J. Membrane Biol. 126, 159–169 (1992)

The Journal of
Membrane Biology

Effects of Osmotic Stress on Mast Cell Vesicles of the Beige Mouse

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Summary. The large size of the vesicles of beige mouse peritoneal mast cells (4 μm in diameter) facilitated the direct observation of the individual osmotic behavior of vesicles. The vesicle diameter increased as much as 73% when intact cells were perfused with a 10 mM pH buffer solution; the swelling of the vesicle membranes exceeded that of the insoluble vesicle gel matrix, which resulted in the formation of a clear space between the optically dense gel matrix and the vesicle membrane. Hypertonic solutions shrank intact vesicles of lysed cells in a nonideal manner, suggesting a limit to the compressibility of the gel matrix. The nonideality at high osmotic strengths can be adequately explained as the consequence of an excluded volume and/or a three-dimensional gel-matrix spring. The observed osmotic activity of the vesicles implies that the great majority of the histamine known to be present is reversibly bound to the gel matrix. This binding allows vesicles to store a large quantity of transmitter without doing osmotic work. The large size of the vesicles also facilitated the measurement of the kinetics of release as a collection of individual fusion events. Capacitance measurements in beige mast cells revealed little difference in the kinetics of release in hypotonic, isotonic, and hypertonic solutions, thus eliminating certain classes of models based on the osmotic theory of exocytosis for mast cells.

Key Words vesicle · gel matrix · tonicity · bulk modulus · capacitance · fusion

Introduction

The secretory vesicles of the mast cell of the beige mouse are unusually large, measuring about 4 μm in diameter (Chi & Lagunoff, 1975). The vesicles are optically dense and the diameters are easily measured with Nomarski optics. These advantages offer an unusual opportunity to observe directly the osmotic behavior of secretory vesicles. Previous studies on small vesicles have relied on light-scattering techniques (Sudhof, 1982) whose results are less accurate and more indirect. The possibility that mast cell vesicles are either impermeable to water or contain a significant water-inaccessible space is suggested by the labeling experiments of Bennett Cockcroft and Gomperts (1981). The isolated matrices of mast cells of the beige mouse can expand or contract depending on the ionic milieu (Curran, 1986; Curran & Brodwick, 1991: Fernandez, Villalon, & Verdugo, 1991). Moreover, these matrices exhibit elastic properties when their shape is transiently deformed. We therefore wished to examine the osmotic behavior of isolated vesicles with the expectation of nonideality.

A second motive for studying the osmotic behavior of vesicles comes from the demonstration of an osmotic step in the fusion of liposomes with artificial membranes (Cohen, Zimmerberg & Finkelstein, 1980; Cohen, Akabas & Finkelstein, 1982) and the suggestion that this may be true in cells as well. For example, hypertonic solutions inhibited exocytosis in sea urchin eggs (Zimmerberg, Sardet & Epel, 1985), chromaffin cells (Hampton & Holz, 1983) and in intact rat mast cells (Curran, 1986). However, in chromaffin cells the inhibition by extracellular hypertonicity is secondary to the increase in intracellular ionic strength that occurs as water leaves the cell (Holz & Senter, 1985). Furthermore, the initiation of fusion in beige mast cells precedes vesicle swelling in hypertonic solutions (Zimmerberg et al., 1987). Despite these findings which argue strongly against a critical osmotic step in fusion, recent evidence suggest that the membrane of secretory vesicles from mast cells is under tension (Monck, Alvarez de Toledo & Fernandez, 1990). If vesicle membrane tension is relevant to fusion as suggested by Monck et al. (1990), it is of interest to examine the properties of vesicles under osmotic stress and the relation of osmotic stress (rather than ionic strength) to the kinetics of fusion.

In brief, our results indicate that the vesicles of mast cells of the beige mouse are indeed osmotically active. However, the relation between osmol-

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ality and volume departs markedly from ideality at high osmolalities consistent with the suggestion that the vesicle gel matrix behaves as a spring resisting osmotic compression. In addition, we show that osmolality *per se* has little effect on secretion kinetics. Some of these results have been presented previously (Curran & Brodwick, 1985; Curran, 1986).

Materials and Methods

Cell Preparation

Beige mice, C57BL/6NCr-bg, (NIH facility and Jackson Laboratories, Bar Harbor, ME) of either sex were sacrificed quickly and painlessly by cervical dislocation. The peritoneium was injected with a modified Krebs' solution containing (in mM): 140 NaCl, 5 KCl, 5 $MgCl_2$, 1–2 $CaCl_2$, 5.6 glucose, 10 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 cover slips. To insure attachment of the cells, especially during solution changes, the cover slips were pretreated with polylysine (50 μg/ml of 100 kDa) for several minutes. Although soluble polylysine is a known secretagogue of mast cells (Padawer, 1970), we found 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 more than 24 hr. In contrast, mast cells, especially from the beige mouse, that were incubated for several hours in a modified Krebs' solution exhibited a high percentage background release. All experiments were performed at room temperature, approximately 22°C.

For the experiments on the osmotic behavior of vesicles, the bathing solution contained 155 mM K glutamate, 5 mM HEPES, 2 mM $CaCl_2$, 0.01% (wt/vol) ruthenium red, pH 7.4, 290 mOsm. Hypertonic solutions were made by the addition of sucrose. Alternatively, for the experiments in which the solutions were made hypertonic with the addition of stachyose, the isotonic bathing solution was Krebs' solution. The vesicles were challenged in a sequence of increasing osmotic strengths. The osmolality was measured with a Wescor 5100 C vapor pressure osmometer (Logan, UT). For the sucrose solutions of 1 molal and higher, osmolalities were taken from Washburn (1928).

Cells with lysed cell membranes but intact vesicle membranes were prepared by carefully pushing individual cells with a pipette controlled by micromanipulators under microscopic observation. The cell membrane was disrupted at the original site of attachment between the cell and the polylysine-coated cover slip. Alternatively, vigorous mixing of the bathing solution with Pasteur pipettes lysed cell membranes but left the vesicle membranes intact. Cell lysis was verified by optical inspection of nuclei stained blue with trypan blue. The integrity of vesicle membranes was verified optically by the absence of red-staining vesicles in ruthenium red solutions (Lagunoff, 1972).

Microscopy

Measurements of cell and vesicle sizes were made under Nomarski differential contrast microscopy with a Reichert inverted microscope or a Zeiss IM inverted microscope with a 1.4 NA objective and a 0.63 NA long working distance condenser. The largest orthogonal axis of the nonspherical vesicles were averaged to obtain one measurement per vesicle. Cell and vesicle images were captured by videocamera (Dage-MTI, 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 was approximately equal to 0.18 μm (Inoue, 1986). The error in the vesicle size measurements was less than 2.5% as determined by the ratio of the standard deviation of the measured diameters to the mean. The microscopic and recording techniques are described in more detail elsewhere (Curran & Brodwick, 1991).

