.84	0.64	54.30
6.83	0	3.85	2.38	12.00
		4.01	2.07	10.50
		5.72	1.04	5.68
		5.23	1.19	12.00
	25	4.19	2.07	7.90
		6.68	1.03	8.37
		5.77	1.03	8.37
		3.38	2.40	10.90
8.99	0	18.90	7.41	3.97
		11.60	3.67	3.16
		18.40	6.76	3.82
		2.54	3.28	1.29
	25	5.10	2.30	1.87
		6.37	1.15	4.71
		7.43	2.78	2.17
		5.18	2.78	1.81

THE EVALUATION OF THERMODYNAMIC FUNCTIONS OF
THE HISTAMINE-HEPARIN INTERACTIONS
BY EQUILIBRIUM DIALYSIS

by

WAYNE BURL ROSE

B. S., Adams State College, 1955

M. Ed., Adams State College, 1957

AN ABSTRACT OF
A THESIS

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

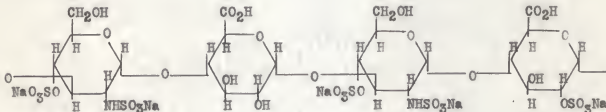
KANSAS STATE UNIVERSITY
MANHATTAN, KANSAS

1961

The medical and biochemical literature of histamine and heparin systems is quite extensive. However practically no attention has been given to the physical chemical properties of these compounds. It is believed that an explanation of some of the reactions of these compounds may come from their thermodynamic quantities.

There appears to be a definite relationship between the physiological chemistry of heparin and histamine. Both of these substances are found in the same tissue, and should histamine be released into the blood of an animal it is not long until heparin also appears in this fluid.

Heparin appears to have a polysaccharids structure, containing two specific rings within a unit, namely glucosamine and glucuronic acid. Below is the sodium salt of one of the reported structures of heparin.



Heparin is known for its ability to prevent the coagulation of blood and medical science has used this substance for this purpose.

The method of binding study used here was developed by Klotz, who derived an equation for the "intrinsic equilibrium constant." This constant is related to experimentally measurable quantities in an actual dialysis.

The expression connecting these quantities assumes first, that the overall binding reaction occurs in a stepwise manner; and second, that there exists no interaction between sites.

The experimental procedure involved the establishment of an equilibrium between a heparin solution, contained in a dialysis bag, and an external solution of histamine, both at a known initial concentration. For each heparin-containing tube, a corresponding blank tube was prepared identical to the first except that it contained only buffer solution in the dialysis bag. The difference in the equilibrium histamine concentrations of the tubes containing heparin and the blank tubes, at a given histamine concentration, was taken to be the concentration of histamine bound by the heparin. The equilibrium concentrations of histamine and of heparin in these interactions were used to calculate the equilibrium constants and the changes in the Gibb's free energy.

It was found that definite binding between heparin and histamine does occur. The free energy values are of the magnitude of 5 Kcal/mole. This is much weaker than the usual covalent bonding, but within the range of values of hydrogen bonding. The final structure of the heparin-histamine complex assumes the heparin sulfate anion is bonded to the protonated amine group of the histamine.

The analytical colorimetric method for the analysis of histamine, using the sulfanilic acid azo-dye, was reinvestigated. The color was found to be more stable than previously suspected, and a modification in the analysis of histamine is suggested.