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Mechanical Properties of Brush Border Membrane Vesicles from Kidney Proximal Tubule

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Abstract. The mechanical properties of brush border membrane vesicles, BBMV, from rabbit kidney proximal tubule cells, were studied by measuring the initial and final equilibrium volumes of vesicles subjected to different osmotic shocks, using cellobiose as the impermeant solute in the preparation buffer.

An elevated intracellular hydrostatic pressure was inferred from osmotic balance requirements in dilute solutions. For vesicles prepared in 18 and 85 mosM solutions, these pressures are close to 17 mosM (290 mm Hg). The corresponding membrane surface tension is 6.0×10^{-5} N cm^{-1} while the membrane surface area is expanded by at least 2.2%. When these vesicles are exposed to very dilute solutions the internal hydrostatic pressure rises to an estimated 84 mosM (1444 mm Hg) just prior to lysis. The corresponding maximal surface tension (pre-lysis) is 18.7×10^{-5} N cm^{-1}, and the maximal expansion of membrane area is 6.8%. The calculated area compressibility elastic modulus was 2.8×10^{-3} N cm^{-1}.

Key words: Kidney vesicles — Hydrostatic pressure — Elastic modulus — Osmotic Behavior — Mechanical properties

Introduction

Evidence implicating mechanical properties of cell membranes in the control of transport has been described by numerous sources. Good examples are provided by mechanosensitive ion channels, where the probability of being opened depends on the stress at membrane surface; these have been reported in several cell systems studied with patch-clamp techniques (Morris, 1990). Coupling of membrane tension and permeation has also been shown in more complex transporters. Using intestinal brush border membrane vesicles Miyamoto, Maeda & Fujime (1988) found a correlation between the membrane elastic modulus and the activation of the Na^+-Glucose cotransport system indicating an increase in membrane flexibility upon activation of this cotransport. The area compressibility elastic modulus decreases 47% from its initial value of 1.5×10^{-3} N cm^{-1} when the D-Glucose concentration rises from 0 to 10 mM in the presence of 10 mM Na^+.

Under physiological conditions, changes in mechanical properties of epithelial cells can be expected because they are often exposed to variable transmembrane osmotic gradients. If membrane tension and permeability are coupled, then the influence of these osmotic gradients on transport and function will be more complex than the simple linear transport of water implied by the Kedem Katchalsky equations.

In view of the above, our goal in this paper is to characterize the mechanical properties of kidney brush border membranes when exposed to transmembrane osmotic gradients of a highly impermeant solute. We studied the mechanical properties of brush border membrane vesicles (BBMV) from rabbit kidney proximal tubule cells by measuring their initial and final equilibrium volumes when subjected to different osmotic shocks. Volumes were measured by two independent methods, quasi-elastic light scattering spectroscopy and the trapped volume of an intravesicular marker, while osmotic gradients were formed with cellobiose as the impermeant solute. Results do not follow a simple Boyle-

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VantHoff relation unless a hydrostatic pressure is operative. The magnitude of this pressure as well as the resulting membrane strain was evaluated. These measurements were used to characterize the mechanical behavior of the vesicles by providing estimates of elastic parameters.

Materials and Methods

Preparation of Brush Border Membrane Vesicles

Renal brush border membrane vesicles were prepared from rabbit renal cortex by the calcium precipitation method (Evers et al., 1978) with some modifications (Thierry, Poujeol & Ripoche, 1981). Briefly, kidneys from young adult male New Zealand White rabbits were removed and washed in ice-cold isotonic buffered solution (300 mM mannitol, 10 mM Tris-Hepes pH 7.4), then decapsulated. From here onwards the whole process was conducted at a temperature of 4°C in the presence of a single buffer containing 16 mM mannitol, 2 mM Tris-Hepes pH 7.4. The external cortex was sliced and homogenized in this buffer (Waring blender, low speed, 1 min) to obtain a ratio of 1 g of tissue to 40 ml buffer. To this homogenate was added a 1 M solution of $CaCl_2$ to reach the final concentration of 10 mM. The mixture was stirred for 15 min and centrifuged at 500 × *g* for 12 min in a Beckman J2-21M/E refrigerated centrifuge. The supernatant was carefully removed and centrifuged at 22,000 × *g* for 15 min. The pellet was then homogenized in the same buffer (1:100 *w/v* ratio) with a glass teflon homogenizer (10 strokes at 1000 rpm) and centrifuged at 48,000 × *g* for 20 min. This step was repeated once, changing the ratio of pellet to buffer to 1:10 w/v, and centrifuged at 2,500 × *g* for 7 min. The brush border membrane vesicles were collected by centrifuging the supernatant at 48,000 × *g* for 20 min and resuspending the final pellet in an appropriate volume of buffer. Other vesicle preparations were obtained using cellobiose Tris-Hepes buffer of 18 and 85 mosM.

The membrane preparations obtained were either immediately used for experiments, or stored in liquid nitrogen for later use.

