

Ionic Control of the Size of the Vesicle Matrix of Beige Mouse Mast Cells

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ABSTRACT Isolated matrices of the giant secretory vesicles of mast cells of the beige mouse were reliably produced by the osmotic lysis of isolated vesicles. These matrices maintained their form, and their sizes were easily measured using Nomarski optics. The size of the matrix depended on the ionic composition of the bathing solution. The physiologically relevant ions, histamine and serotonin, contracted the matrix. Multivalent cations condensed the matrix relative to univalents. Ag^+ , acid pH (below 5), and basic pH (above 9) expanded the matrix. In the presence of 10 mM histamine, lowering the pH from 9 to 5 contracted the matrix more than can be attributed to the pH-dependent matrix contraction in zero histamine. The nontitratable organic cation, dimethonium, contracts the matrix with little effect of pH in the range of 5–9. These results suggest that histamine acts as a matrix contractor in the divalent form. The dose–response (contraction) relation for histamine was gradual from micromolar to 316 mM (millimolar) histamine. Experiments with mixtures of histamine and sodium show antagonistic effects on the matrix but are inconsistent with either a model where ions compete for identical sites or a parallel model where ions interact with separate independent sites. In vigorous histamine washoff experiments, the half time for vesicle expansion in 10^{-4} M pH buffer was ~ 4 s; in isotonic NaCl solution, it was 0.5 s. When 1 M histamine was presented to closely apposed matrices, fusion resulted. The matrix material returned to its initial shape after being mechanically deformed with a glass probe. These results suggest that the matrix size is controlled by its ion exchange properties. The matrix expansion can quantitatively account for the vesicular size increase observed upon exocytosis (as a postfusional event) and the osmotic nonideality of intact vesicles. The mechanical expansion is probably significant in the widening of the exocytotic pore and the dispersal of the vesicular contents.

INTRODUCTION

In addition to transmitter agents, hormones, and various enzymes, secretory vesicles are invested with a matrix of highly charged glycosaminoglycans (acetylcholine vesicles [Stadler and Dowe, 1982], pituitary vesicles [Zanini, Giannattasio, Nussdorfer, Margolis, Margolis, and Meldolesi, 1980], sea urchin egg cortical granules

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[Schuel, Kelly, Berger, and Wilson, 1974], pancreas zymogen granules [Reggio and Palade, 1978], mucous cells of the trachea [Verdugo, 1984], snail mucous cells [Watanabe, 1976], and mast cell granules [Metcalf and Kaliner, 1981]). After exocytosis, these giant polymeric molecules disperse and, in the case of the sea urchin egg, raise an impressive fertilization membrane (Schuel, 1984). Whereas the vesicle matrix dispersion is rapid for most preparations, in mast cells the secreted matrix remains intact for a period of hours (Fawcett, 1955).

The chemical properties of vesicular matrices have been examined for several preparations. Mucous is thought to be in a condensed state before exocytosis. After secretion the mucous hydrates in an ion-dependent manner (Tam and Verdugo, 1981). Calcium ions, for example, induce a congealed form of mucous characteristic of cystic fibrosis (Verdugo, Aitken, Langley, and Villalon, 1987*a*). Indeed, Ca^{2+} is thought to be responsible for the condensation of mucins within the vesicle; Ca^{2+} is released upon secretion (Verdugo, Deyrup-Olsen, Aitken, Villalon, and Johnson, 1987*b*). A similar model has been proposed (Whitaker and Zimmerberg, 1987) to account for the dispersal of granule contents after the fusion of secretory vesicles from sea urchin eggs. The dispersal was inhibited by a polymer at 950 mosM in the absence of ions, but required significantly lower osmolalities in the presence of calcium, magnesium, or sodium ions. Thus the granule contents in sea urchin eggs act like a matrix of interwoven fibrils that form interstices small enough to exclude high molecular weight substances. In a remarkable series of experiments, Uvnas and co-workers studied the ion exchange properties of masses of isolated mast cell granules. They demonstrated that Na^+ or K^+ can exchange for biogenic amines (Uvnas, Aborg, Lyssarides, and Thyberg, 1985*b*) in a manner consistent with the Rothmund-Kornfeld ion exchange equation. Their titration data implicated a carboxyl group as the amine binding site (Uvnas, Aborg, and Bergendorff, 1970). Similarly, superfused chromaffin granules release catecholamines and ATP in exchange for NaCl or KCl solutions in accordance with the ion exchange equation (Uvnas and Aborg, 1988). Moreover, uptake studies indicate that catecholamines and ATP are separately bound to the amphoteric matrix, presumably to carboxyl and amino groups, respectively (Uvnas and Aborg, 1988). These properties have been extended to the adrenergic granules of bovine splenic nerves and rat vas deferens and to the dopaminergic granules of the rat striatum (Uvnas and Aborg, 1984*a*; Uvnas, Aborg, and Gojny, 1985*a*).

In the course of studying the osmotic behavior of the giant vesicles (4 μm diam) of beige mouse mast cells, we noticed that secreted granules shrank when perfused with multivalent cations. We hypothesized that ions control the size of the matrix; our results suggest that the vesicle expansion observed upon secretion is a postfusional event (Breckenridge and Almers, 1987; Zimmerberg, Curran, Cohen, and Brodwick, 1987). In this paper we will show that multivalent ions caused matrix contraction while monovalents caused expansion. The contraction induced by histamine decreased as the pH increased. In the range of 5–9, pH had little effect on the (nontitratable) dimethonium divalent cation-induced contraction. Experiments with mixtures of sodium and histamine reveal that the two ions are antagonistic. Indeed, Na^+ accelerated the expansion in histamine washout experiments. Separate granule matrices fused when presented with histamine. Isolated matrices returned to their

initial shape after mechanical compression. These results suggest that the elastomeric matrix expansion is a consequence of its ion exchange properties.

Some of the results presented here have been reported previously (Curran and Brodwick, 1985; Curran, 1986; Verdugo and Fernandez, 1990; Monck, Oberhauser, Alvarez de Toledo, and Fernandez, 1991).

METHODS

Cell Preparation

Beige mice, C57BL/6Ncr-bg (NIH facility and Jackson Laboratories, Bar Harbor, ME) of either sex were killed quickly and painlessly by cervical dislocation. The peritoneum was injected with a modified Krebs solution containing 140 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1–2 mM CaCl₂, 5.6 mM glucose, 10 mM HEPES, and titrated with NaOH to 7.1 pH. The animal was moved about to insure good mixing of the peritoneal contents. The peritoneum was cut open and the injected solution was aspirated with a pasteur pipette and pipetted onto coverslips. To insure attachment of the cells, especially during solution changes, the coverslips were pretreated with 50 µg/ml of 100 kD polylysine. After several minutes of exposure to the polylysine the coverslips were washed in distilled water to remove excess polylysine. Although soluble polylysine is a known secretagogue of mast cells (Padawer, 1970), we find that mast cells will remain intact when contacting polylysine adsorbed to glass. Before experimentation, cells were incubated in a modified Eagle's medium (without bicarbonate) that contained 10 mM HEPES, pH 7.4, and 1 mg/ml streptomycin and penicillin (ICN Biomedicals, Costa Mesa, CA). We found that modified Eagle's medium preserved intact mast cells for >24 h. In contrast, mast cells, especially from the beige mouse, that were incubated for several hours in a modified Krebs solution exhibited a high percentage of background release. All experiments were performed at room temperature, ~21°C.

