Mechanical properties of vesicles

I. Coordinated analyses of osmotic swelling and lysis

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ABSTRACT To determine how transmembrane osmotic gradients perturb the structure and dynamics of biological membranes, we examined the effects of medium dilution on the structures of osmolyte-loaded lipid vesicles. Our preparations were characterized by dynamic light scattering (DLS) and nuclear magnetic resonance (NMR) spectroscopies. Populations of *Escherichia coli* phosphatidylethanolamine (PE) or dioleoylphosphatidylglycerol (DOPG) vesicles prepared by the pH jump technique were variable and polymodal in size distribution. Complex and variable structural changes occurred when PE vesicles were diluted with hypotonic buffer. Such vesicles could not be used as model systems for the analysis of membrane mechanical properties. NaCl-loaded, DOPG vesicles prepared by extrusion through 100 nm (diameter) pores were reproducible and monomodal in size distribution and unilamellar, whereas those prepared by extrusion through 200-, 400-, or 600-nm pores were variable and polymodal in size distribution and/or multilamellar. Time and pressure regimes associated with osmotic lysis of extruded vesicles were defined by monitoring release of carboxyfluorescein, a self-quenching fluorescent dye. Corresponding effects of medium dilution on vesicle structure were assessed by DLS spectroscopy. These experiments and the accompanying analysis (Hallett, F. R., J. Marsh, B. G. Nickel, and J. M. Wood. *1993. Biophys. J.* 64:000–000) revealed conditions under which vesicles are expected to reside in a consistently strained state.

INTRODUCTION

Interest in the mechanical properties of biological membranes has been both widespread and persistent. The rheological properties of membranes are believed to influence both blood flow and cell fusion. Postulated molecular bases for such phenomena as cellular osmoregulation, blood pressure homeostasis, and fluid translocation through higher plants invoke membrane stretch receptors or sensors. Mechanosensitive ion channels have been revealed through the application of patch clamping techniques to animal, plant, and bacterial cells (Morris, 1990) and biochemical measurements have indicated that turgor sensitive regulatory proteins and enzymes may be present in bacterial cytoplasmic membranes (Csonka and Hanson, 1991). The latter observations have heightened interest in biochemical correlates of membrane elasticity. In spite of that interest, technical difficulties have limited our exploration of the effects of lateral tension on the structure and organization of membrane lipids and proteins. Methodologies that support simultaneous (or coordinated) monitoring of biochemical activities and membrane strain will be essential if we are to test hypotheses linking membrane strain, enzymatic or channel activities, and physiological outcomes. Since membrane vesicle systems are commonly used to study membrane biochemistry (e.g., Kaback, 1986), methodologies applicable to membrane vesicles will be particularly useful.

A variety of techniques have been used to characterize membrane elasticity (reviewed by Rutkowski et al., 1991). Dynamic light scattering (DLS) spectroscopy has recently been applied to measure size changes in vesicles exposed to transmembrane osmotic gradients. Although the latter experiments have been interpreted to yield vesicle expansion limits and membrane elastic moduli, interpretation of DLS data derived from membrane vesicle samples has been complicated by a number of factors. Theoretical treatments of vesicle osmotic swelling have usually been based on the assumption that vesicle populations are unilamellar and monodisperse, though such populations are not readily prepared (for illustrations of this problem see Rutkowski et al., 1991, and this work). Although the elastic properties of vesicles are expected to be directly related to their sizes (for further discussion of this point see Hallett et al., 1993), characterization of vesicle size distributions has proven difficult, even with the application of DLS techniques (e.g., Rutkowski et al., 1991). The interpretation of vesicle swelling data depends strongly on assumptions regarding the leakage of solutes from swollen or lysing vesicles (Rivers and Williams, 1990) and on changes in the structures of vesicles that approach or exceed their elastic limit, but no coordinated measurements of lumenal solute loss and vesicle structural change have been reported.

Our goal is to devise experimental preparations, procedures, and analytical techniques that will allow us to test physical models describing vesicle membrane mechanics. We will apply those methods to correlate biochemical activities with vesicle membrane strain. We identified DOPG vesicles prepared by extrusion as an appropriate model experimental system for these studies. The extrusion technique yielded monodisperse popu-

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lations of unilamellar vesicles whose size distributions could be defined by DLS spectroscopy. Their behavior during osmotic swelling could thus be modeled quantitatively. Vesicles loaded with the self-quenching fluorescent dye, 5(6)-carboxyfluorescein (CF), were used to monitor the release of lumenal solutes during dilution of the vesicle medium. DLS spectroscopy was then used to define changes in vesicle structure accompanying vesicle lysis. In the companion paper (Hallett et al., 1993), we present models for membrane strain and vesicle lysis which explicitly address issues of vesicle polydispersity and solute leakage. We demonstrate that a model based on partial release of lumenal solutes from swollen vesicles best fits our dye release data. That model defines conditions under which vesicle membranes are likely to reside in a consistently strained state and establishes protocols appropriate for the examination of membrane mechanical properties using more complex membrane systems.

EXPERIMENTAL METHODS

Preparation of phospholipid vesicles

E. coli phosphatidylethanolamine (PE) and 1,2-dioleoyl-*sn*-3-phosphatidylglycerol (DOPG) (Avanti Polar Lipids, Inc., Pelham, AL) were stored in powder form at −40°C. PE or DOPG vesicles were prepared by the pH jump technique essentially as described by Aurora et al. (1985). Eight milligrams of PE or DOPG were dissolved in 2 mL chloroform. This solution was washed four times with 2 mL of chloroform/methanol/200 mM HCl (3:48:47, vol/vol). The resulting lower phase was washed once with 2 mL of chloroform/methanol/water (3:48:47, vol/vol). The lower phase was transferred to a 100 mL round-bottomed flask and the solvent was evaporated to dryness by rotary evaporation to form a phospholipid film. The phospholipid film was dried under vacuum for 10 min and then hydrated by stirring in 250 mM sucrose, 2 mM TrisHCl (pH 7.55). The resulting phospholipid suspension was titrated with 100 mM NaOH to a pH of 10.0 ± 0.2 pH units. After 1 min of stirring at the basic pH, the pH of the suspension was readjusted to 8.6 for *E. coli* PE (Li and Haines, 1986) or to 7.55 for DOPG using 100 mM HCl.

