Mechanism of Histamine Binding I: Effect of Calcium on Binding of Histamine to a Plasma Peptide

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Abstract □ A method of isolating a single polypeptide from human plasma, termed peptide H for its histaminopexic or histamine-binding capability, is described. The peptide was obtained by acid precrystallization of human plasma, followed by water extraction of the precipitate and separation on an ion-exchange column. A molecular weight of 4492 was calculated from amino acid analysis. A dynamic dialysis technique was used to study the binding of histamine to peptide H. No binding of histamine to peptide H was observed in the absence of Ca^{+2}; however, in the presence of Ca^{+2}, binding of histamine to peptide H did occur. It was determined that histamine first forms a complex with Ca^{+2}, with subsequent binding of the Ca^{+2}–histamine complex to peptide H. From the dialysis data and kinetic analysis, it was possible to demonstrate that the data are consistent with a peptide having three binding sites for the Ca^{+2}–histamine complex, two fast sites and one slow site.

Keyphrases □ Histamine binding to plasma protein—calcium effect, mechanism proposed, dialysis and kinetic data □ Plasma protein binding of histamine—calcium effect, mechanism proposed, dialysis and kinetic data □ Calcium effect—histamine binding to plasma protein, mechanism proposed □ Dialysis technique, dynamic—used to determine histamine binding to plasma protein

Histaminopexy, or the ability of serum proteins to bind histamine, was first detected by Parrot *et al.* (1) when it was noted that normal human serum reduced the effect of histamine on the isolated guinea pig ileum, the standard bioassay for histamine. Since then, Parrot and coworkers (2–4) presented several reviews relating to various aspects of histaminopexy. Following the detection of histaminopexy, Parrot *et al.* (5) described the preparation of a human serum fraction, termed plasmapexin, in which the active factor was considered to be a globulin fraction and which demonstrated an ability to bind histamine. A further fractionation was developed to yield a histamine-binding fraction termed plasmapexin I (6).

Guirgis (7) prepared a number of serum protein fractions and tested them for histaminopexic activity and protein content. He concluded that the active fraction was not a γ-globulin but was of relatively low molecular weight. Agneray *et al.* (8) also subsequently concluded that the active histaminopexic factor is probably of low molecular weight and may be associated with immunoglobulin. Several reports have appeared which differ somewhat from the findings of Guirgis (7) and Agneray *et al.* (8) in that histamine was suggested to bind to high molecular weight globulin fractions (9, 10). Gecse *et al.* (11), however, found histamine to be bound to a fraction from rat and human serum containing a polypeptide.

Other investigators found negative results with respect to biological and dialysis tests for histaminopexy. Pautrizel *et al.* (12) found that while asthmatic serum values of histaminopexy were generally lower than normal, the results were too variable to be of more than limited value. Kirtcher and Frankland (13) also indicated that histamine binding by serum is highly unselective. Yet Huriez *et al.* (14) ran over 3000 guinea pig ileum tests and confirmed Parrot's initial observation; Wodniansky (15) evaluated the ileum test on 500 serums, each run three times by three different workers in a double-blind study, and also confirmed the validity of Parrot's finding.

Binding studies using γ-globulin were also shown to produce negative results by Beall (16) and Kaplan and Davis (17). Recently, however, Cohen and Nieforth (18) demonstrated histamine binding to γ-globulin.

When the mass of literature is considered as a whole, the evidence is that when whole serum or a proper serum fraction is used low histaminopexy values are diagnostic of allergies. It is possible that variability in histaminopexy may be such that studies with small numbers of subjects may be unrewarding. One reason for variability may be that guinea pig ileum, due to endocrinological changes, may not show histaminopexy from October to March (19). A more important cause of variability in both the biological test and the binding studies involving γ-globulin and other protein fractions is that, without exception, Tyrodes solution is used as the aqueous medium. Tyrodes solution contains a number of mono- and divalent cations that, as will be seen in this and the following report, affect the binding of histamine to serum protein.

Calcium appears to play a fundamental role in both the release of histamine to the body fluids and in the binding of histamine to blood proteins. Much of the body histamine is stored in mast cells bound to a heparin–protein complex by weak electrostatic forces (20). The release of histamine from the mast cells appears to require the presence of Ca^{+2} (21).

Conversely, the binding of histamine to serum protein also appears to require the presence of Ca^{+2} (4, 22, 23). Calcium salts have been successfully used in the clinical treatment of allergic conditions. Single injections of calcium gluconate into asthmatic patients resulted in marked clinical improvement and *in vitro* tests of the patients' serums indicated histaminopexy (24). Other calcium salts have been found beneficial in the treatment of asthma and hay fever upon prolonged administration (25). Guinea pigs or rabbits on high Ca^{+2} diets or dosed orally with calcium gluconate were found resistant to the bronchoconstrictive effects of histamine (26, 27). As might be

expected, addition of edetate (EDTA) to normal human serum *in vitro* prevented the binding of histamine; however, the addition of calcium chloride restored the serum's ability to bind histamine (28).

While it is obvious that Ca^{+2} affects the binding of histamine *in vitro* and *in vivo*, the mechanism by which Ca^{+2} promotes histamine binding has not been determined. The objective of this study is to elucidate the mechanism of the Ca^{+2}-induced binding of histamine to a polypeptide isolated from human plasma. A dynamic dialysis technique was used to study the peptide–histamine interaction. This technique was developed for the study of binding to macromolecules (29, 30) and recently was thoroughly investigated (31).