Microscopy

Measurements of cell and vesicle sizes were made under Nomarski differential contrast microscopy. Nomarski optics give an image with a shadow-cast appearance having boundaries that are sharp, distinct, and free of distortions from halos or out-of-focus objects that plague phase contrast microscopy; thus, the edges of the granules are well defined. Since Nomarski optics give optical cross-sectional images with a minimal depth of field, maximum diameters, measured by visual inspection of the various cross-sections as one adjusts the focus through the image, were recorded. The plane of focus was adjusted before each image was recorded. This procedure insured maximum accuracy especially in determining sizes after swelling or shrinking. Furthermore, because the vesicles often present in nonspherical shapes, only the largest orthogonal axes were considered and averaged to obtain one measurement per vesicle, which we denote as the size (diameter). The size of the vesicles throughout the paper are expressed in terms of percentage expansions and contractions of the vesicle diameter. The vesicles of beige mouse mast cells were large enough (average diameter, ~4 µm) to measure the sizes with a Reichert inverted microscope or a Zeiss IM inverted microscope with a 1.4 n.a. objective and a 0.63 n.a. long working distance condenser. The images of the beige mast cells were captured by video camera (Dage-MTI Inc., Michigan City, IN) and stored on tape by a videocassette recorder (Sony BVU-820 U-Matic or Panasonic AG 6010) for later analysis. The two point limit of resolution of our system, as determined by the ability to separate two overlapping diffraction patterns, was approximately equal to 0.18 µm (Inoue, 1986). The accuracy of the video images as determined by the ratio of the standard deviation of the measured diameters to the mean was <2.5%.

When appropriate, the "images" of beige mast cells were then downloaded to Bernoulli discs (Iomega Co., South Roy, UT) and enhanced with Image Pro II software (Media Cybernetics, Silver Spring, MD). "Enhancement" involves increasing the contrast with digital processing on a pixel by pixel basis, which makes the cell or vesicle more distinct from its environment without changing the dimensions. Positives were processed from 4 × 5 negatives, which were made using a M6000 color graphic recorder (Matrix Instruments, Orangeburg, NY).

To promote access to the granule matrices the following procedures were adopted. Cells were first mechanically lysed with a small wooden probe. The vesicles that remained attached to the ruptured cells waved about when the bathing solution was lightly agitated. Vesicles were then lysed by replacing the bathing solution with distilled water containing only 10⁻⁴ M (molar) MES buffer, pH 5.9. Alternatively, cell and vesicle membranes were ruptured with distilled water containing only 10⁻⁴ M MES buffer, pH 5.9. In those experiments in which the effects of pH were tested on the vesicle matrix the following pH buffers at 0.1 mM were used: citric acid (Sigma Chemical Co., St. Louis, MO) for pH 4 and 5, MES (Sigma Chemical Co.) for pH 6, PIPES (Sigma Chemical Co.) for pH 7, HEPES (Sigma Chemical Co.) for pH 8, and Na₂B₄O₇ for pH 9.

Rapid Perfusion System

To measure the rate of matrix expansion during histamine removal, isolated matrices were prepared and solution changes were introduced by a rapid perfusion system. Lysed vesicles were isolated by mechanical agitation so that the granule matrix separated completely from the cell and attached securely to the polylysine-coated coverslip. Optical fields containing several such granules were selected and a pair of walls (made out of a mixture of Vaseline and silicon grease) were erected with a separation of several millimeters running the length of the coverslip. An inlet tube and an outlet tube under vacuum were placed at either end of the 2.5-cm coverslip. Solutions were pumped at rates as high as 500 cm³/min with a System I fish aquarium pump (Dun-Mar Products, Brighton, MI) through this tiny slip. Many vesicles washed away in the turbulence, but remarkably, many remained attached.

RESULTS

Vesicles of mast cells from the beige mice swell upon exocytosis in isotonic Krebs solution. The average vesicle diameter increase due to exocytotic swelling was 39% ± 11 SD (*n* = 12). Exocytosis can be monitored with the dye ruthenium red (Lagunoff, 1972). This membrane impermeant polycationic dye is concentrated in the polyanionic matrix of only those vesicles that have fused with the surface membrane. We noted that ruthenium red (0.064 mM) caused stained vesicles to contract. Because the postexocytotic matrix is only partially covered by membrane (in some cases the matrix granule is completely externalized) and the ruthenium red interacts directly with the matrix, the contraction must result from chemical interactions of the dye with the matrix rather than by an osmotic mechanism requiring a membrane.

Ion Effects on Isolated Granule Matrices

After vesicle lysis the granule matrix remained intact and had a well-defined, measurable size as seen in Fig. 1. Moreover, if the vesicle had a form before lysis (for example, kidney shaped), the demembrated granule matrix generally maintained this shape with a scale change as illustrated in Fig. 1, *A* and *B*.

