J. Williamson, and B. S. Dzindzio, *Clin. Pharmacol. Ther.*, **19**, 30 (1976).
(22) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1974, pp. 670, 671.
(23) *Ibid.*, p. 435.
(24) G. Cramer and B. Isaksson, *Scand. J. Clin. Lab. Invest.*, **15**, 553 (1963).
(25) P. Sampson, in "BMD, Biomedical Computer Programs," W. J. Dixon, Ed., University of California Press, Berkeley, Calif., 1974, pp. 693–704.
(26) D. U. Huntsberger and P. E. Leaverston, "Statistical Inference in the Biomedical Sciences," Allyn and Bacon, Boston, Mass., 1970, p. 213.
(27) J. G. Wagner, "Fundamentals of Clinical Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton, Ill., 1975, p. 351.
(28) W. J. Westlake, *J. Pharm. Sci.*, **62**, 1585 (1973).
(29) N. M. Downie and R. W. Heath, "Basic Statistical Methods," 2nd ed., Harper and Row, New York, N.Y., 1965, pp. 237–239.
(30) G. A. Portmann, in "Biopharmaceutics," J. Swarbrick, Ed., Lea & Febiger, Philadelphia, Pa., 1970, pp. 4–6.
(31) J. G. Wagner, "Fundamentals of Clinical Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton, Ill., 1975, p. 81.
(32) NLIN, Share Library No. 360 D-13.6.007, Research Triangle Park, NC 27709.
(33) G. A. Portman, in "Biopharmaceutics," J. Swarbrick, Ed., Lea & Febiger, Philadelphia, Pa., 1976, p. 17.
(34) J. G. Wagner, "Fundamentals of Clinical Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton Ill., 1975, pp. 81, 82.
(35) J. G. Wagner, "Biopharmaceutics and Relevant Pharmacokinetics," 1st ed., Drug Intelligence Publications, Hamilton, Ill., 1971, p. 252.
(36) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1974, p. 648.
(37) R. E. Gerhardt, R. F. Knouss, P. T. Thyum, R. J. Luchi, and J. J. Morris, *Ann. Intern. Med.*, **5**, 927 (1969).
(38) A. F. Lyon and A. C. DeGraff, *Am. Heart J.*, **69**, 834 (1965).
(39) K. M. Kessler, D. T. Lowenthal, H. Warner, T. Gibson, W. Briggs, and Reidenberg, *N. Engl. J. Med.*, **290**, 706 (1974).

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Mechanism of Histamine Binding II: Effect of Alkali Metal and Alkaline Earth Cations on Histamine Binding to Peptide H

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Abstract □ The association constants of histamine with Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} were determined in buffer solutions at constant pH using an ion selective electrode. These cations enabled histamine to bind to peptide H. A minimum cation binding concentration was required for histamine binding. A linear relationship existed between the minimum cation binding concentration and the log of the equilibrium constant for the histamine–cation complexes, indicating that the specificity of the alkaline earth cations in promoting histamine binding was due to the difference in their ability to complex with histamine. The monovalent cations, Na^+, K^+, and Cs^+, inhibited histamine binding to peptide H, with the extent of inhibition dependent on cation concentration. An ion-exchange mechanism or a conformation change in the peptide may account for the inhibition.

Keyphrases □ Histamine—binding to peptide H, effect of alkali metal and alkaline earth cations, association constants determined □ Peptide H—binding to histamine, effect of alkali metal and alkaline earth cations, association constants determined □ Alkali metal and alkaline earth cations—effect on binding of histamine to peptide H, association constants determined □ Binding—histamine to peptide H, effect of alkali metal and alkaline earth cations, association constants determined □ Association constants—determined for binding of histamine to peptide H, effect of alkali metal and alkaline earth cations □ Metals—alkali metal and alkaline earth cations, effect on binding of histamine to peptide H

Previous reports indicated that Ca^{2+} is involved in the binding of histamine to serum protein (1, 2). Cations other than Ca^{2+} also appear to affect histaminopexy. Serum Mg^{2+} levels in asthmatics are low during attacks but normal while patients are free of symptoms (3); magnesium sulfate given intravenously to severe asthmatics gives relief for 18–20 hr (3). Injections of magnesium chloride restore histaminopexy in adrenalectomized and ovariectomized rats for up to 3 months, an effect similar to that of Ca^{2+} but longer lasting (4). In contrast to the divalent alkaline earth cations, the monovalent cation K^+ appears to inhibit histaminopexy (2, 5–7).