DOPG vesicles were prepared by extrusion essentially as described by Nayar et al. (1989). DOPG was dissolved in 2 mL chloroform in a 100 mL round-bottomed flask. The solvent was evaporated to dryness by rotary evaporation to form a thin phospholipid film. The DOPG film was dried under vacuum for 10 min. The phospholipid was hydrated by stirring for 30 min in an appropriate buffer (750 mM NaCl, 20 mM NaMops, pH 7.4, unless otherwise stated) to a concentration of 50 mg DOPG/mL buffer. The phospholipid suspension was frozen and thawed 10 times using liquid N_2 and water at 25°C, respectively, and then passed through a stainless steel extrusion device similar to that previously described (Nayar et al., 1989). The extruder employed for these experiments was surrounded by a water-bearing compartment that was attached to a circulating water bath, allowing all extrusions to be performed at a constant temperature of 25°C, which was well above the phase transition temperature of the phospholipid. Each vesicle preparation was made by extruding the phospholipid suspension 10 times through two polycarbonate filters (Nucleopore Corp., Pleasanton, CA) with pore diameters 100, 200, 400, or 600 nm. The final concentration of five representative extruded DOPG vesicle preparations was 37.6 ± 1.3 mg phospholipid/mL buffer (standard deviation), as determined by the procedure for phospholipid phosphorus analysis of Bartlett (1959). Thus, approximately 75% of the total phospholipid used in the extrusion technique was recovered. Vesicle suspensions were maintained at 25°C and used within 24 h of preparation.

Determination of vesicle size distributions by DLS

The apparatus used for the DLS experiments has been described previously (Hallett et al., 1989). The sample, contained in a 3 mL plastic cuvette (Hellma Ltd., Concord, ON), was placed in a thermally jacketed sample holder at 25°C. The light source, a helium-neon laser of wavelength 632.8 nm (model 125; Spectra Physics, Mountain View, CA) was focused onto the sample and scattered light was detected at 90° to the incident beam by a photomultiplier tube (model 9863; EMI Electronics Ltd., Hayes, England). The photomultiplier tube was connected to a quantum photometer (model 1140; Princeton Applied Research, Princeton, NJ). Autocorrelation was completed by a correlator (model 1096; Langley-Ford Instruments, Amherst, MA). Prior to each light scattering experiment, the proper alignment of the light scattering apparatus was verified using polystyrene latex microspheres (Duke Scientific, Palo Alto, CA) with a diameter of 91 nm. Scattering intensity averaged mean vesicle diameters were determined by moments analysis (Koppel, 1972). Number distributions of vesicle diameters and number averaged mean diameters were determined as described by Hallett et al. (1991), employing Rayleigh–Gans–Debye form factors for hollow spheres in a discrete Laplace inversion routine. Published values were used to estimate medium viscosities (Stokes and Mills, 1965).

For vesicle sizing, each sample was diluted, using the buffer in which the phospholipid was originally suspended, to a final concentration of 0.5 mg lipid/mL buffer. This dilution ensured that each sample was sufficiently dilute for light scattering experiments and that lumen and external medium were identical in both osmotic strength and refractive index. For each sample, data were collected during 24 successive 30-min periods. The mean diameters of the phospholipid vesicles could be determined with a data collection period as short as 1 h, but longer data acquisition was required to obtain precise vesicle size distributions. This was achieved by averaging data from several contiguous runs. Analysis of data derived from the individual runs as a function of real time revealed that mean vesicle diameters were stable during the 12-h data acquisition period.

Determination of vesicle lamellarity by ^{31}P-NMR spectroscopy

Proton decoupled ^{31}P-NMR spectra were obtained using a Bruker WP 200 NMR spectrometer (Milton, ON). Spectra were accumulated from 1,000 scans by employing a pulse of 18.5 μs and a relaxation time of 2.0 s, a spectral width of 41.7 kHz, and an acquisition time of 0.197 s. Two spectra were obtained with each sample. For the first spectrum, 3.0 mL of vesicle sample (37.6 ± 1.3 mg phospholipid/mL buffer) were transferred to a 10-mm NMR tube. The second spectrum was obtained under experimental conditions identical to those of the first, except that 1.0 mL of an isotonic $MnCl_2$-containing buffer (20 mM $MnCl_2$, 720 mM NaCl, 20 mM NaMops, pH 7.4) was added to the 3-mL vesicle sample, giving a final $MnCl_2$ concentration of 5 mM. Peak areas were determined by computer integration. The average number of lipid bilayers per vesicle ($[N]$) was computed from the percentage relative loss of ^{31}P signal intensity (RLOS) due to Mn^{2+} addition (Schwartz and McConnell, 1978).

Osmotic lysis of phospholipid vesicles: DLS spectroscopy

E. coli PE vesicles, prepared by the pH jump technique in 250 mM sucrose, 2 mM TrisHCl, pH 8.6, were diluted with the same buffer to a final concentration of approximately 0.5 mg phospholipid/mL buffer. A transbilayer osmotic gradient was created by using a syringe pump (model 255-1; Sage Instruments, Evanston, IL) loaded with hypotonic

buffer (150 mM sucrose, 2 mM TrisHCl, pH 8.6) to dilute the vesicle suspension. The medium external to the vesicles was diluted at a rate of 40 μL/min to a final sucrose concentration of 242 mM. The approximate vesicle size distribution was determined by DLS spectroscopy as described above, except that data were collected for a period of 1 h. The same sample was progressively diluted four more times, to final sucrose concentrations of 234, 226, 218, and 210 mM. After each dilution, DLS data were obtained.