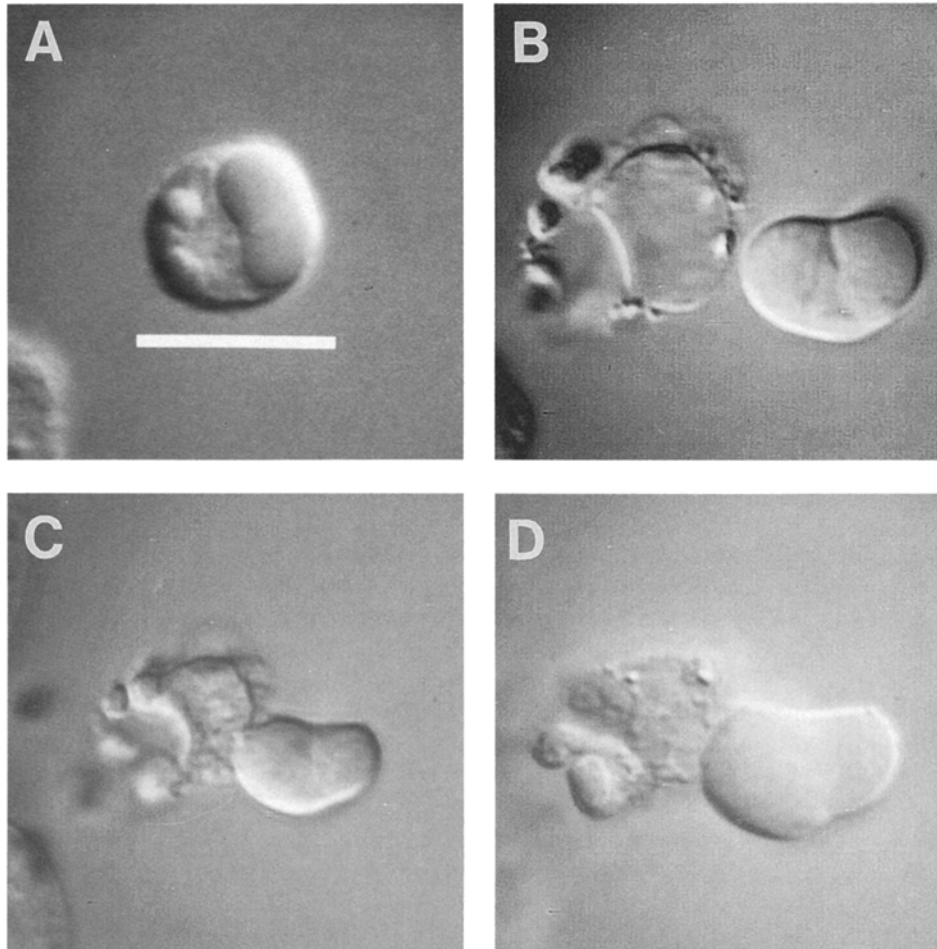


FIGURE 1. The size (diameter) of the lysed vesicle of the beige mouse mast cell is sensitive to the ionic milieu. (A) Intact mast cell in modified Eagle's medium. Note the irregular shape of the vesicle on the right side of the cell. (B) The cell and vesicle have been lysed in a hypotonic solution containing only 10^{-4} M MES buffer, pH 5.9. The position of the lysed vesicle has rotated 90° clockwise. The cell remains are to the left of the lysed vesicle or matrix. Note the increase in size (diameter) compared with A, and the conservation of shape. (C) Approximately 5 min after a solution change to 10^{-2} M histamine (chloride), pH 5.9, the size (diameter) of the matrix has decreased and the shape has been conserved. (D) Approximately 5 min after a solution change to 10^{-1} M NaCl with 10^{-4} M MES buffer, pH 5.9, the size (diameter) of the matrix has increased relative to C and B. The shape again has been conserved. A slight modification is only apparent due to rotation of the matrix about its horizontal axis. Calibration bar = $10 \mu\text{m}$.

The granule matrix expansion upon exocytosis could be due to either the loss of some soluble component of the vesicle or the gain of some component from the extracellular solution. We therefore measured the effects of several substances on the size (diameter) of the granule matrix. Table I summarizes the percent change of matrix size (diameter) from a control condition in 10 mM HEPES buffer at pH 7.1. The following points can be deduced. First, the physiologically significant cations histamine (see Fig. 2) and serotonin caused contraction, whereas their carboxylated precursors, histidine and tryptophan, caused expansion. Second, the imidazole group of histamine as well as a combination of imidazole and histidine were unable to cause contraction. Third, lysine at 125 mM caused expansion, whereas polylysine (even at a comparable molar concentration of lysine monomer) caused contraction.

TABLE I
Lysed Granules Are Sensitive to the Ionic Milieu

Compound	Molar	% Change	SD	n
5-Hydroxytryptamine (serotonin)	0.05	-30	7	20
<i>l</i> -Histidine	0.15	+10	8	13
Tryptophan	0.05	+30	10	8
Imidazole	0.18	-1	4	13
Imidazole/ <i>l</i> -histidine	0.09/0.075	+4	4	5
Lysine	0.125	+6	4	15
Polylysine (100 K mol wt)	10 ⁻⁵	-21	6	2
Polylysine (12 K mol wt)	8.3 × 10 ⁻⁵	-22	7	11
Ruthenium red (on lysed vesicles)	6.4 × 10 ⁻⁵	-27	7	7
Ruthenium red (on exocytosed vesicles)	6.4 × 10 ⁻⁵	-33	7	6
MgCl ₂	0.113	-10	5	9
CaCl ₂	0.113	-15	5	5
Ethylenediamine	0.14	-25	3	9
Hexamethonium	0.124	-9	6	16
Decamethonium	0.124	-4	5	16
ATP	0.10	0	5	13
ZnCl ₂	10 ⁻⁴	-15		2
AgNO ₃	2 × 10 ⁻⁵	+35	6	5

Lysed granules were challenged for 10–15 min with a variety of compounds. The change in granule size (diameter) was calculated relative to control values in 10⁻² M HEPES buffer, pH 7.1.

Fourth, the dye ruthenium red stained and contracted lysed or secreted vesicles. Fifth, the divalent ions Mg, Ca, Zn, dionium, ethylenediamine, hexamethonium, and decamethonium all caused contraction. Sixth, the trivalent cation lanthanum caused substantial contraction. Compared with distilled water controls, 10⁻⁴ M lanthanum caused a 15% contraction ±6 SD, *n* = 8. (Because of the difference in control solutions, La³⁺ was not included in the table.) Seventh, substituting the anion ATP (100 mM) or glutamate for chloride was without significant effect. Lastly, silver nitrate (Ag has a high affinity for sulfonic acid groups [see Smith and Martell, 1976]) caused an impressive expansion even at millimolar concentrations. In summary, these data suggest that multivalent cations contract the granule matrix, whereas

monovalent cations tended to cause expansion. Though less systematically investigated, anions appeared to be much less important in controlling the matrix size.

The Histamine Dose-Response Relation

Because histamine is the most important physiological constituent of mast cell granules, we measured the matrix size (diameter) for lysed vesicles over a concentration range from micromolar to 1 M. The experiment was performed at pH 5.9 with 10^{-4} M MES buffer. The buffer concentration was kept low to reduce possible effects of the sodium counterion. The acidic pH was selected to mimic the presumed normal intravesicular pH of ~ 6 (Johnson, Carty, Fingerhood, and Scarpa, 1980). The

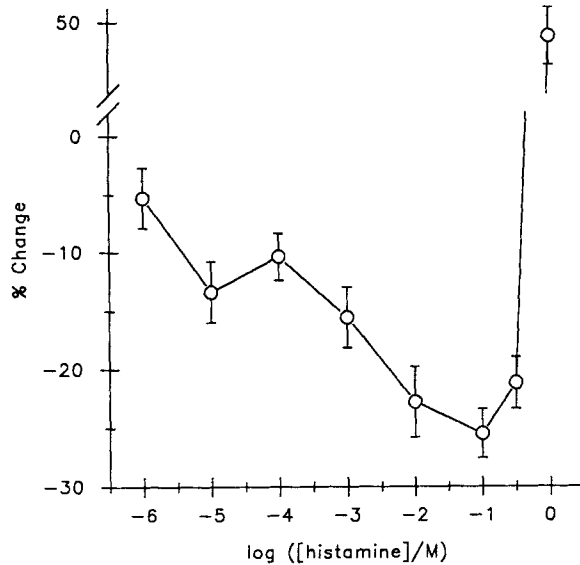


FIGURE 2. Histamine causes lysed granules to shrink. Granules of beige mouse mast cells were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then challenged with histamine at various concentrations with 0.1 mM MES buffer, pH 5.9, for 10 min and the percent change in size (diameter) was calculated. (The 1 M histamine data point was the exception to the 10-min incubation protocol. Because of the complex behavior of gels in 1 M histamine, the incubation time was only 11–20 s, which corresponded to the maximum expansion). Each lysed granule was challenged with only one concentration of histamine. The means and SEMs were calculated from 5–11 observations.

