

Osmotic properties of large unilamellar vesicles prepared by extrusion

Barbara L.-S. Mui,* Pieter R. Cullis,* Evan A. Evans,† and Thomas D. Madden§

Departments of Biochemistry,* Pathology,† and Pharmacology and Therapeutics§ The University of British Columbia, Vancouver, BC V6T 1W5 Canada

ABSTRACT We have examined the morphology and osmotic properties of large unilamellar vesicles (LUVs) prepared by extrusion. Contrary to expectations, we observe by cryo-electron microscopy that such vesicles, under isoosmotic conditions, are non-spherical. This morphology appears to be a consequence of vesicle passage through the filter pores during preparation. As a result when such LUVs are placed in a hypoosmotic medium they are able to compensate, at least partially, for the resulting influx of water by "rounding up" and thereby increasing their volume with no change in surface area. The increase in vesicle trapped volume associated with these morphological changes was determined using the slowly membrane-permeable solute [^3H]-glucose. This allowed calculation of the actual osmotic gradient experienced by the vesicle membrane for a given applied differential. When LUVs were exposed to osmotic differentials of sufficient magnitude lysis occurred with the extent of solute release being dependent on the size of the osmotic gradient. Surprisingly, lysis was not an all-or-nothing event, but instead a residual osmotic differential remained after lysis. This differential value was comparable in magnitude to the minimum osmotic differential required to trigger lysis. Further, by comparing the release of solutes of differing molecular weights (glucose and dextran) a lower limit of about 12 nm diameter can be set for the bilayer defect created during lysis. Finally, the maximum residual osmotic differentials were compared for LUVs varying in mean diameter from 90 to 340 nm. This comparison confirmed that these systems obey Laplace's Law relating vesicle diameter and lysis pressure. This analysis also yielded a value for the membrane tension at lysis of 40 dyn cm^{-1} at 23°C , which is in reasonable agreement with previously published values for giant unilamellar vesicles.

INTRODUCTION

Biological membranes display much higher permeabilities to water in comparison to other molecules and as a result are sensitive to osmotic gradients. Such gradients can have profound influences on cell structure and function and much recent work, therefore, has examined the mechanisms by which cells detect, and respond to, osmotic forces. Mechanosensitive ion channels have been reported in a variety of cell types and it has been suggested that these may control osmotic gradients by coupling bilayer permeability to membrane tension (Martinac et al., 1987; Miyamoto et al., 1988; Yang and Sachs, 1989; Morris, 1990). Osmotic gradients have also been suggested to play a role in membrane fusion-dependent processes such as exocytosis (Cohen et al., 1980; Hampton and Holz, 1983; Pollard et al., 1984).

Liposomes, which exhibit similar permeability properties to biological membranes (Bangham et al., 1967), represent a convenient model system with which to study osmotic stress and osmotically-induced lysis. In this context a number of studies have examined the osmotic sensitivity of large multilamellar vesicles (Bangham et al., 1967; Alhanaty and Livne, 1974; Blok et al., 1976). As model systems for osmotic studies, MLVs¹ are superior to small unilamellar vesicles

(SUVs) prepared by sonication, which are osmotically insensitive (Johnson and Buttress, 1973), however, they present two major difficulties. First, their onion-like structure with multiple internal aqueous compartments necessarily complicates studies involving lysis and solute release. Partial release of entrapped solute upon exposure of MLVs to an osmotic gradient, for example, might result from lysis of only outermost lamellae. The second disadvantage to the use of MLVs results from the non-equilibrium distribution of solutes within the various internal aqueous compartments (Gruner et al., 1985; Mayer et al., 1985). Consequently, when MLVs are placed in hypo- or hyperosmotic media different osmotic gradients will be experienced by different internal lamellae. These limitations can be overcome by the use of large unilamellar vesicles (LUVs) and we have therefore examined the osmotic properties of such vesicles prepared by the extrusion procedure (Olson et al., 1979; Szoka et al., 1980; Hope et al., 1985). While earlier reports described the use of photon correlation spectroscopy to measure osmotic swelling and lysis in LUVs prepared by the pH-adjustment method (Li et al., 1986; Li and Haines, 1986; Haines et al., 1987), these data have recently been retracted (Rutkowski et al., 1991).

