Elasticity of Synthetic Phospholipid Vesicles and Submitochondrial Particles during Osmotic Swelling†

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ABSTRACT: A rapid and accurate method has been developed for measuring the elastic response of vesicle bilayer membranes to an applied osmotic pressure. The technique of dynamic light scattering is used to measure both the elastic constant and the elastic limit of dioleoylphosphatidic acid (DOPA) and DOPA–cholesterol vesicles and of submitochondrial particles derived from the inner membrane of bovine heart mitochondria. The vesicles prepared by the pH-adjustment method are unilamellar and of uniform size between 240 and 460 nm in diameter. The vesicles swell uniformly upon dilution. The observed change in size is not due to any change in the shape of the vesicles. The data also indicate that the vesicles are spherical and not flaccid. The total vesicle swelling in these studies resulted in a 3–4% increase in surface area for vesicles swollen in 0.15 M KCl and a 5–10% increase in surface area for vesicles swollen in 0.25 M sucrose. This maximum represents the elastic limit of the vesicles. Evidence is presented to show that the vesicles release contents after swelling to this maximum, reseal immediately, and reswell according to the osmotic pressure. For DOPA vesicles in a 0.15 M KCl–tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.55), the observed membrane modulus is found to be in the range of 10^8 dyn/cm^2. The modulus was found to be in the order of 10^7 dyn/cm^2 for DOPA vesicles in a 0.25 M sucrose–Tris-HCl buffer (pH 7.55). This is comparable to that of submitochondrial particles in the same sucrose–Tris-HCl buffer. The observed membrane modulus also descreases with vesicle size. Its magnitude and its variation with ionic strength indicate that the major component of bilayer elasticity is neither the inherent elasticity of the bilayer nor the bending modulus. The variation of the membrane modulus with respect to curvature suggests that its principal component may be related to surface tension effects including the negative charges on the vesicle surface. There is considerable variation between vesicles swollen in sucrose and those swollen in KCl in the membrane modulus, in the elastic limit at which the vesicles burst, and in the transbilayer pressure difference at bursting. The latter was found to be 4–6 mosM (10^5 dyn/cm^2) in sucrose solution and 20–40 mosM (10^6 dyn/cm^2) in KCl solution.

Cation transport across biological membranes plays a major role in the handling of energy by cells. The mechanical properties of membranes are presumed to play a significant role in the transport of ions across membranes. Such properties will vary with lipid and protein composition. Recently, Haines (1979, 1982) has proposed a mechanism for the electrical and mechanical coupling of cation transport activities such as transport in mitochondria during oxidative phosphorylation. In this mechanism, the movement of an ion through a cell membrane produces a compaction (or electrostriction) that in turn produces a standing compaction wave. In order to study the effect of stress developed in membranes during an active cation transport event (Parsegian, 1975), a knowledge of membrane elasticity is required.

Many experiments on ion transport and bioenergetics are conducted on closed unilamellar vesicles derived from biological sources or synthetic phospholipids. Ion transport in such vesicles necessarily results in osmotic swelling (or shrinking) of the vesicles. The stresses placed on a bilayer during osmotic swelling will disturb the conformation and dynamics of both the transport proteins and the lipids. Deformations of this sort presumably effect the transport rate and other properties of membrane proteins. Furthermore, living cells must experience such deformations during transport, cation (such as calcium or proton) signaling, etc. At present, investigators in this field do not generally consider osmolarity in bioenergetic studies on vesicles primarily because accurate measurements on the deformations and/or the elastic limit of bilayers have not been available.

Two methods have been used to measure the elastic sonstants of bilayer membranes. In the first method, membrane capacitance was measured as a function of voltage impressed across it. This produces an electrocompression of the membrane. Crowley (1973) and White (1974) reported a thickness compressibility modulus of 10^6 dyn/cm^2. It was, however, pointed out by Alvarez and Latorre (1978) that the mechanisms by which a voltage-dependent capacitance could arise in black lipid membranes are (a) formation of new membrane from the surrounding torus region of the membrane, (b) exclusion of the solvent from the membrane into the torus, which leads to the formation of microlenses, and (c) thinning of membrane at a constant volume of the membrane. Only the third mechanism appears to have some relevance since biological membranes do not have solvents and are not in equilibrium with a torus region. They measured the membrane capacitance in membrane systems in which the solvent flow effects were minimized. In their experiment, a direct measurement of the membrane thickness as a function of electric field indicated a thickness modulus on the order of 10^8 dyn/cm^2.

The second method consists of micropipet aspiration of a small portion of a membrane and measurement of the ensuing change in area. This method has been applied at first to sea

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urchin egg cells (Mitchison & Swann, 1954) and later to red blood cell membranes (Rand, 1964) and more recently to phosphatidylcholine vesicles (Kwok & Evans, 1981). With this method, the membrane modulus is found to vary through the range 7.3×10^6 to 2×10^9 dyn/cm^2. In their review article, Evans and Hochmuth (1975) conclude that the best experiments involving micropipet aspiration yield a thickness compressibility modulus of about 4.5×10^8 dyn/cm^2 for a 10 nm thick membrane. In this method, a fairly large deformation is required to obtain good measurements.

Attempts to study the elasticity of a model bilayer by the osmotic swelling of unilamellar vesicles in the size range of organelles or whole cells (prokaryote) have been frustrated by two problems. The first is that most vesicle preparations, even when they appear to be uniform in size, have sufficient size variation (standard deviation of size) that will not yield elasticity measurements during osmotic swelling that are useful. Furthermore, preparations of unilamellar vesicles having a size uniformity that might be considered appropriate for such studies are obtained by sonication after the method of Huang (1969). Such vesicles are curvature-limited (Chrzeszczyk et al., 1977; Huang & Mason, 1978). Since curvature fixes their size and is the presumed basis for the uniformity, it is not possible to use these vesicles for osmotic swelling measurements of the membrane modulus.

Second, a method for obtaining precise and rapid measurements of vesicle size to an accuracy of within a few angstroms has not been available. Photon correlation spectroscopy (PCS)[1] is a laser light scattering technique capable of measuring the size and shape of particles in solution with high accuracy (Cummins & Pike, 1973; Berne & Pecora, 1976; Chu, 1974). It has been applied by several investigators to the determination of the size and size distribution of both membrane and phospholipid vesicles (Dahneke, 1983; Goll et al., 1982; Sornette & Ostrowsky, 1981; Norisuye & Yu, 1977; Aurora et al., 1985). Taking advantage of PCS and a recent method that allowed for the synthesis of a highly uniform preparation of large unilamellar vesicles of sizes between 200- and 600-nm diameters (Aurora et al., 1985; Li & Haines, 1986), we have studied osmotic swelling of phospholipid vesicles. An important feature of the method of vesicle preparation is that one may choose the size by varying the conditions of vesicle formation and the lipid composition. The method is a modification of the pH-jump method of Hauser and Gains (1982) using principles of acid-anion formation between acidic lipid head groups as laid out by Haines (1983). This work was undertaken with the goal of developing an accurate and rapid method of measuring membrane elastic constants. The elasticity was found to be linear, and the elastic limit was found to be similar in all of the vesicles and the submitochondrial particles. The membrane modulus was found to decrease slightly with the diameter, to depend largely on surface tension, and to be an order of magnitude lower in 0.25 M sucrose than it is in 0.15 M KCl.