contraction was compared with the size (diameter) in low salt buffer alone. All measurements were made at 10 min after perfusion to ensure equilibration. The effect of histamine was reversible, which suggests that the amount of material in the matrix remains constant. It is unlikely that histamine acts osmotically, as a one molal sucrose solution (at pH 5.9 buffered with 10^{-4} M MES buffer) had no appreciable effect on the gel size; i.e., it shrank the gel by only 2% (± 4 SD, $n = 14$). Fig. 2 illustrates the histamine dose-response relation (also compare Fig. 1, B and C). Between micromolar and 316 mM concentrations histamine condensed the matrix in a gradual fashion. A concentration of 10^{-2} M histamine corresponds in size to the intact vesicle size in an intact cell. At 1 M histamine, the matrix expanded $\sim 50\%$

compared with controls. We often observed time-dependent behavior in 1 M histamine: the granule contracted, then expanded, and then contracted again. Unlike the lower concentrations, the 1 M effects were often not reversible. These complex results may have arisen in part from nonuniform mixing, equilibration processes, and the eventual dissolution of the matrix. Granule expansion was also observed in 0.35 M CaCl_2 , which caused a 19% increase in size (diameter) compared with controls containing 10 mM HEPES, pH 7.1 (± 4 SEM, $n = 5$).

Sodium Dose-Response Relation

Because sodium is the major physiological extracellular monovalent cation and some monovalent cations caused expansion of the matrix (Table I), we measured the matrix size (diameter) over a range of sodium concentrations relative to control

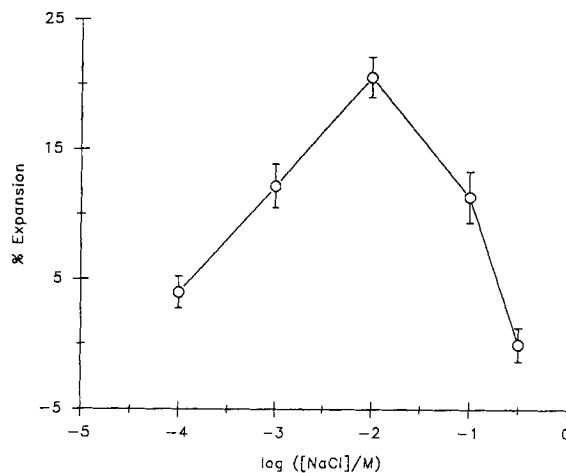


FIGURE 3. The effect of sodium on lysed granules. Granules of beige mouse mast cells were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then challenged for 10 min with sodium at various concentrations with 0.1 mM MES buffer, pH 5.9. The change in granule size (diameter) was calculated relative to controls in 0.1 mM MES, pH 5.9. The means and SEMs were calculated from 9–17 observations.

values in 0.1 mM MES buffer, pH 5.9 (Fig. 3). The matrix expanded as NaCl was increased from 0.1 to 10 mM NaCl. At higher concentrations the trend was reversed. Matrices bathed in 1 M NaCl often expanded irreversibly.

The Effect of Mixtures of Histamine and Na^+ on Matrix Size

Because histamine and sodium exert opposite effects on matrix size, the effect of mixtures of these two ions on matrix diameter was examined. The qualitative difference between histamine and sodium on matrix size is best exemplified in Fig. 1, C and D. In C, 10^{-2} M histamine caused a substantial contraction of the matrix relative to the effect of 10^{-1} M sodium shown in D. We considered two forms of interaction. In the parallel model, the two species were imagined to act upon separate, independent sites; the effects of given concentrations of the two antagonists should then be simply additive. Alternatively, the two species could compete for a common set of sites (though not necessarily with the same stoichiometry) and exert opposite effects; in this case the addition of an antagonist should be “more” than

additive. The dose-response curves for histamine and sodium are unusual. Both curves have no clear saturation point and the Na curve reverses itself at high concentrations of ligand. We therefore selected a restricted range of concentrations between 5×10^{-4} and 10^{-1} M for both ligands. Fig. 4 illustrates a family of dose-response curves for histamine at different sodium concentrations (filled triangles, 5×10^{-4} M; open triangles, 5×10^{-3} M; open circles, 5×10^{-2} M; filled circles, 5×10^{-1} M; the clumping of the three unconnected points at 0.1 M histamine are at 10^{-3} , 10^{-2} , and 10^{-1} M sodium). The complex behavior of these curves is consistent with previous data. The histamine dose-response relation at 5×10^{-4} M sodium is similar to the dose-response relation in Fig. 2. The top three points in the vertical sequence of points at 5×10^{-4} M histamine, the lowest concentration investigated in

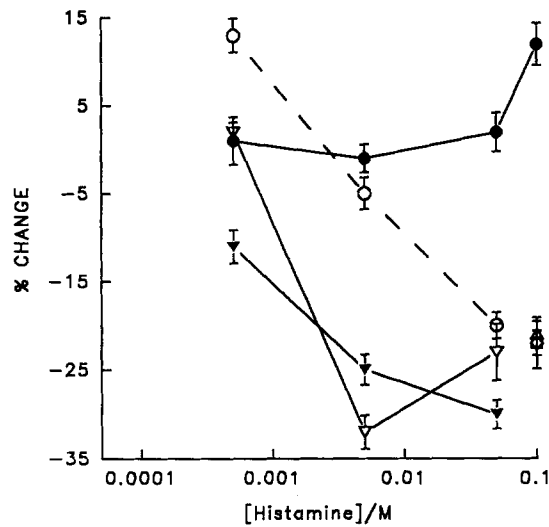


FIGURE 4. Sodium decreases the efficacy of histamine-induced contraction. Granules were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then challenged with mixtures of histamine and sodium to generate a family of dose-response curves for histamine at different concentrations of sodium. The filled triangles are at 5×10^{-4} M sodium, the open triangles at 5×10^{-3} M sodium, the open circles at 5×10^{-2} M sodium, and the filled circles at 5×10^{-1} M sodium, and the

clumping of three unconnected points at 0.1 M histamine have sodium concentrations of 10^{-3} , 10^{-2} , and 10^{-1} M sodium. At 10 min incubation the percent change in size (diameter) was calculated relative to the size (diameter) in 0.1 mM MES buffer alone at pH 5.9. Each lysed granule was challenged with only one experimental solution. The means and SEMs were calculated from 6–13 observations.

the mixture experiment, conform to the sodium dose-response curve of Fig. 3. The individual curves are inconsistent with either of the two simple models. For example, while 5×10^{-3} M NaCl shows an expansion of $\sim 10\%$ relative to 5×10^{-4} M NaCl at 5×10^{-4} M histamine, this expansion reverses or is attenuated at higher histamine concentrations consistent with a competitive model. In contrast, the curve at 5×10^{-2} M NaCl is reasonably parallel to the 5×10^{-4} M NaCl curve, consistent with the independent site model. Worse still, the curve at 5×10^{-1} M NaCl seems utterly independent of histamine except at the highest histamine concentrations. However, except for the unphysiological 5×10^{-1} M NaCl curve, there is no doubt that the effects of histamine and sodium chloride are antagonistic.