Enrichment in specific activity (BBMV/crude homogenate) of the apical markers leucine-aminopeptidase (Haase et al., 1978; Kramers & Robinson, 1979) and alkaline phosphatase (Berner & Kinne, 1976; Quamme, 1990) as well as the basolateral markers Na^+/K^+ ATPase (Quigley & Gotterer, 1969; Schoner et al., 1967) and K^+-stimulated phosphatase (Garrahan, Pouchan & Rega, 1969; Murer et al., 1976), assayed as described, were 16.6 ± 2.7, 8.6 ± 2.3, 1.3 ± 0.04 and 0.4 ± 0.09 ($n = 16$) respectively. The contamination by the lysosomal fraction was evaluated through the enrichment in the acid phosphatase activity (Sacktor, 1968; Scalera et al., 1980). The value obtained was 1.2 ± 0.40 ($n = 16$).

Protein content was determined using the Bradford technique (Bradford, 1976) with bovine albumin as standard.

Osmolarity Measurements

All solution osmolarities were determined from freezing point depression on a cryometric automatic semi-micro osmometer (Knauer GmbH, Germany). Standards of 100 and 400 mosM were analyzed prior to samples, which were measured in triplicate.

Vesicle Size Determination

Vesicle size of all the membrane preparations was determined by two different techniques:

(i) Vesicle size of all the membrane preparations was determined before or after each experiment by the Quasi-elastic light scattering (QELS) technique (Brookhaven Instruments BI-90). Reviews of the theory and the application of this technique in the determination of vesicular sizes, have been published (Chong & Colbow, 1976; Latimer, Moore & Bryant, 1968; Perevucnik et al., 1985). The equilibrium volumes of the vesicles were measured in isosmotic conditions at different inner osmolarities, obtained by incubating the vesicles for at least one hour in different cellobiose solutions.

(ii) Vesicle trapped volume measurements. Changes in trapped volumes of vesicles were measured by loading the vesicles with slowly permeating, labeled glucose and using a rapid filtration technique to separate vesicles from media (Pratz, Ripoche & Corman, 1986; Verkman et al., 1985). The vesicles (1 mg protein/ml) were incubated in several cellobiose buffers of different osmolarities containing D-[^{3}H]-glucose (5 μCi/ml) for 90 min at room temperature, and passed through 0.65 μm Sartorius filters in a rapid filtration system. The filters were washed three times with 3 ml of the buffer used in the experiment, and the radioactivity measured on a β-scintillation counter. The nonspecific binding of the isotope to the surface of the vesicles as well as to the filters was determined by a similar experiment but without incubation. All experiments were carried out in triplicate.

These methods were used to determine the equilibrium volumes of vesicles prepared in 18 and 85 mosM cellobiose buffers, and subjected to several osmotic shocks. The results of both methods were in good agreement (*see* Fig. 4).

Influx of Mannitol and Cellobiose Followed by Stopped-flow Experiments

The influx of the two solutes tested after imposing an osmotic gradient was monitored in a stopped flow apparatus. 0.1 ml of BBMV prepared with mannitol or cellobiose as the internal solute and resuspended in the same buffer (0.4 mg protein/ml), were subjected to a hyper osmotic shock of the same solute (osmolarity increased 4.36 times). The change in 90° scattered light intensity was followed at 400 nm for 2.5 and 10 hr respectively, on a HI-TECH Scientific PQ/SF-53 stopped flow, which has a 2 msec dead time, temperature controlled, interfaced with an IBM PC/AT compatible 80386 microcomputer. Experiments were done at 23°C.

Measurements of Vesicle Hypotonic Lysis

Osmotically induced vesicle lysis was determined by following the release of radiolabeled mannitol initially entrapped within the vesicles. In this experiment, BBMV prepared in 18 and 140 mosM mannitol buffer were tested. The vesicles (3 mg protein/ml) were incubated at room temperature in the preparation buffer containing [^{14}C]-mannitol (5 μCi/ml) for 4 hr, to allow labeled mannitol equilibration on both sides of the membrane. The reaction was started by injecting aliquots of the incubation media (150 μg protein) in different tubes containing 2 ml of isotonic (control experiment, where the release of the marker is due to permeation) or hypotonic mannitol buffers with decreasing osmolarities. At pre-established time intervals, the total volume of each tube was filtered using the same protocol described above (*see* Vesicle Trapped Volume Measurements).

Na^+ and Mannitol Ratios Across the Membrane

The ratios of charged (Na^+) and noncharged (mannitol) species across the membrane vesicles were evaluated to obtain experimental evidence

for the existence of an impermeable ion species (which could contribute to NS) inside the vesicles. BBMV prepared and resuspended in mannitol buffer 18 mosM (2 mg protein/ml) were incubated at room temperature overnight in the same buffer containing [^{14}C]-mannitol 5 μCi/ml, [^{22}Na] 0.5 μCi/ml, and [^{3}H]-inulin 2 μCi/ml. This last compound is considered as totally impermeant solute (MW = 5,000 g mol^{-1}). After the incubation period, vesicles were separated from the external media on an ultra-rapid filtration system Biologic RFS (Dupont, 1984). This ultra-rapid filtration assures a filter time washing sufficiently small (50 msec) to consider that no significant sodium leakage from inside the vesicles occurs. The γ-radiation from [^{22}Na] as well as the β-radiation from [^{14}C]-mannitol and [^{3}H]-inulin retained in the filters (containing the intravesicular media and the nonspecific binding of the isotopes to the external surface of the vesicles as well as to the filters) and in samples of the external media without vesicles, was determined. The nonspecific binding was evaluated by a similar experiment but without incubation. The values for the three isotopes on the filters (internal media and nonspecific binding) and the incubation media without filtration (external media) were obtained using isotope standard solutions counted for γ and β radiation in different channels in order to eliminate interferences.