Experimental Procedures

Materials

L-α-Dioleoylphosphatidic acid (DOPA) was procured from Avanti Polar Lipids, Inc. (Birmingham, AL). Its purity was monitored by thin-layer chromatography (TLC) on silica gel 60 plates (EM Laboratories, Elmsford, NY) with $CHCl_3$/CH_3OH/H_2O (65:35:5 v/v/v) as a solvent system. Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Cholesterol (98% pure) was procured from Aldrich Chemical Co., Inc. (Milwaukee, WI). The submitochondria particles derived from the inner membrane of bovine heart mitochondria were kindly provided by Dr. H. S. Penefsky. All chemicals were reagent-grade unless otherwise indicated. All solvents were redistilled before use.

Methods

Photon Correlation Spectroscopy (PCS). Dynamic light scattering was used to investigate the elastic response of bilayer membrane vesicles to an applied osmotic pressure. The PCS techniques and data analysis have been described elsewhere (Aurora et al., 1985). Dynamic light scattering was used to determine the vesicle size. Light from an argon ion laser at 488 nm was focused onto the vesicle sample in a glass cuvette maintained at constant temperature (20 °C) by a Lauda water circulator. The intensity of the scattered light was detected at 90° to the incident beam with a Hamamatsu (Middlesex, NJ) photomultiplier tube. The radius of the vesicles in a sample is calculated from the correlation function. In PCS, one measures the second-order correlation function $g^{(2)}(\tau)$, where τ is the delay time. For spherical particles in Brownian motion, the correlation function decays exponentially with the delay time according to the expression:

$$g^{(2)}(\tau) = B(1 + Ae^{-\Gamma\tau})$$

where B is the base line, Γ is the inverse correlation time, and the A is a constant between 0 and 1 determined by the optical system. Γ is given by $2Dq^2$, where D is the translational diffusion coefficient and q is the scattering vector. D is related to the particle radius R by the Einstein–Stokes expression:

$$D = kT/(6\pi\eta R)$$

where k = Boltzmann's constant, η = viscosity of the solution, and T = absolute temperature. By fitting a single exponential through the correlation data, one can determine the diffusion coefficient (D) and from this the particle radius (R). The light scattering apparatus was calibrated with a standard, monodisperse sample of polystyrene latex spheres (with diameter 126 nm).

Correlation data obtained in the measurement of vesicle size were analyzed by the standard cumulants method from which the z-average radius $\langle R\rangle_z$ and the polydispersity index μ_2/Γ_0^2 were determined. For vesicles in the size range examined, the standard deviation σ of the size distribution is given approximately by $\sigma/R_0 = x\mu_2/\Gamma_0^2$ with $x \sim 3$ (Aurora et al., 1986). This latter work also compared the PCS method of sizing vesicles and determining polydispersity of preparations to electron microscopy, extrusion column chromatography, and internal volume measurements. The studies concluded that PCS measurements were at least as accurate and reproducible as the above techniques.

Preparation of Vesicles. The modified pH-adjustment procedure for making DOPA and DOPA–cholesterol vesicles has been described (Aurora et al., 1985; Li & Haines, 1986). The DOPA concentration in the vesicle suspension was about 1.5 mg/mL. This suspension (3.0 mL) was diluted by adding the same buffer solution as the vesicles were made in (either 0.25 M sucrose–Tris-HCl buffer or 0.15 M KCl–Tris-HCl buffer) to obtain a total volume of 15.0 mL. In order to see the effect of vesicle size on the elastic properties of bilayer, larger DOPA vesicles were required. Larger vesicles could be made by a slight change of the procedure. When the

[1] Abbreviations: DOPA, dioleoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; MDO, membrane-derived oligosaccharides; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; PE, phosphatidylethanolamine; SOPC, stearoyloleoylphosphatidylcholine; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

phospholipid suspension was brought to a high pH in the pH-adjustment procedure, it was allowed to stay at the high pH for up to 2 min; larger vesicles were formed. We also prepared vesicles with different radii by incorporating different amounts of cholesterol in DOPA. The entire experiment was conducted at 20 ± 0.2 °C. In order to check for the presence of any phase transition in the membrane, we studied the vesicles between 15 and 25 °C without conducting any dilution.

Osmotic Dilution of Vesicle Suspensions. Initially, the vesicle dilution was carried out by two methods. In the first method, 1.0 mL of vesicle suspension was poured into each of a number of cuvettes. Each vesicle suspension was then diluted by adding 1.0 mL of a buffer solution. Thus, buffer solutions of different concentration were used for dilution in order to obtain the desired concentration of the vesicle suspension. In this method of dilution, the number of vesicles remained the same for all the cuvettes. In the second method of dilution, 1.0 mL of vesicle suspension was placed in a cuvette, and different amounts of the same diluted buffer were added to it (either 0.15 M sucrose or 0.086 M KCl solution). In this second method of dilution, the vesicle concentration changed during the process of dilution. In our later work, the second method was adopted since it was more convenient and we did not detect any difference in results obtained by the two methods.

The dilution was carried out very slowly in order to prevent the vesicles from experiencing osmotic shocks. A syringe pump (Razel, Stamford, CT) was employed for conducting slow dilution of vesicle suspensions (0.025 mL/min). The vesicle solution was continuously stirred with a magnetic stir bar during dilution and was kept in a closed cuvette (with a small hole in the Teflon stopper for the syringe needle) to prevent evaporation of water, which might give rise to size change by changing the solution concentration. The diluent was added to the vesicle suspension at the rate of 0.025 mL/min. The vesicle suspension and the buffer solutions were filtered through 1.0- and 0.3-μm pore-size filter paper (Nuclepore, Pleasanton, CA), respectively, prior to dilution.

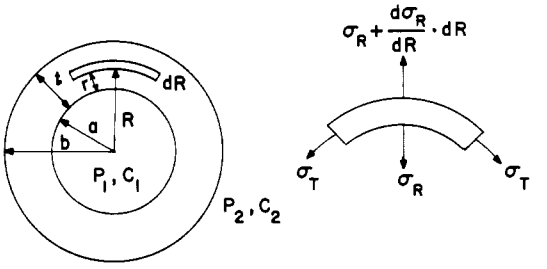
Osmotic Dilution of Submitochondrial Particles. The isolation procedure for bovine heart submitochondrial particles derived from the inner mitochondrial membrane is that of Knowles and Penefsky (1972). The original particle suspension in 0.25 M sucrose–Tris-HCl buffer (pH 7.5) was diluted 40 times by adding 0.25 M sucrose–Tris-HCl (2.0 mM, pH 7.55) buffer then filtered with 1.0-μm pore-size filter paper (Nuclepore, Pleasanton, CA). The dilution procedure was the second one described above. Comparable studies on osmotic swelling in KCl solutions are not possible because the nature of the protein composition of the particles would change on exposure to 0.15 M KCl (Huang et al., 1973).