Address correspondence to Dr. Thomas D. Madden, Department of Pharmacology and Therapeutics, Faculty of Medicine, 2176 Health Sciences Mall, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

¹ *Abbreviations used in this paper:* (FAT)MLV, (frozen and thawed) large multilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; EPC, egg phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine;

POPC, palmitoylphosphatidylcholine; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine; RHO-PE, N-(lissamine rhodamine B-sulfonyl)-dioleoylphosphatidylethanolamine; Chol, cholesterol; CF, 5(6)-carboxyfluorescein; HEPES, N-(2-hydroxyethyl)-piperazine-N-2-ethylsulfonic acid; QELS, quasi-elastic light scattering.

MATERIALS AND METHODS

Egg phosphatidylcholine (EPC), N-(lissamine rhodamine B-sulfonyl)-dioleoylphosphatidylethanolamine (RHO-PE), and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine (NBD-PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (standard for chromatography) was from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-citrate, [^{14}C]-glucose and [^3H]-glucose were purchased from NEN while [^3H]-dextran (avg. mol. wt. 70,000) and [^{14}C]-dipalmitoylphosphatidylcholine ([^{14}C]-DPPC) were obtained from Amersham Corp. (Arlington Hts., IL). 5(6)-carboxyfluorescein was purchased from Eastman Kodak Co. (Rochester, NY) and purified according to Weinstein et al. (1984). Gold 700 mesh bare EM grids were from Marivac Ltd. (Halifax, Canada).

Preparation of Lipid Vesicles. Lipid mixtures were prepared by co-lipophilization from benzene:methanol (95:5 vol/vol) under high vacuum (<60 millitorr) for a minimum of 4 h, protected from light. Unless otherwise stated, mixtures of phospholipid and cholesterol were prepared in a 55:45 molar ratio. Large multilamellar vesicles were prepared by hydration of the dry lipid in an appropriate solution and the dispersion was then freeze-thawed five times employing liquid nitrogen to promote equilibrium transmembrane solute distributions (Mayer et al., 1985). Large unilamellar vesicles were then prepared by extruding the frozen and thawed MLVs ten times through two stacked polycarbonate filters (Nuclepore) using an Extruder (Lipex Biomembranes, Inc., Vancouver, Canada) as described previously (Hope et al., 1985). Unless otherwise stated 100 nm pore size filters were used.

Measurements of Vesicle Lysis. Osmotically-induced vesicle lysis was determined by following the release of either carboxyfluorescein or a radiolabeled solute initially entrapped within the lipid vesicles. These marker molecules were encapsulated by including them in the buffer solution used to prepare the vesicles. Unencapsulated carboxyfluorescein or radiolabel was then removed by gel exclusion chromatography. Details of individual experiments are given below.

Time Course of Carboxyfluorescein Release From Vesicles. EPC:Chol vesicles (50 mg ml $^{-1}$, 120 nm average diameter) were prepared in 100 mM carboxyfluorescein, 600 mM NaCl, 60 mM HEPES, pH 7.4, and an aliquot (1 ml) passed down a Sephadex G-50 (medium) column (1.5 \times 10 cm) pre-equilibrated with an isoosmotic solution, 750 mM NaCl, 75 mM HEPES, pH 7.4. The peak lipid fraction (approximately 20 mM lipid) was collected. The high intravesicular concentration of carboxyfluorescein results in essentially complete fluorescence quenching. Only following release of the fluorophore into the external solution can a fluorescent signal be detected (for review see Weinstein et al., 1984). To determine the time course and extent of osmotically-induced lysis, vesicles were diluted into buffered saline solutions of various osmolarities at 22°C, and the appearance of fluorescence monitored using a SLM Aminco SPF 500C spectrofluorimeter at 492 nm excitation (bandwidth 1 nm) and 520 nm emission (bandwidth 10 nm). Control vesicles were diluted into isoosmotic saline solutions. Complete release of the fluorophore was achieved by adding the detergent octylglucopyranoside (final concentration 25 mM) to the sample. Similar protocols were used in other studies in which carboxyfluorescein release was followed.