Mannitol Partial Molar Volume

The partial molar volume of mannitol was estimated using the technique described by Shoemaker (Shoemaker, Garland & Nibler, 1989). A pycnometer was filled alternatively with water or with mannitol solutions of increasing concentrations, and all weight measurements were performed on an analytical balance inside a thermostated chamber at 25°C.

Osmotic Balance

The total concentration of solutes inside the vesicles is equivalent to the total inner osmolarity osm_{in}, and is given by the sum of the concentrations of mannitol or cellobiose used in the buffer preparation as the impermeant species (*S*), and of any other species (*NS*) that remained inside the vesicles during the preparation. If the solute *S* is the only solute species of the external medium then its concentration equals the medium osmolarity, osm_{out}.

$$osm_{in} = C_{S\_in} + C_{NS\_in} \text{ and } osm_{out} = C_{S\_out} \tag{1}$$

The osmotic equilibrium conditions for vesicles in a suspension is:

$$\Delta P = \Delta\Pi = RT[osm_{in} - osm_{out}] \tag{2}$$

where $\Delta P = (P_{in} - P_{out})$ and $\Delta\Pi = (\Pi_{in} - \Pi_{out})$ are the hydrostatic and osmotic pressure differences between the inside and the outside of the vesicles. At time $t = 0$, the osmolarity of the external medium is changed from $(osm_{out})_o$ to a new value $(osm_{out})_\infty$. The intravesicular volume and osmolarity respond, moving from the initial values of V_o and $(osm_{in})_o$ to their final equilibrium values V_∞ and $(osm_{in})_\infty$. Solving for osm_{in} at $t = 0$ and $t = \infty$

$$(osm_{in})_o = \frac{\Delta P_o}{RT} + (osm_{out})_o \tag{3}$$

$$(osm_{in})_\infty = \frac{\Delta P_\infty}{RT} + (osm_{out})_\infty \tag{4}$$

Noting that the impermeability of all solutes requires $V_o(osm_{in})_o = V_\infty (osm_{in})_\infty$, multiply (3) by V_o, and (4) by V_∞ to arrive at

$$V_o \left(\frac{\Delta P_o}{RT} + (osm_{out})_o\right) = V_\infty \left(\frac{\Delta P_\infty}{RT} + (osm_{out})_\infty\right) \tag{5}$$

We define the tonicity of the osmotic shock, Λ, as the ratio of $(osm_{out})_\infty$ to $(osm_{out})_o$, i.e., $(osm_{out})_\infty = \Lambda(osm_{out})_o$. Substituting this into the above, and solving for Λ, we arrive at

$$\Lambda \equiv \frac{(osm_{out})_\infty}{(osm_{out})_o} = \frac{V_o}{V_\infty}\left(1 + \frac{\frac{(\Delta P)_o}{RT}}{(osm_{out})_o}\right) - \frac{\frac{(\Delta P)_\infty}{RT}}{(osm_{out})_o} \tag{6}$$

where $(\Delta P)_o/RT$ is constant and $(\Delta P)_\infty/RT$ is itself a function of V_o/V_∞. If $(\Delta P)_\infty/RT = 0$, the equation predicts a straight line plot of the observables, Λ vs. V_o/V_∞. If both hydrostatic pressure differences are zero, then Eq. (6) reduces to the simple $\Lambda = (osm_{out})_\infty/(osm_{out})_o = V_o/V_\infty$. The initial and final concentrations of solutes *S* and *NS* are obtained by the following: Since the solute *S* is in equilibrium in the preparation buffer at time zero (before the shock), then

$$\frac{(C_{S\_in})_o}{(C_{S\_out})_o} = e^{-\bar{V}_S \frac{(\Delta P)_o}{RT}} \tag{7}$$

where $\bar{V}_S$ is the partial molar volume of solute *S*. From (7) and (1),

$$(C_{S\_in})_o = (osm_{out})_o\, e^{-\bar{V}_S \frac{(\Delta P)_o}{RT}} \tag{8}$$

During the relatively short time course of an osmotic shock, *S* appears impermeable so that

$$(C_{S\_in}))_\infty = (C_{S\_in})_o \frac{V_o}{V_\infty} \tag{9}$$

Similarly *NS* is impermeable so that

$$(C_{NS\_in})_\infty = (C_{NS\_in})_o \frac{V_o}{V_\infty} \tag{10}$$

Finally from (1) and (2)

$$(C_{Ns\_in})_o = (osm_{out})_o - (C_{S\_in})_o + \frac{(\Delta P)_o}{RT} \tag{11}$$

Results

Permeability of Mannitol and Cellobiose

Computer simulations results have indicated that the use of a highly impermeant solute in vesicles subjected to osmotic shocks is a critical prerequisite for making valid determinations of mechanical behavior (Rivers & Williams, 1990). To fulfill this requirement we tested the permeability of two commonly employed ''impermeable'' solutes, mannitol and cellobiose. Figure 1 shows the volume changes observed in BBMV prepared with mannitol and cellobiose buffers (18 mosM) and subjected