Calculations

The principle of an osmotic swelling experiment is illustrated in Chart I. Vesicles with initial inner radius a_0 are prepared in an aqueous solution containing C_i (mol/L) of solute (sucrose, KCl, ...). Water is then added to the sample, reducing the final external concentration to C_f, thus producing an osmotic pressure difference across the vesicle wall. Assuming that the vesicle is permeable to water but not to the solute, water will flow into the vesicle, which expands until the final internal concentration C_1 is reached with final radius a, at which point the osmotic pressure difference $P_1 - P_2$ is just balanced by the elastic forces produced by the expansion. Since for dilute solutions the osmotic pressure is proportional to the concentration

$$(P_1 - P_2) = K(C_1 - C_2) = K(C_1 - C_f) \qquad (1)$$

Chart I



where K is the experimentally determined proportionality constant.

Since the total solute content of a vesicle is conserved, the product of concentration and volume is constant. Thus, $C_1a^3 = C_ia_0^3$ or $C_1 = C_i(a^3/a_0^3)$, and eq 1 becomes

$$(P_1 - P_2) = K\left[\frac{C_i}{(a/a_0)^3} - C_f\right] \qquad (2)$$

Two limiting cases can be analyzed immediately. (1) If the membrane is infinitely rigid, the vesicle will not expand, so $a = a_0$ for all C_f. (2) If the membrane is infinitely flexible, the vesicle will expand until the inner and outer pressures are equal, so

$$a = a_0(C_i/C_f)^{1/3} \qquad (3)$$

For real vesicles, the final radius a will increase as C_f is described by dilution, but not as rapidly as in the limit of eq 3. To compute a as a function of C_f, we need to consider the elastic forces in detail.

In Chart I, a small segment of the vesicle wall is shown on the right. It is located at a distance r from the inner wall (or $R = a + r$) and has thickness dR. By symmetry, the segment is subjected to two stresses: a tangential stress σ_T, which is the same in any direction in the plane of the membrane, and a radial stress σ_R, which varies with r from P_1 at the inner surface to P_2 at the outer surface. The requirement that all such elements be in static equilibrium leads to the result (Timoshenko & Goodier, 1951)

$$\sigma_R = \frac{1}{R^3(a^3 - b^3)}[P_2b^3(R^3 - a^3) + P_1a^3(b^3 - R^3)] \qquad (4a)$$

$$\sigma_T = \frac{1}{2R^3(a^3 - b^3)}[P_2b^3(2R^3 + a^3) - P_1a^3(b^3 + 2R^3)] \qquad (4b)$$

For vesicles with thin walls, $b = a + t$, $t \ll a$, and eq 4 reduce to

$$\sigma_T = \frac{a}{2t}(P_1 - P_2) \qquad (5a)$$

$$\sigma_R = \frac{1}{t}[P_1(t - r) + P_2r] \qquad (5b)$$

Note the $\sigma_R/\sigma_T \sim t/a$, and σ_R can therefore usually be neglected. Equation 5a can be derived simply by considering a plane bisecting the vesicle. The net force pushing the two hemispheres apart is $\pi a^2(P_1 - P_2)$. This force is distributed over the intersection of the plane and the wall, an area of $2\pi at$. The tangential stress is therefore

$$\sigma_T = \frac{\pi a^2(P_1 - P_2)}{2\pi at} = \frac{a}{2t}(P_1 - P_2)$$

in agree with eq 5a (Southwell, 1941).

Here we present the central conclusions obtained by assuming that the membrane is isotropic in its plane and that the elastic response is linear. The in-plane logitudinal strain e, which equals the fractional increase in radius $(a - a_0)/a_0$,

is related to the tangential stress σ_T by

$$e = \frac{\sigma_T}{2M_e} \quad (6)$$

where the vesicle elastic modulus M_e is related to the elastic constants C_{ij} (assuming hexagonal symmetry) by

$$M_e = \left(\frac{1}{2}\right)\left(C_{11} + C_{12} - \frac{2C_{13}^2}{C_{33}}\right) \quad (7)$$

Combining eq 2, 5a, and 6 and replacing the final radius a by R_f, we obtain

$$e(1 + e)^3 = \left(\frac{R_f K}{2t}\right)\left(\frac{1}{2M_e}\right)[C_i - C_f(1 + e)^3] \quad (8)$$

which can be solved numerically as a function of C_f for different assumed values of M_e, as shown in Figure 1.

The total membrane modulus contains two additional contributions besides the purely elastic part of eq 7: one is a surface tension effect due to the increase in surface area (and hence surface energy) as the vesicle swells (Evans & Simon, 1975); the second is a bending term due to the decrease in curvature as the vesicle swells (Evans & Hochmuth, 1978). The total membrane modulus M_{total}, which replaces M_e in eq 7, is then

$$M_{total} = M_e + \frac{\gamma}{2t_0} + \frac{10B}{t_0 R_0^2} \quad (9)$$

where t_0 and R_0 are the initial thickness and radius of the vesicle.

Numerical estimates show that the bending term is $\sim 3 \times 10^5$ dyn/cm^2, while the surface tension term is $\sim 10^8$ dyn/cm^2. The values of the observed membrane modulus were found to be in the range of 1×10^7 to 1×10^{10} dyn/cm^2, so the bending term can be neglected. But the surface tension term must be retained and may play a more important role than the elastic term in determining the total swelling of vesicles in response to osmotic pressure changes.

Evans (Evans & Waugh, 1977; Kwok & Evans, 1981) reported an elastic area compressibility modulus K for red cell membranes of 450 dyn/cm at 25 °C. Their K is defined by $T = K\Delta\alpha$, where $\Delta\alpha$ is the fractional change in area and T is the membrane isotropic tension. T is related to the in-plane stress σ_T by $T = \sigma_T t$, where t is the thickness of the membrane bilayer. In eq 7 and 9, we show that the in-plane strain e_1 is related to the in-plane stress σ_T by $e_1 = \sigma_T/2M_{total}$, where M_{total} is the total membrane modulus determined from osmotic swelling experiments. Since $\Delta\alpha = 2e_1$, $\sigma_T = M_{total}\Delta\alpha$ or $T = tM_{total}\Delta\alpha$. Therefore, our total membrane modulus M_{total} is just Evans' area compressibility modulus K divided by the membrane thickness t. For $t = 3.0$ nm, $K = 450$ dyn/cm corresponding to $M_{total} = 1.5 \times 10^9$ dyn/cm^2, slightly larger than the largest values in our experiments (Table III).