Influence of Solute Molecular Weight on Osmotically-Induced Leakage. Vesicles of EPC:Chol (50 mg ml $^{-1}$) were prepared in either 300 mM sodium citrate, 30 mM HEPES, pH 7.4, or 800 mM sodium citrate, 80 mM HEPES, pH 7.4, containing 4 $\mu\text{Ci ml}^{-1}$ [^3H]-dextran (average mol wt 70,000) and 2 $\mu\text{Ci ml}^{-1}$ [^{14}C]-glucose. Untrapped radiolabels were then removed by passing the vesicles down a Sephadex 2B-CL column (1.5 \times 12 cm) pre-equilibrated in the same buffer used to prepare the vesicles. A hypoosmotic gradient was established by passing the vesicles down a similar 2B-CL column pre-equilibrated with 10 mM HEPES, pH 7.4. Following incubation at 22°C for 15 min

the amount of radiolabel retained was determined following passage of an aliquot (500 μl) down a Sephadex 2B-CL column (1.5 \times 10 cm).

Vesicle Trapped Volume Measurements. Changes in trapped volumes of extruded vesicles were characterized using EPC:Chol (labeled with 9×10^{-4} μCi [^{14}C]-DPPC per μmol lipid) LUVs prepared in 10 mM NaCl, 1 mM glucose (1.5 $\mu\text{Ci ml}^{-1}$ [^3H]-glucose). Glucose is slowly membrane permeable (half-times for equilibration into 100 nm EPC:Chol vesicles are 14 h and 1 h at 30°C and 45°C, respectively) and can therefore be used to follow changes in vesicle trapped volume over time. Vesicles were prepared by extrusion at 45°C and then diluted 5-fold into 1.5 $\mu\text{Ci ml}^{-1}$ [^3H]-glucose spiked solutions of either 1 mM glucose or 10 mM NaCl, 1 mM glucose. Samples were filtered sterilized using 0.22 μm polycarbonate filters (Nuclepore Corp.) and incubated at 45°C. At various times, 500 μl aliquots were withdrawn and vesicle trapped volumes determined after removal of the external radiolabel using pre-packed 9 ml Sephadex G-25 columns (Pharmacia) pre-equilibrated with 10 mM sodium citrate, 5 mM HEPES, pH 7.4.

Solute Equilibration in FATMLVs. To determine the influence of solute equilibration in frozen and thawed MLVs on extruded vesicle morphology, EPC:Chol was hydrated at 50 mg ml $^{-1}$ in 1 mM glucose (1.5 $\mu\text{Ci ml}^{-1}$ [^3H]-glucose) and, following five freeze-thaw cycles, incubated at 30°C in sterilized Eppendorf tubes. At various times aliquots were taken and LUVs prepared by extrusion through 100 nm filters at 30°C. These vesicles were then filter-sterilized through 0.22 μm Nuclepore filters and incubated under sterile conditions at 30°C for 75 h. Initial and final (after 75 h) trapped volumes of the extruded vesicles were determined as described above.

Cryo-Electron Microscopy. A drop of the liposomal suspension was placed on a bare 700 mesh gold EM grid, which had been glow-discharged for 15 s before use, and excess sample was removed by blotting the grid with filter paper. The grid was then plunged into liquid propane cooled to -187°C and transferred to a Gatan 126 cold stage at liquid nitrogen temperatures using a Reichart Jung Universal Cryo-fixation system. The sample was visualized using a Zeiss EM10C STEM.