The Effects of pH with and without Histamine

The valence of histamine is dependent upon the titration of its two ionizable groups: the amino group, which has a pK of 9.7, and the imidazole group, which has a pK of 5.8 (see Fasman, 1975). In view of the differential effects of mono- and divalent cations on matrix size, we wished to determine which form of histamine was responsible for the matrix contraction. Solutions were therefore prepared at several pH values at 10^{-4} M buffer (see Methods). Experiments with lysed vesicles at various values of pH in the presence (open symbols) and absence (closed symbols) of 10 mM histamine are illustrated in Fig. 5A. A positive percent change is equivalent to expansion, a negative percent change to contraction. In the absence of histamine, from pH 5 to pH 9 there was a gradual expansion to a maximum of ~20%. Below pH 5 and above pH 9 the matrix expanded indefinitely as though it were dissolving (data not shown). In the presence of 10 mM histamine the matrix expanded for pH greater than 7 and contracted for pH less than 7. In Fig. 5B the difference curve between the titration in solutions containing histamine and the control titration in buffer alone is indicated by the solid line. The different charged species resulting from the titration of histamine is illustrated in three curves representing the fraction of divalent (dashed line), monovalent (dash-dotted line), or neutral species (dotted line) of histamine. The histamine titration curve for the different charged species is consistent with the suggestion that the divalent form of histamine caused contraction and the monovalent form caused expansion. For example, note that at pH 6 and below the divalent form of histamine became significant, as did the contraction of the matrix. On the other hand, note that at pH 8, where the monovalent form of histamine was >90% and the divalent form was negligible, matrix expansion resulted. At pH 9 the monovalent form decreases and the uncharged form increases, resulting in a decrease in the monovalent expansion (i.e., a net contraction compared with pH 8). Moreover, note that during a physiological secretion event the matrix pH would change from pH 6 to 7.4, yielding an 8% net expansion itself. In another series of experiments using a 10 mM Na MES buffer system (used above) we examined the matrix dimensions at various values of pH. The matrix remained constant between pH 5 and 10 and then expanded at lower pH values. These data are consistent with the observation that monovalents tended to expand the matrix. The pK of isolated rat mast cell vesicles in the vicinity of pH 4 suggests a role for carboxyl groups (Uvnas et al., 1970).

The Effects of Dimethonium on the Matrix Size

The results of the experiments reported above suggest that pH alone cannot account for the substantial contraction of the matrix as pH is varied and histamine is held constant. Rather, the experiments suggest that the valency of histamine is crucial in determining matrix size. However, the above experiments do not test whether pH has an indirect role by increasing the sensitivity of the matrix to divalent-induced contraction. To test this hypothesis we tested the effects of the nontitratable organic cation, dimethonium. The solid curve of Fig. 6 shows a gradual matrix contraction as the concentration of dimethonium is increased from 1 μ M to 0.1 M. As with the histamine dose-response experiments, the dimethonium dose-response experiments

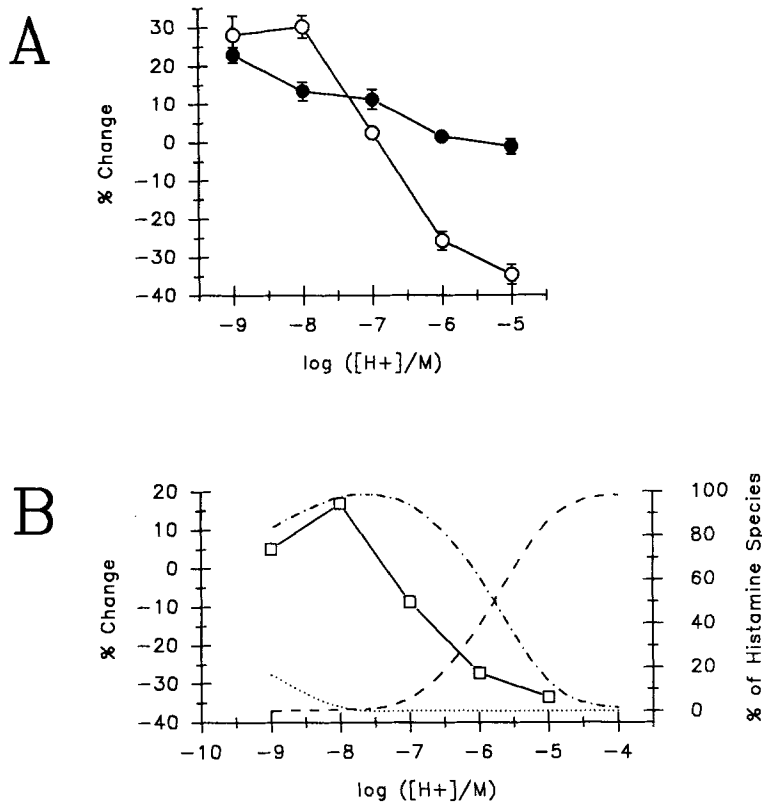


FIGURE 5. The effect of histamine on lysed granules was dependent on pH. (A) Granules of beige mouse mast cells were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then challenged for 10 min with solutions of various pH values (buffered with 0.1 mM of appropriate buffer) with (*open circles*) and without (*closed circles*) 10^{-2} M histamine. The percent change in granule diameter (ordinate) was calculated relative to control values in 0 M histamine and 0.1 M MES, pH 5.9. Each lysed granule was challenged with only one experimental solution. The means and SEMs were calculated from 5–11 observations. (B) The data of A are presented as a difference curve (*solid line*), which was calculated by subtracting the data obtained in the absence of histamine (*closed circles* in A) from the data in the presence of histamine (*open circles* in A). Based on the two pK's of histamine, 9.7 and 5.8, the relative percent of the divalent (*dashed line*), monovalent (*dash-dotted line*), and neutral (*dotted line*) species of histamine as a function of pH are calculated and presented for comparison (*right ordinate*).

were performed at pH 5.9 with 10^{-4} M MES buffer. All measurements were made at 10 min after perfusion and the contraction was compared with the size (diameter) in low salt buffer alone. We chose 10^{-2} M dimethonium as the most matrix-sensitive point in the curve, varied the pH with 10^{-4} M buffer (see Methods), and tested the effects of these solutions on the matrix size. As the dashed curve in Fig. 6 shows, there is little change in matrix size (diameter) from pH 9 to pH 5 at a constant concentration of dimethonium. These results suggest that pH does not change the sensitivity of the matrix to divalent ions.