Kinetics of Fusion

Patch pipettes were pulled from thin-walled, hard glass (World Precision Instruments, New Haven, CT) on a P-80/PC Flaming-Brown Micropipette puller (Sutter Instruments, San Rafael, CA), coated with Sylgard (Dow Chemical, Midland, MI) and heat polished on a CPZ-101 pipette forge (Adams and List Associates, Great Neck, NY). The pipette filling solution contained one of three solutions depending upon the experimental protocol; for isotonic conditions, 155-160 mM KCl, 7 mM $MgCl_2$, 1.6 mM $CaCl_2$, 0.2 mM EGTA, 5 mM HEPES, 10 μM guanosine 5'-[3-thio]-triphosphate (GTP-δ-S), 0.5 mM inosine triphosphate (ITP), pH 7.2, 285 mOsm. Low intracellular concentrations of calcium and GTP-δ-S are known to promote fusion in mast cells (Fernandez, Neher & Gomperts, 1984). For hypotonic conditions KCl was reduced to 72 mM to achieve an osmolality of 155 mOsm and for hypertonic conditions 300 mM sucrose was added to achieve an osmolality of 600 mOsm. Similarly, the external bathing milieu consisted of one of three solutions depending upon the experimental protocol; for isotonic conditions, 140 mM NaCl, 5 mM KCl, 1 mM $Mgcl_2$, 6 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2, 285 mOsm. For hypotonic conditions NaCl was reduced to 60 mM to achieve an osmolality of 153 mOsm and for hypertonic conditions 300 mM sucrose was added to achieve an osmolality of 600 mOsm. Solutions made hypertonic with sucrose had the same ionic strength as isotonic solutions. Cells were incubated for 10 min in the test solution before the whole cell configuration was established. We found that 5 min were sufficient for the cell volume to reach a steady-state volume and therefore assumed that 10 min was sufficient time for osmotic equilibration.

A patch electrode filled with solutions of the same osmolality as the external solution was applied to the cell. The patch-clamp technique permits control of intracellular ionic strength as the cell interior is continuous with the contents of the pipette. Thus the ionic strength remains constant as the osmotic stress is increased with added sucrose.

Cell admittance was measured with the quadrature technique of Neher and Marty (1982). A stimulating sine wave of 1600 Hz and 90 mV peak-to-peak amplitude was applied across the membrane with a patch clamp (EPC-7, List Electronics, Darmstadt, Germany) in the voltage-clamp mode at −50 mV holding potential. The current output of the patch clamp was applied to a two-channel lock-in amplifier (Lothar Meyer, Elme Electronik, Gottingen-Nikolausberg, Germany). The output of the lock-in amplifier, the in-phase (cell conductance) and the out-of-phase

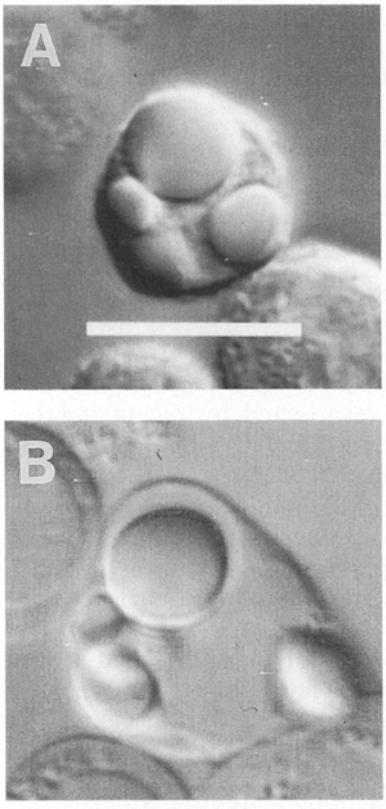

Fig. 1. Hypotonic solution causes the vesicle membrane to separate and lift off the vesicle gel matrix. (*A*) An intact beige mast cell in isotonic saline solution (pH 7.1). (*B*) The same cell a few minutes after challenge with hypotonic solution containing only 10^{-4} M MES buffer, pH 5.9. Note the clear space between the vesicle gel matrix and the vesicle membrane of the vesicle at the 12 o'clock position. Calibration bar = 10 μm microns.

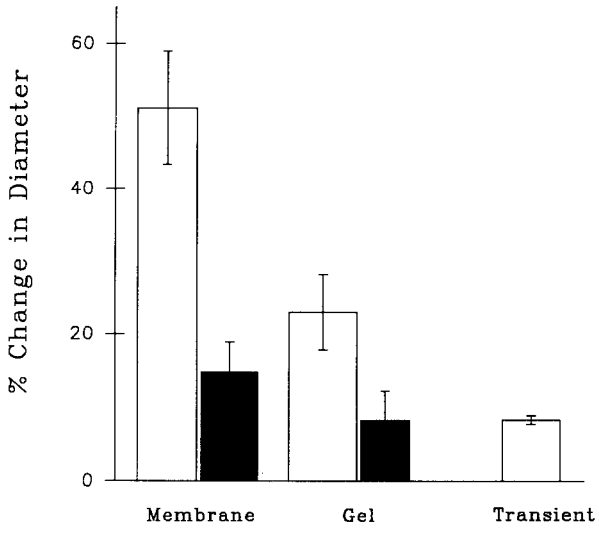

Fig. 2. Vesicles are osmotically active in hypotonic solution. The effects of hypotonic challenge on the size (diameter) of intact vesicles of intact mast cells of the beige mouse. The blank bar labeled *Membrane* shows the expansion of vesicle membranes in *intact* cells exposed to a hypotonic solution containing 10 mM HEPES, pH 7.1 (<20 mOsm) (presented as percent change in diameter); the controls were in isotonic Krebs' solution (300 mOsm, 7.1 pH). The reversibility of the vesicle membrane expansion after return to isotonic solution is presented by the filled bar labeled *Membrane*. The gel-matrix expansions (blank bar) and shrinkages (filled bar) of the same vesicles are shown in a similar manner. The means and SEM (error bars) were calculated from five observations. The bar labeled *Transient* represents the maximum percent increase in diameter of vesicles of lysed beige mast cells that were exposed to the same control and hypotonic solutions above. *Transient* denotes the observed brief interval between vesicle expansion and vesicle lysis; the latter is determined by the sudden increase in vesicle diameter. The means and SEM were calculated from five observations.

(cell capacitance) signals were filtered by an 8-pole Bessel filter at a corner frequency of 400 Hz, PCM encoded, and stored on magnetic tape.