Vesicle Fusion Analysis. A fluorescent assay based on resonance energy transfer between NBD-PE (the energy donor) and RHO-PE (the energy acceptor) was used to probe for vesicle fusion (Uster and Deamer, 1981). 1% NBD-PE or 1% RHO-PE labeled EPC:Chol vesicles containing 400 mM sodium citrate, 40 mM HEPES, pH 7.4, were initially mixed together in equal molar ratios and then osmotically shocked by dilution with 50 mM NaCl, 20 mM HEPES, pH 7.4, buffer. A decrease in fluorescence of NBD-PE (excitation 460 nm, emission 530 nm) would be indicative of vesicle fusion. A standard curve was constructed by measuring NBD-PE fluorescence in vesicles containing various ratios of the two fluorophores and total fluorescence determined following addition of Triton X-100 (2% final concentration). Fusion was probed under a variety of assay conditions including lipid concentration (0.5 to 10 mM EPC:Chol), lipid composition (EPC and EPC:Chol) and the magnitude of the applied osmotic gradient (870–1640 mOsm/kg).

Vesicle Size Analysis. The size distribution of extruded vesicles was determined by quasi-elastic light scattering (QELS) using a Nicomp 370 submicron particle sizer (Nicomp Instruments, Goleta, CA) operating at 632.8 nm and 5 mW. Aliquots of the vesicle suspension were diluted approximately 1:100 in 150 mM NaCl, 10 mM HEPES, pH 7.4, which had previously been filtered through a 0.22 micron polycarbonate filter. Measurements were made at 22°C.

Osmolarity Measurements. Solution osmolarities were determined from freezing point depression using an Advanced Digimatic 3C2 Osmometer. Standards (100, 290, and 900 mOsm/kg) were analyzed prior to samples which were measured at least in duplicate.

Analytical Procedures. In some experiments phospholipid concentrations were determined by phosphate assay (Fiske and Subbarow,

RESULTS

While the assumption is often made that exposure of LUVs to a hypoosmotic solution will result in the creation of an osmotic pressure, this assumption is not necessarily valid. In the case, for example, of vesicles that are not initially spherical, the influx of water resulting from an applied osmotic differential will first be accommodated by a change in shape as the vesicles maximize their volume to surface area ratio. Only if the applied differential exceeds the volume increase resulting from vesicles assuming a spherical shape will the lipid bilayer experience an osmotic pressure. Before examining the osmotic properties of LUVs prepared by extrusion, therefore, it is appropriate to first characterize their morphology.

Morphological and associated volume changes for extruded LUVs

Earlier studies by Talmon et al. (1990) employing the lipid dioleoylphosphatidylethanolamine (DOPE) at pH 9.9 indicated that vesicles prepared by extrusion are non-spherical. We therefore examined EPC:Chol LUVs prepared in 150 mM NaCl, 20 mM HEPES, pH 7.4, using the technique of cryo-electron microscopy. In agreement with this earlier study we observe that LUVs maintained under isoosmotic conditions exhibit a variety of morphologies (Fig. 1 *A*). While the oblong and tubular images present clearly arise from non-spherical vesicles, some circular images are also observed. Based on results to be presented later, however, it is likely that many of these circular images arise, not from spherical vesicles but from systems which are discoid in shape and which at certain orientations would appear circular when viewed from above. Exposure of extruded vesicles to a hypoosmotic solution should result in an influx of water, causing the vesicles to adopt a spherical shape (which maximizes their volume to surface area ratio). This behavior is illustrated in Fig. 1 *B*. While vesicles prepared in saline solutions are non-spherical, similar systems made in distilled water and viewed by cryo-electron microscopy (Fig. 1 *C*) are spheres. This would indicate that in the absence of an impermeant solute membrane bending forces are sufficient to establish a spherical shape. These forces are relatively small, however (Evans and Hochmuth, 1978), and for vesicles containing an impermeant solute are readily opposed by the osmotic forces resulting from any volume change. It should be noted that earlier studies, which employed the technique of freeze-fracture electron microscopy to visualize extruded vesicles, did not report the presence of non-spherical systems (Hope et al. 1985; Mayer et al. 1986). This discrepancy may result from the use of high concentrations (approximately 3.4 M) of the membrane-perme-

able solute, glycerol, as a cryoprotectant in samples prepared for freeze-fracture.