EXPERIMENTAL

Materials—Histamine dihydrochloride[1], chromatographically homogeneous, and tromethamine hydrochloride[1], ultrapure, were used in this investigation. The maximum Ca^{+2} limit in the ultrapure tromethamine hydrochloride was 0.00001%. All other chemicals used were reagent grade.

The cellulose membrane[2] (average pore diameter 24 Å) was prepared for use by first rinsing with distilled water and then boiling for 15 min in a 10% acetic acid solution, followed by repeated rinsings with boiling distilled water. The distilled water used throughout was twice distilled, the second time in an all-glass still.

Preparation of Peptide Fraction—Outdated, frozen, pooled human plasma was obtained from a hospital[3] using professional donors and was used routinely. Freshly frozen and fresh pooled human plasma were used for comparisons with the outdated, frozen material. Donors were screened for allergic conditions, and the plasma used was considered to be normal human plasma. A sample of plasma was diluted with 15 times its volume of distilled water. When frozen plasma was used, it was first thawed at room temperature and strained through cotton to remove precipitated material. Hydrochloric acid (0.1 *M*) was then added to the diluted plasma with constant stirring, and the pH was continuously monitored. Any precipitate at pH 6.5 was removed by decanting and centrifugation, and 0.1 *M* HCl was again added to the supernate. At pH 5.2 the precipitate was collected by decantation and centrifugation. The precipitate was centrifuged until very dry, and the tubes were turned upside down on a towel to allow any fluid to drain off. The pellet was washed with a small volume of distilled water and again drained. The precipitate was then removed from the centrifuge tubes and mixed with a volume of distilled water equal to one-half the original plasma volume. The mixture was stirred with a magnetic stirrer for 1 hr and then allowed to settle. After centrifuging, the supernate was collected. Sodium chloride was added to make the solution 0.9% in sodium chloride. This solution was stable with refrigeration for up to 2 weeks.

Column Chromatography—The resin[4] was swollen for 2 hr on a boiling water bath in the 0.1 *M* NaCl used as eluant; it was then cooled and packed into a 35 × 2.5-cm column. A sample of the acid-precipitated fraction already described was applied by pipet, and 5-ml fractions were collected and assayed at 280 nm for protein content. A flow rate of 3 ml/min was used.

Determination of Protein Concentration—The protein concentration was determined at 280 nm, and the following equation was found to apply (32):

$$C = 1.55A \qquad \text{(Eq. 1)}$$

where C = concentration of protein in milligrams per milliliter, and A = absorbance at 280 nm. To check Eq. 1, protein solutions *having known absorbances in* 0.1 *M* NaCl were pipeted into tared aluminum foil cups and dried to constant weight. The cups were weighed and the calculated weight of sodium chloride was subtracted to allow comparison with weights calculated using Eq. 1. A maximum difference of 2.5% between concentrations determined by Eq. 1 and by weighing was observed.

Table I—Results of Amino Acid Analysis of Acid Precipitated Fraction

Amino Acid[a]	(*a*) Milligrams Percent	(*b*) Molecular Weight	(*a*)/(*b*)	(*c*) Number of Residues	(*b*) × (*c*)
Alanine	1.30	89.09	0.015	3	267.27
Valine	1.15	117.15	0.010	2	234.30
Leucine	1.95	131.17	0.015	3	393.51
Proline	0.85	115.13	0.007	2	230.26
Threonine	0.95	119.12	0.008	2	238.24
Serine	0.90	105.09	0.009	2	210.18
Phenylalanine	1.15	165.19	0.011	2	330.38
Aspartic acid	1.85	133.10	0.014	3	399.30
Glutamic acid	3.15	147.13	0.021	4	588.52
Tyrosine	0.90	181.19	0.005	1	181.19
Lysine	1.90	146.19	0.013	3	438.57
Histidine	0.80	155.16	0.005	1	155.16
Arginine	0.55	174.20	0.003	1	174.20
Cystine	0.70	240.30	0.003	1	240.30
Glycine	0.40	75.07	0.005	1	75.07
Isoleucine	0.30	131.13	0.002	1	131.13
Methionine	0.10	149.21	0.0007	0	—
					4288.00
Tryptophan[b]	—	204	—	1	204
					4492

[a] Amino acid determination by Analytical BioChemistry Laboratories, Columbia, MO 65201. [b] Tryptophan presence was confirmed by the glyoxylic acid test, and one residue is assumed.

Freezing-Point Depression Determination—The freezing-point depression of the fraction already described was determined using a freezing-point apparatus[5] with a differential thermometer and an ice–salt water bath. The freezing-point depressions of two 30-ml samples at a concentration of 0.21 mg/ml (in distilled water) were determined, as were the freezing points of two distilled water blanks.

Amino Acid Analysis—A GLC amino acid analysis was conducted on a sample of the fraction already described. Hydrolysis was effected by 6 *N* HCl, thus destroying any tryptophan present. The results (Table I) were used to calculate a molecular weight by the least-ratios method. The respective milligram percentages of each amino acid were divided by the molecular weight of the amino acid. The common divisor was 0.005, and methionine was eliminated to give the results shown in Table I.

Tryptophan Determination—Tryptophan was tested for using the Hopkins–Cole or glyoxylic acid reaction (33). One milliliter of 5% glyoxylic acid in water was mixed with 1 ml of a 0.19-mg/ml fraction in distilled water and layered over 1 ml of concentrated sulfuric acid. The appearance of a violet ring is indicative of the presence of an indole ring system. A distilled water blank was also run.