Results

Hypotonic Expansion of Mast Cell Vesicles from the Beige Mouse

The possibility that rat mast cell vesicles might be water impermeable was suggested by the apparent existence of a large water-inaccessible space (Bennett et al. 1981). However, beige mouse mast cells swelled when placed in a solution containing only 10^{-4} M MES at 5.9 pH. After a delay the vesicles swelled as well. Visually, the vesicle membrane appeared to separate from the more opaque gel-matrix material (Fig. 1). The average size (diameter) of the vesicles expanded by about 50% (with a maximum of 73%), while the average diameter increase of the gel-matrix material was about 20% (Fig. 2). The expansions probably did not result from vesicle lysis, since both expansions were mostly reversible after return to isotonic solution. However, it is possible that reversibility of expansion could have occurred in vesicles that have resealed after lysis. The expansions do not represent steady-state values as both cell and vesicle lysis always followed during prolonged exposure to hypotonic solutions, i.e., more than 5 min. The probability of lysis rapidly approaches 100% when vesicles expand more than 50% in diameter. Vesicle lysis was never observed in intact cells.

In preparations where the cell membrane was mechanically removed and the vesicles were incubated in a solution containing only 10 mM HEPES at pH 7.2, vesicle expansion was limited to an increase in diameter of 8.3 ± 0.6% SEM, $n = 5$ (see bar

labeled *transient* in Fig. 2) before the vesicle lysed (as noted by a sudden increase in vesicle size). This expansion is in marked contrast to the 50% expansion observed in intact cells.

The difference between the amount of swelling of the intact and radically disrupted preparation is not due to an effect of the lysis procedure on control vesicle size. Furthermore, if the osmotic driving force in the disrupted preparation is decreased by applying a sequence of solutions of decreasing osmolality in 5 mOsm steps, for a 2–3 min incubation period to allow for osmotic equilibrium, the average vesicle expansion is not significantly altered, i.e., 8.5 ± 1.4% SEM (n = 4).

Hypertonic Compression

That vesicles are osmotically active was also demonstrated by experiments in which isolated vesicles were subjected to hyperosmotic solutions. The vesicle size (volume) decreased monotonically in solutions made hypertonic with sucrose (filled circles) or stachyose (open circles) (Fig. 3). However, the observed shrinkage was less than that predicted for an ideal osmometer as indicated by the short-dashed line (Fig. 3). The long-dashed line represents an excluded volume model constrained to go through the initial control point at 290 mOsm and 100% volume. The other curves represent models that treat the vesicular contents as a three-dimensional spring (*see* Discussion). Departures from ideal osmotic behavior have been observed for the red blood cell (*see* Sachs, Knauf & Dunham, 1975) and hepatocytes (Gleeson, Corasanti & Boyer, 1990).

The nonideal osmotic activity observed in hypertonic solutions could also be the result of an increase in the osmotic coefficient of the internal solutes with increasing concentration. Indeed, hemoglobin in red blood cells appears to have this property (McConaghey & Maizels, 1961; Adair, 1929). The contents of mast cell granules have not yet been fully characterized. However, it is known that heparin comprises about 30% and histamine 10% of the dry weight (Metcalfe & Kaliner, 1981). We therefore measured the osmolality of these constituents and calculated the osmotic coefficients and, for heparin (Sigma brand), the apparent osmotic coefficients, which we define as the ratio of observed osmolality to concentration (mg/ml). The osmotic coefficient for histamine *decreased* with increasing concentration, while the ratio of the apparent osmotic coefficients for heparin increased by only 13% for a doubling of concentration (in the relevant range of 246 to 558 mOsm). Thus changes in the osmotic coefficients of histamine and heparin cannot account for the observed departure from ideality; nor can the normal cytoplasmic inorganic ions, all of which have osmotic coefficients that decrease with concentration (Lide, 1990). The osmotic coefficient of the protein component of the gel matrix is unknown.

Kinetics of Fusion

The proposal (Cohen et al., 1980, 1982) that the vesicle membrane must be under tension for fusion to occur has been resurrected by recent indirect evidence presented by Monck et al. (1990). The demonstration of osmotic activity of the vesicles from the mast cells of the beige mice suggests that this system may be suitable to test the osmotic hypothesis. A simple version of this hypothesis predicts that the kinetics of fusion should be slowed or stopped in a solution that decreases vesicle membrane tension, i.e., a hypertonic solution; by similar logic the rate of release would be accelerated in a hypotonic solution. We measured the kinetics of release from the capacitance increase that accompanies vesicle membrane fusion using the patch-clamp technique in the whole cell configuration (Zimmerberg et al., 1987). After the patch membrane ruptures the time for the solution in the electrode to fill the cell is approximately 30 sec to 1 min (Pusch & Neher, 1988). This time was less than the delay of release as measured by the interval between obtaining a whole cell configuration (membrane rupture) and the first increase in capacitance. The delay was highly variable in all three osmotic solutions; that for isotonic conditions was 85 ± 19 sec SD (n = 5); for hypertonic conditions, 134 ± 62 sec (n = 5); for hypotonic conditions, 81 ± 46 sec (n = 4). The differences in the delays among these three groups were not significant at the 0.05 confidence level. The capacitance increase, as a function of time for the hypo-, iso-, and hypertonic solutions, was approximately exponential (Fig. 4) as previously described (Fernandez et al., 1984; Neher, 1988). The standard deviations for exponential fits of the kinetics for each cell ranged from 1 to 8 sec. The mean values for tau and their standard deviations were, for the hypotonic condition, 62 ± 12 sec SD (n = 4), for the isotonic condition, 34 ± 9 sec (n = 6), and for the hypertonic condition, 48 ± 15 sec (n = 5). The difference between the isotonic and hypertonic case was not significant at the 0.05 confidence level. However, the mean of the hypotonic group was greater than that for the isotonic case at that confidence level; this result