Before characterizing the influence of osmotic gradients on extruded LUVs it is important to first quantitate the change in intravesicular volume associated with "rounding up" of such systems. The influx of water associated with this process will dilute the intravesicular solution, and to determine the osmotic gradient across the vesicle membrane it is necessary to correct for this effect. We therefore prepared EPC:Chol vesicles in 10 mM NaCl and applied a small osmotic pressure by adjusting the external salt concentration to 2 mM. Both solutions also contained 1 mM glucose ($1.5 \mu\text{Ci ml}^{-1}$ [^3H]-glucose). As glucose is slowly membrane permeable ($t_{1/2} = 1$ h, at 45°C), it can be used to measure any change in vesicle trapped volume following extrusion without impeding the process. The trapped volumes of 200 nm vesicles, incubated for up to 18 h under isoosmotic conditions or exposed to a small osmotic gradient, are shown in Fig. 2 *A*. In the absence of a gradient (10 mM NaCl inside and outside) little change in trapped volume is observed with an average value of $1.3 \mu\text{l}/\mu\text{mole}$ lipid obtained over the time course followed. In contrast, a fairly rapid increase in trapped volume is shown by vesicles exposed to a small hypoosmotic gradient with a value of $2.0 \mu\text{l}/\mu\text{mole}$ lipid obtained after 18 h. It should be noted that this volume increase cannot be the result of osmotically-induced swelling of initially spherical vesicles. Based on an elastic area expansivity modulus of $1,000 \text{ dyn cm}^{-1}$ (Evans and Needham, 1987), an osmotic differential of 16 mOsm/kg would generate a volume increase of less than 0.2%. Similar changes in trapped volume upon "rounding up" were observed for vesicles prepared through 50, 100, and 400 nm pore size filters. To confirm that the measured increases in trapped volume reflected a change in vesicle shape, samples were examined by cryo-electron microscopy. As predicted, micrographs of vesicles examined shortly after extrusion show a variety of non-spherical vesicle morphologies (Fig. 3 *A*). In contrast, the same vesicles exposed to a small osmotic differential (10 mM NaCl inside; 2 mM NaCl outside), while of similar size, are clearly spherical (Fig. 3 *B*).

Two hypotheses can be advanced to account for the observation that extruded vesicles are non-spherical. First, it may be suggested that full solute equilibration is not achieved during preparation of frozen and thawed MLVs (FATMLVs) and the extruded vesicles simply reflect this condition. Alternatively, it is possible that the extrusion process generates non-spherical systems and osmotic forces then prevent "rounding up". To determine which of these two explanations applies, EPC:Chol FATMLVs were prepared at 30°C in 1 mM glucose ($1.5 \mu\text{Ci ml}^{-1}$ [^3H]-glucose) and incubated at 30°C for up to 75 h. At various times aliquots were taken, 100 nm extruded vesicles prepared and vesicle trapped volumes determined immediately following extrusion or after incu-

