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The Elasticity of Uniform, Unilamellar Vesicles of Acidic Phospholipids during Osmotic Swelling Is Dominated by the Ionic Strength of the Media

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ABSTRACT: Uniform, unilamellar vesicles have been prepared by the pH-modification technique. The initial sizes of the vesicles were from 200 to 700 nm and were measured to within 1–3% by photo correlation spectroscopy. Vesicles were made of the dioleoyl esters of phosphatidic acid, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, the diphytanyl ethers of phosphatidylglycerol, *Escherichia coli* lipids, and lac permease reconstituted into *E. coli* lipids. The vesicle suspensions were prepared and then diluted with electrolyte (KCl) and/or nonelectrolyte (sucrose, trehalose, pentaerythritol) impermeants. The amplitude of the swelling is linearly proportional to the osmotic pressure difference across the bilayer. We have determined the elastic modulus, the elastic limit (percent surface expansion at bursting), and the transbilayer pressure difference at bursting for each of these vesicles at constant osmolarity but at different ionic strengths. We find that the elastic properties of the bilayer vary by a factor of 10 in electrolyte media as compared to isosmolal nonelectrolyte media and that this variation appears to be related to both the charge density at the surface and the ionic strength of the media. Anionic lipid vesicles in 150 mM KCl have a significantly higher modulus (50×10^7 dyn/cm^2) and transbilayer pressure difference (40 mosM) at bursting with a small capacity to stretch (3–4% surface expansion) compared to the same vesicles suspended in nonelectrolyte impermeants. The latter vesicles undergo a significant surface expansion (8–10%), display a low modulus (3×10^7 dyn/cm^2), and burst at 3–4 mosM bilayer pressure difference. Vesicles suspended in media of constant osmolarity at various ionic strengths display properties with proportional values. Vesicles burst when diluted from 150 to 120 ± 5 mM KCl or from 250 to 215 ± 5 mM sucrose. This is a considerably narrower range of dilutions than has been examined by studies on the effects of osmotic stress on bilayers in the literature. A comparison of the mechanical properties of bilayers comprised of different lipid head groups shows that dioleoylphosphatidylethanolamine displays the properties of an acidic lipid with an unusually high modulus, low surface expansion, and high transbilayer pressure difference at bursting. This contrasts with dioleoylphosphatidylserine, which displays an unusually low modulus, moderate surface expansion, and a low transbilayer pressure difference at bursting. Vesicles of reconstituted *E. coli* lactose carrier into the cell's lipids display an even higher modulus than phosphatidylethanolamine, which dominates the bacterium's lipid composition.

Vesicles of pure phospholipids have been used as models for an understanding of the properties of biological membranes since the early experiments of A. D. Bangham and his coworkers, who showed that synthetic phospholipids could form spontaneous bilayers (Bangham et al., 1965; Bangham, 1968, 1972) with osmotic properties (Bangham et al., 1967). Most of this work has been conducted on multilayered liposomes, a complicated system for studying osmotic swelling as has recently been shown by comparing the osmotic properties of standard preparations of open-ended rolls to multilamellar closed concentric spheres made by a new preparation procedure (Gruner et al., 1986). Very recently, osmotic studies have been conducted on unilamellar vesicles including giant vesicles (10 μm) (Kwok & Evans, 1981) of DMPC, sonicated (SUV)[1]

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[1] Abbreviations: BLM, bilayer lipid membrane; ΔP, transbilayer pressure difference at bursting; DMPC, 1,2-dimyristoyl-3-*sn*-glycerophosphocholine; DOPA, 1,2-dioleoyl-3-*sn*-phosphatidic acid; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DOPE, 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine; DOPG, *rac*-1,2-dioleoyl-3-*sn*-phosphatidylglycerol; DΦPC, diphytanoylphosphatidylcholine; DΦPG, diphytanylphosphatidylglycerol; EM, electron microscope; EPC, egg phosphatidylcholine; HDTA, hexadecyltrimethylammonium chloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LUV, large unilamellar vesicles; MDO, membrane-derived oligosaccharides; PCS, photon correlation spectroscopy; SUV, sonicated (small) unilamellar vesicles; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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vesicles (40–60 nm) (Milon et al., 1986; Sun et al., 1986) of DMPC, and large (LUV) vesicles of DOPC or DMPC (160–180 nm) (Hantz et al., 1986) or of DOPA (200–600 nm) (Li et al., 1986). The significance of osmotic pressure effects on membranes can hardly be understated since ion transport is central to bioenergetics. Most eukaryotic cells are 10 μm, and the organelles in which the energy is transduced are about 200–600 nm in size. Osmotic stress on membranes has also been of practical interest in the cryobiology of plants (Steponkus & Wiest, 1979) and the minimization of hemolysis after the storage of frozen erythrocytes (Takahashi et al., 1986).

Osmotic swelling of biological membranes has been a subject of investigation since Leeuwenhoek recognized the erythrocyte shape in 1675. The literature of erythrocyte swelling is voluminous [for a recent review see Mohandas et al. (1983)]. As Mohandas et al. state, "...most of the deformations occurring in vivo, as well as most observed in vitro, involve no increase in surface area. The normal red cell can undergo large linear extensions of up to 230% of its original dimension, but an increase of even 3% or 4% in surface area results in lysis." A subtle distinction that comes forward in this summary of the elasticity of the erythrocyte membrane is that the observations on erythrocyte swelling are by and large observations on the cytoskeleton of the erythrocyte and that the 3–4% surface area increase appears only after this spectrin network has been disrupted so that it is no longer in intimate contact with the lipid bilayer. This distinction of the bilayer's expansion as a material as opposed to increased erythrocyte volume was experimentally sorted out by Evans (Kwok & Evans, 1981; Evans & Waugh, 1980; Evans et al., 1976). Bilayer tension has been studied in other biological membranes including sea urchin eggs (Cole, 1932; Mitchison & Swann, 1954), giant algal cells (Kozuge & Tazawa, 1979), neutrophils (Sung & Chien, 1978; Sung et al., 1982), isolated rye protoplasts (Wolfe & Steponkus, 1981, 1983), and submitochondrial particles (Li et al., 1986).