Results

Several preliminary experiments were conducted to establish the parameters of the vesicle preparation. The number of vesicles in a given sample was calculated from a knowledge of the amount of phospholipid in the sample, which could be obtained either by phosphate analysis or from the total counts per fraction measured on PCS (Aurora et al., 1985), and the vesicle radii, which were determined from light scattering. It was found to be about 10^{12}/mL in both cases. The two methods of dilution (slow dilution with a low gradient and more rapid dilution with a high gradient) yielded the same

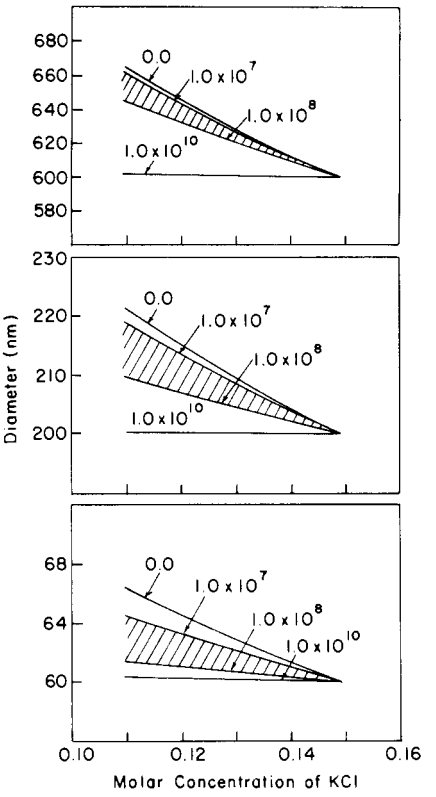


FIGURE 1: Theoretical curves of variation of vesicles with change of concentration of KCl solution (e.g., change of osmotic pressure). These are calculations from eq 8. The elastic membrane moduli (M_e) are indicated on the curves as dyn/cm^2.

results for the diffusion coefficient (and hence the vesicle radius) of a given sample. Since the two methods yield different concentrations of vesicles, this indicates that the diffusion coefficient does not depend on the vesicle concentration in the concentration range that this work was carried out. The transition temperature is below 0 °C for DOPA. The vesicle radii did not change with temperature over the range 15–25 °C, confirming the absence of any gel to liquid-crystal phase transition (Evans & Kwok, 1982) at the temperature this work was conducted.

Theoretical Osmotic Swelling Curves of Vesicles according to Size. Theoretical curves of the variation of vesicle radii with the concentration of the solution are shown in Figure 1. These curves are obtained from eq 8 for different values of the membrane modulus. It is seen from these curves that the percent change in vesicle radii is larger for vesicles that have a large initial radius for a given amount of dilution. The vesicles with radii close to 50 nm are harder to swell. The situation is similar to that of blowing up a balloon, in the sense that it is harder to blow it up when it is small but as the radius increases it becomes comparatively easier to blow it up further. Vesicles with radii of about 200 nm are seen to be better suited for osmotic swelling studies where the expected vesicle elastic modulus is in the range of 10^8 dyn/cm^2. For this reason, we chose vesicles in this size range for our studies.

Vesicle Swelling in 0.15 M KCl Solution. A typical set of osmotic swelling data is given in Table I. It is seen that the vesicle radius steadily increases on swelling. For dilution steps of about 0.004 M KCl each, the swelling is linear for 8–10 dilutions. The index of polydispersity does not change significantly and did not display a trend that related to the swelling in any of our studies.

Figure 2 shows typical results obtained for osmotically swelling vesicles made from DOPA and from DOPA–chole-

Table I: Typical Set of Osmotic Swelling Data[a]

KCl concn (M)	vesicle diameter after swelling (nm)	index of polydispersity
0.150	389.8 ± 1.7	0.08
0.143	390.8 ± 0.9	0.08
0.139	391.6 ± 1.3	0.08
0.135	392.4 ± 1.7	0.08
0.131	393.0 ± 1.9	0.08
0.128	393.8 ± 1.2	0.08
0.125	395.2 ± 1.4	0.08
0.121	396.0 ± 1.2	0.09
0.118	397.0 ± 1.2	0.08
0.115	396.0 ± 1.4	0.08
0.111	395.6 ± 1.3	0.08

[a] The DOPA–cholesterol (15%) vesicles were prepared by the pH-adjustment method in 0.15 M KCl–Tris-HCl buffer. Dilution was made by adding 0.086 M KCl solution at the rate of 0.025 mL/min. KCl concentration in the table refers the concentration outside the vesicle after dilution. The vesicle radii and the index of polydispersity were measured with PCS. These data are also presented in Figure 2, curve E, and in Figure 5.

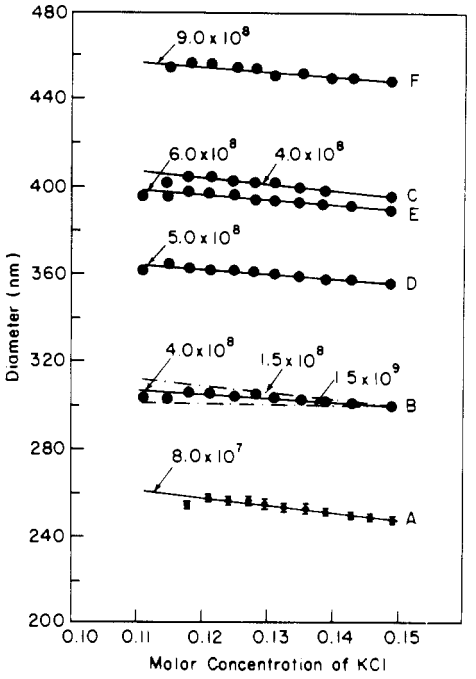


FIGURE 2: Osmotic swelling curves of DOPA (A–C) and DOPA–cholesterol (D–F) vesicles in 0.15 M KCl–Tris-HCl (2.0 mM) buffer (pH 7.55). The vesicles were made with the pH-adjustment method. The DOPA vesicles with diameter of 395.0 nm (C) were made by increasing the period of exposure to high pH during vesicle formation. Curves D–F were 10, 15, and 25% (mol %) cholesterol in DOPA, respectively. Dilutions were made by adding 0.086 M KCl–Tris-HCl (2.0 mM) buffer (pH 7.55) to each sample at the rate of 0.025 mL/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered with the 1.0- and 0.3-μm (pore size) filter paper, respectively. Solid lines are the "observed" membrane modulus calculated from the osmotic swelling curve with eq 8. The error bars (shown on curve A only) represent the error of measurement with PCS and not the standard deviation in vesicle size. The calculated dashed lines on curve B show two calculated membrane moduli so that error in this calculation can be estimated. The heavy line drawn through the first 8–10 dilution steps of each dilution was calculated from eq 8. The subsequent dilution steps were not included in the fit as they fall below the line and are assumed to represent burst vesicles (see Table II). The dashed lines (shown in curve B only) permit an estimate of the error involved in assigning a "best fit" value to the modulus.

sterol in a solution of 0.15 M KCl. In the DOPA–cholesterol vesicles, the amount of cholesterol varies from 10 to 25 mol %. For DOPA vesicles (curves A–C), the observed membrane modulus is found to be in the range (0.8–4.0) × 10^8 dyn/cm^2