DOPG vesicles prepared by extrusion through 100 nm pores in 20 mM NaMops, pH 7.4, 750 mM NaCl were diluted with the same buffer to achieve a phospholipid concentration of approximately 0.2 mg/mL. Paired samples of the resulting suspension were diluted again with either the same buffer or 20 mM NaMops, pH 7.4, to yield samples that were identical in all respects except post-dilution extravesicular NaCl concentration. The size distributions of the vesicles present in the paired samples were determined by collecting DLS data (as described above) during alternate 1-h sample acquisition periods over a period of 24 h (12 h per sample). Analysis of the resulting data as a function of time indicated that the vesicle mean diameters were stable over the 24-h data acquisition period. Vesicle size distributions were therefore determined (Hallett et al. (1991)) on the basis of the pooled data for each sample.

Osmotic lysis of phospholipid vesicles: fluorescence spectroscopy

The osmotic properties of DOPG vesicles were examined by monitoring the release of the self-quenching fluorescent dye, CF (Sigma Chemical Company, St. Louis, MO), from the lumen of vesicles exposed to hypotonic stress. CF fluorescence is quenched and fluorescence emission is reduced by self-absorption when the dye is present in solution at concentrations greater than approximately 10 μM (Figure 1). DOPG vesicles were prepared by extrusion through 100-nm pores as described above, except that a dye-containing buffer (100 mM CF [Na^+ salt], 600 mM NaCl, 20 mM NaMops, pH 7.4) was used in vesicle formation. The free dye was separated from dye-containing DOPG vesicles by gel filtration using Bio-Gel P-6DG desalting gel (Bio-Rad, Mississauga, ON) in a column with dimensions 30 cm (height) by 1.5 cm (diameter). Dye-containing vesicles were eluted with a CF-free, isotonic buffer (750 mM NaCl, 20 mM NaMops, pH 7.4) at a flow rate of 1.0 mL/minute. The phospholipid concentration of the vesicle sample eluted from the column was typically 13.9 ± 0.3 mg/mL buffer (standard deviation), as determined by the procedure for phospholipid phosphorus determination of Bartlett (1959), modified by Kates (1985). Prior to the osmotic lysis experiment, vesicles obtained from the column were diluted to a final concentration of 0.5 mg phospholipid/mL buffer using isotonic buffer. This dilution ensured that the concentration of dye obtained after complete lysis of the vesicle population was approximately 1 μM.

For paired vesicle samples in 1 cm × 1 cm cuvettes, the medium external to the vesicles was diluted with isotonic (750 mM NaCl, 20 mM NaMops, pH 7.4) or hypotonic (20 mM NaMops, pH 7.4) buffer. A syringe pump (model 255-1; Sage Instruments, Evanston, IL) was used to dilute the medium external to the vesicles continuously, at a rate of 160 μL/minute. Alternatively, samples were allowed to stir for 5 min following stepwise additions of buffer. The fluorescence of each suspension was monitored with a Hitachi F-2000 fluorescence spectrophotometer (Mississauga, ON) throughout the continuous dilution experiments and after each stepwise dilution. An excitation wavelength of 479 nm, an emission wavelength of 520 nm, a response time of 0.5 s, and excitation and emission bandpath widths of 10 nm were used to monitor the fluorescence intensity of the probe. Results are presented as percent CF release from vesicles (%F), the total fluorescence intensity having been determined after addition of Triton X-100 to a final concentration of 0.1% (vol/vol) to the sample that had been diluted with hypotonic buffer. Triton X-100 disrupts vesicle bilayers but does not itself influence the detected fluorescence intensity (Sila et al., 1986, and Fig. 1). The fluorescence intensity of suspensions diluted with hypotonic buffer was corrected for dilution, and further adjusted by subtracting the fluorescence of the original, maximally quenched sample. That correction was approximately 5% of the fluorescence intensity measured after lysis with Triton X-100.

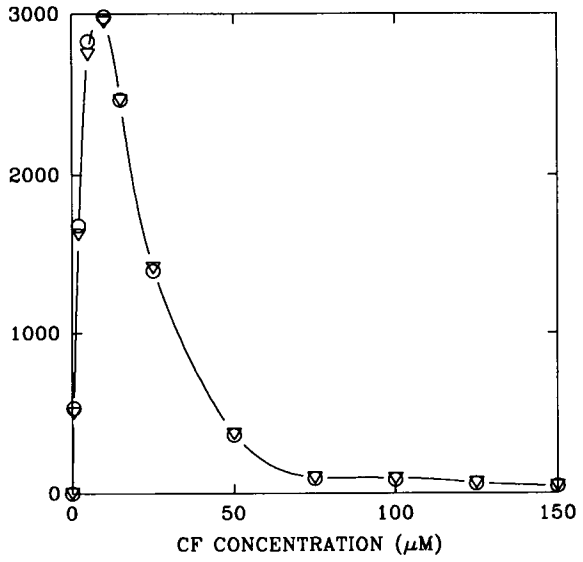

FIGURE 1 Concentration dependence of CF fluorescence. The fluorescence of CF (Na^+ salt, pH 7.4) was determined in the absence (○) and the presence (▽) of 0.1% (vol/vol) Triton X-100. Spectrophotometer settings included excitation and emission wavelengths of 479 and 520 nm, respectively, a response time of 0.5 s, and excitation and emission bandpath widths of 10 nm. Samples were contained in 1 cm × 1 cm cuvettes.