Histamine Wash-off from Isolated Matrices

Using sequential single frame analysis, we followed the rate of expansion of isolated matrices upon the washout of histamine using the rapid perfusion system (see Methods), as shown in Fig. 6 (also compare Fig. 1 C with 1 D). Because zero time could not be determined absolutely, the onset was noted by the turbulent flow patterns which we defined as a nominally zero time. When the matrices were incubated in a solution of 100 mM histamine, pH 5.9, and washed in distilled water containing 10^{-4} M MES buffer, pH 5.9, the half-time for matrix expansion (indicating loss of histamine) was $3.8 \text{ s} \pm 2.6 \text{ SD}$ ($n = 5$). When isotonic NaCl was used with

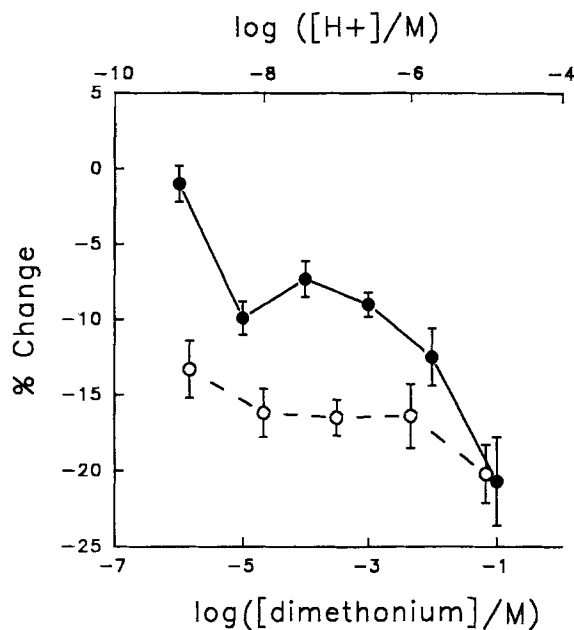


FIGURE 6. Dimethonium causes matrix contraction in a pH-independent way. (*Upper solid curve*) Granules were lysed by exposure to a solution containing only 0.1 M MES buffer, pH 5.9. The lysed granules were then challenged with dimethonium at various concentrations (*lower abscissa*) with 0.1 mM MES buffer, pH 5.9, for 10 min and the percent change in size (diameter) was calculated. The means and SEMs were calculated from 9–11 observations. (*Lower dashed curve*) Granules were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then challenged for 10 min with solutions with various pH values

with 10^{-2} M dimethonium (*upper abscissa*). The percent change in granule size (diameter) was calculated relative to control values in 0 M dimethonium. The means and SEMs were calculated from 6–18 observations. For both experiments each lysed granule was challenged with only one experimental solution.

10^{-4} M MES buffer, pH 5.9, the matrices expanded to half-maximal size (diameter) in $0.5 \text{ s} \pm 0.2 \text{ SD}$ ($n = 5$). Merely for descriptive purposes, we found that a sum of two exponentials of the form $C - Ae^{-t/\tau_1} - Be^{-t/\tau_2}$ (where t is time, τ_1 and τ_2 are the time constants, and A , B , and C are constants) adequately fit the data as illustrated in Fig. 7. The best-fit time constant estimations were made by allowing the computer to redefine zero time; the calculated zero time for the isotonic sodium washout was 135 ms and the sodium-free washout was zero relative to the nominal zero time. The difference in the zero time for the two data sets is presumably attributable to complexities of the “true” underlying kinetic processes of the histamine washoff, or to errors in the judgment of the turbulent flow patterns.) For the isotonic NaCl

solution we find that $\tau_1 = 0.12$, $A = 27$, $\tau_2 = 2.6$, $B = 3.8$, and $C = 31$. The errors for both the time constant and amplitudes for the first exponential are $\sim 10\%$; for the second, 100–300%. The large errors for the second exponential weaken the rationale for using two exponentials, even though two exponentials fit the data better than one. However, it is clear that the majority of the expansion happened by the faster process. The corresponding data for the NaCl-free solution (with 0.1 mM MES buffer) were $\tau_1 = 0.56$, $A = 14$, $\tau_2 = 18$, $B = 13$, and $C = 28$. Here the use of two exponentials is validated by the low errors (5–10%) for the time constants and amplitudes for both exponentials. These data suggest that the sodium chloride solution did not merely expand the matrix in parallel to the histamine washoff, but rather that sodium accelerated the dissociation of histamine from the matrix.

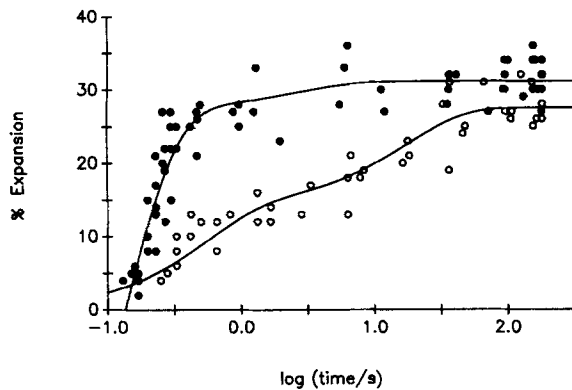


FIGURE 7. Sodium accelerates swelling of lysed granules. Five granules of beige mouse mast cells were lysed by exposure to a solution containing only 0.1 mM MES buffer, pH 5.9. The lysed granules were then incubated for at least 10 min with 10^{-1} M histamine. The change in granule size (diameter) was monitored during vigorous continuous washings with either a 10^{-1} M sodium solution and 0.1 mM MES, pH 5.9 (closed

circles) or with only 0.1 mM MES, pH 5.9 (open circles), and expressed as percent expansion as a function of the "log time" in seconds. The data were fitted to the sum of exponentials of the form $C - Ae^{-t/\tau_1} - Be^{-t/\tau_2}$, where t is time, τ_1 and τ_2 are the time constants, and A , B , and C are constants.

Fusion of Isolated Matrices in the Presence of Histamine

When matrices were in close apposition ($\sim 1\text{--}2 \mu\text{M}$) and 1 M histamine was added, the matrices expanded and fused together ($n = 7$) (Fig. 8). Upon close scrutiny, tiny filaments were seen connecting the two matrices. Though clearly visible under light microscopy, these filaments did not reproduce well in photographs of the video-recorded image. The pair of fused matrices did not remain in their initial figure eight or merged ellipsoidal structures, but rather formed spheres. The minimization of the surface to volume ratio for a sphere would be expected of a system maximizing the number of "crossbridge" bonds within the matrix; i.e., the matrix likes itself better than water. Note, however, that the failure of many intact vesicles or isolated granules to present as spheres implies that other structural forces are also operating.