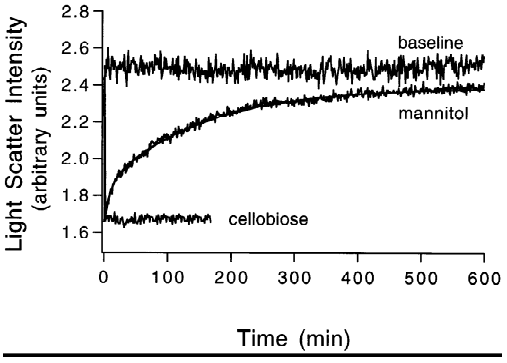

Fig. 1. Mannitol and cellobiose influx studied with a stopped-flow technique. BBMV prepared in mannitol and cellobiose 18 mosM were subjected to osmotic shocks of tonicity 1 (baseline), 4.36 (mannitol) and 4.40 (cellobiose). No reswelling was detected for the vesicles prepared in the cellobiose buffer in contrast with the mannitol buffer where the initial vesicular volume was reestablished after 10 hr. With mannitol, water equilibrates rapidly, establishing a quasi-equilibrium soon after the osmotic shock. At this point the calculated relative volume and mannitol concentration gradient responsible for subsequent mannitol influx are $V_o/V_\infty = 2.3$ and $\Delta C_{man} = 38.5$ mosM. The volume estimate is taken from data in Fig. 5 (*below*), while the concentration gradient estimate is based on Eqs. (8), (9), (10) and (11) together with $\bar{V}_{Man} = 120\ cm^3\ mol^{-1}$ (measured, *see* Materials and Methods), $(\Delta P)_o/RT = 17.3$ mosM, and $(\Delta P)_\infty/RT = 0$. Actual concentrations estimated with these equations are $(C_{Man\_in})_o = 17.6$ mosM, $(C_{Man\_in})_\infty = 40.1$ mosM, $(C_{NS\_in})_o = 16.5$ mosM, $(C_{NS\_in})_\infty = 38.4$ mosM and $(C_{Man\_out})_\infty = 78.5$ mosM.

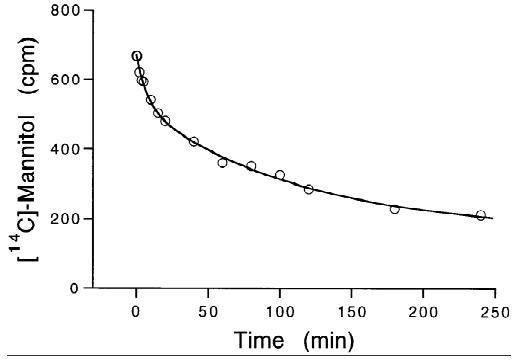

Fig. 2. Mannitol efflux from BBMV loaded with ^{14}C-mannitol. The efflux of mannitol was studied in vesicles prepared in 18 mosM. The data was used to calculate the mannitol permeability $P_{man} = 1.7 \times 10^{-9}$ cm sec^{-1}. This estimate is based on the time constant τ of the exponential fit to the experimental data and was calculated using $P_{man} = V_o/A\tau$ where V_o/A is the vesicular volume to membrane area ratio (vesicular diameter 300 nm, measured by QELS).

to osmotic shocks of $\Lambda = 4.36$ and 4.4, respectively. It can be seen that the vesicles prepared with cellobiose maintain their final volume after shrinkage during at least two and a half hours, while the vesicles prepared in mannitol start reswelling a few minutes after the osmotic shock, reaching their initial volume after several hours.

Interpretation of these results would be compromised if mannitol was actually impermeable but was slowly hydrolyzed into smaller permeable molecules. This was tested by incubation of BBMV (prepared in 18 mosM mannitol buffer) in two different hypertonic mannitol solutions of 41 ± 4 and 141 ± 5 mosM ($n = 4$). After 48 hr, the final osmolarity of the supernatants was not significantly different from their original values (44 ± 2 and 146 ± 4 mosM) indicating negligible mannitol hydrolysis occurred; reswelling of the vesicles (Fig. 1) was apparently only due to mannitol reequilibration.

Internal Hydrostatic Pressure

Results (Fig. 1) with cellobiose show that water equilibrates very rapidly. With mannitol, water also equilibrates rapidly, establishing a quasi-equilibrium soon after the osmotic shock. The position of this quasi-equilibrium slowly shifts as mannitol leaks into the vesicle. The results imply that after the osmotic shock, the inner and outer mannitol concentrations must be different so that mannitol influx occurs. This phenomena is only possible if other molecular species besides mannitol exist inside the vesicles to help support the quasi-equilibrium of water. According to Eq. (11), these species can account for the existence of an initial hydrostatic pressure differences $(\Delta P)_o$ between the inner and outer medium.

Verification that mannitol influx occurs over this time period is shown by data in Fig. 2 which plots the efflux of [^{14}C]-mannitol from vesicles pre-loaded with this tracer. Figures 1 and 2 show similar time courses, but they are not quantitatively comparable because they represent different experimental conditions; Figure 1 reflects the interaction of water and mannitol flux which is complicated by the fact that vesicle volume is continuously changing, while Fig. 2 is a simpler equilibrium exchange at constant volume. As expected, the plot in Fig. 1 is faster because mannitol is entering shrunken vesicles. Using the data of Fig. 2 we estimate the mannitol permeability at $P_{man} = 1.7 \times 10^{-9}$ cm sec^{-1}.