Dialysis Apparatus and Techniques—The dynamic dialysis technique used in this investigation closely follows that of Meyer and Guttman (31). The apparatus consisted of a 4-cm diameter, 7 cm high, cylindrical plastic cell suspended in a 100-ml jacketed beaker. The cellulose membrane was secured across the lower opening of the plastic cell. Seventy-five milliliters of tromethamine buffer at 37° was placed in the jacketed beaker and comprised the external solution. Ten milliliters of histamine, 9.0×10^{-4} *M*, in tromethamine buffer was pipeted into the plastic cell along with 10 ml of the peptide solution in tromethamine buffer or a blank solution of tromethamine buffer. Whenever used, the calcium chloride concentration was the same in the internal and external solutions. The plastic cell containing the internal solution was placed in the external solution and leveled with the external solution to prevent a hydrostatic head. A magnetic stirrer rotat-

[1] Schwarz/Mann, Orangeburg, NY 10962
[2] Sargent Welch, Skokie, Ill.
[3] St. Elizabeth's Hospital, Lafayette, IN 47904
[4] Sephadex G-25, *Pharmacia Fine Chemicals, Inc.*, Piscataway, N.J.
[5] Molecular weight apparatus, Beckmann, Sargent Welch Co., Skokie, Ill.

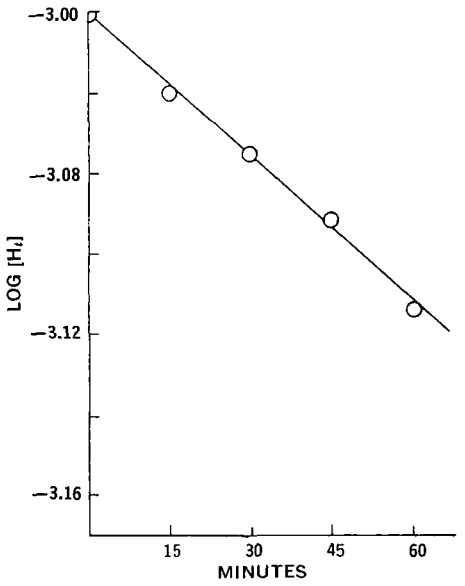


Figure 1—*Plot of log of total histamine* $[H_t]$ *remaining in the dialysis cell at time* t *as a function of time. The membrane constant,* K_m*, is determined from the slope of the line.*

ing at 200 rpm agitated the external solution. The internal solution was stirred by a twisted glass rod at 50 rpm. Temperature was maintained at 37° by pumping water through the jacketed beaker from a controlled-temperature bath.

Samples of 25 ml were taken by pipet at 15-min intervals with replacement. The sample volume maintained sink conditions in the external solution for histamine. The concentrations were corrected for the replacement volumes. Dynamic dialysis determinations were made for histamine and mixtures of histamine and Ca^{+2}. Histamine was assayed spectrophotometrically at 211 nm. There was no observable binding of histamine to the cellulose membrane or plastic container in the presence or absence of Ca^{+2} during the 1-hr dynamic dialysis determination.

Determination of Membrane Constant—The constant was determined from the dialysis of histamine through the membrane in the absence of peptide H. The basic equation for dynamic dialysis was given by Meyer and Guttman (31):

$$\frac{-d[H_t]}{dt} = K_m[H_f] \qquad \text{(Eq. 2)}$$

where $[H_f]$ is the free histamine concentration at any time, $[H_t]$ is the total histamine concentration remaining in the dialysis cell or internal solution at time t, $d[H_t]/dt$ is the dialysis rate, and K_m is the membrane constant. Plots of log $[H_t]$ *versus* time were found to be linear (Fig. 1), and K_m was calculated from the slope.

The nature and design of the experimental apparatus permitted the same cellulose membrane to be used throughout this study. Since all dialysis determinations were made through a constant area of the same membrane, there were no significant differences between replicate determinations.

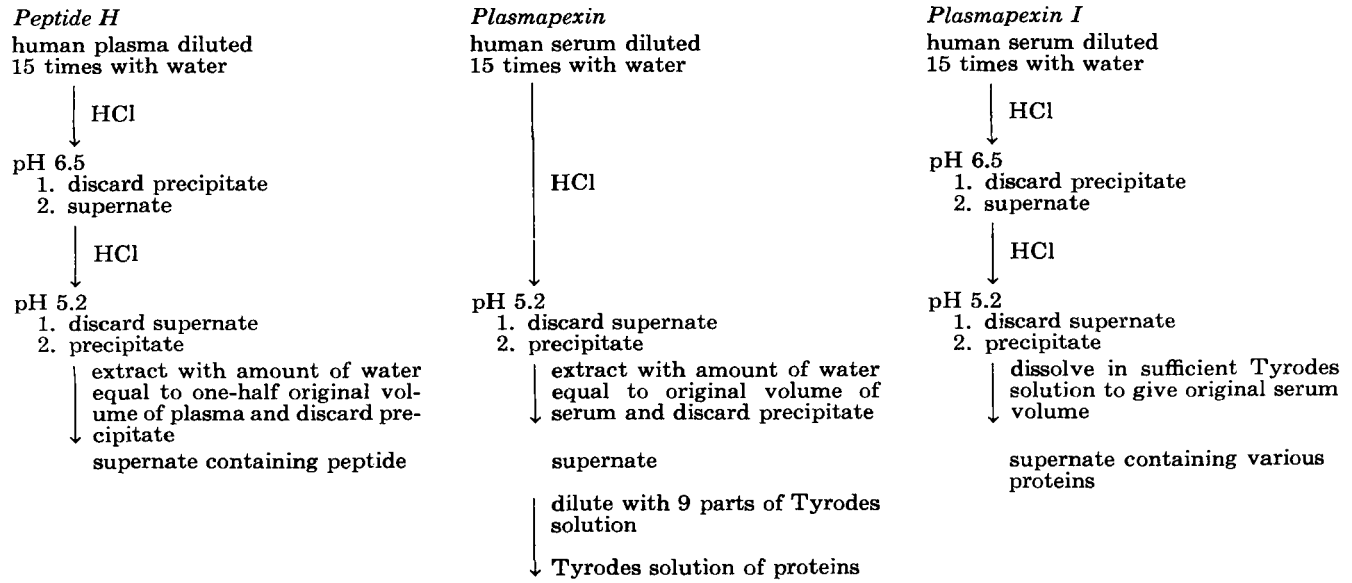
Determination of Initial Free Histamine Concentration—Solutions of histamine (4.5×10^{-4} *M*), Ca^{+2} (9×10^{-3} *M*), and peptide (8.6×10^{-5} *M*) in pH 7.4, 0.9% NaCl-tromethamine buffer were incubated in a 250-ml volumetric flask suspended in a 37° water bath. Twenty-milliliter samples were withdrawn by pipet at appropriate intervals over 24 hr. The samples were then used as the internal solutions of dynamic dialysis runs. The total histamine remaining in the cell was plotted against time, and from these plots the initial rate could be determined. By knowing the initial rate and the membrane constant, the initial free histamine concentration as a function of time was calculated by Eq. 2.