Previously (8), histamine was found to bind to a plasma peptide, termed peptide H, through the formation of a Ca^{2+}–histamine complex. Apparently, only one extensive investigation of histamine–alkaline earth complexes has been reported. Chawla (9) measured association and other thermodynamic constants for Be^{2+}, Ca^{2+}, and Mg^{2+} complexes with histamine, antistine, and similar molecules at several temperatures. Several studies determined association constants for histamine complexes with various other metals (10–12), and the method of pH titration generally has been used. For accurate results, this technique requires extensive experimental precautions such as those used by Chawla (9).

This report describes the determination of histamine–cation constants at constant pH, using a cation selective electrode. The purpose of this investigation was to deter-

Table I—Values for the Stoichiometry and Association Constants for Alkaline Earth Cation–Histamine Complexes at 37° and pH 7.4

Cation	n	log K	K, M^{-1}
Mg^{2+}	1.00	2.50	3.2×10^2
Ca^{2+}	1.02	3.07	11.8×10^2
Sr^{2+}	0.88	2.96	9.2×10^2
Ba^{2+}	0.80	2.87	7.4×10^2

mine the effect of the alkaline earth and alkali metal cations on the binding of histamine to peptide H.

EXPERIMENTAL

Materials—Histamine dihydrochloride[1] and tromethamine hydrochloride, ultrapure[1], were used. All other chemicals were reagent grade. The isolation of peptide H and the preparation of the tromethamine buffer were described previously (8).

Minimum Detectable Binding Concentration—To determine the minimum concentration of the alkaline earth cation necessary for detectable histamine–peptide binding, a series of dynamic dialysis runs was carried out with increasing concentrations of the alkaline earth cation added to internal and external solutions. The apparatus and experimental procedure for dynamic dialysis were described previously (8). Plots of log total histamine remaining in the internal cell *versus* time were made. When no binding occurred, the slopes of these linear plots were the same as the slope with no peptide present. When binding occurred, the slope decreased, indicating less free histamine present.

Association Constants for Histamine and Alkaline Earth Cations—Calcium-ion and calomel electrodes were used with a pH meter[2]. Solutions were maintained at 37 ± 0.1° in a jacketed beaker and agitated with a magnetic stirrer. A titrant, alkaline earth chloride solution in isotonic tromethamine buffer (pH 7.4), was added in 0.1-ml increments to either 50 ml of 1.81×10^{-3} M histamine in the same tromethamine buffer or 50 ml of a buffer blank containing no histamine. Each 0.1-ml increment of titrant produced a 0.0005 M increment of cation.

Plots of cation concentration *versus* millivolt reading, both in the presence and absence of histamine, were made. From these plots, the concentration of free cation and, by subtraction, the concentration of bound cation were determined.

Millivolt Titrations with Histamine, Peptide, and Calcium: Effect of Alkali Metal Cation—Alkali metal chloride was added to 25 ml of peptide H, 7.8 mg/ml, in 0.9% NaCl. Sodium, potassium, and cesium chlorides were used. Since 0.9% NaCl was used in the buffer system throughout all investigations, 0.154 M, corresponding to 0.9% NaCl, was the lowest concentration of potassium and cesium chlorides used. The peptide H solution was also made 0.770 and 1.1540 M in sodium chloride; 0.308, 0.616, and 1.232 M in potassium chloride; and 0.308 and 0.770 M in cesium chloride. To these solutions, histamine dihydrochloride solution was added in 0.1-ml increments such that each addition produced a 0.001 M increment in histamine.