The capacity of bacteria to deal with osmotic swelling has also come to the attention of biochemists with the recent identification of at least three operons in *E. coli* that are specifically regulated by osmotic changes in the culture media: the *kdp* operon, which responds to osmolarity changes by altering the potassium ion concentration in the cell (Laimins et al., 1981); the *omp* operon, which among other responses regulates the size of the pore formed by the protein porin in response to changes in osmolarity (Comeau et al., 1985); the *mdo* operon, which initiates the biosynthesis of MDO (membrane-derived oligosaccharides), polyanionic oligoglucosides that regulate osmolarity in the periplasmic space protecting the inner membrane (Weissborn & Kennedy, 1984). Several bacteria that are halotolerant respond to an increase of the salinity of the culture medium by increasing the anionic component of their lipid composition. This includes both halophilic Gram-negative bacteria (Ohno et al., 1976, 1979; Kogut & Russell, 1984; Hanna et al., 1984) and halotolerant Gram-positive bacteria (Kanemasa et al., 1972; Komaratat & Kates, 1975; Miller, 1985). A well-investigated eukaryote, *Dunaliella salina*, has a unique osmoregulatory system that allows it to survive in a wide range of salinities by synthesizing glycerol in response to increased salt in the culture medium (Ben-Amotz & Avron, 1983) so that the glycerol content may exceed 50% of the total dry weight of the organism.

Membrane elasticity and curvature are both features of the response of a bilayer to osmotic stress, and information about both may be obtained from studies on osmotic swelling. Several groups have begun in recent years to develop a picture of membrane elasticity and of curvature. A number of reviews and theories from different points of view are now available (Helfrich, 1973, 1974; Scheetz & Singer, 1974; Deuling & Helfrich, 1976; Israelachvili et al., 1976; Petrov et al., 1978; Evans & Hochmuth, 1978; Evans & Skalak, 1979; Haines, 1979; Doniach, 1979; Israelachvili et al., 1980; Dimitrov, 1981; Gruner et al., 1985b). Most of these reviews and theories focus on fundamental features of the biological membrane as a material sheet. Osmotic swelling of sized unilamellar vesicles permits the study of both elasticity and curvature. These are related in turn to the area per lipid molecule in the membrane, which is a fundamental parameter in the statistical mechanical models that have been proposed for bilayers (Nagle, 1973, 1980; Marcelja, 1974; Jacobs et al., 1975; Berde et al., 1980; Scott, 1977; McCammon & Deutch, 1975; Jackson, 1976; Pink, 1982; Kirk et al., 1984; Gruner et al., 1985b). Many of these models can be probed by imposing an enforced increase in the area per molecule by membrane stretching during osmotic stress. Quantitation of the stressed state may permit measurement of the intermolecular forces of lipids and the role of lipid structure in those forces.

One would expect, for example, that lipid dynamics would be altered by osmotic swelling. Several studies have been conducted on the effect of osmotic stress on "fluidity" (chain motion) in the bilayer using both fluorescent and ESR probes. With two exceptions (Rigaud et al., 1974; Nunes, 1981) it is found to increase with an increased osmotic pressure difference across the bilayer (Borochov & Borochov, 1979; Surewicz, 1983; Curtain et al., 1983; Barkai et al., 1983; Takahashi et al., 1986). Araki and Rifkind (1981) have shown that the rate-limiting step of hemolysis of erythrocytes correlates with membrane fluidity. The T_m of a bilayer decreases as both osmotic pressure and external pressures are applied (Barkai et al., 1983). In all of these studies relatively large osmotic gradients (>300 mosM), which we have shown to be well in excess of that necessary to burst vesicles (Li et al., 1986), have been impressed on the bilayers.

We have developed a method (Aurora et al., 1985; Li & Haines, 1986) for making uniform vesicles of anionic phospholipids and of mixtures containing anionic phospholipids in the size range from 200 to 600 nm. Sun et al. (1986) have shown that osmotic swelling cannot be conducted on vesicles smaller than 40 nm and that such studies in the 40–50-nm (the largest they examined) range are dominated by the curvature. Hantz et al. (1986) studied the elasticity of DMPC and DOPC vesicles that ranged in size from 160 to 180 nm. They found that the response was not linear, showing slight deviation suggesting that curvature was affecting the modulus measurement. DOPA vesicles in the 200–450-nm range could be swollen linearly (Li et al., 1986) during six to eight dilutions of impermeants; after this linear expansion, the mean size of the preparation no longer increased nor did the vesicles return to the size (the relaxed state) at which the dilution began. The end of the linear expansion of the vesicles represented the elastic limit of the DOPA bilayer. It was possible to calculate (Li et al., 1986) the sizes obtained in the region of the dilution curve once the elastic limit was reached by assuming the following: (1) The vesicles contained the original impermeant before the swelling experiment began. (2) Upon exceeding the elastic limit, the vesicles burst and relieved themselves of sufficient contents so that they returned to their original size (the relaxed state of the bilayer). (3) The concentration of the solution expelled was that of the interior at the time of bursting. (4) The vesicles immediately resealed. (5) The vesicles reswelled to the new size determined by the new

difference in concentration between the inside and the outside of the vesicles.