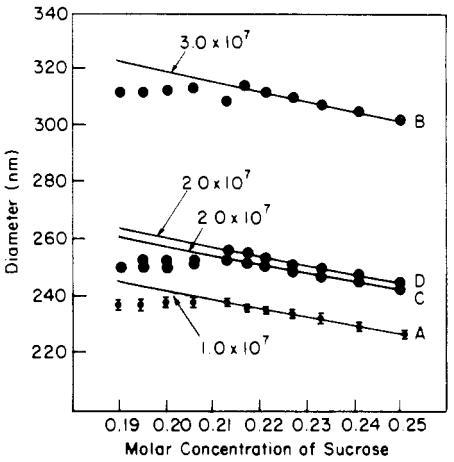


FIGURE 3: Osmotic swelling curves of DOPA (A and B) and DOPA–cholesterol (C and D) vesicles in 0.25 M sucrose–Tris-HCl (2.0 mM) buffer (pH 7.55). The vesicles were made with the pH-adjustment method. The DOPA vesicles with diameter of 300.8 nm (B) were made by increasing the period of exposure to high pH during vesicle formation. Curves C and D were 10 and 15% (mol %) cholesterol in DOPA, respectively. Dilutions were made by adding 0.15 M sucrose–Tris-HCl (2.0 mM) buffer (pH 7.55) to each sample at the rate of 0.025 mL/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered with the 1.0- and 0.3-μm (pore size) filter paper, respectively. Solid lines are the "observed" membrane modulus calculated from the osmotic swelling curve with eq 8. The error bars (shown on curve A only) represent the error of measurement with PCS and not the standard deviation in vesicle size.

for vesicles in the size range 110–200 nm. The addition of cholesterol to DOPA increases slightly the observed membrane modulus (curves D–F); however, addition of cholesterol also increases the size of the vesicles that contain it. Two calculated membrane moduli are shown in curve B to exhibit the allowable error made by this method.

Vesicle Swelling in 0.25 M Sucrose Solution. The results of osmotic swelling of vesicles in a sucrose–Tris-HCl buffer (pH 7.55) are shown in Figure 3. The observed membrane modulus is found to be of the order of 10^7 dyn/cm^2 and is persistently an order of magnitude smaller than that measured in the KCl–Tris-HCl buffer (pH 7.55).

Submitochondrial Particles Swelling in 0.25 M Sucrose. The variation of the size of submitochondrial particles on dilution is shown in Figure 4. The observed membrane modulus is found to be about 3 × 10^7 dyn/cm^2 for the particles that have an average diameter of 200 nm. These particles are also found to rupture after an expansion of 5% of initial diameter (see below).

Vesicle Bursting and Reswelling. In Figures 2–4, it may be seen that the vesicles swell according to the observed membrane modulus (eq 8) to a maximum, at which point the size no longer increases but appears to plateau (or slightly decline). If one considers that the vesicle has reached its elastic limit, then it would be expected to burst, release solution, and return to the unstressed state of the bilayer, which is the original size. The amount of solution released by the vesicle can be estimated by the difference between the vesicles' volume at the maximum (bursting) size and its original (unstressed) volume. The concentration of the interior at its maximum size is presumably the concentration of the released fluid. We may therefore calculate the concentration difference (inside vs. outside) and estimate the osmolarity of the vesicle when it reseals at the original unstressed size. The vesicle having resealed at a new osmolarity, we find that the vesicle reswells to a new size that may be calculated from the observed membrane modulus. Let C_i and R_i be the initial concentration

Table II: Experimental and Calculated Mean Vesicle Sizes after Rupture and Reswelling[a]

vesicle prepn	initial vesicle diam	max vesicle swelling diam	vesicle diam after reswelling	calcd vesicle diam after reswelling	size difference (found − calcd)
Figure 4	186.6	191.4	189.8	188.0	−1.8
Figure 3A	226.0	237.4	233.0	229.8	−3.2
Figure 3C	242.0	252.6	247.6	248.0	0.4
Figure 3D	243.2	254.0	248.0	244.4	−3.6
Figure 2A	247.0	257.4	254.0	251.8	−2.2
Figure 2B	299.0	305.8	303.0	303.0	0
Figure 3B	300.8	313.0	308.0	306.4	−1.6
Figure 2D	355.4	363.4	362.4	306.8	−1.6
Figure 2E	389.8	397.0	396.0	395.0	−1.0
Figure 2C	395.0	404.4	401.2	401.6	0.4
Figure 2F	448.2	456.0	455.2	453.8	−1.4

[a] Sizes are expressed in nm and are only significant to three figures.

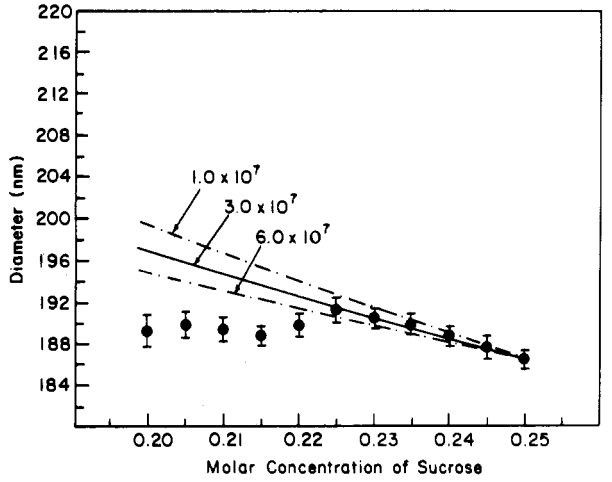


FIGURE 4: Osmotic swelling curve of submitochondrial particles derived from the inner membrane of bovine heart mitochondria in 0.25 M sucrose–Tris-HCl (2.0 mM) buffer (pH 7.0). The particles were a gift of Dr. H. Penefsky. Before osmotic dilution, the original particle suspension was diluted 40 times (volume) in 0.25 M sucrose–Tris-HCl (2.0 mM) buffer (pH 7.55) and then filtered through a 1.0-μm (pore size) filter. Concentration dilutions were made by adding 0.15 M sucrose–Tris-HCl (2.0 mM) buffer (pH 7.55) [filtered through 0.3-μm (pore size) filter] to each sample at the rate of 0.025 mL/min at room temperature. The solid line is the "observed" membrane modulus calculated from the osmotic swelling curve with eq 8. The broken lines allow for an estimation of the error in the observed membrane modulus. The error bars represent the error of measurement with PCS and not the standard deviation in vesicle size.

(both sides of the bilayer) and initial vesicle radius, respectively. When the vesicle swells during dilution, the radius increases until the maximum radius R_f obtains. The concentration outside the vesicles C_f at the maximum vesicle swelling state is known from the amount of diluted solution added. C_{vi}, the concentration inside the vesicles at this state, is given in the equation:

$$C_i\left(\frac{4\pi R_i^3}{3}\right) = C_{vi}\left(\frac{4\pi R_f^3}{3}\right) \qquad (10)$$

This gives

$$C_{vi} = C_i(R_i/R_f)^3 \qquad (11)$$

The vesicle reswells according to its observed membrane modulus, using C_{vi} as the initial concentration (C_i, eq 8) for the reswelling. The calculated radii after vesicle reswelling for each first point on the plateaus in Figures 2–4 are shown in Table II.