RESULTS

Properties of vesicles prepared by the pH jump technique

Application of the pH jump technique to a variety of lipids has been reported to yield large unilamellar vesicles with mean diameters in the 300–600 nm range (Hauser and Gains, 1982; Rutkowski et al., 1991). Since we are interested in *E. coli* membrane biochemistry (Milner et al., 1988), we were particularly interested in examining the mechanical properties of *E. coli* lipids PE and PG. Moments analysis of our DLS data seemed to corroborate previous work by suggesting that the pH jump method yielded PE and PG vesicles with mean diameters close to 300 nm (Table 1). In contrast, further analysis of our light scattering data by the method of Hallett et al. (1991) revealed marked polydispersity in each preparation (Fig. 2 and Table 1). Transbilayer osmotic gradients were created by stepwise dilution of sucrose-loaded *E. coli* PE vesicles. Assuming no loss of lumenal solutes, the maximum transbilayer gradient present in these experiments would have been 40 mOsm. The apparently simple pattern of vesicle swelling obtained when moments analysis was applied to light scattering data obtained from such preparations (Fig. 3) was

TABLE 1 **Properties of phospholipid vesicles prepared by the pH jump technique**[a]

LIPID	EXP. #	MOMENTS ANALYSIS Mean diameter (*nm*)	NUMBER DISTRIBUTION Mean diameter (*nm*)	NUMBER DISTRIBUTION Relative fraction of population
E. coli PE	I	298	28	0.51
			80	0.44
			306	0.06
	II	311	29	0.82
			238	0.17
			672	0.01
	III	350	73	0.47
			288	0.47
			704	0.06
DOPG	I	301	31	0.49
			104	0.42
			324	0.09
	II	325	29	0.50
			70	0.39
			350	0.11
	III	333	28	0.50
			82	0.50
			688	≪0.01

[a] Vesicle diameter was determined by DLS spectroscopy as described in Materials and Methods. Three replicate experiments were performed with each lipid (Exp. #I, II and III). MOMENTS ANALYSIS and NUMBER DISTRIBUTION refer to the parameters determined by the method of Koppel (1972) and of Hallett et al. (1991), respectively (see text).

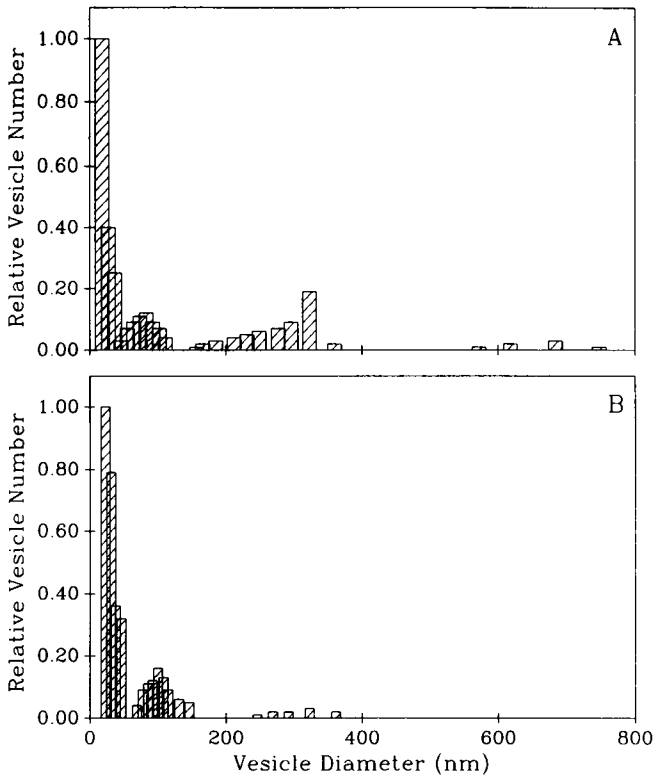

FIGURE 2 Vesicle number average diameter distributions, determined as described by Hallett et al. (1991), for phospholipid vesicles prepared by the pH jump technique. (*A*) *E. coli* PE. (*B*) DOPG.

contradicted when the corresponding distributions of vesicle sizes were examined (Fig. 4). For example, moments analysis of DLS data derived from vesicles diluted to an extravesicular sucrose concentration of 226 mM suggested that they had a mean diameter of approximately 330 nm (Fig. 3) whereas the corresponding vesicle number distribution (Fig. 4, panel D) was clearly polymodal with vesicle populations centered at mean diameters less than 100 and greater than 400 nm. We concluded that the complexity of these vesicle preparations would preclude detailed, quantitative analysis of their swelling behavior.

Properties of vesicles prepared by the extrusion technique

The extrusion technique has been employed in preparing unilamellar vesicles with diameters in the 30–200 nm range (Nayar et al., 1989). We applied that technique to DOPG, which more readily forms stable lipid bilayers than PE. NaCl was used as primary osmolyte in these experiments because NaCl solutions were more readily extruded than sucrose solutions of corresponding osmolarity.

When DOPG vesicles were prepared by extrusion through 100- or 200-nm pores, reproducible preparations with unimodal size distributions were obtained (Fig. 5 and Table 2). Mean vesicle diameters close to 100 and 200 nm, respectively, were obtained regardless of the analytical technique applied to the light scattering data (Table 2). Extrusion through 400- or 600-nm pores yielded DOPG vesicle preparations with variable, polymodal size distributions (Fig. 5 and Table 2). No obvious relationship between extrusion pressure and vesicle

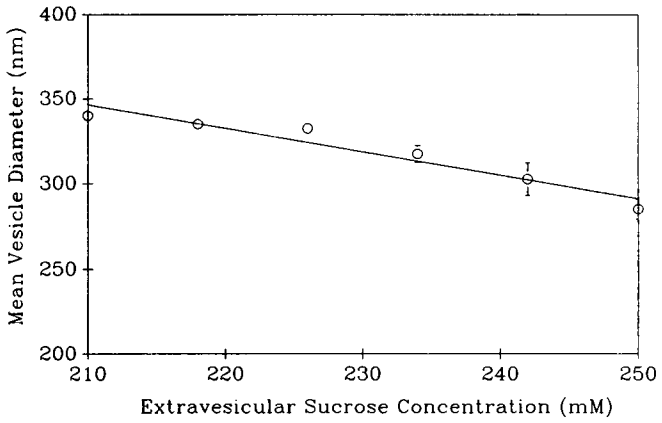

FIGURE 3 Change in mean diameter of *E. coli* PE vesicles due to dilution of the extravesicular medium. Vesicles prepared by the pH jump technique in 250 mM sucrose, 2 mM Tris HCl, pH 8.6 were diluted with 150 mM sucrose, 2 mM TrisHCl, pH 8.6 to create the indicated changes in extravesicular sucrose concentration. Mean vesicle diameters were determined by moments analysis (Koppel, 1972) of DLS data.