To obtain additional evidence for the existence of non-mannitol internal solutes, the Donnan ratios $(Na^+)_{in}/(Na^+)_{out}$, $(\text{mannitol})_{in}/(\text{mannitol})_{out}$, and $(\text{inulin})_{in}/(\text{inulin})_{out}$ were measured. Their values were respectively 0.068, 0.016, and 0. The ratio $(Na^+)_{in}/(Na^+)_{out}$ is 4.25 greater than the ratio $(\text{mannitol})_{in}/(\text{mannitol})_{out}$, the distribution of the charged species among the two sides of the membrane is asymmetric, presumably due to fixed charge within the vesicle.

Vesicles prepared in 18 mosM mannitol seem remarkably resistant to hypotonic shock. To estimate the maximum hydrostatic pressure difference that the vesicles can support before lysis occurs requires a larger range of test shocks than is accessible with these vesicles. Therefore we used vesicles prepared in 140

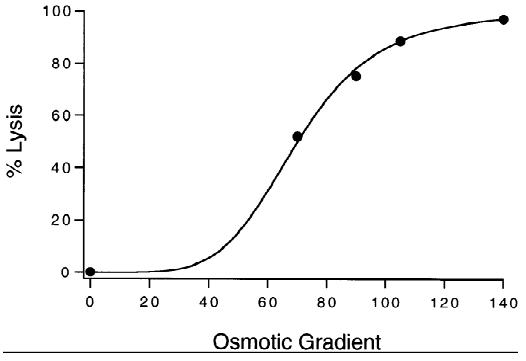

Fig. 3. Hypotonic lysis of BBMV loaded with ^{14}C-mannitol. Vesicles were prepared in 140 mosM mannitol buffer, injected in hypotonic media and filtered immediately (15 sec). The amount of radioactive mannitol that remained inside the vesicles was measured and used to calculate the isotope leakage in the first 15 sec. As the permeability of mannitol is low ($P_{man} = 1.7 \times 10^{-9}$ cm sec^{-1}), this rapid leakage is assumed to indicate lysis.

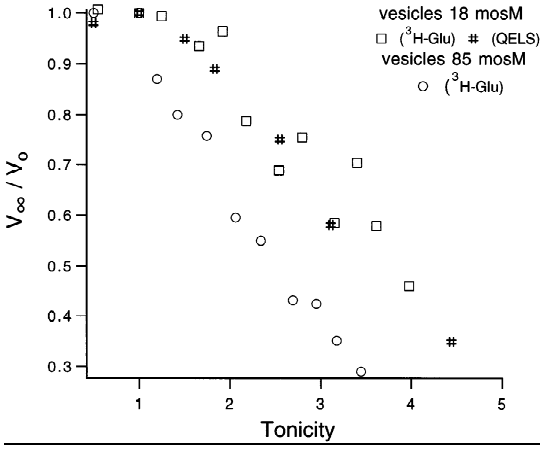

Fig. 4. Equilibrium volume ratio (V_∞/V_o of BBMV subjected to osmotic shocks of different amplitudes as a function of the tonicity of the shock. Equilibrium volumes were measured by vesicle trapped volume experiments and QELS. BBMV were prepared in 18 and 85 mosM cellobiose buffers.

mosM mannitol and assayed the efflux of [^{14}C]-mannitol immediately (first data point at 15 sec) after subjecting them to large hypotonic shocks of different magnitudes. Vesicles prepared in 140 mosM and subjected to an osmotic gradient of 0, 70, 90, 105 or 140 mosM show respectively a 0.1, 52, 75, 89 or 97% isotope leakage within the first 15 sec. Since the permeability of mannitol is so low, we attribute this rapid leakage to lysis of 0.1, 52, 75, 89 or 97% of the vesicles. These results are plotted in Fig. 3.

Volume Dependence on External Osmolarity

When the vesicles are in equilibrium in the preparation buffer (initial volume V_o), they keep a spherical shape (Haase et al., 1978) and a $(\Delta P)_o > 0$ that causes an increment in initial membrane surface tension; a hyposmotic shock will induce a build up of this initial pressure to its final value, so that $(\Delta P)_\infty > (\Delta P)_o$, with a consequent increase in membrane surface tension. On the other hand, a hyperosmotic shock has the opposite effect, dissipating the initial hydrostatic pressure difference so that $(\Delta P)_\infty < (\Delta P)_o$ and decreasing the membrane surface tension.

The mechanical properties of BBMV were determined by following the change in vesicular volume induced by several osmotic shocks using cellobiose as the impermeant solute. Figure 4 shows the dependence of the experimental volume ratio V_∞/V_o on Λ, for BBMV prepared in 18 and 85 mosM cellobiose buffer, measured by vesicle trapped volume experiments. The initial vesicular diameters measured by the QELS technique for the 18 and 85 mosM vesicles were 373 ± 16 and 360 ± 20 nm respectively.