Preequilibration of Calcium Chloride with Histamine and Peptide H—Calcium chloride was equilibrated separately with histamine and peptide H for 8 hr at 37°. Solutions were in pH 7.4, 0.9% NaCl-tromethamine buffer. The calcium chloride was at twice the concentration used during the dynamic dialysis since the equilibrated solution was diluted with an equal amount of either histamine or peptide H solution prior to dialysis.

RESULTS AND DISCUSSION

Isolation of Peptide H—The method used to isolate a single polypeptide from human plasma was similar to methods previously used to isolate two histaminopexic fractions, plasmapexin and plasmapexin I, from human serum (5, 6). The isolated polypeptide was termed peptide H for reference purposes. The preparation of plasmapexin, plasmapexin I, and the acid-precipitated fraction used throughout the rest of this study is outlined in Scheme I. Plasmapexin differs from plasmapexin I in that for plasmapexin I the precipitate at pH 6.5 is discarded and the precipitate at pH 5.2 is dissolved in Tyrodes solution, not water. The fraction used in this study was prepared from plasma, not serum. The precipitate was taken only between pH 6.5 and 5.2 as for plasmapexin I but was extracted with water instead of Tyrodes solution. When Tyrodes solution was used, the entire precipitate went into solution, as it also did with pH 7.4 tromethamine buffer containing 0.9% NaCl. When water was used, however, the amount of material extracted from the precipitate was very small.

If the blood fraction responsible for histaminopexy was a polypeptide and was contained in the precipitate at pH 5.2, its water solubility should be greater than the solubility of the higher molecular weight globulins also present in the precipitate. For this reason, a selective solubilization of the pH 5.2 precipitate with



Scheme I—Isolation of plasmapexin, plasmapexin I, and peptide H

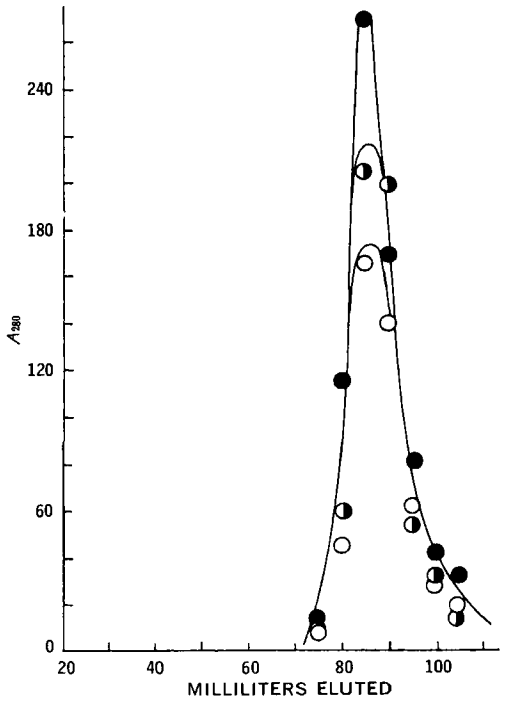


Figure 2—*Absorbance at 280 nm of the eluted peptide H from the ion-exchange column. Peptide H was prepared from fresh* (○)*, fresh frozen* (●)*, and outdated frozen* (◑) *pooled human plasma.*

distilled water, containing no salts, was used to exclude the high molecular weight proteins and isolate the polypeptide. The acid precipitation and water extraction procedure was used on fresh, pooled human plasma; fresh frozen, pooled human plasma; and outdated, frozen, pooled human plasma. In preparation it was noted that the outdated frozen plasma contained large amounts of precipitated matter when thawed. At pH 6.5, however, there was little or no precipitate present. The fresh frozen plasma contained some precipitated material when thawed and a moderate amount at pH 6.5. The fresh plasma had no precipitated material until the pH was lowered to pH 6.5 when considerable precipitate was noted. When the precipitate between pH 6.5 and 5.2 was extracted with water, most of the precipitate remained undissolved in all three cases.