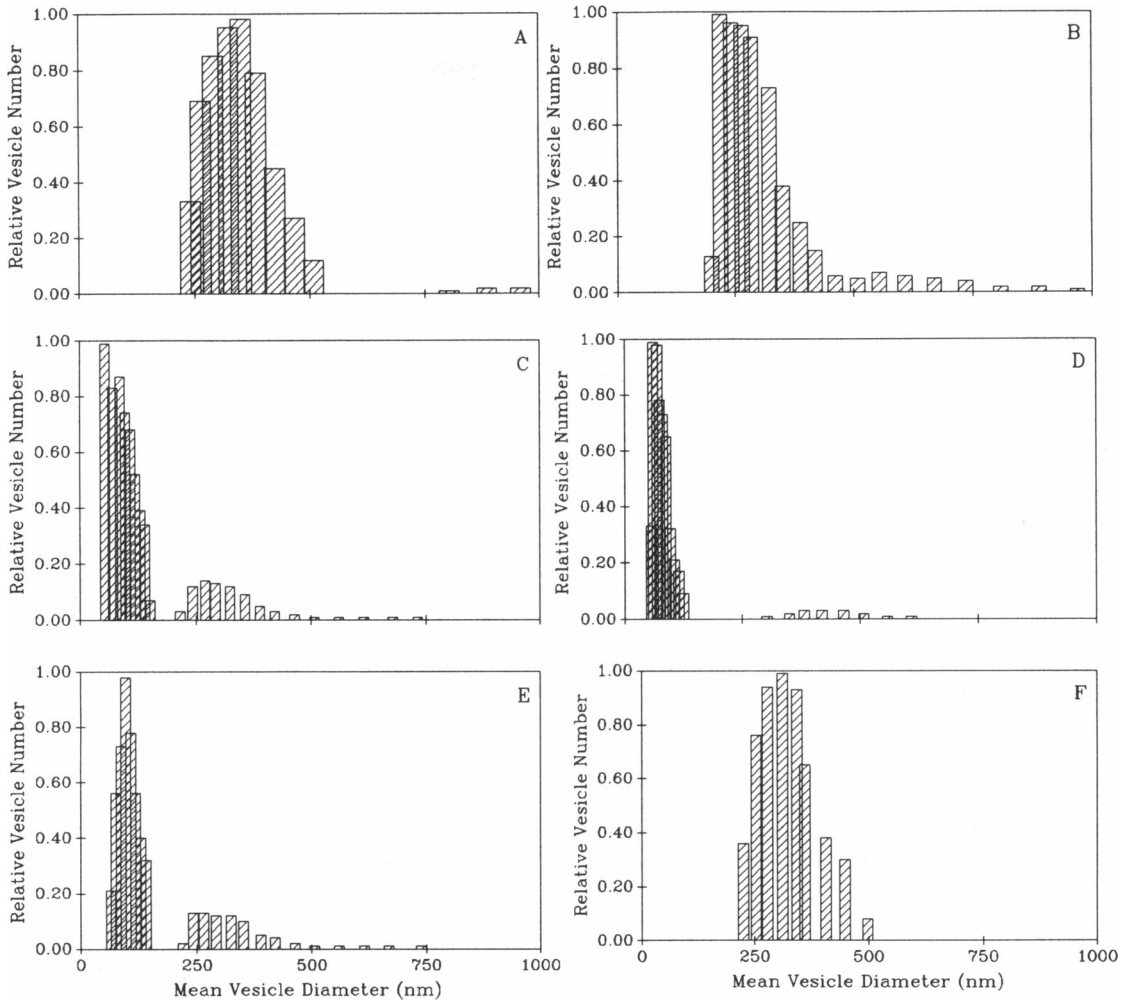

FIGURE 4 Change in diameter distribution of *E. coli* PE vesicles due to dilution of the extravesicular medium. Vesicles prepared by the pH jump technique in 250 mM sucrose, 2 mM TrisHCl, pH 8.6 were diluted with 150 mM sucrose, 2 mM TrisHCl, pH 8.6 to create the indicated changes in extravesicular sucrose concentration. Vesicle number average diameter distributions were determined as described by Hallett et al. (1991) for vesicles diluted with isotonic buffer (250 mM sucrose, 2 mM TrisHCl, pH 8.6) (*A*) or with hypotonic buffer (150 mM sucrose, 2 mM Tris HCl, pH 8.6) to reduce the extravesicular sucrose concentration to 242 mM (*B*), 234 mM (*C*), 226 mM (*D*), 218 mM (*E*), or 210 mM (*F*).

diameter was observed (Table 2). The lamellarity of extruded DOPG vesicle preparations was assessed using ^{31}P-NMR spectroscopy as described by Schwartz and McConnell (1978) (Table 2). Only the vesicles with diameters near 100 nm were unilamellar ($[N] = 1$). The variability of the observed lamellarities ($[N]$) of the vesicles prepared by extrusion through larger pores correlated with the variability of their size distributions as determined by DLS spectroscopy (Table 2). We concluded that DOPG vesicles prepared by extrusion through 100-nm pores could be employed in further studies of membrane mechanical properties based on vesicle osmotic swelling and lysis.