Data of Fig. 4, for hyperosmotic shocks where we can expect dissipation of any internal pressure, provides further evidence for an initial hydrostatic pressure gradient. Setting this final pressure gradient $(\Delta P)_\infty$ equal to 0 in Eq. (5) and rearranging to express the final volume V_∞ in terms of the initial volume V_o, yields

$$V_\infty = V_o \left(\frac{(\Delta P)_o/RT}{(osm_{out})_\infty} + \frac{1}{\Lambda}\right) \qquad (12)$$

It follows from Eq. (12) that V_∞ decreases with increasing $(osm_{out})_\infty$ whenever $(\Delta P)_o > 0$. If $(\Delta P)_o = 0$, the final equilibrium value for V will be identical for all vesicles. This decrease of V_∞ with $(osm_{out})_\infty$ can be seen in Fig. 4 for osmotic shocks with $\Lambda > 2$. By definition (Eq. 6), for any given Λ, vesicles prepared in higher osmolar solutions will have a larger $(osm_{out})_\infty$ than those prepared in more dilute solutions. Figure 4 shows that at any $\Lambda > 2$, the volume of vesicles prepared in 85 mosM is less than those prepared in 18 mosM.

Figure 5 shows the data points of Fig. 4 plotted as suggested by Eq. (6). The linear component of this fit, when $(\Delta P)_\infty/RT$ has collapsed (≈0), is extrapolated by the dashed lines. According to Eq. (7) the slopes of these straight lines is given by $(1 + (\Delta P_o/RT/(osm_{out})_o)$. Using this expression together with measured values of the slopes yields the value of $(\Delta P)_o/RT = 17.3$ mosM. Knowing the value of $(\Delta P)_o/RT$, we can rearrange Eq. (6) to calculate $(\Delta P)_\infty/RT$ as a function of the volume ratio, i.e.,

$$\frac{(\Delta P)_\infty}{RT} = (osm_{out})_o \left(\Lambda - \frac{V_o}{V_\infty}\left(1 + \frac{\frac{(\Delta P)_o}{RT}}{(osm_{out})_o}\right)\right) \qquad (13)$$

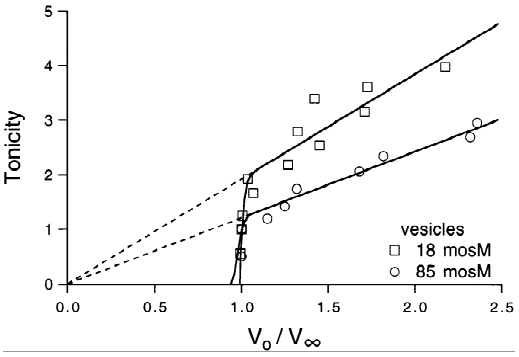

Fig. 5. *Tonicity as a function of the initial to final volume ratio.* From the slopes of the linear fits to the experimental data points where $(\Delta P)_\infty/RT \approx 0$, an evaluation of the $(\Delta P)_o/RT = 17.3$ mosM was obtained (*see* first term of Eq. (6)). Volumes were determined by vesicle trapped volume measurements.

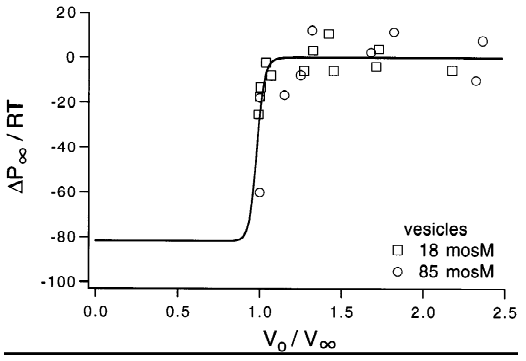

Fig. 6. Influence of the volume ratio on the hydrostatic pressure difference across the membrane. The experimental data points, rearranged according to Eq. (13), using the value of $(\Delta P)_o/RT = -17.3$ mosM were plotted as a function of V_o/V_∞. These points, $(\Delta P)_\infty/RT$, were fit to Eq. (14). The estimated values were $(\Delta P)_{max}/RT = -84$ mosM, $c = 0.98$ and $n = 42$. Volumes were determined by vesicle trapped volume measurements.

Figure 6 shows the calculated values of $(\Delta P)_\infty/RT$ as a function of V_o/V_∞. The fact that both data sets (18 and 85 mosM) are resolved into a single curve lends credibility to this interpretation. To expedite discussion we characterize this relation by an empirical function as

$$\frac{(\Delta P)_\infty}{RT} = \frac{(\Delta P)_{max}}{RT}\left(1 - \frac{(\frac{V_o}{V_\infty})^n}{c^n + (\frac{V_o}{V_\infty})^n}\right) \qquad (14)$$

where $(\Delta P)_{max}/RT = 84$ mosM, $c = 0.98$ and $n = 42$ are adjustable parameters determined by curve fitting the data of Fig. 5. For small values of V_∞, when $V_o/V_\infty \gg c$, $(\Delta P)_\infty/RT$ will tend asymptotically to zero. On the other hand, with large values of V_∞, when V_o/V_∞ tends to zero, $(\Delta P)_\infty/RT$ will tend asymptotically to $(\Delta P)_{max}/RT$. The estimated value of the maximal sustainable pressure, $(\Delta P)_{max}/RT = 84$ mosM, corresponds to approximately 70% lysis (*see* Fig. 3).

Although no special significance is attached to the functional form of Eq. (14), it's sigmoid shape does capture the essential behavior pattern observed in elastoplastic materials under similar conditions (Mendelson, 1968).

Elastic Properties

Mechanical properties of membrane vesicles are reflected by the changes in ΔP that accompany changes in vesicular volume or radius r. Let the membrane surface tension be denoted by σ(N cm^{-1}). Then LaPlace's law for a sphere is given by

$$\sigma = \Delta P\, r/2 \qquad (15)$$

which allows us to compute σ via Eq. (14). These have been plotted in Fig. 7. Note that unlike Fig. 6, the horizontal axis in Fig. 7 increases with r or V).