We have now made vesicles of a variety of phospholipids and mixtures of phospholipids and studied the expansion of the bilayer during osmotic swelling under a variety of conditions. We find that the charge density at the bilayer surface and the ionic strength of the surrounding water, taken together, dominate (1) the membrane modulus, (2) the maximum surface expansion at bursting, and (3) the pressure difference across the bilayer at bursting (ΔP). These effects are independent of the osmolarity of the medium.

Materials and Methods

Materials. DOPA, DOPG, DOPE, DOPS, DOPC, *E. coli* PE, and *E. coli* lipids were procured from Avanti Polar Lipids, Inc. (Birmingham, AL). They were checked for purity by TLC. DΦPG was isolated from cells of *Halobacterium cutirubrum* by Laura Stewart in Dr. Morris Kates laboratory, Ottawa, Canada, using his procedure (Kates, 1978). Lactose carrier was prepared and reconstituted into vesicles in H. Ronald Kaback's laboratory at the Roche Institute for Molecular Biology, Nutley, NJ, according to Viitanen et al. (1985). The purity of the lipids was monitored by TLC on silica gel 60 plates (EM Laboratories, Elmsford, NY) using $CHCl_3/CH_3OH/H_2O$ (65:35:5 v/v/v) as a solvent system. Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Trehalose and pentaerythritol were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals were reagent grade, and solvents were redistilled before use.

Preparation of Vesicles. The modified pH-adjustment procedure for making unilamellar and uniform vesicles was described previously (Aurora et al., 1985; Li & Haines, 1986). The lipid (5–10 mg) was dissolved in 2 mL of chloroform. In order to remove sodium (or ammonium) ions, this solution was washed 4 times with 2 mL of chloroform/methanol/0.2 M HCl (3:48:47 v/v/v). The upper phase was discarded each time. The resulting lower phase was washed with a 2-mL mixture of chloroform/methanol/water (3:48:47 v/v/v). In the vesicle preparations of lipid mixtures, the lipids were mixed at this point. The lower phase was then rotary evaporated in a round-bottom flask to form a phospholipid film. The film was dried under a water aspirator for about 10 min. A vacuum pump with a longer exposure of the film can be used if solvent-free vesicles are desired. The dried film (total sample of 5–10 mg) was then suspended in 3.0 mL of the desired unbuffered medium (e.g., 150 mM KCl) for vesicle formation. The mixture was stirred with a stirring bar for up to 30 min at room temperature. This suspension was titrated with 0.1 N NaOH to pH 7.55–11.0 (generally 10.0). It was then immediately adjusted to pH 7.55 with 0.1 N HCl.

Photon Correlation Spectroscopy (PCS). Dynamic light scattering was used to determine vesicle size (Aurora et al., 1985; Li & Haines, 1986) on an apparatus described by Hwang and Cummins (1982). Light from an argon 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 mean radius of the vesicles in a sample is calculated from the correlation function. By fitting a single exponential through the correlation data, one can determine the diffusion coefficient and, from this, calculate the radius.

Vesicle sizes determined by PCS show very good agreement with measurements using negative-staining EM on the same vesicle preparation. We found that the sizes of curvature-limited SUV's measured by PCS to those obtained by EM showed excellent agreement (Grzesiek & Dencher, 1986) whereas large unilamellar vesicles (~240 nm) show slightly larger hydrodynamic diameters in PCS than in electron microscopy probably due to slight shrinkage during the negative staining (Aurora et al., 1985). Sun et al. (1986) were able to measure 60-nm vesicles to within 2 Å using PCS.

Osmotic Dilution of Vesicle Suspensions. The vesicle suspension (1.0 mL) was placed in a cuvette and diluted with either 150 mM sucrose (or other nonelectrolyte impermeant) or 86 mM KCl at 0.025 mL/min on a syringe pump (Razel, Stamford, CT). The vesicle solution was continuously stirred with a magnetic stir bar. The cuvette was sealed with a Teflon stopper containing a small hole for the needle to prevent evaporation (and consequent concentration) during the addition. In early experiments, the vesicle suspension and the buffer solutions were filtered through 1.0- and 0.3-μm pore size filter paper (Nucleopore, Pleasanton, CA), respectively, prior to dilution. In later experiments it was found that if a filtered (0.3-μm) buffer was used to make vesicles and if filtered buffer was used for all dilutions, it was not necessary to filter the solution containing the vesicles. As this seemed less deleterious to the vesicle preparation and less likely to distort the sizing of the vesicles, this procedure was used for subsequent work.

The calculation of the modulus and the assumptions on which the calculation is based are described by Li et al. (1986). The total membrane modulus contains contributions due to the elasticity, bending, and surface tension. The bending term (Evans & Hochmuth, 1978) is negligible ($\sim 3 \times 10^5$ dyn/cm^2); however, the surface tension term is significant ($\sim 10^8$ dyn/cm^2) and must be considered since our observed membrane moduli are in the range of 10^7–10^9 dyn/cm^2.

For each swelling experiment the data are fitted to a best fit modulus calculated according to Li et al. (1986) in order to assign a modulus. The data are plotted to establish the point beyond which the vesicles no longer expand but "plateau". This point is the elastic limit (Li et al., 1986). From the size of the vesicles just before that dilution the surface expansion is calculated to show the percent surface expansion at the elastic limit. In order to calculate the pressure difference across the bilayer at that point, it is assumed that no impermeant has left the interior compartment of the vesicle but that the impermeant has been diluted according to the new radius of the vesicle as measured just prior to bursting. From that radius is calculated the volume containing the original amount of impermeant. The concentration on the interior of the vesicle is thus calculated. The concentration on the exterior is fixed by the dilution. The difference between these two is used to calculate the pressure difference across the bilayer at bursting (mosM).