Osmotic lysis of DOPG vesicles

We wish to detect strain in biological membrane vesicles. To precisely model the process of vesicle swelling and hence to define conditions under which vesicle membranes are strained, it has become necessary to define the strain end point, vesicle leakage or lysis. We have used the self-quenching fluorescent dye, CF (Weinstein et al., 1977; Fig. 1), to indicate solute leakage from our membrane vesicles. DOPG vesicles (100-nm diameter) were loaded with CF (100 mM) and extravesicular dye was removed by gel filtration chromatography. The intensity of CF fluorescence was monitored as samples of dye-loaded vesicles were diluted, either continuously or stepwise, with hypotonic buffer, creating trans-membrane osmotic gradients (see Methods). In the case of stepwise dilution, the CF fluorescence intensity reached a stable level during a 5-min equilibration period after each dilution step. Each dilution technique was applied during three replicate experiments.

Dye began to leak from the vesicles when the NaCl concentration of the extravesicular medium had been reduced to approximately 600 mM (an osmolarity ap-

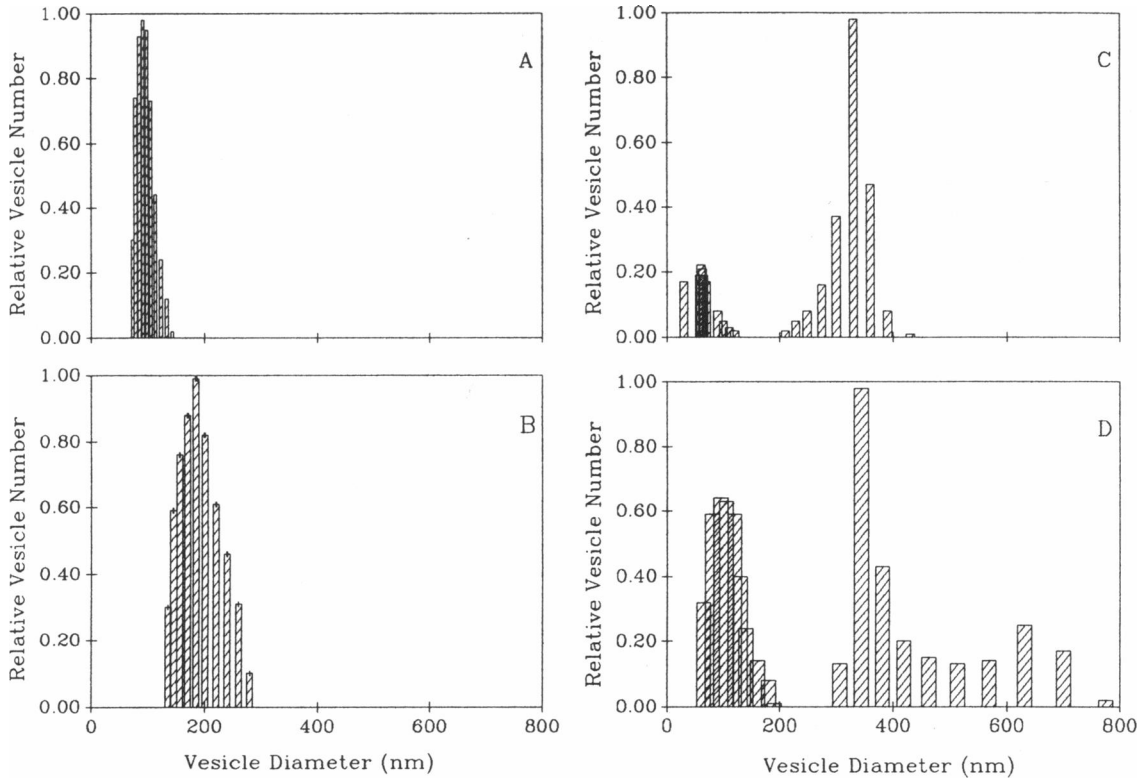

FIGURE 5 Vesicle number average diameter distributions of DOPG vesicles prepared by the extrusion technique. Vesicles were extruded through filters with 100 nm (*A*), 200 nm (*B*), 400 nm (*C*), or 600 nm (*D*) pores, and vesicle diameters were determined by DLS spectroscopy as described by Hallett et al. (1991).

proximately 300 mOsm lower than the initial osmolarity of the vesicle lumen) (Fig. 6). At extravesicular NaCl concentrations below 600 mM, more dye was released from vesicles allowed to equilibrate after stepwise dilution of the extravesicular medium than from vesicles that were diluted continuously. Each protocol yielded a distinct, reproducible CF release profile, however (Figs. 6 and 7, and data not shown). The two fluorescence profiles converged at extravesicular NaCl concentrations lower than ~250 mM. In this dilution range, the absolute osmolarity change created by each dilution step was much smaller than the absolute osmolarity change created by steps early in the dilution sequence. Thus, shorter adaptation periods are expected during the later steps. These results suggested that the continuous dilution rate and the rate at which vesicles responded to dilution by swelling and losing lumenal solutes were in a similar range. On average, the total dilution time for a stepwise dilution experiment was approximately 1.5-h, whereas the total dilution time for a continuous dilution experiment was approximately 1 h.

The stepwise dilution protocol was assumed to provide the soundest basis for further analysis of vesicle swelling and lysis. As indicated by CF release, very little solute was lost from the vesicles until an extravesicular NaCl concentration of 400 mM (a transmembrane osmotic gradient of approximately 700 mOsm) had been created. From that point, the CF fluorescence intensity increased linearly over a broad range of extravesicular osmolarities until approximately 50% of the dye had been released at an extravesicular NaCl concentration of ~100 mM. Further dilution of samples beyond that point was impractical. Fluorescence data derived from the experiments illustrated in Fig. 7 have been analyzed to further characterize the processes of vesicle swelling and lysis (Hallett et al., 1993).