A pH meter[3] was used with combination electrode to determine the millivolt readings. Identical runs were conducted with calcium chloride added to the peptide H–alkali metal chloride solutions.

RESULTS AND DISCUSSION

Cation–Histamine Association Constants—Total valence electron calculations show that histamine, in both its neutral and monoprotonated forms, exists in a configuration theoretically favorable to metal and intramolecular hydrogen binding (13). The equilibria involved are shown in Schemes I and II:

$$n_1M^{2+} + Hi^+ \overset{K}{\rightleftharpoons} M_{n_1}Hi^+$$

Scheme I

$$n_2M^{2+} + Hi \overset{K'}{\rightleftharpoons} M_{n_2}Hi$$

Scheme II

[1] Schwarz/Mann, Orangeburg, NY 10962.
[2] Orion model 801, Orion Research, Cambridge, MA 02139.
[3] Sargent model NX, Sargent-Welch Co., Skokie, IL 60076.

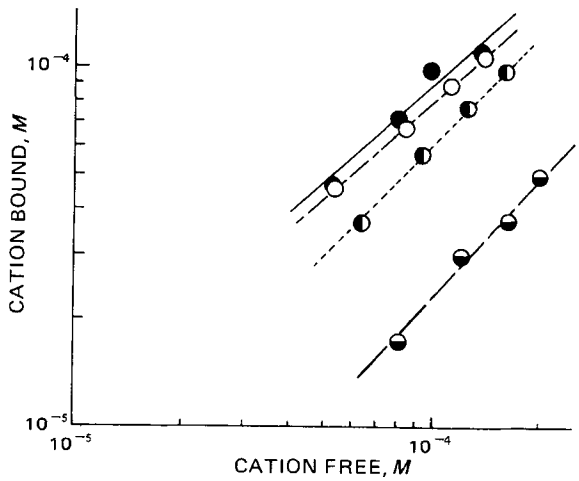

Figure 1—*Log–log plot of the bound alkaline earth cation* versus *the free alkaline earth cation for various histamine–alkaline earth cation complexes. The slope is equal to the number of cations bound per histamine molecule. Key:* ◒, *Mg;* ◐, *Ba;* ○, *Sr; and* ●, *Ca.*

where Hi and Hi^+ are the unionized and monoprotonated forms of histamine, M^{2+} is the alkaline earth divalent cation concentration, n is the number of moles of cation bound per mole of histamine, and $M_{n_1}Hi^+$ and $M_{n_2}Hi$ are the concentrations of the complexes formed.

If $n_1 = n_2 = n$, then:

$$K'/K = [M_nHi]/[M_nHi^+]\{[Hi^+]/[Hi]\} \qquad \text{(Eq. 1)}$$

Since the side-chain pKa of histamine is about 9.4, the ratio Hi^+/Hi is between 30 and 100 at a physiologic pH of 7.4. Therefore, the species M_nHi makes only a minor contribution to the total bound alkaline earth cation at pH 7.4 unless the affinity constant, K', for the unionized histamine–metal complex is an order of magnitude higher than that for ionized histamine, K. Assuming Scheme I represents the primary association, one can calculate the cation–histamine$^+$ association constant, K, for Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} from determinations of free and bound alkaline earth cation concentrations at pH's where Hi^+ predominates. The equilibrium expression for Scheme I is:

$$K = \frac{[M_nHi^+]}{[M^{2+}]^n[Hi^+]} \qquad \text{(Eq. 2)}$$

which may be written as:

$$\log K = \log [M_nHi^+] - \log [Hi^+] - n \log [M^{2+}] \qquad \text{(Eq. 3)}$$

or:

$$\log [M^{2+}_{\text{bound}}] = \log K + \log [Hi^+] + n \log [M^{2+}_{\text{free}}] \qquad \text{(Eq. 4)}$$

where $\log [M^{2+}_{\text{bound}}] = \log [M_nHi^+]$. A plot of log cation bound *versus* log cation free was linear (Fig. 1), and the slope, n, was approximately one for all four cations, indicating a 1:1 complex (Table I). The association constants may then be calculated using Eq. 2, since the concentrations of bound and free cation, and thus of complex and free histamine, are known (Table I). The complexes of histamine with Ca^{2+}, Sr^{2+}, Ba^{2+}, and Mg^{2+} were found to be 1:1 with association constants for Ca^{2+}, Sr^{2+}, and Ba^{2+} with histamine being fairly close at about 10^3 M^{-1} and for the histamine–Mg^{2+} complex being considerably lower.