When samples of these three fractions were chromatographed[4] and eluted with 0.1 *M* NaCl, only single sharp peaks with volumes of elution of 87 ml were noted (Fig. 2). The fractionation range of the resin is 1000-5000 for peptides and globular proteins.

The single sharp peak for fractions prepared from fresh, fresh frozen, and outdated frozen plasma demonstrated that the material is not a decomposition or fragmentation product due to the age or freezing of the plasma. Furthermore, it suggested that a single protein was present. The fractionation range of the resin indicated that it was actually a peptide, and the elution volume indicated that it was in the upper weight range.

Amino Acid Analysis and Molecular Weight Determination—The acid hydrolysis of peptide H, followed by amino acid analysis, resulted in the identification of 32 amino acid residues (Table I). The molecular weight of peptide H was found to be 4288. This was calculated by the least-ratios method assuming 32 amino acid residues and a milligram per molecular weight ratio of 0.005 equal to one residue.

Tryptophan was not determined in the amino acid analysis because it was destroyed by acid hydrolysis. When the peptide H-glyoxylic acid mixture was layered onto concentrated sulfuric acid, a violet ring appeared at the interface, indicating the presence of tryptophan. While the glyoxylic acid test detected tryptophan, it is a qualitative test and is not capable of quantitatively determining the number of tryptophan residues present in the peptide molecule. Therefore, the molecular weight of peptide H was also determined by measuring the freezing-point depression of a peptide solution of known concentration (the concentration was determined by Eq. 1). For two determinations the freezing points of the peptide solutions were identical, as were those of the distilled water blanks, and gave a molecular weight of 4340. If one tryptophan residue is assumed in the peptide H molecule, a molecular weight of 4492 is obtained (4288 + 204), differing from the value obtained by the freezing-point method by only 3.4% and correlating with the fractionation range of the resin. The value of 4492 was used in subsequent calculation of molar concentrations of peptide H. The agreement of the freezing-point depression molecular weight and that calculated from the amino acid analysis and added tryptophan, together with the chromatography results, substantiates the conclusion that the water extract of pH 6.5 and 5.2 acid-precipitated plasma is a single peptide.

Demonstration of Histaminopexy and Its Requirement for Divalent Calcium—From the results of dynamic dialysis it was determined that peptide H binds histamine, but only in the presence of Ca^{+2}. Figure 3, where the log of total histamine remaining in the cell is plotted *versus* time, shows that the blank run of histamine and calcium is linear following first-order kinetics. The plot for histamine in the presence of peptide H with no Ca^{+2} coincides with the blank run showing no binding. However, the plot of histamine and peptide H with Ca^{+2} shows a decreased slope, indicating binding. It also is linear over this time period, obeying apparent first-order kinetics.

There are essentially two pathways by which Ca^{+2} may promote the binding of histamine to peptide H. One pathway is through an initial Ca^{+2}-peptide H association resulting in a direct or indirect change of the charge density at the binding sites or a change in the conformation of peptide H. A conformation change for bovine serum albumin accompanying histamine binding has been reported (34). The second possible pathway is through an initial Ca^{+2}-histamine association, with subsequent binding of the Ca^{+2}-histamine complex to peptide H.

The possibility of Ca^{+2} influencing the peptide in some manner was investigated by equilibrating Ca^{+2} with peptide H prior to the addition of histamine, while the preequilibration of Ca^{+2} with histamine prior to the addition of peptide H was used to elucidate the possible requirement for the Ca^{+2}-histamine complex prior to binding. Figure 4 shows the results of the preequilibration studies. When Ca^{+2} was preequilibrated with peptide H, there was no increase in binding as compared to the binding evidenced when the Ca^{+2}, histamine, and peptide H were simultaneously mixed prior to dialysis. Thus, it is evident that a conformation change such as that reported for histamine-bovine serum albumin bind-

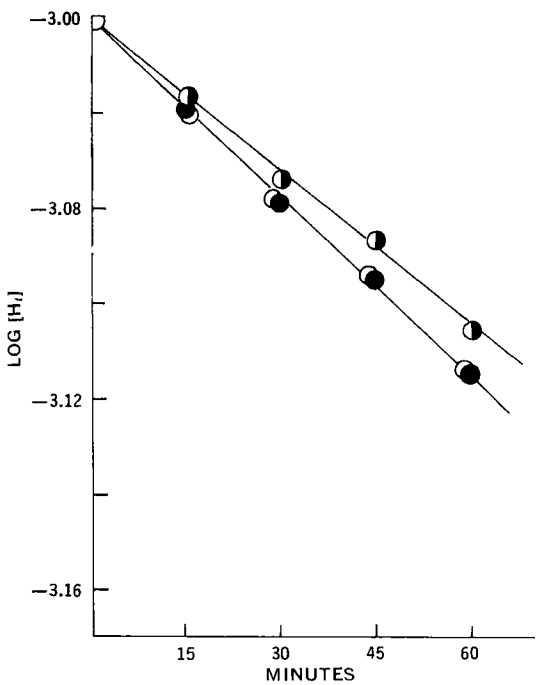


Figure 3—*Dynamic dialysis results showing the effect of Ca^{+2} on the binding of histamine to peptide H. Key:* ○*, histamine and Ca^{+2};* ●*, histamine and peptide H; and* ◑*, histamine, Ca^{+2}, and peptide H.*

ing is not involved in the mechanism of histamine binding to peptide H. However, the preequilibration of Ca^{+2} with histamine resulted in a lower dialysis rate, indicating increased histamine binding to peptide H. It is then evident that the complexation of histamine and Ca^{+2} is the essential step in binding to peptide H and that the Ca^{+2}–histamine complex is the molecular species responsible for histamine binding to peptide H.