We used DLS spectroscopy to detect structural changes in lipid vesicles, which had been subjected to transmembrane osmotic gradients sufficiently large to cause leakage of lumenal solutes (defined above). Small but reproducible increases in mean vesicle diameter were observed when the extravesicular NaCl concentration was reduced by stepwise dilution of vesicle suspensions. The post-dilution vesicle diameter distributions remained monomodal (data not shown), but they were shifted to slightly higher ranges (Table 3). Vesicle expansion was stable for many hours (see Methods) and it approached a limit as the imposed transmembrane gradient increased.

DISCUSSION

Vesicle preparation and analysis

Until recently, only moments or cumulants analysis (Koppel, 1972) had been applied to DLS data for vesicle

TABLE 2 **Properties of DOPG vesicles prepared by the extrusion technique**[a]

Extrusion pore size (*nm*)	Extrusion pressure (*psi*)	MOMENTS ANALYSIS Mean diameter (*nm*)	NUMBER DISTRIBUTION Mean diameter (*nm*)	Relative fraction of population	Lamellarity [*N*]
100	180	106	97	1.0	1.0
	250	97	97	1.0	0.9
	240	106	106	1.0	ND
200	110	195	187	1.0	1.4
	110	203	198	1.0	1.7
	140	200	194	1.0	ND
400	50	357	77	0.34	4.3
			328	0.66	
	60	499	27	0.94	1.4
			193	0.06	
	60	205	148	0.44	ND
			428	0.56	
600	40	728	111	0.58	2.2
			472	0.42	
	40	592	180	0.62	2.6
			540	0.38	
	50	454	41	0.92	ND
			222	0.05	
			632	0.03	

[a] Data are derived from three replicate experiments performed at each extrusion pore size. Vesicle diameter was determined by DLS spectroscopy and vesicle lamellarity was determined by NMR spectroscopy as described in Materials and Methods. MOMENTS ANALYSIS and NUMBER DISTRIBUTION refer to the parameters determined by the methods of Koppel (1972) and of Hallett et al. (1991), respectively (see text). ND is Not Determined.

size determination. Moments analysis can yield scattering intensity-average values for particle diameters, and for the width and skewness of particle diameter distributions. It accurately describes preparations in which all particles are solid spheres and particle size distributions are both monomodal and relatively monodisperse. There has been considerable concern that misleading conclusions could result from application of moments analysis to data derived from membrane vesicles with polydisperse, polymodal size distributions (Hope et al., 1986). By incorporating particle form factors and applying a discrete Laplace inversion routine, Hallett et al. (1991) have developed analytical procedures, which allow particle number average size distributions to be extracted from DLS data obtained from populations of particles that are not solid spheres and that have polydisperse, polymodal size distributions.

Early work suggested that the pH jump technique, applied to a variety of lipids including PE and PG, could yield populations of large unilamellar vesicles appropriate for the analysis of vesicle swelling by DLS spectroscopy (Aurora et al., 1985; Li and Haines, 1986; Li et al., 1986; Haines et al., 1987). That work was based on moments analysis of DLS data. Other analyses have revealed that vesicles made by the pH jump technique are variable, polydisperse, and polymodal in size distribution (Hauser and Gains, 1982; Rutkowski et al., 1991; this work, Table 1 and Fig. 2). Furthermore, the apparent simplicity of their behavior after osmotic swelling and/or lysis indicated by moments analysis of DLS data (Fig. 3) is contradicted when those data are analyzed by the procedure of Hallett et al., 1991 (Fig. 4). Re-evaluation of earlier data and comparison of the results of light scattering and electron microscopic analyses has also led Rutkowski et al. (1991) to suggest that earlier light-scattering studies of vesicle swelling were confounded by vesicle polydispersity.

Like others, we have been successful in preparing populations of relatively large, unilamellar vesicles by the extrusion technique. That technique involved shearing frozen and thawed multilamellar vesicles by passing them through polycarbonate filters. Although vesicles obtained by extrusion are not monodisperse, full analysis of our spectroscopic data has allowed us to define the size distribution and lamellarity of the vesicles present in each preparation (Fig. 5 and Table 2). We have therefore provided the first analysis of DOPG vesicle size distribution and lamellarity as a function of filter porosity. We obtained unilamellar vesicles that were monomodally distributed in size only by extrusion through filters with 100-nm pores. The smallest vesicles within preparations that were polymodally distributed may have been those present in the frozen and thawed suspension that were much smaller than the filter pore size. They would then pass through the pores without structural alteration.

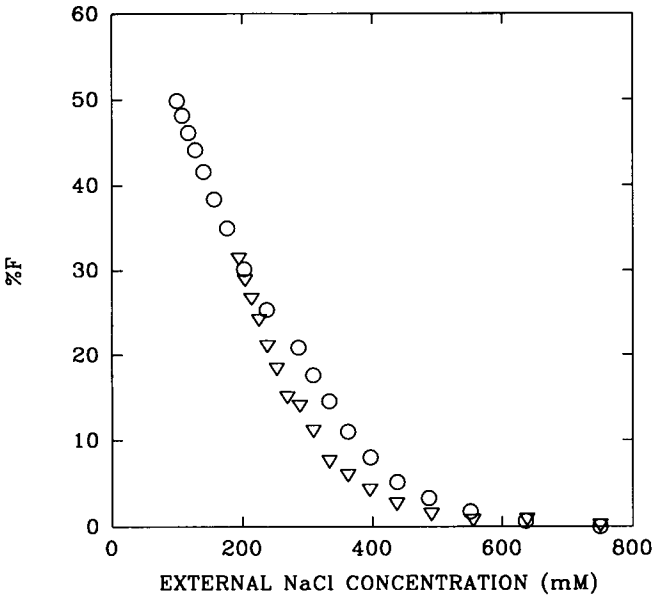

FIGURE 6 Release of CF from 100 nm DOPG vesicles prepared by extrusion in 100 mM CF, 600 mM NaCl, 20 mM NaMops, pH 7.4. Changes in relative CF fluorescence intensity (%F) were monitored as transbilayer osmotic gradients were created by either continuous (160 μL/min) (▽) or stepwise (○) dilution of the extravesicular medium (750 mM NaCl, 20 mM NaMops, pH 7.4) with 20 mM NaMops, pH 7.4. In the case of stepwise dilution, the system was allowed to equilibrate for 5 min after each dilution step before the fluorescence intensity was recorded.