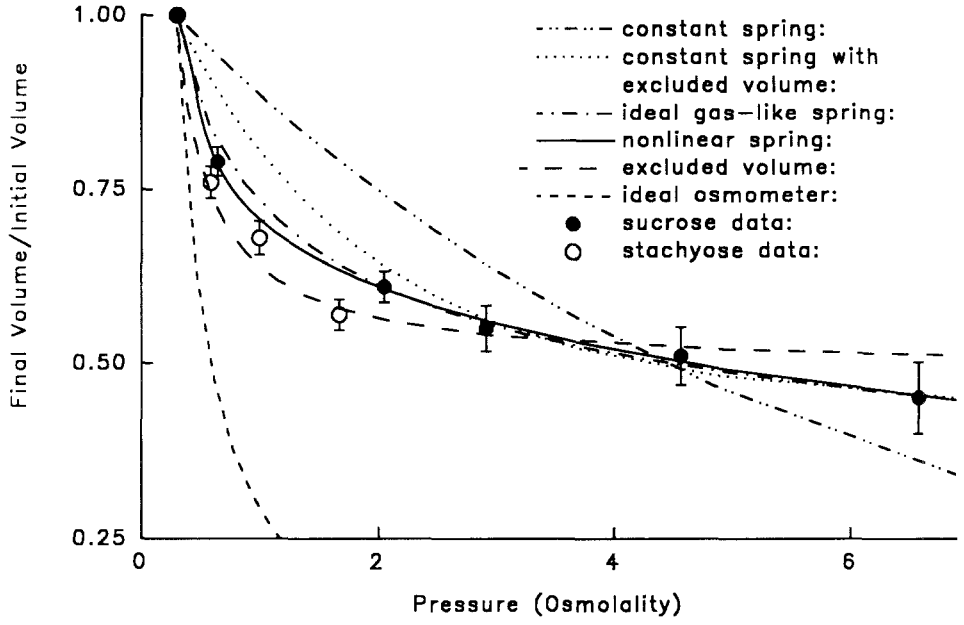

Fig. 3. Isolated vesicles under hypertonic conditions behave like springs. Vesicle sizes of *lysed* beige mice mast cells were measured in isotonic solutions (290–300 mOsm) and in solutions made hypertonic with sucrose (filled circles) or stachyose (open circles) after 15–20 min incubation. (The vesicle sizes reached equilibrium under hypertonic conditions quickly as sizes at 2 and 15 min incubation for stachyose and 5 and 20 min incubation for sucrose were comparable.) The volumes of the vesicles were calculated assuming that the vesicles presented as perfect spheres. The experimental values of vesicle volumes in hypertonic solution were normalized to their volumes in isotonic solution. The osmotic pressure is in mOsm. The means and SEM were calculated from 10–13 observations. Six theoretical models of vesicle compression fitted to the sucrose data are also presented. The short-dashed line is the prediction of an *ideal* osmometer. The long-dashed line is the prediction of an *excluded solute volume* model in which the solute density is so great that it occupies a significant proportion of the total vesicular volume. The remaining four theoretical curves are the predictions of *spring* models which assume that the osmotic contraction of the vesicle is opposed by an oppositely directed outward force due to a three-dimensional spring. For the *linear (constant) spring* model the spring constant is independent of the volume change (dot-dot-dash line). For the *linear (constant) spring with an excluded volume* (dot line) model the gel matrix occupies a significant fraction of the total volume. For the *ideal gas-like spring* model (dot-dash line) the bulk modulus varies with the volume change. Finally, for the *nonlinear spring* model (solid line) the spring compliance decreases with increasing pressure.

is in the reverse direction to that predicted by the osmotic hypothesis. We conclude that the kinetics of release do not support the osmotic hypothesis.

Discussion

The main findings of this paper concern the effects of tonicity on vesicle size which have possible functional consequences as discussed below. In addition we have demonstrated that osmotically active vesicles do not behave as predicted by the osmotic hypothesis of exocytosis as described by Cohen et al. (1980, 1982) and recently championed by Monck et al. (1990). In its simplest form, the osmotic hypothesis posits that tension in the vesicle membrane is an essential driving force in membrane fusion. Thus an increase in vesicle tension would accelerate the process while a decrease would have the opposite effect. Our kinetic data on exocytosis in mast cells in the whole-cell patch-clamp mode are contrary to the predictions of the osmotic hypothesis.

Osmotic Behavior of Secretory Vesicles

The large size of the vesicles of beige mouse mast cells permitted direct optical investigation of the osmotic properties. Other vesicular preparations are osmotically active. In hypertonic solutions the vesicles of chromaffin cells behave as ideal osmometers as determined by a light-scattering technique (Sudhof, 1982). Sea urchin cortical vesicles appear to shrink in hypertonic solutions (Zimmerberg et al., 1985). The distinction between the excitatory (round) and inhibitory (flattened) vesicles in electron micrographs of the central nervous system is probably also a reflection of osmotic properties expressed in the course of tissue preparation (Peters, Palay & Webster, 1976). During nerve terminal degeneration, vesicle volume appears to increase by as much

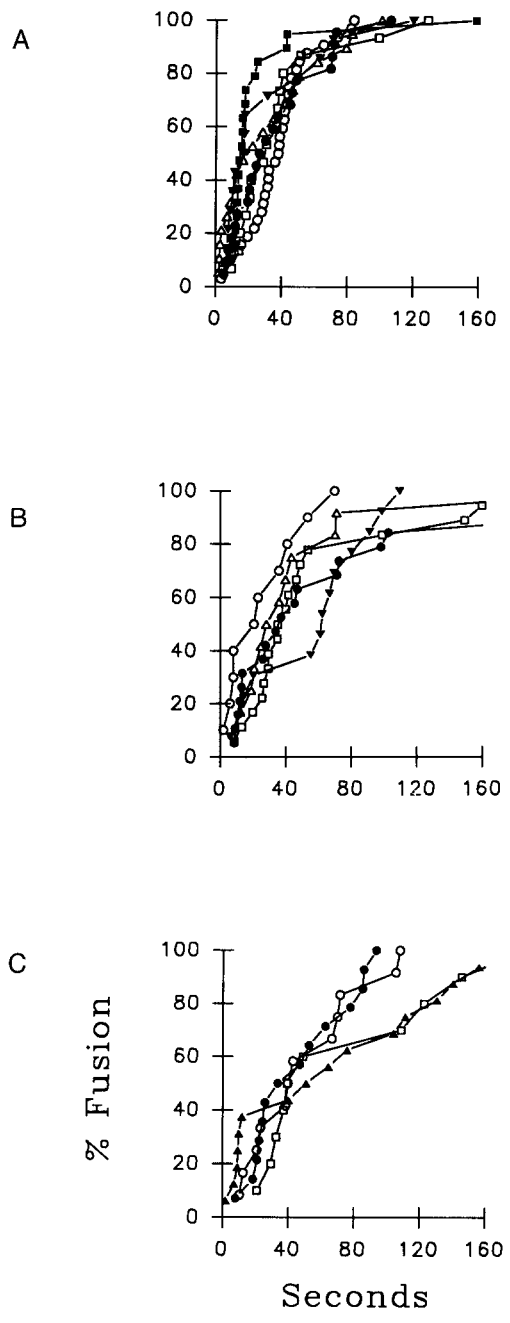

Fig. 4. Capacitance measurements reveal that osmotic strength has little effect on rates of release. Individual fusion events were monitored over time by measuring discrete changes in capacitance in mast cells from beige mice as a function of the osmotic strength. In all cases the intracellular and the extracellular osmotic pressures were equal. Each graph, *A-C*, has four to six plots representing the rates of release in a single beige mast cell stimulated with 10 μM intracellular GTPγS. Each graph has an identical time range (abscissa) for easy comparison. The ordinate is the normalized % of the fusion events for each cell. Each point of each graph represents a single fusion event. Zero time was defined as the time of the first capacitance change after the whole cell mode was established. (*A*) Isotonic conditions (285 mOsm). (*B*) Hypertonic conditions (600 mOsm) (*C*) Hypotonic conditions (153 mOsm).

as 174% (a 40% increase in diameter), an effect Peters et al. (1976) ascribed to osmotic effects secondary to permeability changes in the nerve membrane. These electron microscopic studies did not follow individual vesicles sequentially but represent conclusions based on statistical sampling. Our direct observations on single vesicle sizes disclose two departures from ideal osmotic behavior.