Results

Previous work (Li et al., 1986) on DOPA vesicles suggested that the concentration of the salt might have a significant effect on the modulus, the elastic limit, and the transbilayer pressure at bursting. As a test for this concept we conducted a series of experiments swelling vesicles of DOPG in isoosmolar solutions of 150 mM KCl, 250 mM sucrose, and a 50:50 mixture of each solution (Figure 1). The figure also shows the error bars of each measurement (curve A) and the error involved in the calculation of a best fit of the modulus (curve B). It can be seen by inspection of the slopes as well as by the calculated moduli (straight lines) that the modulus increases significantly with the salt concentration between 0 and 75 mM

Table I: Observed Membrane Moduli, Surface Area Increase, and Pressure Difference across the Membrane at Bursting (ΔP) of DOPA and DOPG Vesicle Preparations in 150 mM KCl, 125 mM Sucrose/75 mM KCl, 250 mM Sucrose, 250 mM Trehalose, and 250 mM Pentaerythritol

lipid	diluent	size (nm) initial	size (nm) max	% surface increase	modulus ($\times 10^{-7}$ dyn/cm^2)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm^2)
DOPA	KCl	299.0	305.8	4.8	40.0	[40.0] (1.02)
DOPA	sucrose/KCl	294.0	308.4	10.0	10.0	[26.9] (0.68)
DOPA	sucrose	226.0	237.4	10.3	1.0	[4.0] (0.10)
DOPA	trehalose	290.0	302.0	8.4	4.0	[9.2] (0.23)
DOPA	pentaerythritol	176.8	184.8	9.3	1.0	[6.5] (0.16)
DOPG	KCl	456.3	467.2	4.8	50.0	[39.3] (1.00)
DOPG	sucrose/KCl	389.8	400.0	5.3	30.0	[30.0] (0.72)
DOPG	sucrose	334.5	348.4	8.5	5.0	[9.1] (0.23)
DOPG	trehalose	268.3	280.0	8.8	3.0	[7.8] (0.20)
DOPG	pentaerythritol	201.9	211.8	10.0	1.0	[3.9] (0.10)

Table II: Observed Membrane Moduli, Surface Area Increase, and Pressure Difference across the Membrane at Bursting (ΔP) of DOPG Vesicles at Constant Osmolarity and in Variable Ionic Strength in Mixtures of KCl and Pentaerythritol (penta)

diluent (mM)	size (nm) initial	size (nm) max	% surface increase	modulus ($\times 10^{-7}$ dyn/cm^2)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm^2)
KCl (150)	456.3	467.2	4.8	50.0	[39.3] (1.00)
sucrose/KCl (125/75)	389.8	400.0	5.3	30.0	[30.0] (0.72)
penta/KCl (167/50)	358.9	372.2	7.4	15.0	[24.4] (0.61)
penta/KCl (201/30)	337.2	352.2	9.1	13.0	[24.6] (0.62)
penta/KCl (242/5)	218.4	228.1	9.1	5.0	[19.2] (0.49)
pentaerythritol (250)	201.9	211.8	10.0	1.0	[3.9] (0.10)

KCl whereas the difference in the modulus at 75 and 150 mM KCl is minor. Of course it is also possible that the *decrease* observed with sucrose is due to specific interactions between sucrose and the phospholipid head groups. Considerable speculation in the literature has been focused on the unique qualities of sucrose and trehalose (Crowe et al., 1984, 1985) at phospholipid surfaces. In order to establish whether it was the sucrose or the ionic strength that was altering the modulus, we used 250 mM trehalose and 250 mM pentaerythritol as impermeants to measure the modulus. The results are shown in Table I for both DOPA and DOPG vesicles, which have essentially the same pattern. It is interesting to note that the initial sizes of the vesicles from our pH-adjustment method show a rather wide range for the nonelectrolyte impermeants. That vesicles made in 250 mM trehalose have nearly the same diameter as those made in 150 mM KCl and yet display a dramatically different modulus, elastic limit, and ΔP shows that these properties are not dependent on the radius of the vesicles but primarily on the ionic strength. Nonetheless, a slight increase in the modulus, decrease in tthe percent surface increase, and increase in ΔP for trehalose as compared to sucrose and pentaerythritol suggests that the radius *might* have a small but noticeable affect on the three properties.

Because DOPG contains no more than a single charge per head group whereas DOPA can maximally have two charges per head group [however, see Haines (1983) for a discussion on this matter], one would expect the DOPA vesicles to have a greater surface charge density and therefore a greater modulus if the modulus was based solely on the charge density. It should also be noticed that the DOPG vesicles are significantly larger than the DOPA vesicles in each medium in which they are made (except for trehalose). In 150 mM KCl these vesicles display a slightly larger modulus—consistent with the suggestion that size may have a small effect—but they show identical percent surface increase and ΔP. At lower ionic strength they show a smaller percent surface increase at bursting and a larger modulus than the DOPA vesicles in the sucrose/KCl mixture but otherwise have the same properties within the error of our measurements.