The matrix fusion appears to require an initial expansion step. The fusion in Fig. 8 was generated in 1 M histamine, which induced sufficient expansion for adjacent matrices to touch. To test whether mere apposition or low concentrations of histamine could induce the matrix fusion, we pushed two matrices together with a glass microprobe. In the absence of histamine or at a concentration of histamine that

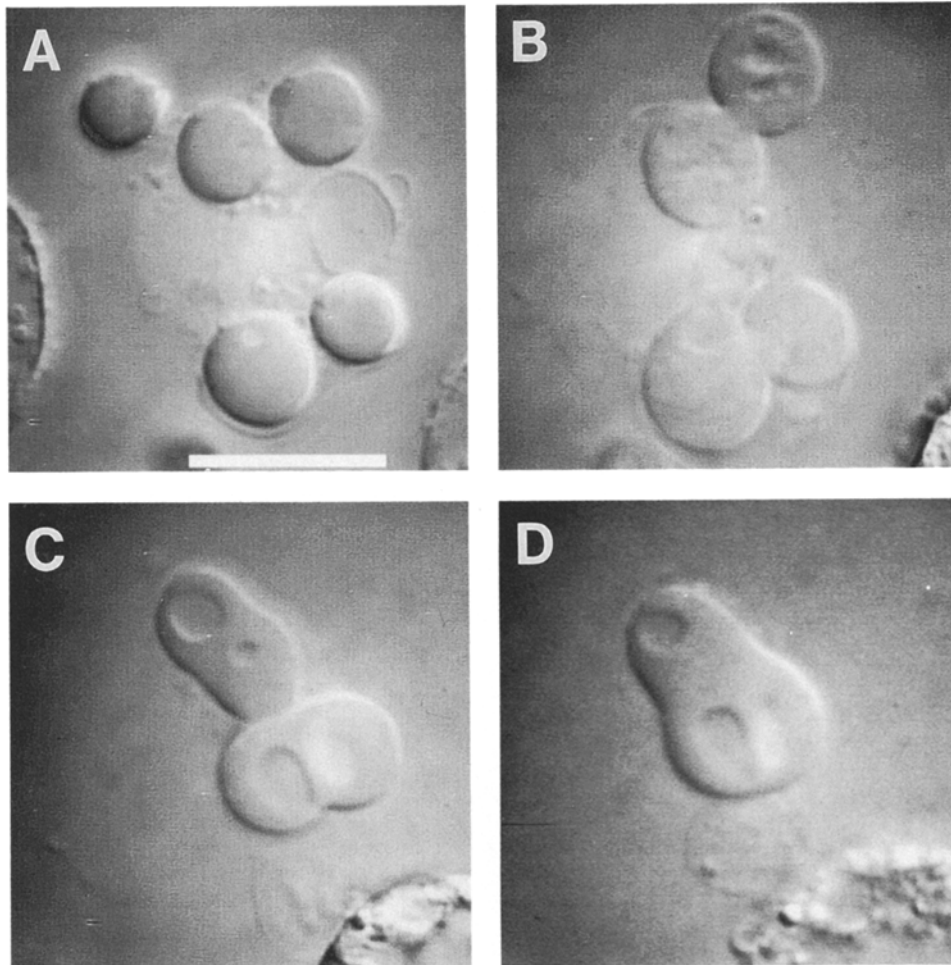


FIGURE 8. 1 M histamine causes lysed vesicles to fuse. (A) Matrices of lysed vesicles in hypotonic solution containing only 10^{-4} M MES buffer, pH 5.9. (B) The same matrices ~ 1 min after challenge with 1.0 M histamine (chloride), pH 5.9. Note that two closely apposed matrices on the top had started to meld together as had the two on the bottom. The two melding or fusing pairs were separated by cellular debris. A fifth matrix seen in the upper left-hand corner in A had been washed away by the solution change. (C) Approximately 5 min after challenge with histamine the two fused pairs were starting to fuse together. Note the internal structural detail of the matrices. (D) Approximately 8 min after challenge with histamine the four fused matrices approached a more spherical configuration. Calibration bar = 10 μm .

induced matrix contraction (10^{-1} M histamine at pH 5.9), no fusion was observed within a half hour. These results suggest that matrix polymer strands must unravel before they can reassemble and intermesh.

Matrices Are Springlike

To examine the mechanical properties of isolated matrices, we performed three elementary physical manipulations. First, lysed vesicle matrices were sucked into

pipettes of much smaller size ($\sim 1 \mu\text{m}$) and then discharged by applying pressure. The granule elongated in the pipette and returned to its original structured shape when expelled ($n = 2$). A second manipulation entailed the poking of the matrix with a blunt tip glass probe ($\sim 2 \mu\text{m}$ tip diameter). The probe was advanced onto the side of the granule with a micromanipulator and the granule was dimpled to about half its size ($\sim 2 \mu\text{m}$). During the mechanical compression the diameter increased, indicating a certain amount of volume redistribution. Because our z axis is uncalibrated we could not assess the magnitude of this redistribution. The probe was then immediately retracted and the matrix was observed to return to its original size with two distinct velocity components ($n = 4$); the first was instantaneous with the removal of the pipette and accounted for the majority of the relaxation; the second lasted for ~ 1 s for a displacement of $< 0.5 \mu\text{m}$. Third, when the granule matrix was ripped apart by vigorous manipulation with a pipette, the fractured end was sharp and irregular, in contrast to the smooth periphery. These observations demonstrate that the matrices behaved like bulk, elastic springs rather than plastic, deformable masses. We have no measure of the volume of the granule or the magnitude of the applied pressures required for a proper quantitative description. Consequently, we cannot discriminate mechanical volume contraction from mechanical volume deformation at constant volume. Nevertheless, these observations can account, at least in part, for the self-expulsion of some granule matrices during exocytosis.

DISCUSSION

Matrices Are Sensitive to Ions

The central result of this paper is that the size of the matrix of lysed granules is dependent on the composition of the perfusion solution. Vesicles of beige mouse mast cells expand $\sim 40\%$ during exocytosis in isotonic Krebs solution. This expansion was probably postfusional since capacitance jumps (indicating the increase in membrane surface area associated with vesicle fusion) preceded the increase in vesicle size (Breckenridge and Almers, 1987; Zimmerberg et al., 1987). This postfusional matrix expansion could be due to either the loss of internal vesicular constituents or the gain of extracellular ions. Our results and the requirement that the matrix remains macroscopically net neutral suggest both processes. Upon secretion the loss of histamine yielding a 40% expansion would imply that intact vesicles contain between 10 and 100 mM free histamine (see Fig. 2). Osmotic experiments performed on intact vesicles indicate that the great majority of histamine is in a bound, osmotically inactive form (Brodwick, M. S., M. J. Curran, and C. Edwards, manuscript in preparation). The amount of histamine bound would then be determined by the dissociation constant and the free histamine. Upon vesicle fusion with the surface membrane, the externalized granule matrix would lose histamine by mass action and expand. The expansion was also aided by pH changes. Mast cell vesicles maintain an acidic intravesicular pH of 6 (Johnson et al., 1980). After exocytosis into a medium of pH 7.4 the histamine-filled vesicle would expand 8–10% by a pH change alone (see Fig. 5). The gain of external Na^+ also induced expansion as illustrated in the Na^+ dose–response relation (Fig. 3), the effects of sodium and histamine on the matrix size (diameter) (Fig. 4), and the histamine washoff in Na^+ -containing solution (Fig. 7). While these observations can quantitatively account for the observed matrix

expansion, it is possible that other more complex changes were happening in parallel.