On the other hand letting k (N cm^{-1}) be the area compressibility elastic modulus, and assuming a linear relation between membrane tension and area expansion (Evans, Waugh & Melnik, 1976) we have

$$\sigma = k\frac{\Delta A}{A} = k\frac{2\Delta r}{r} = 2k\frac{r - r_{min}}{r_{min}} = (2k\frac{r_o}{r_{min}})\frac{r}{r_o} - 2k \qquad (16)$$

where Δr is assumed to be small, and r_{min} denotes the radius where ΔP and σ vanish. It follows that the area compressibility modulus is easily obtained from the slope and intercept of the linear plot of σ *vs.* r.

The plot shown in Fig. 7 yields a value of $k = 2.8 \times 10^{-3}$ N cm^{-1}. The membrane surface tension for vesicles equilibrated with their preparative medium was $\sigma_o = 6.0 \times 10^{-5}$ N cm^{-1}. This corresponds to an initial deformation $(r_o - r_{min}/r_{min} = 0.011$ and an increase in membrane surface area $(\Delta A)_o/A_{min} = 2.2\%$ (Fig. 7). The lower limit of r_{max} (point where membrane lysis can occur) is shown in Fig. 7 and corresponds to an elastic deformation $(r_{max} - r_{min})r_{min} = 0.033$ and to a maximal area expansion $(\Delta A)_{max}/A_{min} = 6.8\%$. The maximal surface tension obtained is $\sigma_{max} = 18.73 \times 10^{-5}$ N cm^{-1}.

Discussion

BBMV prepared in two different solutes (mannitol and cellobiose) and subjected to an osmotic shock with the same solute, behave differently (Fig. 1). While a mannitol influx after the osmotic shock was detected with a

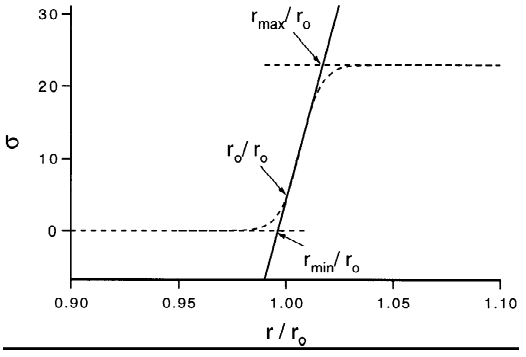

Fig. 7. Change of the membrane tension (σ) as a function of vesicular radius (r/r_o). A straight line fit using equation (16) gave a value of the area compressibility elastic modulus (k) of 5.5×10^{-3} N cm^{-1}. The arrows indicate the radius ratio correspondent to the point where surface tension starts raising from zero (r_{min}/r_o), reaches its equilibrium value for vesicles in the preparation buffer (r_o/r_o), and its maximal value before lysis (r_{max}/r_o).

consequent reswell of the vesicles to their initial volume with a time constant of 7.69×10^3 sec (128 min), a cellobiose influx was not observed for at least two hr. By following the equilibrium exchange of radioactive mannitol, a permeability coefficient for mannitol equal to 1.7×10^{-9} cm sec^{-1} was approximated. This is about one order of magnitude larger than the glucose permeability measured in lipid bilayers (Bresseleers, Goderis & Tobback, 1984; Brunner et al., 1980).

The influx of mannitol observed after the osmotic shock is due to its concentration gradient, implying that other species besides mannitol are present inside the vesicles. At true equilibrium, these species (*NS*) inside the vesicles are presumed to be balanced by a hydrostatic pressure difference between the two sides of the membrane. Further evidence for this hydrostatic pressure difference is derived from detailed measurements of volume changes of BBMV prepared in cellobiose 18 mosM buffer which showed a non linear dependence of volume on the reciprocal of the tonicity of the osmotic shock (Figs. 4 and 5); analysis of the data suggests the existence of an internal hydrostatic pressure (Eq. (6)) with $(\Delta P)_o/RT = 17.3$ mosM (290 mm Hg). The results of Fig. 4 and Eq. (12) show that volume changes induced by the same tonicity shocks ($\Lambda > 2$) depend on the preparation buffer osmolarity in a predictable way.

Evidence for *NS* species is provided by the Donnan ratios; we found a Na^+_{in}/Na^+_{out} ratio (charged species) 4.25 times greater than the ratio $mannitol_{in}/mannitol_{out}$ (noncharged species), thus indicating the existence of counter ions inside the vesicle. Measurement of this ratio by isotopic techniques required the introduction of approximately 0.7 μM of carrier NaCl to the 1 mM charged buffer salt already present. Under these conditions the Donnan ratio of 4.25 requires an internal impermeable anion concentration of 4.02 mEq. Assuming a univalent impermeable anion, this corresponds to an excess internal osmotic pressure of some 6.5 mosM (i.e., beyond the corresponding osmotic pressure in the external medium) which must be balanced by an internal hydrostatic pressure. The remaining (17.3 − 6.5) = 10.8 mosM are presumably contributed by zwitterions or neutral species.