Hypotonic Expansion

The first departure from ideal osmotic behavior is found in the difference between the hypotonic swelling of vesicles in intact cells to approximately 50% (diameter) compared to that in disrupted cells of approximately 8%. This expansion cannot be limited by membrane stretching, which is limited to a 2–3% increase in surface area (1% increase in diameter), before the bilayer rips apart (Kwok & Evans, 1981). We have also shown that the difference between the disrupted and intact cells cannot be due to the rate of expansion or in some mechanism that preexpanded the vesicles in disrupted cells (*see* Results). Preliminary data (*not shown*) suggest that the difference may arise as a consequence of the loss of histamine from the vesicles of disrupted cells; we have previously shown that histamine contracts the gel matrix (Curran & Brodwick, 1991).

Hypertonic Compression

Osmotic ideality was also violated on the hypertonic limb of the osmotic pressure-volume relationship of isolated vesicles. The departure from ideality increased with increasing hypertonicity. This nonideal behavior was not a consequence of the osmoticant's (sucrose or stachyose) having a reflection coefficient less than one since the vesicle sizes remained constant over one hour. The nonideal behavior was consistent with the existence of a vesicle gel matrix's playing a role in vesicle size determination. In general, experiments purporting to demonstrate regulatory volume compensation in response to osmotic stresses may involve changes in the gel-matrix spring properties. Curran and Brodwick (1991) have demonstrated that the size of the gel martix is under ionic control. The size of a cell could then represent the balance of gel-matrix spring forces and transmembrane osmotic pressure.

Three physical properties of the gel matrix could contribute to the vesicle's osmotic nonideality. First the density of solute could be so great that an appreciable fraction of the total volume (V_t) could be occupied by the solute (V_s). The remaining aqueous vol-

ume (V_{os}), though osmotically ideal, would be less than the observed V_t. This V_{os} would then vary inversely with osmolality, π. The short-dashed line of Fig. 3 represents the *ideal* case

$$V_t = K/\pi \tag{1}$$

(where $K = 0.29$ Osm to satisfy the initial condition of 100% volume), whereas the long-dashed line represents the case where *excluded solute volume* is considered, i.e.

$$V_t = (K/\pi) + V_s. \tag{2}$$

Because the initial control point at 100% volume and 290 mOsm makes K depend on V_s the solute volume model predicts

$$V_t = [0.29(1 - V_s)/\pi] + V_s. \tag{3}$$

The calculated values of V_s for the sucrose and the stachyose solutions were 49 and 51%, respectively. These values of V_s are greater than the 28% value calculated for the red blood cell solutes (*see* pp. 67 and 78, Lentner, 1984) or the 38% "nonsolvent" volume calculated for rat hepatocytes (Gleeson et al., 1990).

Second, the gel matrix could behave as an internal spring resisting compression. Three-dimensional spring properties are described by the bulk modulus, B, which is given by

$$B = -\Delta P/(\Delta V/V) \tag{4}$$

where ΔP is the pressure change and $\Delta V/V$ is the normalized volume change. In the appendix we have recast this equation to account for osmotic compression of the gel matrix, i.e., the linear spring model, as

$$-B\ln(V_f/V_i) = RT\{C_{\text{out},f} - C_{\text{out},i}/(V_f/V_i)\} \tag{5}$$

where $C_{\text{out},f}$ and $C_{\text{out},i}$ are the final and initial concentrations of external osmoticants (assumed to be impermeant and ideal) and V_f and V_i represent the volumes of the vesicle bathed in $C_{\text{out},f}$ and $C_{\text{out},i}$, respectively. The internal osmoticants are assumed to behave ideally. The dashed-double dotted line of Fig. 3 is the best fit. The calculated bulk modulus is 1.36×10^7 NM^{-2}.

If we combine the excluded volume and the linear spring models, the relation becomes

$$-B\ln[(V_f - V_s)/(1 - V_s)] = RT\{C_{\text{out},f} - C_{\text{out},i}/[(V_f - V_s)/(1 - V_s)]\}. \tag{6}$$

(*See* Appendix.) For the more complete sucrose data the excluded volume is calculated to be 39% and the bulk modulus, B, is 3.59×10^6 NM^{-2}. The dotted line of Fig. 3 is the best fit.

The bulk modulus is usually calculated for small changes of volume, and in the above models, B is treated as a constant. However, the bulk modulus could be a function of volume. We offer two such models. In the first the bulk modulus is treated as a sum of successive terms that depend on increasing powers of the volume in the manner of the virial expansion (Adamson, 1986)

$$B = B_0 + B_1(1 - V) + B_2(1 - V)^2 + \ldots \tag{7}$$

Recasting the equation in terms of the experimental variables gives (*see* Appendix)

$$(B_0 + B_1 + B_2)\ln V_f - (B_1 + 2B_2)(V_f - 1) + (B_2/2)(V_f^2 - 1) = RT(C_{\text{out},f} - C_{\text{out},i}/V_f). \tag{8}$$

This nonlinear equation produces an excellent fit as illustrated by the solid line of Fig. 3. The squared term alone can reasonably fit the data. Note that this model transforms into the excluded volume model at high osmolarities where the squared term becomes very large, i.e., where B becomes large.

A second nonlinear model, suggested to us by Dr. David Baker, treats the gel matrix as though its compressibility were proportional to the volume, i.e.,

$$B = AP \tag{9}$$

where A is the proportionality constant and P is the applied pressure. Such a property would be expected if the gel matrix behaved as a gas or if the vesicle contained a solution with impermeant solutes. For the ideal case A equals unity. The "ideal gas-like" model reduces to the following simple equation

$$V_i/V_f = (P_f/P_i)^{1/A} \tag{10}$$

where P_f and P_i are the external osmotic pressure for the final and initial solutions, respectively. This model also fits the data admirably, as illustrated by the dash-dotted line of Fig. 3, with $A = 3.94$, i.e., the gel matrix behaves as though it were four times less compressible than the ideal case. This model implies that the volume of the vesicle is determined by the difference between osmotic and spring forces. If we assume that the osmotic component is ideal, then the remaining A (approximately 3) gives a bulk modulus [*see* C of Eq. (A 13)] that varies about 20-

fold between 2.08×10^6 NM^{-2} and 4.73×10^7 NM^{-2} for the isotonic control volume and the hypertonic compression to 41%, respectively. The calculated bulk moduli from all the above models range between 10^5 and 10^8 NM^{-2}. These values are about four orders of magnitude more compliant than iron, 9.6×10^{10} NM^{-2}, or quartz, 2.7×10^{10} NM^{-2}, (Weidner & Sells, 1973), but are comparable to values found for hydrated gelatin 0.66×10^5 NM^{-2} (recalculated from Northrop, 1927). The main point of this analysis is that the nonideality at high osmotic strengths can be adequately explained as the consequence of an excluded volume and/or a three-dimensional gel-matrix spring. Moreover, a structural substrate, in the form of an elastomeric gel matrix contained within lysed vesicles has been demonstrated (Curran & Brodwick, 1991).