We find that di- and multivalent cations contract the matrix, while monovalents tend to expand the matrix. The ability of histamine to contract the matrix depends on the ambient pH. Thus, for example, when histamine is primarily in the divalent form at acid pH's, contraction results. When, on the other hand, the monovalent form prevails, net expansion occurs. Consistent with this suggestion is the observation that lanthanum at 0.1 mM caused a substantial contraction. Close inspection of Fig. 5 *B* reveals an apparent shift of the histamine titration curve with respect to the mono- and divalent forms of histamine. We suggest two explanations for this discrepancy. First, the pH in the matrix ought to be more acidic than the bulk aqueous solution bathing it. At low ionic strength the negative charges of the matrix would create a Donnan equilibrium concentrating not only divalent cations, but also protons. Second, the divalent contraction and monovalent expansion could have different potencies. Thus the relative contraction between pH 8 and 7 could result from the small increase in the divalent form while the monovalent form remains almost constant.

Matrix Binding Theory

The ion exchange properties of isolated rat mast cell matrices have been studied by Uvnas et al. (1985*b*) by measuring released histamine in a flow apparatus. Their binding curves are consistent with simple ion exchange kinetics (Uvnas, 1985); ours are not. We would like to relate our dose-response data on matrix size to those of an actual binding theory. Because of the complexity of the actual dose-response curve we do not feel confident that a quantitative model is justified. Indeed, a meaningful dose-response relation must include a relationship between site occupancy and the observed response. We would, however, like to propose a qualitative model that accounts for the various phenomena. The matrix is composed of soluble components such as histamine, serotonin, and inorganic ions, and a larger insoluble portion composed of various proteins and heparin (Metcalf and Kaliner, 1981). Heparin, a giant polyanionic glycosaminoglycan, is bound to a protein core (see Stevens, 1986). In the vesicle many of these feather duster molecules are interwoven, giving the appearance of a randomly wound ball (Chandler, 1986) which constitutes the matrix. Within the matrix cations either bind to or screen the anionic sites. Heparin has two to three negative charges per disaccharide repeating unit (Franssen, 1985). We propose that divalent cations interact with the two adjacent anionic sites to form a two-site, one-ligand stoichiometry, i.e., a crossbridge. Monovalent cations, on the other hand, are incapable of forming effective crossbridges because of their relatively low charge density. Entry of monovalent cations is accompanied by anions and water molecules. When the ligand concentration is increased, more ions enter the matrix and the accompanying water exerts an osmotic pressure. The elastic spring properties of the matrix then permit it to expand. However, as the valence of the cationic ligand is increased, the attractive force between the two anionic sites and its crossbridge ligand is increased. When the coulombic attractive forces exceed the expansive osmotic forces, a net contraction of the matrix results. At higher divalent concentrations the multiple occupancy of both sites by ligands results in a coulombic repulsion force that adds to the osmotic force and produces a net expansion.

Such a model is supported quantitatively by relationships between the histamine dose–response relations, the Na^+ dose–response relation, and the histamine titration experiment. For example, when histamine is largely in the divalent form, at pH 6–5 the contraction is large and corresponds to the contraction on the histamine dose–response relation, $\sim 25\%$. At about pH 8, where the monovalent species dominates, the expansion of the matrix is maximal, $\sim 30\%$; the expansion in the monovalent 10^{-2} M histamine corresponds reasonably to the 20% expansion observed in 10^{-2} M Na^+ . Experiments with mixtures of divalent histamine and Na^+ also show that the two species act antagonistically. Unfortunately these competition experiments do not allow us to discriminate between a model where mono- and divalent cations compete for identical sites, and a parallel site model where mono- and divalent ions interact with separate independent sites. However, the washoff experiments show that Na^+ enhances the rate of the removal of preloaded histamine, suggesting a competitive model (assuming that the removal of histamine requires the replacement by a substitute cation, either Na^+ or H^+ for electroneutrality). As the ligand concentration is increased, the binding shifts to a one-site, one-ligand stoichiometry (or “multiple” occupancy, which would now exhibit coulombic repulsion). At sufficiently high concentrations the net forces holding adjacent polymer strands together is dominated by repulsive and osmotic forces and the expanded matrix would appear to melt irreversibly.

The ligand-induced “matrix fusion” could account for the observation that matrices from mature lysed vesicles are intact entities. The vesicles of the mast cell of the rat and beige mouse originate from the fusion of smaller precursor granules (Hammel, Lagunoff, Bauza, and Chi, 1983; Hammel, Lagunoff, and Kruger, 1988; Alvarez de Toledo and Fernandez, 1990). After unit vesicle membrane fusion, the matrices would presumably fuse in the presence of histamine. The stability of such fusion may be the consequence of complex double-helix formation and the formation of higher order aggregates of double helices (see Figs. 14–31 of Alberts, Bray, Lewis, Raff, Roberts, and Watson, 1989).

Physiological Roles for Matrix Size Changes

The complete removal of diffusible vesicular contents or mediators from a small vesicular volume through an exocytotic pore would be much faster than for a beige mouse mast cell vesicle; the volume ratio is about one million. The expanding matrix would open the exocytotic pore, allowing a faster egress of secretory products. Moreover, in view of the ion exchange nature of the granule matrix described in this paper and by Uvnas and Aborg (1984*a, b*), matrix expansion is probably regenerative. A second indirect consequence results from the observation that histamine condenses the matrix. The mast cell is optimally engineered to release histamine. In a rat mast cell 60% of the cell volume is occupied by vesicles. During release the vesicle size (diameter) increased $\sim 40\%$, which represents a 174% increase in vesicular volume. Thus the matrix in the condensed state allows more vesicles to be stored in the limited cell volume.

The mechanical coupling described in this paper has interesting thermodynamic consequences. Just as ligand binding promoted matrix contraction, so should matrix expansion promote ligand unbinding in a thermodynamically reversible system. Thus an expanding matrix would exhibit a decreasing affinity for ligand. Moreover, the

ligand unbinding with its subsequent release of the matrix spring would not only dilate the exocytotic pore but would also provide the mechanical energy that would expulse some of the matrix granules beyond the cell boundary. These mechanical consequences undoubtedly aid in the dispersal of the vesicle contents, many of which are destined for distant targets.

Note added in proof: During the course of the preparation of this manuscript for resubmission, many of the results presented here have been reproduced in Fernandez, Villalon, and Verdugo (1991).

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