It can be seen that for all but 2 of the 11 preparations the vesicles reswell to within 1 nm of the calculated size. The agreement between the calculated and the observed size may be taken as evidence that the plateau begins at the elastic limit of the vesicles.

Discussion

Measurement of Membrane Modulus by Osmotic Swelling. Living cells transport ions as a major component of their energy transduction. Such ion movements necessarily generate concentration gradients with their resultant osmotic pressure deformations of the entrapping membrane. Knowledge of the elasticity of lipid bilayers and of their elastic limit is necessary for a full understanding of ion transport in cells. Measurement of the membrane modulus had previously been undertaken by two methods, membrane capacitance (Alvarez & Latorre, 1978) and micropipet aspiration of a small portion of a membrane (Rand, 1964). The latter method has greater applicability to stress in living cells, but a large deformation is required in order to obtain the measurement. The Rand approach has been used by Kwok and Evans (Kwok & Evans, 1981; Evans & Kwok, 1982; Evans & Needham, 1986) on large (10–20-μm) vesicles made from DMPC (sometimes mixed with DMPE). These investigators used the expression "compressibility modulus".

Osmotic swelling of vesicles is a useful method for studying the membrane modulus because (1) it has immediate applicability to the membranes of living cells, (2) bilayers made of synthetic lipids can be used to explore the impact of specific molecular features on the membrane modulus, elasticity, and its linearity, (3) solution conditions (ionic strength, range of osmolarity, pH, etc.) can be varied to establish their impact on elasticity, (4) the impact of stress on lipid (and protein) conformation and dynamics can be studied by physical methods on uniform preparations, and (5) many fundamental features of *membrane* structure and dynamics can be explored.

Osmotic pressure has only recently been considered from the point of view of its effect on membranes (Gruner et al., 1985). It is so important to living cells that *Escherichia coli* regulates its osmolarity in the periplasmic space by a group of membrane-derived oligosaccharides (MDO) (Kennedy, 1982), which in turn regulate phospholipid turnover (van Golde et al., 1973).

Obtaining the membrane modulus by osmotic swelling requires a uniform preparation of unilamellar vesicles in a size range appropriate for such studies. Calculations showed (Figure 1) that the appropriate vesicle size for measuring a modulus of 10^7 or 10^8 is in the 100–400-nm diameter range. Uniform preparations of unilamellar vesicles have only been available in much smaller sizes (20–100-nm diameter). We recently developed a procedure for making uniformly sized unilamellar vesicles in a range of choice from 200- to 600-nm diameter (Aurora et al., 1985; Li & Haines, 1986). Uniform vesicles were obtained by exposing protonated anionic lipids

to a pH jump in 150 mM KCl or in 250 mM sucrose. The high pH removes protons, which, according to the acid-anion theory of head group interactions (Haines, 1983), represent attractive forces between head groups. The greater the repulsion on the inner monolayer, the greater the curvature of the resulting vesicle. Vesicle sizes were further increased (Aurora et al., 1985) by inclusion of a significant fraction of cholesterol in the phospholipid during vesicle formation. The cholesterol serves, in this model, to increase the average head group distance (Rand & Luzzati, 1968) in phospholipid bilayers that contain it and thereby decreases H bonding and increases the repulsion on the inner monolayer.

The vesicle size was found to increase linearly in discreet increments during 8–10 steps of concentration dilution (Figures 2–4). The observed membrane moduli shown in each of these dilution experiments are calculated from the experimental results with eq 8. They were chosen because they align closely with the data points ("best fit"). It is possible, nonetheless, that the increase in the average size of the vesicles during dilution is not due to the stress of swelling but to a shape variation that results in spheres at its maximum size. Such shape changes were suggested during an osmotic swelling study of vesicles in the size range of 40.0-nm diameter (Lichtenberg et al., 1981). It should be noted that the KCl concentration range used in that study was of the order of 10 times that of this study. Furthermore, the elastic limit as established in this study appears to be exceeded in the Lichtenberg et al. study. Finally, the vesicle size they used was so much smaller than that of this work that the difference may not be applicable. Kwok and Evans (1981; Evans and Kwok, 1982) found a surface area increase of 2–3% for DMPC vesicles in the 10–20-μm size range. We find the surface area increase to be 3–4% for the DOPA (anionic lipid) vesicles that are of the 0.2–0.6-μm size range in 0.15 M KCl. The vesicles in the same size range show a 5–10% surface area increase in 0.25 M sucrose. It should also be noted that the observed membrane modulus increases with the size of the vesicle in our study and the percent surface area increase decreases with the increasing vesicle size. All of these data are consistent with the work of Evans and Kwok.

Before concluding that the linear increments in observed size are due to surface expansion and not due to a nonspherical equilibrium configuration that become spherical upon dilution of the KCl, we examined the polydispersity during swelling. In the typical results of a swelling experiment shown in Table I, it may be seen that the polydispersity remained constant throughout the experiment. Identical results were obtained in all of the swelling experiments. Had large changes occurred in shape of the vesicles upon dilution, the polydispersity would have changed. We therefore conclude that the vesicles were spherical throughout the experiments. This is consistent with our observations in electron microscopy (Aurora et al., 1986). The observed increase in internal volume is therefore assumed to be due to stretching of the bilayer.

Experiments designed to examine the impact of cholesterol on the observed membrane modulus in both KCl (Figure 3) and sucrose (Figure 4) preparations showed that neither the presence of cholesterol nor its concentration had a significant effect. Because vesicle size in KCl was dependent upon cholesterol content, a special preparation of vesicles (Figure 2, experiment C) was made (400-nm diameter) in the size range of cholesterol-containing preparations as a control; these exhibited an only slightly smaller observed membrane modulus than the cholesterol-containing preparation (Figure 2, experiment E) of equivalent size. Likewise, cholesterol-containing vesicles made in sucrose displayed the same observed membrane modulus (Figure 3) as those lacking cholesterol.

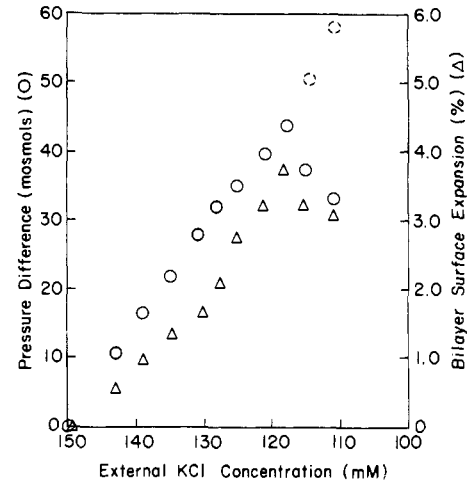


FIGURE 5: Transbilayer pressure difference (O) during osmotic swelling of vesicles described in Table I (also Figure 2, curve E). The KCl that was trapped inside the vesicles at the start of the swelling experiment was assumed to remain in the vesicle as it expanded. The concentration difference is used to calculate the transbilayer pressure difference with eq 2; the dashed circles assume that the vesicle did not burst whereas the corresponding solid circles are calculated based on the assumptions used in Table II for bursting and reswelling. The surface area expansion (Δ) suggests by the decrease in size at 43 mosM that the vesicles have burst. The vesicles (390 nm) are DOPA containing 15 mol % cholesterol.