Kinetics of Interaction of Ca^{+2}–Histamine Complex with Peptide H—The result of allowing peptide H, Ca^{+2}, and histamine to react in a volumetric flask and following the reaction using dynamic dialysis runs to determine the concentration of free histamine is shown in Fig. 5. The time scale for the disappearance of free histamine is hours for this ternary mixture of the three components. While preequilibration of Ca^{+2} and histamine was shown previously to be required for binding to peptide H, the preequilibration time was chosen as 8 hr without elucidating how much of that 8 hr might actually be required for Ca^{+2}–histamine interaction and/or conformational change and how much is a time-dependent adaptation of the peptide. It can be concluded from the data presented that the 8 hr of preequilibration of Ca^{+2} and histamine is sufficient to produce essentially maximal binding, that 8 hr of incubation of the three components is also sufficient, and that no binding is observed without preequilibration. At 24 hr, the ratio of bound histamine (2.18×10^{-4} *M*) to peptide H concentration (0.86×10^{-4} *M*) was 2.54 (Fig. 5), suggesting at least three histamine binding sites per peptide molecule.

Two kinetic mechanisms have been considered to account for the disappearance of histamine observed in Fig. 5. If the data are plotted semilogarithmically, a straight line is obtained during the initial stages of the reaction (Fig. 6). If the reaction is indeed controlled by a first-order mechanism, the final bimolecular reaction must be fast compared to a slower intermediate process. The mechanism shown in Scheme II may be proposed to account for an apparent first-order reaction:

$$Ca^{+2} + \text{histamine} \overset{\text{fast}}{\rightleftharpoons} \text{CaHi} \qquad \text{(Step 1)}$$

$$\text{CaHi} \overset{\text{slow}}{\rightleftharpoons} \text{CaHi}' \qquad \text{(Step 2)}$$

$$\text{CaHi}' + \text{peptide} \overset{\text{fast}}{\rightleftharpoons} \text{CaHi}P \qquad \text{(Step 3)}$$

Scheme II

Chawla (35) obtained an association constant of $10^3\ M^{-1}$ for Step 1 using a titrimetric method, indicating a very rapid approach to equilibrium relative to the time scale depicted in Fig. 5. The slow step, Step 2, must therefore involve a conformational change in the CaHi complex. Kier (36) reported two conformers of histamine in solution, one having an intranitrogen bond distance of 4.55 Å and the other 3.6 Å. The conformation of the CaHi complex is, however, unknown. To be consistent with a first-order mechanism, there must be a binding specificity for one conformer, with the rate-determining step being the slow conversion of the CaHi complex to the desired conformer. While such a mechanism is consistent with observed kinetics, it seems unlikely to the authors that such a rearrangement of an organic ion–metal ligand complex would occur.

It seems more likely that whatever the structure of the CaHi complex, different sites on the peptide might be available for binding at different rates. The kinetics of Fig. 5 were thus interpreted in terms of a bimolecular reaction involving sites with different rate constants for binding the CaHi complex. This is not in conflict with Fig. 6, because the linear portion of the line is over only slightly more than 2 half-lives, during which time it is difficult to distinguish between first- and second-order reactions. The following assumptions were made concerning the binding process:

1. There are three histamine binding sites per peptide H molecule (from Fig. 5).
2. The sites are independent.
3. The reaction is second order and can be treated as irreversible.
4. [Hi] is approximately equal to [CaHi] due to the high association constant, $K_{[\text{CaHi}]} = 1.18 \times 10^3\ M^{-1}$ (35).

Bimolecular reactions of the calcium-histamine complex with peptide H are depicted in Scheme III for two types of sites having different rate constants, where [CaHi] represents the concentration of the calcium–histamine complex, [*P*] represents the concentration of unoccupied peptide binding sites, and [CaHi*P*] represents the concentration of peptide-bound calcium–histamine complex, all at a given time *t*. The subscripts *f* and *s* refer to fast and slow binding sites on the peptide. In all subsequent discussion, the subscript 0 shall be used to denote the initial concentration of any one of these species; k_s and k_f are the rate constants associated with binding to the slow and fast peptide sites, respectively.

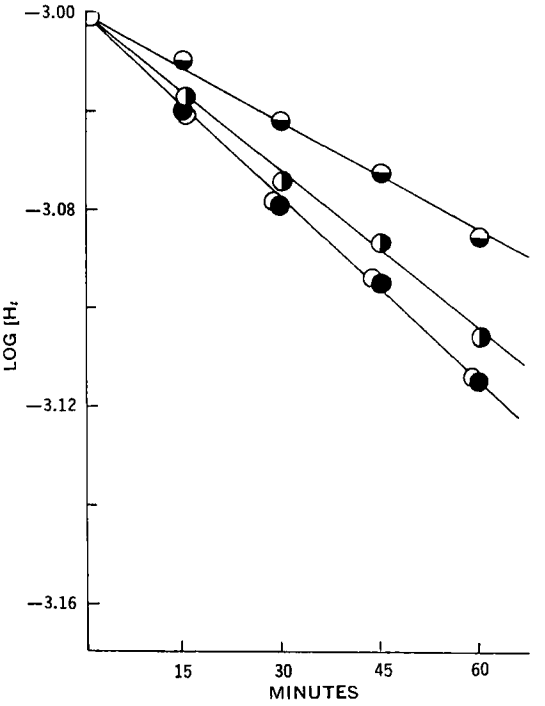


Figure 4—*Dynamic dialysis results showing the effect of preequilibration on the binding of histamine to peptide H. Key:* ○, *histamine and* Ca^{+2} *blank;* ●, *histamine and peptide H;* ◑, *histamine,* Ca^{+2}, *and peptide H (no equilibration) and histamine added to preequilibrated* Ca^{+2} *and peptide H; and* ◒, *peptide H added to preequilibrated* Ca^{+2} *and histamine.*