Analysis of mechanical properties of BBMV vesicles revealed an initial membrane surface tension $\sigma_o = 6.0 \times 10^{-5}$ N cm^{-1}, corresponding to an elastic deformation ($\Delta r_o/r_{min}$) of 0.011 and to an increase in membrane surface area ($(\Delta A)_o/A_{min}$) of 2.2%. The elastic deformation ($(r_{max} - r_{min})/r_{min}$) corresponding to the point where membrane lysis can occur is at least 0.033, and the maximal tangential tension is 18.7×10^{-5} N cm^{-1}. Similar values of 10 to 12×10^{-5} N cm^{-1} and 40 $\times 10^{-5}$ N cm^{-1} were found respectively for RBC (Evans et al., 1976) and for membrane tension at lysis in liposomes (Mui et al., 1993), where bilayer defects corresponding to the opening of pores of 12 nm diameter were postulated (Hallett et al., 1993). The relative change in area required to lyse our BBMV preparation was found to be 6.8%, a value similar to the one required to lyse intestinal BBMV (3.6 to 6.6% (Miyamoto et al., 1988)), for hemolysis (2 to 4% (Evans et al., 1976)), and to lyse lipid vesicles (2 to 25% reported from different authors and preparations (Hallett et al., 1993; Hantz et al., 1986; Li et al., 1986; Mui et al., 1993; Rutkowski et al., 1991)). The Table summarizes these values for different systems.

Eq. 14 was introduced as an empirical function to represent the data. The constants c and n have no significance; they are simply adjustable parameters that allow a convenient interpolation as the function runs through the data points. But, $(\Delta P)_{max}/RT$ requires further discussion. It's position on the plots lies beyond the data points and its value is used to estimate r_{max}. However, the estimated value of $(\Delta P)_{max}/RT$ corresponds to 70% lysis, a figure which seems reasonable; e.g., 75% lysis is a common endpoint in studies of red cell hemolysis (Jacobs et al., 1950). Further, our qualitative conclusions are not perturbed by allowing reasonable variations in this parameter. For example, if we fix $(\Delta P)_{max}/RT$ at 108 mosM corresponding to 90% lysis (Fig. 3) and carry out the same analysis then the initial membrane surface tension is 4.8×10^{-5} N cm^{-1}, corresponding to an elastic deformation ($(\Delta r)_o/r_{min}$) of 0.004 and to an increase in membrane surface area ($(\Delta A)_o/A_{min}$) of less then 1%. The elastic deformation ($(r_{max} - r_{min})/r_{min}$) corresponding to the point where membrane lysis can occur is at least 0.017, and the maximal tangential tension is 24.7×10^{-5} N cm^{-1}. These values for 90% lysis also compare favorably with those reported in the literature.

Evidence for the existence of an internal hydrostatic

Table. Comparison of mechanical properties of several membrane systems

Author	Membrane	Area compressibility k (N cm^{-1})	Max. area expansion ΔA_{max} (%)	Pre-lysis membrane tension (σ_{max}) (N cm^{-1})
Our results	BBMV kidney	2.8×10^{-3}	6.8	18.7×10^{-5}
(Miyamoto, Maeda & Fujime, 1988)	BBMV intestine	$8–1.6 \times 10^{-3}$	3.6–6.6	
(Miyamoto & Fujime, 1988)	Cromaffine granules	2×10^{-3}		
(Mui et al., 1993)	Liposomes		3.4	40×10^{-5}
(Hantz et al., 1986)	Liposomes	6.3×10^{-3}	25	
(Rutkowski et al., 1991)	Liposomes	$3.9–5.2 \times 10^{-3}$	2–3	
(Hallett et al., 1993)	Liposomes	3.4×10^{-3}	10	
(Li et al., 1986)	Liposomes	$0.15–2 \times 10^{-3}$		
(Evans, Waugh & Melnik, 1976)	RBC	$0.95–2.88 \times 10^{-3}$	2–4	$10–12 \times 10^{-5}$
(Hochmuth & Waugh, 1987)	RBC	$3.2–5.6 \times 10^{-3}$		
(Waugh & Evans, 1979)	RBC	4.55×10^{-3}		

pressure within BBMV prepared in 18 and 85 mosM solutions includes:

(i) the influx of mannitol that follows an osmotic shock,
(ii) the nonlinear dependence of volume on the reciprocal tonicity,
(iii) the dependence of the volume changes induced by the same tonicity shocks ($\Lambda > 2$) on the preparation buffer osmolarity,
(iv) a Donnan ratio that departs significantly from unity,
(v) the good agreement of consequent mechanical properties with those cited in the literature for similar systems.

Classical thermodynamics predicts that hydrostatic pressures will develop in osmotically balanced systems. The prudent position is to include pressure in any analysis; it is the neglect of pressure that requires justification. In some cases, e.g., osmotic balance of red cells, this justification can be made in terms of geometry and membrane tensile strength. In the case of BBMV, it cannot. Significant internal pressures can be sustained with intact vesicles because of their small radius, *see* Eq. (15).

In addition to providing an additional driving force that could influence transport (especially water transport), the resulting hydrostatic pressure gradient expands the membrane surface area and subjects the membrane to increased tension. This could affect the activity of mechanosensitive channels (Morris, 1990) and play a role in transport regulation. An example of this is suggested in our companion paper on kinetics of water transport in BBMV. Since epithelial transport is very often studied by the use of membrane vesicle systems, kinetic characterization of transporters may need to take these phenomena into account.

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