The association constants determined for the histamine–alkaline earth complexes by the ion selective electrode at constant pH are in good agreement with Chawla's (9) results (for Mg^{2+}, a log K of 2.26; for Ca^{2+}, a log K of 2.91) determined at 25° using the pH titration method. The

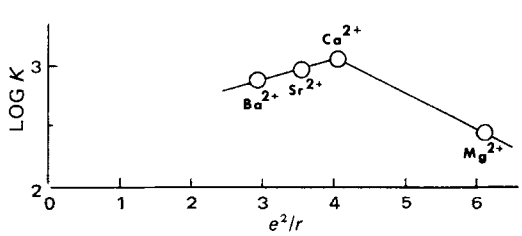

Figure 2—*Log* K versus e^2/r *for histamine–alkaline earth cation complexes.*

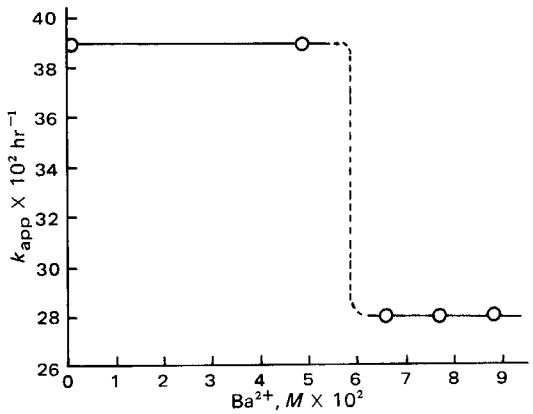

Figure 3—*Relationship between apparent first-order dialysis rate constant, k_{app}, and concentration of Ba^{2+} indicating the minimum detectable binding concentration of Ba^{2+}.*

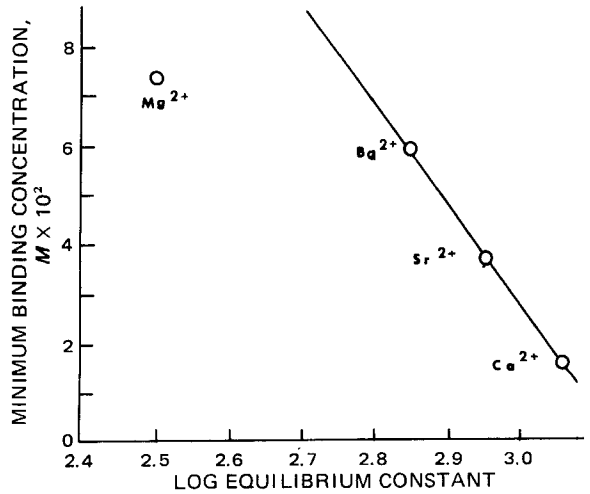

Figure 4—*Relationship between the minimum detectable binding concentration and log of the histamine–alkaline earth cation association constants.*

pH titration method, however, approaches its limit of application for association constants under 10^3, limiting the accuracy of Chawla's values for Ca^{2+}–histamine and, particularly, for Mg^{2+}–histamine (9). While it is difficult to draw conclusions based on only these two systems, the larger deviation of Chawla's log K for Mg^{2+}–histamine from the log K determined by the ion selective electrode was most likely due to the pH titration limitation, with the ion selective electrode measurement of log K being the more accurate.