$$[\text{CaHi}] + [P_f] \xrightarrow{k_f} [\text{CaHi}P_f]$$

$$[\text{CaHi}] + [P_s] \xrightarrow{k_s} [\text{CaHi}P_s]$$

Scheme III

Let us consider first the second-order rate expression for the reaction of CaHi with a *single* type of peptide site, *P*:

$$\frac{d[\text{CaHi}P]}{dt} = k[\text{CaHi}][P] \qquad \text{(Eq. 3)}$$

Since:

$$[\text{CaHi}] = [\text{CaHi}]_0 - [\text{CaHi}P] \qquad \text{(Eq. 4)}$$

and:

$$[P] = [P]_0 - [\text{CaHi}P] \qquad \text{(Eq. 5)}$$

Eq. 3 can be rewritten as:

$$\frac{d[\text{CaHi}P]}{dt} = k([\text{CaHi}]_0 - [\text{CaHi}P])([P]_0 - [\text{CaHi}P]) \qquad \text{(Eq. 6)}$$

Integrating Eq. 6 between [CaHi*P*] = 0 at $t = 0$ and [CaHi*P*] = [CaHi*P*] at $t = t$ yields:

$$kt = \frac{2.303}{[\text{CaHi}]_0 - [P]_0} \log \frac{[\text{CaHi}]_0 - [\text{CaHi}P]}{[P]_0 - [\text{CaHi}P]} - K \qquad \text{(Eq. 7)}$$

A plot of $\log([\text{CaHi}]_0 - [\text{CaHi}P])/([P]_0 - [\text{CaHi}P])$ *versus* time

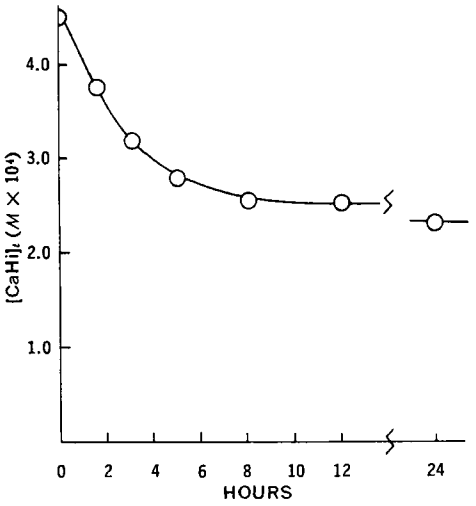


Figure 5—*Free histamine concentration as a function of time, as determined by dynamic dialysis, from which the total bound histamine can be determined.*

will, therefore, be linear for a single type of binding site with an intercept equal to K. Figure 7 shows such a plot where [CaHiP] is the difference between [CaHi]$_0$ and [CaHi] and all the peptide binding sites are considered equivalent. The plot is *not* linear, showing that an assumption of a single type of peptide site is not valid.

If one now considers the postulation of two types of peptide binding sites such that:

$$[P]_0 = [P_f]_0 + [P_s]_0 \qquad \text{(Eq. 8)}$$

then the appropriate rate constants are k_s and k_f, as given in Scheme III, and the logarithmic extrapolation method of Mark and Rechnitz (37) may be used to determine the initial number of fast and slow sites and the rate constants k_s and k_f.

The initial curved portion of Fig. 7 can now be interpreted as a time period when both fast and slow sites are involved in peptide binding of the calcium–histamine complex, and the linear portion of the curve represents a time period when all fast sites are occupied and a single type of site, namely the slow sites, is being

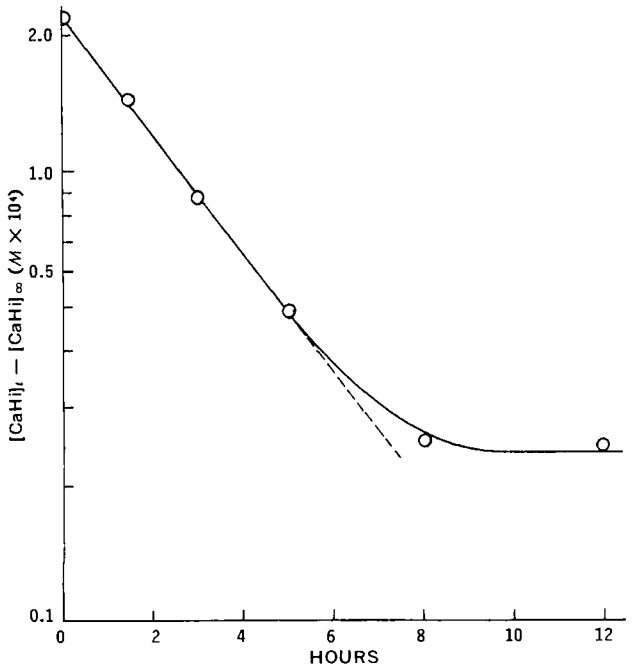


Figure 6—*Semilogarithmic plot of the data in Fig. 5 showing the apparent first-order behavior over approximately 2 half-lives.*