Measurement of Elastic Limit. After 8–10 size measurements during dilution in which stepwise swelling was observed, each of the samples displayed a break in the swelling curve—a plateau. The vesicles appear to have been ruptured at the break, but they did not return to their original unstressed size. Measurement of the sizes at each point on the plateau allowed us to calculate the size expected if the appearance of the plateau did indeed represent the elastic limit; the calculation is based on the following assumptions: (1) that the initial quantity of diluent (KCl or sucrose) that was trapped in the vesicles remained there throughout the swelling; (2) that the vesicle is ruptured at its elastic limit and returned to its unstressed (initial) size; (3) that the solution ejected at the rupture contained the same concentration of diluent as was present in the vesicle at the time of the rupture; (4) that the amount of solution ejected is equal to the change in volume from the swollen state (just before the elastic limit) to the unstressed vesicle state; (5) that the vesicle reseals immediately at the unstressed state and reswells to the appropriate size predicted by its observed membrane modulus.

The results of this calculation are shown in Table II. We find that the amount of ejected solution is insufficient, at the inside concentration of the diluent, to equilibrate the vesicle with the external concentration. Each of the points in Table II represents a ruptured vesicle preparation that swells beyond its elastic limit. The calculated sizes based on the above assumptions, all of which are reasonable, show remarkable agreement with the experimental data. We have therefore taken the last dilution on the observed membrane modulus as the size of the vesicle at its elastic limit.

Figure 5 uses the sample data in Table I to display the surface expansion and the transbilayer pressure difference during an expansion of the vesicles. The transbilayer pressure difference is calculated with the assumption that the internal quantity of KCl remains that which was trapped in the vesicles at the start of the experiment and diluted by the vesicle expansion. The surface expansion reaches a maximum at the

Table III: Observed Membrane Moduli, Surface Area Increase, and Pressure Difference (ΔP) across the Membrane at Bursting of Vesicle Preparations in KCl and Sucrose Solution

vesicle prepn	diluent	initial mean diam (nm)	mol % cholesterol	% surface increase	obsd modulus ($\times 10^{-7}$ dyn/cm^2)	ΔP mosM	ΔP $\times 10^{-6}$ dyn/cm^2
Figure 2F	KCl	448.2	25	3.5	90	44	1.1
Figure 2C	KCl	395.0		4.8	40	41	1.0
Figure 2E	KCl	389.8	15	3.7	60	43	1.1
Figure 2D	KCl	355.4	10	4.6	50	46	1.2
Figure 3B	sucrose	300.8		8.4	3	4	0.1
Figure 2B	KCl	299.0		4.7	40	40	1.0
Figure 2A	KCl	247.0		8.6	8	23	0.6
Figure 3D	sucrose	243.2	15	9.0	2	6	0.2
Figure 3C	sucrose	242.0	10	9.0	2	6	0.2
Figure 3A	sucrose	226.0		10.3	1	4	0.1
Figure 4	sucrose	186.6		*a*	3	*a*	*a*

[a] The initial size of the submitochondrial particles does not permit a calculation of the percent surface increase as they may already have been in a partially swollen state at the beginning of a swelling experiment.

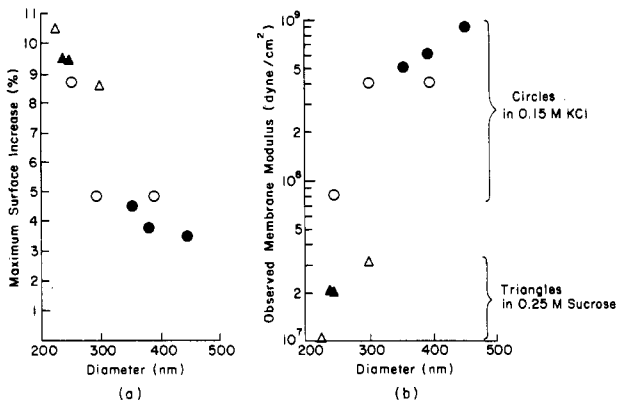


FIGURE 6: Variation of elastic limit (a) and observed membrane modulus (b) with initial vesicle size. The data are shown in Table III. The vesicle preparations were made in 0.15 M KCl solution with DOPA (○) and DOPA–cholesterol (●), respectively. The vesicle preparations made in 0.25 M sucrose solution are with DOPA (△) and DOPA–cholesterol (▲), respectively.

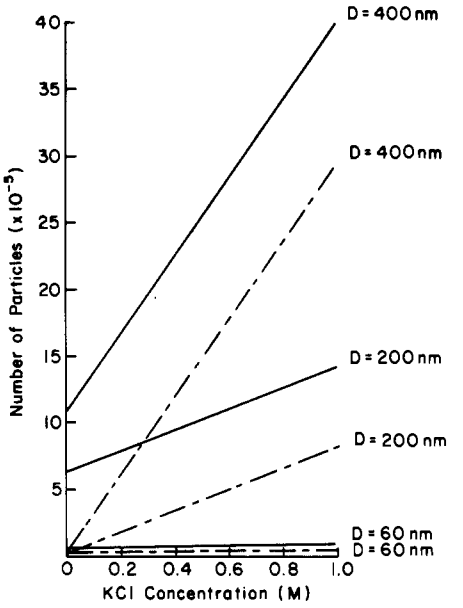


FIGURE 7: Vesicle bursting curves. Vesicles burst above the lines indicated in the figure. Vesicle preparations derived from either biological sources or reconstituted lipids with ion-transport proteins could be made in KCl solution or other salt solutions. Vesicles swell under osmotic pressure produced by ions that are transported into the vesicle. Knowledge of the elastic limit allows an assessment of bursting during experiments in which ions are pumped in. The number of ions needed to burst a vesicle is calculated according to eq 8. The elastic limits for 60-, 100-, and 200-nm (diameter) vesicles are based on the experimental results in this work. They are 5.0, 5.0, and 2.3% overall increases in diameters, respectively.

transbilayer pressure difference of 43 mosM. We have assumed that the vesicles have burst releasing trapped KCl (see Table II and discussion above) in order to to obtain the decreased transbilayer pressure difference (eq 2) shown in the full circles. The dashed circles assume no KCl has been removed from the vesicles, but the decreased size of the vesicles suggests that this is not the case.