A plot of the data according to the Born equation, as described by Martell and Calvin (14), provides some insight into the influence of the charge and radius of the alkaline earth ion on the binding constant:

$$E = \frac{e^2}{2r}\left(1 - \frac{1}{D}\right) \qquad \text{(Eq. 5)}$$

where E = energy of solvation for gaseous ions, r = ionic radius, e = charge, and D = dielectric constant of solvent. If the energy of solvation is assumed to be directly related to log K, then a linear relationship should exist between log K and e^2/r. When the log of the association constants for histamine–alkaline earth cations are plotted *versus* e^2/r, it is seen (Fig. 2) that while Ba^{2+}, Sr^{2+}, and Ca^{2+} follow the expected order, Mg^{2+} is low. It is not unusual, however, for Ca^{2+} complexes to have higher log K's than Mg^{2+}, particularly with ligands forming a highly chelated structure.

One might also expect a relationship between the proton affinity (pK) of the chelating agent and the free energy of the binding process as represented by log K. Martell and Calvin (14) showed a plot of log K *versus* pK for the alkaline earth complexes of a series of structurally similar weak acid chelating agents. A linear relationship was observed for each alkaline earth cation studied. For chelating agents with pK's above 9, the affinity constants were in the order $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$. At pK's below 7.5, the order seemed to be altered; this same order, $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$, was observed for histamine affinities. The present system seems to exhibit the expected metal specificity for a species with a conjugate acid dissociation constant below 7.5, *i.e.*, the monoprotonated form of histamine, the conjugate acid of which has a pK of 5.8, and for a species that predominates at pH 7.4. The binding affinities of the alkaline earth cations for vitamin D-induced calcium binding protein also follow the order $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ (15). This effect may be due to ionizable groups of pK less than 7.5 on the binding protein.

Effect of Alkaline Earth Divalent Cations—When Sr^{2+}, Ba^{2+}, or Mg^{2+} was substituted for Ca^{2+} in the dynamic dialysis runs, it also enabled the peptide to bind histamine. The difference was only in the concentration of cation necessary to promote binding.

When the apparent first-order rate constants were determined from the slopes of plots of log total histamine remaining in the dialysis cell *versus* time and plotted against the concentration of cation at which they were obtained, the apparent first-order rate constants were the same whether the cation was present or not until a minimum cation concentration was reached. At this point, the rate constants decreased, indicating that binding, perhaps mediated by a conformational change, had occurred (Fig. 3). The minimum concentrations of the alkaline earth cation required for binding are shown in Table II. As the divalent cation concentration was increased beyond the minimum for binding, a rate of dialysis independent of cation concentration was reached (Fig. 3). Since the concentration of peptide H was much smaller than the histamine or divalent cation concentrations and was constant, the peptide H concentration probably became a limiting factor, resulting in the apparent constancy of the binding.

When association constants for histamine–alkaline earth cations were logarithmically plotted *versus* their respective minimum detectable binding concentrations (Fig. 4), a linear relationship was observed between the threshold concentration and the free energy of the binding process. Thus, the specificity of the alkaline earth cations in promoting binding of histamine to peptide H is due to the difference in their ability to complex with histamine as indicated by their association constants.

Botre *et al.* (16) studied the binding of histamine to bovine serum albumin at various ionic strengths and reported that, for a given ionic strength, the protein required a threshold concentration of histamine for binding and that this threshold varied with the nature of the inorganic cations present in the system. The explanation given is that the albumin undergoes a "conformational perturbation that is induced by histamine and dependent on ionic strength." Apparently, a similar phenomenon occurs for peptide H, where the conformational change is induced by the alkaline earth cation–histamine complex.

Effect of Monovalent Cations on Histamine–Ca^{2+}–Peptide H Binding—Peptide H in distilled water, with varying concentrations of alkali metal chloride added, was titrated with histamine dihydrochloride, and the millivolt readings were recorded using a pH electrode. A plot of the millivolt readings *versus* the log of the concentration of histamine added was linear (Fig. 5). Since histamine does not bind to peptide H in the absence of Ca^{2+} (8) (or other divalent cations), the pH electrode was responding to free histamine hydrochloride. The addition of calcium chloride resulted, as expected, in a decrease in free histamine due to binding to peptide H, as evidenced by a decreased slope of the millivolt titration curve. This Ca^{2+} effect was most noticeable at the lower concentrations of the alkali metal chlorides, 0.154 M, with the effect of the alkali metal chloride increasing as its concentration increased.