As the results indicate, both an excluded volume model and bulk spring models adequately fit the data. In fact, these two types of models are not unrelated. In the Appendix we show that in the limit the nonlinear spring models reduce to the excluded volume model. The excluded volume is not a well-defined concept; it surely must include more than the volume occupied by the atoms themselves. We suggest that at least part of the apparent excluded volume includes repulsive forces between the strands of the negatively charged matrix.

In a third type of model the osmotic nonideality is a consequence of an increase in the osmotic coefficient, ϕ, of the internal solutes with concentration. For example, the osmotic coefficient of hemoglobin in red blood cells increases from 3 at 7 mmolal to 12 at 20 mmolal (Sachs et al., 1975; McConaghey & Maizels, 1961; Adair, 1929). With such large changes of ϕ, solutes present in relatively low concentrations can play an important role in osmotic behavior. If such a mechanism is operating in mast cells it cannot be generated by the major vesicular solutes: heparin, histamine, or the inorganic ions of biological systems (this study and Lide, 1990). However, other presently unknown matrix substances could behave in this way.

Even at rest the gel matrix may provide outward tension and internal scaffolding that helps determine vesicle shape. When vesicles were expanded in hypotonic solutions a watery zone formed and the boundary of the more optically dense gel matrix was clearly discernible. In the intact cell bathed in isotonic solution, no such clear zone was ever observed. Moreover, assuming that vesicle membrane is neither created nor destroyed during osmotic challenge, then the extra membrane must exist in a folded state upon the vesicle gel matrix. The maintenance of the folded vesicular membrane implies that either (i) the vesicle is structurally constrained by a cytoplasmic exoskeleton or an intravesicular endoskeleton, (ii) the vesicle membrane can perform volume regulation, or (iii) the cytoplasm is hypertonic with respect to the "vesiculoplasm" and squeezes the membrane against the resistance of the gel-matrix spring. A similar role for the axoplasmic gel has been proposed to account for departures from osmotic ideality in squid giant axons (Spyropoulos, 1977).

Physiological Implications

The demonstration that the mast cell vesicles are osmotically active has important functional consequences. A mast cell contains as much as 30 picograms of histamine per cell (Moran, Uvnas & Westerholm, 1962). Each 12 μm cell has a vesicle compartment that comprises about 60% of its volume (Helander & Bloom, 1974). Assuming all of the histamine is stored in vesicles, each vesicle would contain a solution of 0.5 M histamine. For comparison, Wagner, Carlson and Kelly (1978) estimate that Narcine synaptic vesicles contain 0.52 M acetylcholine, while adrenal medullary vesicles contain 0.4–0.6 M catecholamine (Winkler, 1976; Winkler & Westhead, 1980). Since most of the histamine is in the divalent form (Jencks & Regenstein, 1975), with univalent anions the vesicle solution would be 1500 mOsm. This figure underestimates the osmolality, since other ionic solutes such as K^+ (the vesicle membrane has no known K^+ pump), and organic solutes are also present. However, the vesicle *is* osmotically active and is in equilibrium with the cytoplasm which is in equilibrium with extracellular fluids at approximately 300 mOsm. There is at least five times too much histamine to exist in a free state and also maintain osmotic equilibrium. We therefore propose that the heparin-protein gel-matrix of the mast cell vesicle binds, and therefore decreases the osmotic activity of, the great majority of the histamine. Indeed, Aborg, Novotny and Uvnas (1967) have demonstrated that isolated vesicle matrices bind histamine. Thus the vesicle can store more transmitter than would be possible osmotically. There are sufficient binding sites to accommodate 0.5 M histamine. Histamine (mol wt 111.1) constitutes 10% of the dry weight of the granule gel matrix (Metcalfe & Kaliner, 1981). The dry weight "density" of the entire gel matrix is then approximately 500 g/liter, and the isolated gel matrix possesses 1000 nanoequivalents of binding sites per gram (Uvnas & Aborg, 1977). The product of these two independent measurements produces a binding site concentration of 0.5 equiv/liter, a value equal to the total concentration of histamine calculated above.

The high dry weight density of the matrix (500

g/liter) helps explain the ready separation of mast cells from other cells through a density gradient (*see* Materials and Methods). Indeed, the mast cell granules are denser than red blood cells whose dry weight density is 364 g/liter (calculated from 334 g/liter × 1.094 kg/liter, pp. 67 and 78 of Lentner, 1984). The dry mass of leukocytes is approximately 20% less than for the red blood cell (Lentner, 1984). The density of synaptic vesicles from Narcine electric organ isolated in glycerol are 1.119 g/ml, somewhat higher than the RBC value (Wagner et al., 1978). Solute concentrations can be so high as to produce crystalline inclusions that seem to occupy a significant fraction of the vesicular space (*see* pp. 319–336, Fawcett, 1966).

Once exocytosis has occurred the stored histamine would leave the gel matrix by exchanging with other cations by the law of mass action. Such a scheme makes sense for an effector cell whose targets are distant and disperse. The demands on transmitter concentration for such a shotgun approach for mediator delivery obviously exceed that for local, punctate synaptic transmission. Thus the vesicle gel-matrix functions to store transmitter optimally while the mast cell itself is densely packed with vesicles. In chromaffin cells catecholamine and ATP are similarly bound to the vesicular gel matrix (Uvnas & Aborg, 1988). Many vesicular preparations have a gel-matrix material most commonly identified as a glycosaminoglycan similar to heparin. Whether other secretory systems utilize such a storage system is not known at present.

The authors wish to thank Drs. John Kasianowicz, Sergei Leikin, V. Adrian Parsegian, Bruce Simon, David Baker, Donald Rau, and Joshua Zimmerberg for helpful discussions and comments on the manuscript. We also gratefully acknowledge the support from the James W. McLaughlin Fellowship Fund, the Cystic Fibrosis Fellowship (F083 0-1) and the support of the Division of Computer Research and Technology of NIH for use of the image analysis.