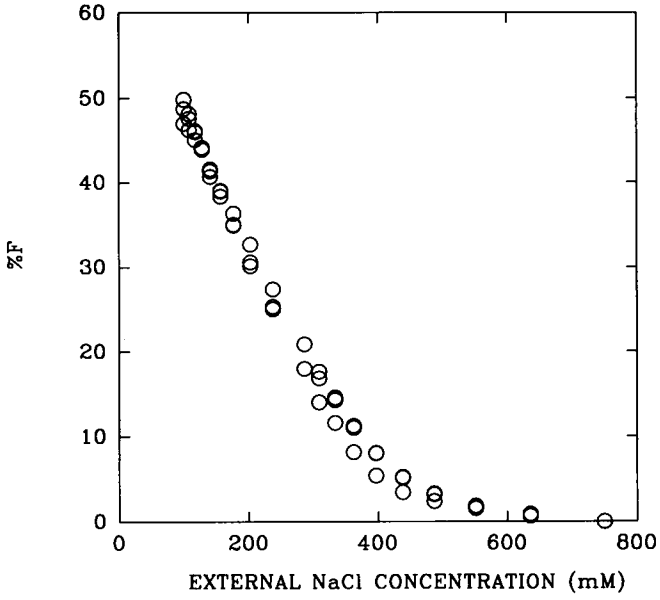

FIGURE 7 Release of CF from 100 nm DOPG vesicles prepared by extrusion in 100 mM CF, 600 mM NaCl, 20 mM NaMops, pH 7.4. Changes in relative CF fluorescence intensity (%F) were monitored as transbilayer osmotic gradients were created by stepwise dilution of the extravesicular medium (750 mM NaCl, 20 mM NaMops, pH 7.4) with 20 mM NaMops, pH 7.4. Data derived from three replicate experiments are plotted.

Our goal has been to devise experimental preparations and analytical techniques that would allow us to detect strain in vesicle membranes and to correlate biochemical activities with vesicle membrane strain. We have now identified DOPG vesicles, prepared by extrusion through 100-nm pores and characterized as described by Hallett et al. (1991), as an appropriate model system for these studies.

Vesicle expansion and leakage/lysis

Membrane vesicles swell when they are exposed to media of lower osmolarity than their lumenal contents. The resulting transmembrane osmotic gradient may be dissipated by a variety of processes. The term leakage is usually used to describe gradual loss of osmolytes from the vesicle lumen without alteration in gross vesicle structure. The term lysis usually evokes a process of vesicle rupture, involving at least transient alterations in gross vesicle structure, with consequent loss of osmolytes from the vesicle lumen. Models that predict the degree of vesicle swelling that will ensue from dilution of the vesicle medium must be based on assumptions regarding the stability (or instability) of the vesicle lumen osmolarity (Rivers and Williams, 1990). Models have been employed to deduce membrane elastic moduli from DLS measurements of vesicle expansion, although changes in lumen osmolarity were not estimated.

We employed DLS spectroscopy to monitor changes in the distribution of vesicle diameters (Table 3) and CF release to indicate changes in vesicle lumen composition (Fig. 6) ensuing from dilution of the vesicle medium. Vesicles diluted with hypotonic buffer showed small increases in diameter, which reached a limit of approximately 6% as dilution increased. CF release accelerated and then proceeded linearly as the osmolarity of the vesicle medium was reduced from ~1500 mOsm to ~100 mOsm. After stepwise dilution, a 5-min equilibration period was required before vesicles attained a new structure and lumenal solute composition that were then stable for many hours. These observations indicate that the terms swelling and solute leakage best describe the processes that result from dilution of the medium external to our DOPG vesicles.

TABLE 3 Post-dilution diameters of DOPG vesicles[a]

Dilution factor	Extravesicular NaCl concentration (Hypotonic) (*mM*)	Mean vesicle diameter (*nm*)		Swelling (%)
		Isotonic	Hypotonic	
3.0	250	115.0	118.2	3
4.6	163	115.2	121.4	5
7.3	103	115.0	122.0	6

[a] Scattering intensity average mean vesicle diameters were determined by DLS as described by Hallett et al. (1991). Estimated standard errors of vesicle diameters were 2 nm.

We used DLS spectroscopy to characterize the effects of osmotic gradients on the structures of large, polydisperse *E. coli* PE vesicles loaded with sucrose (Fig. 4) and of smaller, monodisperse DOPG vesicles loaded with NaCl (text and Table 3). Comparison of those systems suggests that variables such as lipid composition, medium composition, and vesicle size may all influence the response of membrane vesicle structure to osmotic gradients. We have met our immediate objectives by devising an experimental system whose behavior could be precisely defined and interpreted. To gain further knowledge of the membrane strain associated with these phenomena, our data have been fit to a physical model for vesicle expansion and leakage. Derivation of the model and its application to our data are presented in the companion paper (Hallett et al., 1993). Now that we have established a rigorously defined model system, it will likely be possible to apply these methodologies to more complex systems that have more direct biological relevance. Indeed, others have applied DLS and fluorescence techniques to characterize membrane elasticity (see, for example, Nagamachi et al., 1992). Our results suggest that meaningful interpretation of such experiments must be based on full analysis of DLS data in order that experimenters may recognize and deal with issues of vesicle polydispersity and structural lability.

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