Table II—Minimum Detectable Binding Concentrations for the Alkaline Earth Cations

Cation	Minimum Binding Concentrations, M
Mg^{2+}	7.4×10^{-2}
Ca^{2+}	1.6×10^{-2}
Sr^{2+}	3.7×10^{-2}
Ba^{2+}	5.9×10^{-2}

Table III—Minimum Alkali Metal Chloride Concentrations for Complete Inhibition of Histamine Binding

Cation	Inhibitory Concentration, M
Cs^+	0.52
Na^+	0.86
K^+	1.43

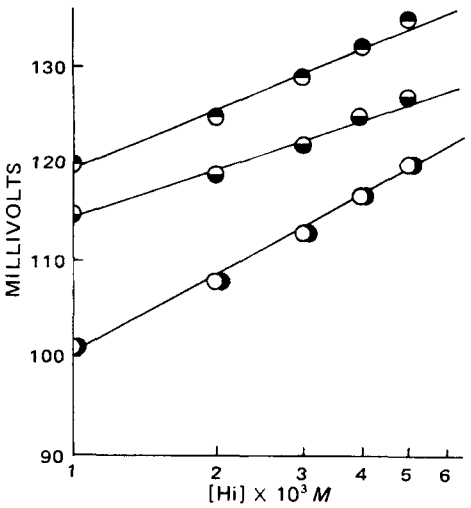

Figure 5—*Relative millivolt readings* versus *concentration of histamine for determining the effect of Na^+ on peptide–histamine–Ca^{2+} binding. Key: ◓, peptide, low Na^+; ◒, peptide, Ca^{2+}, low Na; ○, peptide, high Na^+; and ●, peptide, Ca^{2+}, high Na^+. The concentrations were: peptide, 1.7 × 10^{-4} M; Ca^{2+}, 1.8 × 10^{-2} M; low Na^+, 0.154 M; and high Na^+, 0.770 M.*

As seen in Fig. 5, the curves for both the peptide H and peptide H–calcium chloride solutions containing the higher alkali metal concentration approach one another. Apparently, an alkali metal chloride concentration can be reached that completely inhibits histamine binding to peptide H. That this is indeed the case is shown in Fig. 6, where the millivolt difference between the peptide H and peptide H–calcium chloride solutions was plotted *versus* the concentration of alkali metal chloride present in the solution. An extrapolation to the abscissa gives the concentration of alkali metal chloride necessary to inhibit histamine binding completely. This finding is verified by the complete inhibition of binding by Na^+ and Cs^+ at concentrations over the predicted inhibitory concentration.

The alkali metal cations may inhibit the histamine–peptide H binding

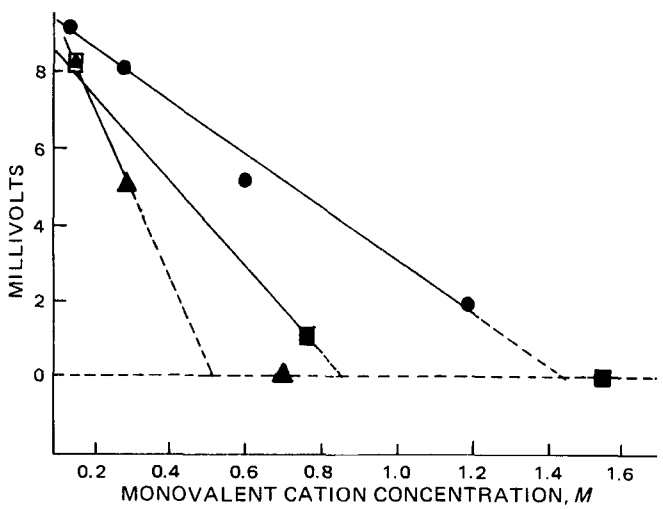

Figure 6—*Relationship between the change in millivolt reading produced by added Ca^{2+} and the concentration of the monovalent cation present in solution. The intercept at 0 mv is predictive of the minimum inhibitory concentration of the monovalent cation. Key: ▲, Cs^+; ■, Na^+; and ●, K^+.*