References

Aborg, C.H., Novotny, J., Uvnas, B. 1967. Ionic binding of histamine in mast cell granules. *Acta Physiol. Scand. Suppl.* **69:**276–283

Adair, G.S. 1929. Thermodynamic analysis of the observed osmotic pressure of protein salts in solutions of finite concentrations. *Proc. R. Soc London A* **126:**16–24

Adamson, A.W. 1986. A Textbook of Physical Chemistry. (3rd ed.) Academic Press College Division, Orlando (FL)

Bennett, J.P., Cockcroft, S., Gomperts, B.D. 1981. Cells permeabilized with ATP secrete histamine in response to calcium ions buffered in the micromolar range. *J. Physiol.* **317:**335–345

Chi, E.Y., Lagunoff, D. 1975. Abnormal mast cell granules in the beige (Chediak-Higashi Syndrome) mouse. *J. Histochem. Cytochem.* **23:**117–122

Cohen, F.S., Akabas, M.H., Finkelstein, A. 1982. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science.* **217:**458–460

Cohen, F.S., Zimmerberg, J., Finkelstein, A. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes: II. Incorporation of a vesicular membrane marker into the planar membrane. *J. Gen. Physiol.* **75:**251–270

Curran, M.J. 1986. Putative gel swell and osmotic steps of exocytosis in mast cells. Ph.D. Thesis. University of Texas, Graduate School of Biomedical Sciences at Galveston, Texas

Curran, M., Brodwick, M.S. 1985. Mast cell exocytosis and the gel-swell of granules. *Biophys. J.* **47:**172a

Curran, M.J., Brodwick, M.S. 1991. Ionic control of the size of the vesicle matrix of beige mouse mast cells. *J. Gen. Physiol.* **98:**771–790

Fawcett, D.C. 1966. *In:* An Atlas of Fine Structure. The Cell. Its Organelles and Inclusions. W.B. Saunders, Philadelphia

Fernandez, J.M., Neher, E., Gomperts, B.D. 1984. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* **312:**453–455

Fernandez, J.M., Villalon, M., Verdugo, P. 1991. Reversible condensation of mast cell secretory products *in vitro*. *Biophy. J.* **59:**1022–1027

Gleeson, D., Corasanti, J.G., Boyer, J.L. 1990. Effects of osmotic stresses on isolated rat hepatocytes: I. Ionic mechanisms of cell volume regulation. *Am. J. Physiol.* **258:**290–298

Hampton, R.Y., Holz, R.W. 1983. Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *J. Cell Biol.* **96:**1082–1088

Helander, H.F., Bloom, G.D. 1974. Quantitative analysis of mast cell structure. *J. Microsc.* **100:**315–321

Holz, R.W., Senter, R.A. 1985. Effects of osmolality and ionic strength on secretion from adrenal chromaffin cells permeabilized with digitonin. *J. Neurochem.* **46:**1836–1842

Inoue, S. 1986. Video Microscopy. Plenum, New York

Jencks, W.P., Regenstein, J. 1975. Ionization constants of acids and bases. *In:* Handbook of Biochemistry and Molecular Biology. (3rd ed.) Physical and Chemical Data. Vol. 1, p. 319. G.D. Fasman, editor. C.R.C., Boca Raton (FL)

Kwok, R., Evans, E. 1981. Thermoelasticity of large lecithin bilayer granules. *Biophys. J.* **35:**637–652

Lagunoff, D. 1972. Vital staining of mast cells with ruthenium red. *J. Histochem. Cytochem.* **20:**938–944

Lentner, C. (editor) 1984. The Geigy Scientific Tables. Vol. 3. American ed. Medical Education Division, CIBA—Geigy Corporation. West Caldwell (NJ)

Lide, D.R. (editor) 1990. CRC Handbook of Chemistry and Physics. (71st ed.) The Chemical Rubber Co., Cleveland

McConaghey, P.D., Maizels, M. 1961. The osmotic coefficients of haemoglobin in red cells under varying conditions. *J. Physiol.* **155:**28–45

Metcalfe, D.D., Kaliner, M. 1981. The mast cell. *CRC Crit. Rev. Immunol.* **3:**23–74

Monck, J.R., Alvarez de Toledo, G., Fernandez, J.M. 1990. Tension in secretory membranes causes extensive membrane transfer through the exocytotic fusion pore. *Proc. Natl. Acad. Sci. USA* **87:**7804–7808

Moran, N.C., Uvnas, B., Westerholm, B. 1962. Release of 5-hydroxytryptamine and histamine from rat mast cells. *Acta Physiol. Scand.* **56:**26–41

Neher, E. 1988. The influence of intracellular calcium concentration on degranulation of dialyzed mast cells from rat peritoneum. *J. Physiol.* **395:**193–214

Neher, E., Marty, A. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* **79:**6712–6716

Northrop, J. 1927. The swelling of isoelectric gelatin in water: I. Equilibrium conditions. *J. Gen. Physiol.* **10:**893–904

Padawer, J. 1970. The reaction of rat mast cells to polylysine. *J. Cell Biol.* **47:**352–372

Peters, A., Palay, S.L., Webster, H. DeF. (editors) 1976. The Fine Structure of the Nervous System: The Neurons and Supporting Cells. pp. 148–150. W.B. Saunders, Philadelphia

Pusch, M., Neher, E. 1988. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pfluegers Arch.* **411:**204–211

Sachs, J., Knauf, P., Dunham, P. 1975. Transport through red cell membranes. *In:* Red Blood Cell. D. Surgenor, editor. pp. 625–629. Academic, New York

Spyropoulos, C.S. 1977. Osmotic relations of nerve fiber. *J. Membrane Biol.* **32:**19–32

Sudhof, T.C. 1982. Core structure, internal osmotic pressure and irreversible structural changes of chromaffin granules during osmometer behavior. *Biochim. Biophys. Acta* **684:**27–39

Uvnas, B., Aborg, C.-H. 1977. On the cation exchanger properties of rat mast cell granules and their storage of histamine. *Acta Physiol. Scand.* **100:**309–314

Uvnas, B., Aborg, C.-H. 1988. Catecholamines (CA) and adenosine triphosphate (ATP) are separately stored in bovine adrenal medulla, both in ionic linkages to granule sites, and not as a nondiffusible CA-ATP-protein complex. *Acta Physiol. Scand. Suppl.* **132:**297–312

Wagner, J.A., Carlson, S.S., Kelly, R. 1978. Chemical and physical characterization of cholinergic synaptic vesicles. *Biochemistry* **17:**1199–1206

Washburn, E.W. (editor) 1928. International Critical Tables of Numerical Data, Physics, Chemistry, and Technology. Vol. 4, pp. 429–430. National Research Council of the USA. McGraw-Hill, New York

Weidner, R.T., Sells, R.L. 1973. Mechanical properties of solids and fluids. *In:* Elementary Classical Physics. Vol. 1, pp. 281–301. Allyn and Bacon, Boston

Winkler, H. 1976. The composition of adrenal chromaffin granules: An assessment of controversial results. *Neuroscience* **1:**65–80

Winkler, H., Westhead, E. 1980. The molecular organization of adrenal chromaffin granules. *Neuroscience* **5:**1803–1823

Zimmerberg, J., Curran, M., Cohen, F.S., Brodwick, M. 1987. Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc. Natl. Acad. Sci. USA* **84:**1585–1589

Zimmerberg, J., Sardet, C., Epel, D. 1985. Exocytosis of sea urchin egg cortical granules in vitro is retarded by hyperosmotic sucrose: Kinetics of fusion monitored by quantitative light-scattering microscopy. *J. Cell Biol.* **101:**2398–2410

Received 19 July 1991; revised 17 October 1991

Appendix

When an intact vesicle is osmotically shrunk, departures from ideality can result from several sources. In this appendix we derive the equations of a series of models in which the vesicular gel matrix is considered as a three-dimensional spring with or without a term for the excluded volume resulting from an unspecified compartment of the gel matrix.