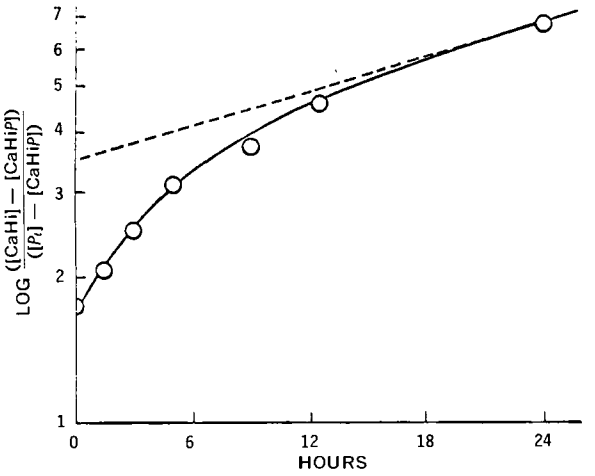


Figure 7—*Graphical representation of Eq. 8 representing the extrapolation method of kinetic analysis. The extrapolation of the linear portion of the curve* (-----) *to* t = *0 gives the numerical value of* K.

filled. For the linear portion of the curve:

$$k = k_s \text{ and } [P]_0 = [P_f]_0 + [P_s]_0 \qquad \text{(Eq. 9)}$$

$$[\text{CaHi}P] = [\text{CaHi}P_f] + [\text{CaHi}P_s] = [P_f]_0 + [\text{CaHi}P_s] \qquad \text{(Eq. 10)}$$

Equation 7 describing the linear portion of Fig. 7 can then be written:

$$k_s t = \frac{2.303}{[\text{CaHi}]_0 - [P]_0} \log \frac{[\text{CaHi}]_0 - [P_f]_0 - [\text{CaHi}P_s]}{[P]_0 - [P_f]_0 - [\text{CaHi}P_s]} - K \qquad \text{(Eq. 11)}$$

This expression enables one to calculate an intercept K for the extrapolation of the linear portion of Fig. 7 to the ordinate. At t = 0, [CaHiP_s] = 0 and Eq. 11 becomes:

$$K = 2.303 \log \frac{[\text{CaHi}]_0 - [P_f]_0}{[P]_0 - [P_f]_0} \qquad \text{(Eq. 12)}$$

From Fig. 7, an extrapolated intercept of log 3.5 is obtained; from Eq. 12, $[P_f]_0$ was calculated to be 1.80×10^{-4} M. This value is almost exactly twice the molar concentration of peptide (1.72×10^{-4} M) and is excellent evidence that there are two sites with the faster binding rate, leaving one site with the slower rate.

The rate constant for the slow reaction was determined from the slope of Fig. 7 beyond 12 hr where the two faster sites are essentially saturated, leaving only the slower site P_s. The slower constant, k_s, was calculated to be 1.19×10^2 M^{-1} hr^{-1}. To obtain the fast rate constant, k_f, the value of k_s was subtracted from the apparent constant calculated from the first linear portion of Fig. 7; k_f was calculated to be 5.08×10^2 M^{-1} hr^{-1}. To verify the accuracy of the calculated rate constants and also that there are two fast sites and one slow site, the rate of histamine-Ca^{+2} binding to peptide H was calculated by substitution of k_f and k_s into the rate equation:

$$\text{rate of binding} = \frac{d[\text{CaHi}P]}{dt} = k_f[\text{CaHi}][2P] + k_s[\text{CaHi}][P] \qquad \text{(Eq. 13)}$$

and this value for the rate of binding (5.1×10^{-5} M hr^{-1}) compared with the rate calculated by two other methods.

First the rate of binding was calculated from dynamic dialysis data, assuming that the difference between the initial rate of appearance of histamine in the external solution when no binding occurred and when binding did occur was equal to the rate at which histamine was being irreversibly bound to peptide H. This was found to be 5.9×10^{-5} M hr^{-1}. In the second method, a tangent was drawn to the initial slope of Fig. 5 and an initial rate, 6.0×10^{-5} M hr^{-1}, was calculated. These two values are in very good agreement with the rate calculated from k_f and k_s.

Recently, Gecse *et al.* (38) reported the isolation of a peptide from human serum having histaminopexic properties. They also used the same resin to isolate the peptide which must then, as they indicate, have a molecular weight between 1000 and 5000. The peptide was isolated from 30 healthy human subjects. Of significance was the fact that serum from eight allergic patients lacked the ability to bind histamine. Their isolation is in accord with the present findings.

No attempt was made in this study to determine if other plasma fractions bound histamine, and certainly other histaminopexic fractions may be isolated in further investigations. The characteristic dependence of histamine binding on the presence of Ca^{+2} is good evidence, however, that peptide H is at least in part responsible for histaminopexy. It must be realized that this conclusion is based entirely on *in vitro* evidence. Extensive additional studies will be required with allergic and nonallergic patients to evaluate the extension of this conclusion to human subjects.

While the binding of histamine to peptide H is most likely an equilibrium phenomenon, the initial assumption that the binding can be treated as irreversible appears reasonable. The excellent agreement between rates calculated using the second-order equation and those observed experimentally indicates that the rate of the back-reaction does not appreciably affect the assumption of irreversibility over the time period studied. It is important to note that the histamine binding to peptide H differs from most drug–protein interactions in that it is not a rapidly reversible process. This is evidenced by the time scale in Fig. 5 in which the concentration of unbound histamine [CaHi] is changing through 24 hr. As such, it was necessary to utilize a kinetic analysis of the data rather than the equilibrium analysis more common to drug–protein binding studies.

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