Having established that the sucrose was not acting in a unique way to alter the properties of the bilayer during osmotic swelling, we then proceeded to examine more closely the alteration of the modulus with ionic strength (Table II). The table shows the vesicle size and the ionic strength of each preparation. It shows a sharp distinction in the three properties in the narrow range between the absence of KCl and its presence at the 5 mM level. It might be noted from Table II that the initial vesicle size increases with the ionic strength, a feature of this method of making vesicles (Aurora et al.,

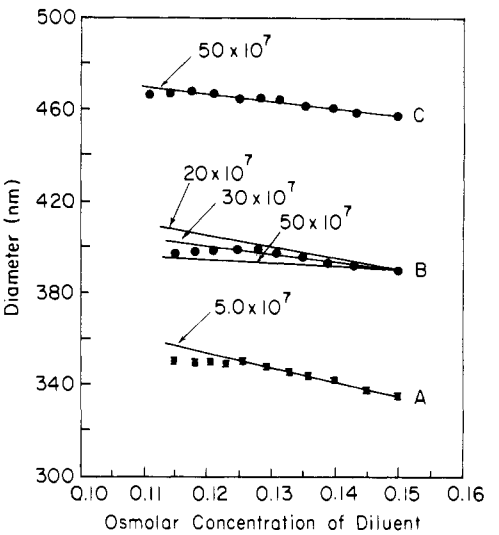

FIGURE 1: Osmotic swelling curves of DOPG vesicles in 250 mM sucrose (A), 125 mM sucrose/75 mM KCl (B), and 150 mM KCl–Tris buffer (2 mM, pH 7.55) (C). The vesicles were made with the pH-adjustment method (Li & Haines, 1986). Dilutions were made by adding 150 mM sucrose (A), 75 mM sucrose/43 mM KCl (B), and 86 mM KCl–Tris buffer (2.0 mM, pH 7.55) (C) to the 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. The error bars (shown on curve A only) represent the error of measurement with PCS and not the standard deviation in vesicle size. The heavy line (modulus) drawn through the first 7–10 dilution steps of each dilution is a best fit calculated from the equation of Li et al. (1986). The subsequent dilution steps were not included in the fit as they fall below the line and represent burst vesicles [see Li et al. (1986)]. Curve B shows two calculated moduli in addition to the best fit to permit estimation of the error in the best fit.

Table III: Observed Membrane Moduli, Surface Area Increase, and Pressure Difference across the Membrane at Bursting (ΔP) of Vesicle Preparations Comprised of Different Lipids[a]

lipids	diluent	size (nm) initial	size (nm) max	% surface increase	modulus ($\times 10^{-7}$ dyn/cm^2)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm^2)
DOPS	KCl	277.0	292.6	5.6	5.0	[16.6] (0.42)
DOPE	KCl	544.0	553.8	3.6	80.0	[41.8] (1.06)
oleic acid	KCl	308.7	329.4	13.0	1.0	[5.9] (0.15)
DΦPG	KCl	656.4	665.6	2.8	50.0	[16.3] (0.41)
E. coli lipids	KCl	537.4	550.4	5.8	60.0	[30.2] (0.77)
E. coli reconstituted vesicles	KP_i/K_2P_i	400.8	410.2	4.7	90.0	[70.8] (1.78)

[a] All vesicles were prepared with the pH-adjustment method in 150 mM KCl except for the *E. coli* lactose carrier reconstituted vesicles, which were prepared with the detergent dilution procedure in 50 mM KP_i/K_2P_i (pH 7.5). The vesicles were made by adjustment of pH to maximum pH 10 except for DOPS, which was adjusted to pH 8.5. All vesicles were restored to pH 7.55 except DOPE, which was restored to pH 8.60.

1985). Our earlier work shows that the very large differences in the three properties cannot be explained on the basis of the size differences. Indeed the size differences are greatest in the higher ionic strength range in Table II whereas the mechanical property differences are greatest in the low ionic strength range.

In order to assess the significance of lipid structure on the modulus we examined several lipids containing different head groups and measured the membrane modulus, the percent surface increase, and ΔP at bursting. Table III shows measurements comparing DOPS, DOPE, DΦPG, and oleic acid vesicles to DOPA and DOPG. It should be noticed that DOPE vesicles cannot be made below pH 8.6 by this method (Li & Haines, 1986) so that measurements on that lipid were made at that pH although all of the other measurements were made at pH 7.55. DOPS vesicles cannot be made if the pH is raised above pH 8.6 so that the vesicles are not made in the usual way (raising the pH to 9 or 10 before lowering it to 7.55). This may explain why the DOPS vesicles are smaller than the others although they are only slightly smaller than the DOPA vesicles. The method of preparation is unlikely to have affected our measurements on the DOPS vesicles. They display a remarkably low modulus for anionic vesicles in salt. These vesicles are easily stretched (low modulus) and yet require a moderately high ΔP at bursting, doing so at a low surface expansion. In contrast, DOPE displays the largest modulus we have obtained with any lipid bilayer, the lowest percent surface expansion at bursting, and the highest ΔP at bursting. The effect of reconstituting the lactose carrier into *E. coli* lipids seems to have a significant impact on the three properties of the membrane. The reconstituted vesicles display an even greater modulus than that of DOPE, a low surface expansion, and the highest ΔP at bursting of any bilayer system tested to date.

Unilamellar oleic acid vesicles were first synthesized by the pH-adjustment approach (Li & Haines, 1986). Oligolamellar liposomes had been synthesized earlier [Gebicki & Hicks, 1973; Hargreaves & Deamer, 1978; Michael Heller as reported in Haines (1983)] where it had been shown that they are only stable in the pH range between 7.0 and 9.5. They are shown to be unilamellar by their swelling properties in this system, confirmed by negative staining electron microscopy (Li & Haines, 1986). They display an extremely low modulus for anionic lipids in 150 mM KCl, a low ΔP at bursting, and the greatest surface expansion at bursting of any vesicles we have tested. These vesicles behave very much like phospholipid bilayers in nonelectrolyte media. One possible explanation for the unusual expansion is that the fatty acid is considerably more soluble than the double-chain phospholipids and soluble monomers of oleate may move from solution into the bilayer as it expands, thereby increasing the amount of bilayer substance. Such an explanation seems unlikely to explain the properties of double-chain phospholipids in nonelectrolyte media.