Table III shows the elastic limit in terms of increased surface area for all of the experiments reported here. Vesicles swelled in sucrose display substantially larger elastic limits (Figure 6a) than those in KCl. This includes both the synthetic phospholipid vesicles and the submitochondrial particles. By and large the elastic limit (maximum area increase before bursting) was found to decrease with increasing initial size (Figure 6a) while the observed membrane modulus was found to increase with increasing size (Figure 6b). Therefore, the elastic limit varies inversely with the observed membrane modulus. The elastic limit of the vesicles is relatively constant, however, in the range of 3–10%. With one exception the vesicles swollen in 150 mM KCl showed surface expansion of 3–5%, and those swollen in 250 mM sucrose showed 8–10% expansion. The stiffer the vesicle the smaller the expansion before it bursts (i.e., a large modulus implies a smaller elastic limit). The transbilayer pressure difference at bursting is significantly greater for vesicle swollen in KCl than for those swollen in sucrose.

The studies of "flicker phenomena" on synthetic vesicles by Servuss et al. (1976) first obtained a bilayer bending modulus on synthetic vesicles from which an elastic modulus could be obtained. The recent work of Evans (Kwok & Evans, 1981; Evans & Kwok, 1982; Evans & Needham, 1986) has expanded this work and applied it to zwitterionic model membrane systems. Our work cannot quite be compared to the work of Evans since our vesicles are not made of zwitterionic lipids. The equivalent membrane modulus obtained for ~10-μm phosphatidylcholine vesicles by Kwok and Evans (1981) ranges from 5×10^8 to 2×10^9 dyn/cm^2 for DOPC and SOPC with or without cholesterol (assuming the membrane is 3.0 nm thick) whereas those for our largest polyanionic vesicles (0.45 μm) are 9×10^8 dyn/cm^2. The data show that the ranges are not inconsistent. The present method has the disadvantage of being restricted to negatively charged lipids, while it has the advantages of speed of obtaining results with choice of vesicle sizes in the range of cells and organelles and synthetic ion-pumping systems.

Figures 7 and 8 are designed to show the bursting limits expected for vesicles as the salt concentration and the vesicle

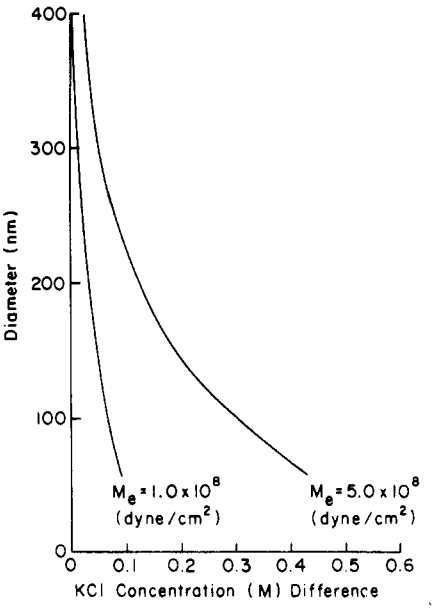


FIGURE 8: Vesicle bursting limit curves. Vesicles burst to the right of the lines indicated by their membrane elastic modulus. It can be seen that the elastic limit varies with the vesicle size and the salt concentration. The minimum concentration difference (inside vs. outside) required to burst a vesicle is calculated from eq 8. The elastic limits for 60-, 100-, and 200-nm (diameter) vesicle are based on the experimental results in this work. They are 5.0, 5.0, and 2.3% overall increases in diameters, respectively.

size varies. Such curves will be useful for the design of experiments involving vesicles in which ions are pumped into the vesicle. It should be noted that bursting does not release the entire contents of the vesicle but only sufficient contents to relieve the pressure so that the vesicle membrane returns to its relaxed state. This is demonstrated by Table II.

Curvature and Membrane Modulus. There is also a trend shown in the data (Table III; Figure 6) that the observed membrane modulus varies with the curvature. Although the trend is by no means linear, it is apparent in both the 150 mM KCl and the 250 mM sucrose swelling experiments. We have attempted to explain these data under Calculations considering the elastic modulus, the bending elastic modulus, and surface tension. Tolman (1949) showed that the surface tension of a spherical surface varies with its curvature. He showed the relationship to be

$$\frac{\sigma}{\sigma_0} = \frac{1}{1 + \frac{2\delta}{R}}$$

where σ is surface tension, δ is a constant, and R is the radius of the sphere. To obtain a lowering of surface tension of say 4%, a value of 0.02 is necessary for δ/R; thus, δ is of the order of magnitude of 10^{-8} cm, too small to explain our observations (and in the wrong direction!). The application of Tolman's theory to our system therefore cannot explain our data on the variation of the membrane modulus with curvature.

Nonetheless, our data suggest that the magnitude of the observed membrane modulus is best explained by surface tension and that it is not due to the inherent elastic modulus nor to the bending energy.

The energy per unit area at the surface (surface tension) includes both hydrophobic interactions and electrostatic contributions due to the surface charges. All of our measurements are conducted on negatively charged lipid systems (although the submitochondrial particles contain both zwitterionic and anionic lipids and proteins, the net surface charge is negative). The fact that the observed membrane modulus is substantially (an order of magnitude) higher in 150 mM KCl than in 250 mM sucrose suggests that charges may dominate the magnitude of the modulus we are observing. The suggestion that anionic charges have much to do with membrane elasticity is consistent with the observations of Kogut and Russell (1984) that the adaptation of *Vibrio costicola* to high-salt medium is associated with an increase in the PG component of the membrane phospholipids at the expense of PE, a zwitterionic lipid. They also noted that reduction of the salinity reversed this adaptation. Furthermore, all of the obligate halophiles contain only anionic lipids, no zwitterionic. A halotolerant Gram-negative organism, *Planococcus* sp., increases its anionic lipids component in response to increase osmolarity (Miller, 1985). This paper contains many references to other work indicating similar observations.

A second area of interest with respect to cell osmolarity and negative charges on the surface is the MDO system of *E. coli* (Weissborn & Kennedy, 1984; Kennedy, 1982). A decrease in the osmolarity of the culture medium [from 300 mosM, which is the osmolarity of the cytosol (Stock et al., 1977) to 50 mosM] provokes *E. coli* to produce polyanionic oligosaccharides on the periplasmic side of the plasma membrane. Comparisons of the membrane modulus of lipid vesicles with different surface charge densities and variations in the ionic strength and surface pH may help to resolve the contributions to the surface energy of hydrophobic interactions, double-layer effects, spherical field effects, and head group interactions. Such experiments will require systematic studies.

ADDED IN PROOF

A study of the elastic modulus of curvature-limited vesicles (<40.0-nm diameter) showed no osmotic swelling (Sun et al., 1986). Slightly larger DMPC vesicles displayed moduli ranging from 33.0×10^7 dyn/cm^2 (42.6-nm vesicles) to 3.4×10^7 dyn/cm^2 (53.0-nm vesicles) decreasing incrementally with each increase in diameter. These results are of the same order of magnitude as those reported herein.

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Registry No. L-α-DOPA, 61617-08-1; KCl, 7447-40-7; cholesterol, 57-88-5; sucrose, 57-50-1; Tris, 77-86-1.

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