by affecting either the Ca^{2+}–histamine complex formation or the binding of the complex to peptide H. To determine if Na^+ affected the complexation between histamine and Ca^{2+}, histamine was titrated with Ca^{2+} solution using a Ca^{2+} ion selective electrode in the same manner as was used to determine the histamine–Ca^{2+} association constant, except that more sodium chloride was added to increase the Na^+ concentration from 0.154 to 0.770 *M*. There was no difference in the concentration of bound Ca^{2+} in the presence of increased Na^+. However, the increase of Na^+ to 0.770 *M* did interfere with the Ca^{2+}-mediated binding of histamine to peptide H (Fig. 5); therefore, the Na^+ effect must occur in the interaction between the histamine–Ca^{2+} complex and peptide H.

Two possible mechanisms for the alkali metal cation inhibition of histamine binding may be considered. One possibility is an ion exchange between the alkali metal cation and the bound calcium–histamine complex, similar to that reported by Aborg and Uvnas (17) for Na^+ inhibition of histamine binding to a heparin–clupeine complex. The inhibition by Na^+ was due to a competition with histamine for carboxyl groups on the clupeine. Peptide H provides nine weak acid groups per peptide molecule, and a similar competitive displacement from these anionic sites is a possible explanation of the present observations. Alternatively, conformational changes in peptide H favorable to binding may be induced by the Ca^{2+}–histamine complex and, above a critical alkali metal salt concentration, these conformational changes cannot be effected. It was reported that alkali metal cations prevent a conformational change in bovine serum albumin that is essential for albumin–histamine binding (16). The order of specificity of this inhibitory effect was $K^+ > Rb^+ > Na^+ > Li^+ = Cs^+$, which is the reverse of the order observed for the inhibition of Ca^{2+}–histamine binding to peptide H (Table III).

REFERENCES

(1) J. L. Parrot and M. Mordelet-Dambrine, *J. Physiol.*, **53,** 439 (1961).

(2) J. L. Parrot, M. Mordelet-Dambrine, and G. Trinquet, *Bull. Acad. Nat. Med. (Paris)*, **146,** 335 (1962).

(3) V. G. Haury, *J. Lab. Clin. Med.*, **26,** 340 (1940).

(4) M. Mordelet-Dambrine and J. L. Parrot, *J. Physiol.*, **58,** 303 (1966).

(5) J. L. Parrot, M. Laborde, and M. Mordelet-Dambrine, *C. R. Soc. Biol.*, **149,** 2094 (1955).

(6) J. L. Parrot and M. Mordelet-Dambrine, *ibid.*, **153,** 750 (1959).

(7) J. L. Parrot and M. Mordelet-Dambrine, *J. Physiol.*, **53,** 439 (1961).

(8) J. R. Hazlett and D. O. Kildsig, *J. Pharm. Sci.*, **63,** 701 (1974).

(9) I. D. Chawla, Ph.D. thesis, Kansas State University, Manhattan, Kans., 1962.

(10) M. Karapetyants and E. Slenskaya, *Zh. Fiz. Khim.*, **38,** 1312 (1964).

(11) P. Kiralo and R. V. Schalien, *Suom. Kemistil.*, **35B,** 7 (1962).

(12) J. Zarembowitch, *J. Chim. Phys.*, **63,** 438 (1966).

(13) J. P. Green, S. Kang, and S. Margolis, *Mem. Soc. Endocrinol.*, **19,** 727 (1971).

(14) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, New York, N.Y., 1952, p. 191.

(15) R. J. Ingersoll and R. H. Wasserman, *J. Biol. Chem.*, **246,** 2808 (1971).

(16) C. Botre, M. Marchetti, and S. Borghi, *Biochim. Biophys. Acta*, **154,** 360 (1968).

(17) C. H. Aborg and B. Uvnas, *Acta Physiol. Scand.*, **74,** 552 (1968).

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