Discussion

In 1925, Gorter and Grendel (1925) on the basis of lipid analyses and the then-recent measurements of Langmuir on the area occupied by a chain in a monolayer concluded that the erythrocyte membrane was a bilayer of lipids two molecules thick. In the same year Fricke (1925) measured its low-dielectric thickness with impedance and found it to be about 3.5 nm. These two experiments, largely ignored, had to be rediscovered for their validation and acceptance; yet together they describe our present-day concept of membranes as lipid bilayers. Mechanical measurements had been conducted on erythrocyte membranes (Seifriz, 1926; Norris, 1939) and on the membranes of sea urchin eggs (Cole, 1932; Mitchison & Swann, 1954) using ingenious methods and what have turned out to be reasonable assumptions. Only recently, however, has the recognition appeared that many of the membrane's properties that were being examined were actually derived from the cytoskeleton rather than from the bilayer (Evans & Waugh, 1980; Mohandas et al., 1983).

In a biological membrane the bilayer includes not only the lipids but also those proteins (transport proteins, receptors, etc.) that occupy a significant area in the membrane continuum. Studies of the mechanical properties of biological membranes in which the cytoskeleton is not considered but only the membrane bilayer examined are comparable to studies on bilayer vesicles made of synthetic or extracted lipids as in the present work. This can be seen from the observed membrane moduli obtained for a number of biological membranes (Table IV) using a variety of techniques and comparing these data to those in Table V obtained on synthetic bilayers. All of these studies report moduli in the range (1–90) $\times 10^7$ dyn/cm^2. This consistency is striking because Waugh and Evans (1979) found that the elastic bilayer modulus for the erythrocyte is 4 orders of magnitude higher than the elastic shear modulus of the cell, a measure of the deformation of the cytoskeleton during osmotic and mechanical stress. We have avoided the term "elastic compressibility modulus" used in Evans' papers since Lis et al. (1982) measured the compressibility of bilayers and found the problem of bilayer compressibility far more complicated than bilayer expansion and quite different in its properties. We therefore refer only to the "elastic modulus" of membranes or bilayers.

The elastic modulus of the bilayer has been measured in a variety of ways. The kinetics of osmotic swelling just prior to hemolysis was used by Katchalsky et al. (1960) to calculate a modulus. An elastimeter (drawing a membrane into a micropipet under a microscope) was developed by Mitchison and Swann (1954). This device was used on erythrocytes by Rand (1964), Burton and Rand (1964), Evans et al. (1976),

Table IV: Membrane Modulus of Biological Membranes[a]

membrane examined	method used	modulus ($\times 10^{-7}$ dyn/cm^2)	reference
erythrocytes	kinetics of osmotic swelling	2.4	Katchalsky et al. (1960)
erythrocytes	pipet aspiration	22.0	Evans et al. (1976); corrected by Waugh & Evans (1979)
erythrocytes	pipet aspiration	22.0	Dimitrov et al. (1978)
rye protoplasts	pipet aspiration	12.0	Wolfe & Steponkus (1983)
submitochondrial particle	osmotic swelling (PCS)	3.0	Li et al. (1986)
brush border vesicles	osmotic swelling (PCS)	20.0–29.0	S. Miyamoto (personal communication)
E. coli lac permease vesicles	osmotic swelling (PCS)	90.0	this work

[a] The membrane modulus as reported in each cited work is recalculated to dyn/cm^2 for comparison. Studies on membrane tension that are not included are Mitchison and Swann (1954) on sea urchin eggs; Weiss (1968) on transformed cells; Rand (1964; Burton & Rand, 1964) on erythrocytes; Sung et al. (1983) on neutrophils; and Kosuge & Tazawa (1979) on giant algal cells. All of these were conducted with the pipet aspiration technique of Mitchison and Swann (1654), but none reported values that could be directly converted (without assumptions) to a comparable membrane modulus. Neither ΔP nor percent surface expansion is accessible by osmotic swelling experiments with biological membrane derived vesicles since the initial interior osmolarity of any such vesicle preparations cannot be known with precision. Evans et al. (1976) found 2–4% for the erythrocyte, and Wolfe and Steponkus (1983) found 2% surface expansion for rye protoplasts. Both groups used the pipet aspiration technique. Shigeaki Miyamoto prepared vesicles of rat small intestine brush border membranes using 0.2 mM $MgSO_4$ in 200 mM mannitol buffer (1 mM Hepes) at pH 7.4. Surface expansion (4–6%) of these vesicles was observed at bursting.