The Gel-Matrix as a Linear Spring

In this model the osmotic contraction of the vesicle is resisted by an oppositely directed outward force due to the compression of a three-dimensional spring whose constant is independent of the volume change. The equivalent spring constant is given by the bulk modulus, B, as

$$B = -\Delta P/(\Delta V/V) \tag{A1}$$

where ΔP is the change in the pressure and $\Delta V/V$ is the normalized change in volume. Separating variables, changing differences for differentials and taking the integral of both sides gives

$$-B \int dV/V = \int dP. \tag{A2}$$

The integrals are then evaluated between the limits f and i for the final and the initial states, respectively, producing

$$-B\ln(V_f/V_i) = P_f - P_i = \Delta P \tag{A3}$$

where V_f/V_i is the normalized value of V after contraction. In these experiments the pressure difference is osmotic, π,

$$\pi = RT(C_{out} - C_{in}) \tag{A4}$$

where R is the gas constant, T is the absolute temperature and C_{out} and C_{in} are the concentrations of ideal impermeable solutes on the outside and the inside. For a change in C_{out}, the change in osmotic pressure would be

$$\Delta\pi = RT(C_{out} - C_{in})_f - RT(C_{out} - C_{in})_i. \tag{A5}$$

Initially we assume that the vesicle is at equilibrium with the spring, offering no resistance. Under these conditions C_{in} equals C_{out} and thus the second term of the right side of Eq (A5) is zero. In these experiments $C_{out,i}$ is 0.290 Osm. The concentration of solutes inside the vesicle, $C_{in,f}$, must be corrected for the change in volume after compression

$$C_{in,f} = C_{in,i}/(V_f/V_i) = C_{out,i}(V_f/V_i). \tag{A6}$$

Substituting these values of the corrected osmotic strength into Eq. (A3) we arrive at the final equation for a linear spring

$$-B\ln(V_f/V_i) = RT[C_{out,f} - C_{out,i}/(V_f/V_i)]. \tag{A7}$$

(For ease in calculation, if B is in units of newtons/meter^2 then RT(Osm) = 2.27×10^6 N/M^2.)

The Gel-Matrix as a Linear Spring with an Excluded Volume

As pointed out in the text, it is reasonable to suppose that the gel matrix is sufficiently dense to occupy an appreciable fraction of the total volume. This excluded volume, V_{ex}, would reduce the

total volume available for osmotic contraction. To correct Eq. (A7) for the excluded volume we define V_{ex} to be normalized excluded volume, referred to V_i as 100%, and subtract this value from all the volume terms

$$-B\ln[(V_f - V_{ex})/(1 - V_{ex})] = RT\{C_{out,f} - C_{out,i}/[(V_f - V_{ex})/(1 - V_{ex})]\}. \quad \text{(A8)}$$

Note that at the limit of infinite osmotic pressure, i.e., as $C_{out,f}$ approaches infinity, V_f approaches V_{ex}. The excluded volume is assumed to be osmotically incompressible. If the spring properties are disregarded, i.e., $B = 0$, Eq. (A8) reduces to

$$V_f = k/\pi - V_{ex} \quad \text{(A9)}$$

where $k = (1 - V_{ex})RTC_{out,i}$ (as determined by initial conditions when $V_f = 1$).

The Gel-Matrix as an "Ideal Gas"-Like Spring

The linear spring models developed above are applicable to systems where the volume changes are small. In general for systems exhibiting appreciable volume changes, the bulk modulus is not a constant, but varies with the volume change. For an ideal gas

$$V = K/P \quad \text{(A10)}$$

where K is a constant. For such a system the bulk modulus equals the pressure as can be seen from Eq. (A1). For a vesicle filled with ideal osmotic particles the bulk modulus is similarly equal to the external osmolarity. These systems share the property that the bulk modulus is proportional to the volume change which is, itself, proportional to the applied pressure

$$B = AP. \quad \text{(A11)}$$

Substituting Eq. (A11) into (A1), separating variables, integrating between the initial and final conditions, we find that

$$V_i/V_f = (P_f/P_i)^{1/A}. \quad \text{(A12)}$$

For an ideal "gas," $A = 1$. Thus for a vesicle filled and bathed in ideal osmotic solutions Eq. (A12) reduces to the ideal osmometer Eq. (A10). If to this ideal case we add a "gas-like" gel-matrix, then the total bulk modulus will have contributions from both pressure sources, thus

$$B = P_{ideal} + CP_{ideal} \quad \text{(A13)}$$

where P_{ideal} is the pressure arising from the ideal osmotic case and C is a constant coefficient of the ideal case to model the gel-matrix pressure in units of the ideal case. Note in this model the osmotic forces operate to compress the gel-matrix spring; in the absence of osmotic forces the gel matrix should expand without limit.

The Gel-Matrix as a Nonlinear Spring

Real materials exhibit linear properties only over a restricted range. When a spring is sufficiently compressed the compliance decreases. There are many ways to model departures from ideality. One convenient method utilizes the virial expansion (*see* Adamson, 1986) of the bulk modulus as a function of volume, $B(V)$, where V is the percent change of volume

$$B(V) = B_0 + B_1(1 - V) + B_2(1 - V)^2 + \ldots \quad \text{(A14)}$$

Note that the higher order terms are of the form $(1 - V)^n$ and therefore increase with compression. We now set up the integral as in Eq. (A2) as

$$\int B(V)\, dV/V = \int dP. \quad \text{(A15)}$$

After substituting Eq. (A14) into (A15), expanding the product, and collecting like terms, the left-hand integral becomes

$$\int (B_0 + B_1 + B_2)\, dV/V - \int (B_1 + 2B_2)\, dV + \int B_2 V\, dV. \quad \text{(A16)}$$

Evaluating the integrals between the final and initial states, f and i, and normalizing the volumes to V_i gives

$$(B_0 + B_1 + B_2)\ln V_f - (B_1 + 2B_2)(V_f - 1) + (B_2/2)(V_f^2 - 1) = RT(C_{out,f} - C_{out,i}/V_f). \quad \text{(A17)}$$

When the coefficients B_1 and B_2 are zero, Eq. (A17) reduces to the equation for a linear spring, Eq. (A7). Note from Eq. (A14) that the nonlinear equation behaves as though there were an incompressible component at reduced volumes as the equivalent bulk modulus becomes very large. In this paper only the first three terms of the expansion were selected for modeling. No physical interpretation of the various coefficients is possible at this time.