Table V: Comparison of the Membrane Modulus, the Pressure Difference across the Membrane at Bursting (ΔP), and the Surface Expansion of Synthetic Lipid Bilayers

method	bilayer	medium	modulus ($\times 10^{-7}$ dyn/cm^2)	ΔP (mosM)	% surface expansion	reference
stressed BLM	chol–HDTA	40 mM KCl	7.5–15.0			Wobschall (1971)
BLM capacitance	PS	100 mM NaCl	10–15			Alvarez & Latorre (1978)
	PE	1 M KCl				
pipet aspiration, 10-μm vesicles	EPC	100 mM NaCl	7		2–3	Kwok & Evans (1981)
42.6-nm vesicles, PCS	DMPC	350 or 150 mM LiCl	33.0		<1	Sun et al. (1986)
53.0-nm vesicles, PCS	DMPC	350 or 150 mM LiCl	3.4		>5	Sun et al. (1986)
PCS	DMPC	150 mM KCl	15			Hantz et al. (1986)
	DOPC					
PCS	DOPA	150 mM KCl	40–90	40–46	3–5	Li et al. (1986)
PCS	DOPA	250 mM sucrose	1–3	4–6	8–10	Li et al. (1986)

Sung and Chien (1978), and Dimitrov et al. (1978). It was used on cancer cells by Weiss (1968), on neutrophils by Sung et al. (1979, 1982), on giant algal cells by Kosuge and Tazawa (1979), and on rye protoplasts by Wolfe and Steponkus (1981, 1983). Only those studies that reported a modulus in a manner that could be compared to the others are reported in Table IV. Spectral analysis of the surface undulations (flicker phenomenon) of erythrocytes has been used by Brochard and Lennon (1975) to measure their curvature elastic properties, including a study under osmotic stress (Fricke & Sackmann, 1984). The curvature elasticity can be converted to the elastic modulus with good agreement with a few reasonable assumptions (Evans & Hochmuth, 1978). This method has been used to study synthetic phospholipid bilayers (Servuss et al., 1976), which displayed precisely the same modulus as that of the intact erythrocytes. The finding is striking since the live erythrocyte's membrane deformability (elastic shear modulus) appears to be dominated by its contact with the cytoskeleton, and yet the undulations observed by Brochard and Lennon (1975; Fricke & Sackmann, 1984) appear to be due to the bilayer alone and are equivalent to that of the synthetic lipid bilayers of Servuss et al. (1976). Deformation of the erythrocyte may have to do exclusively with the spectrin network and have nothing to do with the contact between the cytoskeleton and the bilayer. Such a model has been suggested (Stokke et al., 1986).

Osmotic swelling of the erythrocyte membrane allowed Katchalsky et al. (1960) to estimate the modulus as 2.4×10^7 dyn/cm^2 as quoted in Table IV. This value is lower than the other values reported in the table except for the submitochondrial particles (Li et al., 1986). The latter may be due to the fact that the submitochondrial particles are isolated in 250 mM sucrose, which consistently gives a lower modulus. All of the other results given in the table were obtained in 100–150 mM salt. The values obtained in these studies are intermediate between our results for nonelectrolyte impermeants $(1–5) \times 10^7$ dyn/cm^2 and the values of $(30–80) \times 10^7$ dyn/cm^2 that we get for vesicles swollen in 150 mM KCl. [The exceptions to this statement are DOPS (measured at high pH) and oleic acid.] One would expect that the charge density on these natural membranes would be lower than that on synthetic bilayers made of a single anionic lipid. Furthermore, from the calculation of Evans and Hochmuth (1978), the quantitative agreement of Brochard and Lennon (1975) and Fricke and Sackmann (1984) both using the flicker phenomenon approach enhances the general agreement. Finally, the statistical mechanical theory of Marcelja (1974) predicts an elastic modulus of $(7.5–10) \times 10^7$ dyn/cm^2 and Israelachvili et al. (1976) derived from surface tension a modulus of 10×10^7 dyn/cm^2 (values recalculated to dyne per square centimeter).

Measurements on the elastic constants of synthetic or extracted lipid bilayers shown in Table V include those of Wobschall (1971) (changes in capacitance of a BLM as a function of time during sinusoidal expansion), Servuss et al. (1976) (flicker phenomenon calculated from the curvature elasticity as indicated), Kwok and Evans (1981), Sun et al. (1986) (for this group of the five measurements of the modulus for vesicles, only the 48.5- and 53.0-nm-diameter vesicles that appear not to be curvature-limited are reported), Hantz et al. (1986), and Li et al. (1986). All of these studies have yielded the same range of values as that found for the natural membranes or membrane vesicles as in Table IV for natural membranes. Wobschall (1971) obtained the result using the detergent HDTA in combination with cholesterol. Thus, the modulus of natural membranes seems indeed to be that of the lipid bilayer. It is therefore useful to examine the bilayer modulus to learn about the energetics of a lipid bilayer under

stress and to seek information about lipid dynamics and lateral head group interactions.

Taking advantage of the precision and sensitivity of PCS to measure the sizes of particles and of the fact that we have developed a method for making uniform vesicles, we have measured the modulus of vesicles made up of a variety of anionic phospholipids and of oleic acid. Recognition of the end of the linearity of the swelling as an elastic limit of the vesicle preparation (Li et al., 1986) has permitted us to obtain the percent surface expansion and the pressure difference (ΔP) across the bilayer at bursting. These three parameters are independent of each other in materials science although many materials in the macroscopic world that have a large elastic modulus undergo limited expansion and require a significant shear force to break. In general, the polyanionic bilayers we have studied have shown this combination of properties in 150 mM KCl. The same bilayers in a medium of low ionic strength have a different set of properties that are also common in the macroscopic world, namely, a low modulus of elasticity, a large surface expansion at breaking, and a small shear force necessary for breaking.

The studies of Wolfe and Steponkus (1983) on the modulus and mechanical properties of rye protoplasts have been followed up with an interesting series of papers (Wolfe et al., 1985, 1986) on the time-dependent responses of the membrane bilayers to osmotic stress. Special emphasis in the most recent paper (Wolfe et al., 1986) has been on the greater percent surface expansion observed in vesicles derived from cold-acclimated plants. Protoplasts derived from nonacclimated plants display a surface expansion of from 2 to 3%, whereas protoplasts derived from acclimated seedlings show 6–8% surface expansion before bursting. The combination of the greater expansion and the fact that the kinetics of the expansion is different, taken together with the irreversibility of the slower expansion, provoke them to conclude that there exists in the protoplast a reservoir of lipids that enter the bilayer during osmotic stress only. Some of these data could be interpreted, in the context of our results, as due to a change in the ionic strength of the medium due, for example, to accumulation of sugar in the medium of the cold-acclimated protoplasts and/or a change in the lipid head group composition.

The fact that a vesicle expands on swelling leaves at least two alternative molecular descriptions of the expanded state. In one, the bilayer thins as a consequence of its expansion, retaining a constant membrane volume, analogous to a balloon. This is the basis of much of the calculations of Evans and Hochmuth (1978) and is suggested by the very small thickness changes found in bilayers under pressure (Lis et al., 1982 and references therein) and in the capacitance measurements of bilayers (Alvarez & Latorre, 1978). Thinning the bilayer means that the distance between the head group sheets decreases. This is only possible if there is either an increase in the tilt of the chains, which would increase the order, or an increase in the statistical number of t–g isomerizations per chain, which implies an enthalpy increase. The latter would be expected to decrease the order parameter of each methylene. Only this latter description is consistent with an increase in the fluidity (see introductory paragraphs) during vesicle expansion while a constant membrane volume is maintained (thinning the bilayer). A second possible consequence of vesicle expansion is that the bilayer does *not thin* but remains at constant thickness. In this set of events, the bilayer increases its volume as a response to osmotic swelling of a vesicle. This can only be possible if water enters the bilayer since there is no other apparent way for the bilayer volume to increase. Although such a prospect might seem unlikely, it is consistent with the increase in fluidity that accompanies lateral expansion of the bilayer and should be ruled out experimentally rather than assumed.

Zimmerman et al. (1977) have shown that the electrical breakdown voltage decreases with an increase of osmotic pressure gradients across bilayers. The application of a uniform hydrostatic pressure also decreases the electrical breakdown voltage (Zimmerman et al., 1980).

The finding that the modulus of bilayers comprised of anionic phospholipids is dominated by the ionic strength of the bathing solution is unexpected. It offers an explanation as to why microbes that are tolerant to high concentrations of salt increase the anionic lipid mole fraction when exposed to the higher salt concentration (Kanemasa et al., 1972; Komaratat & Kates, 1975; Ohno et al., 1976; Thirkell & Summerfield, 1977; Ohno et al., 1979; Hiramatsu et al., 1980; Kogut & Russell, 1984; Hanna et al., 1984; Miller, 1985). *E. coli* at the moderate increases of salinity that are permissible showed no change in the phospholipid head groups, but an increase in the cyclopropane chains (McGarrity & Armstrong, 1975).

The question of why microbes increase the anionic lipid fraction of the plasma membrane when faced with an increase in salt concentration arises. The increase in the charge density of the bilayer surface would, according to the data presented here, restrict the expansion of the bilayer (reduce the increase of surface area per lipid molecule) during osmotic stress and at the same time increase the transbilayer pressure necessary to burst it. Both are important for retaining the integrity of the cell. At present it is not known whether a bilayer thins during expansion due to osmotic stress. Whether or not it does, an expansion of its surface area may be expected to alter the conformation of proteins embedded in it. Resistance to expansion by altering the modulus is a defense against the distortion of protein conformations in the plasma membrane. Obviously the simultaneous increase in the ΔP resulting from the greater surface charge density implies that greater transbilayer pressure differences are necessary to burst the bilayer and leak proteins from the cell. It might also be noted that a high content of negatively charged lipids is generally found in halophilic bacteria (Peleg & Tietz, 1971; Stern & Tietz, 1973) including the extreme halophiles (Kates et al., 1966; Hancock & Kates, 1973; Evans et al., 1980).

Another surprising result we obtained is the unusually high modulus of DOPE. This lipid is not normally considered an anionic lipid. The unusual modulus could result from H bonding between adjacent head groups (Hitchcock et al., 1974; Prats et al., 1986). DOPE does not form vesicles above pH 8.6 (Li et al., 1986), which implies a pK in that region and that below this pH the head group is protonated. In the studies just cited on lipid compositional changes in the membranes of Gram-negative bacteria exposed to high salinity, cardiolipin was increased at the expense of phosphatidylethanolamine (Kanemasa et al., 1972; Ohno et al., 1976, 1979; Hiramatsu et al., 1980; Kogut & Russell, 1984; Hanna et al., 1984; Miller, 1985).

A clear implication of the present work is that the surface charge density is dominating the membrane modulus and the mechanical properties of anionic lipid bilayers by virtue of the double diffuse layer. The Gouy–Chapman theory for the structure of the double layer at the surface of negatively charged lipids is generally considered to be a good approximation (McLaughlin, 1977) of the surface energy. The displacement and rearrangement of charges in the double layer that accompanies the expansion of the charged surface requires

energy. Measurements of surface charge density accompanied with its comparison to the measured modulus appears to be necessary to sort out the energetics of the surface expansion. The charge density at the surface of anionic lipid bilayers and monolayers has been the subject of vigorous investigations in the past few years (Papahadjopoulos et al., 1972; Haydon & Myers, 1973; Watts et al., 1978; Eibl & Blume, 1979; Kell & Morris, 1980; Lakhdar-Ghazal et al., 1983; Girault & Schiffrin, 1986), but no one has suggested to date that it might relate to the elasticity of the bilayer.

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