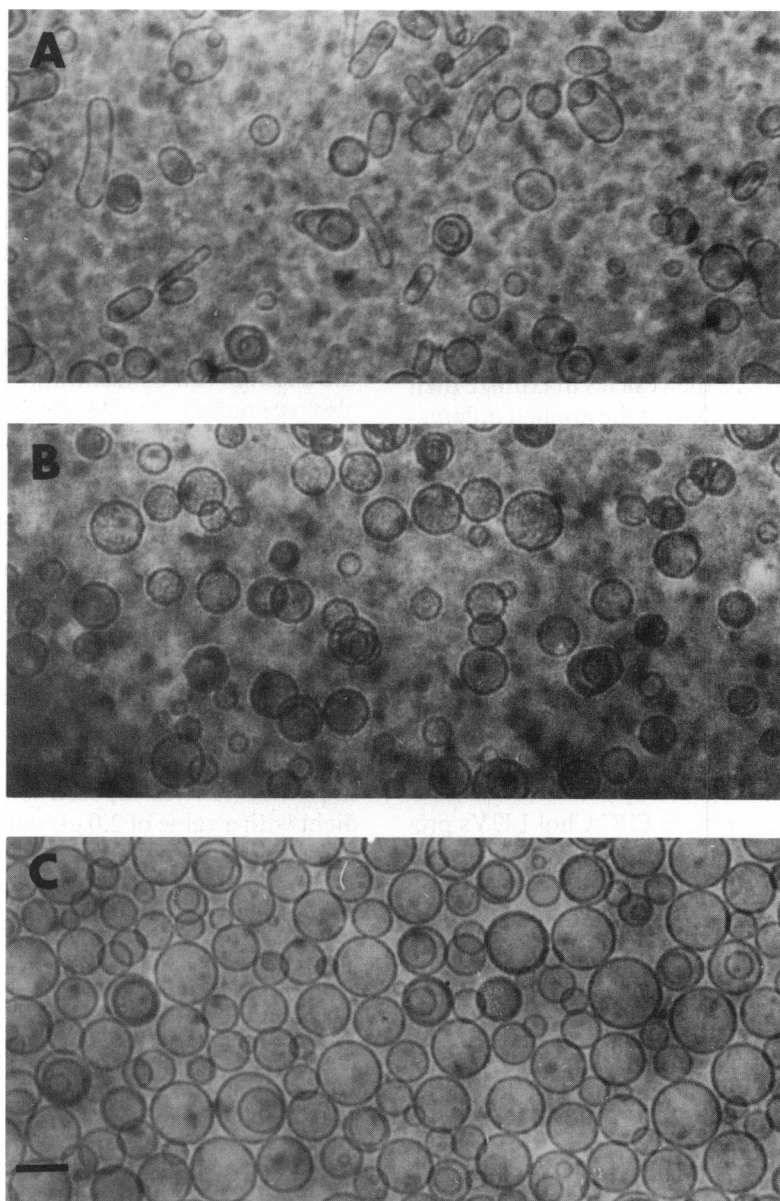


FIGURE 1 Cryo-electron micrographs of extruded vesicles. Vesicles of EPC:Chol were made in 150 mM NaCl, 20 mM HEPES, pH 7.4, and exposed to an (A) isoosmotic or (B) hypoosmotic (75 mM NaCl, 20 mM HEPES, pH 7.4) solution. The lipid concentration is 20 mg ml⁻¹. Vesicles of EPC:Chol prepared in distilled water are shown in (C) at a lipid concentration of 60 mg ml⁻¹. The bar represents 100 nm.

bation at 30°C for 75 h. Control experiments and cryo-electron microscopy verified that initially non-spherical extruded vesicles are able to “round up” in the presence of 1 mM glucose. The experimental rationale is that if the initial low trapped volume of extruded vesicles reflects a non-equilibrium solute distribution in the FATMLVs from which they are prepared, then incubation of FATMLVs in the presence of a permeant solute (glucose) will alleviate this non-equilibrium situation. As shown in Fig. 2 B, however, the discrepancy between initial and final trapped volumes of extruded vesicles is unaffected by pre-incubation of the FATMLVs. This indicates that it is the extrusion process itself which is responsible for the observed vesicle morphology. Addi-

tional support for this contention is provided by experiments in which LUVs prepared in 1 mM glucose (containing 1.5 $\mu\text{Ci ml}^{-1}$ [³H]-glucose), using 100 nm pore size filters, were allowed to fully “round up” and were then reextruded through filters of the same pore size. As shown in Table 1, the trapped volume of vesicles allowed to “round up” following extrusion increases from 1.45 to 2.08 $\mu\text{l}/\mu\text{mole}$ lipid in agreement with previous experiments (c.f. Fig. 2 A). If these spherical vesicles are reextruded, however, their trapped volume is significantly reduced, to 1.74 $\mu\text{l}/\mu\text{mole}$ lipid. That this reduction is a consequence of a change in vesicle shape rather than a decrease in average diameter is illustrated by the fact that upon subsequent “rounding up” the

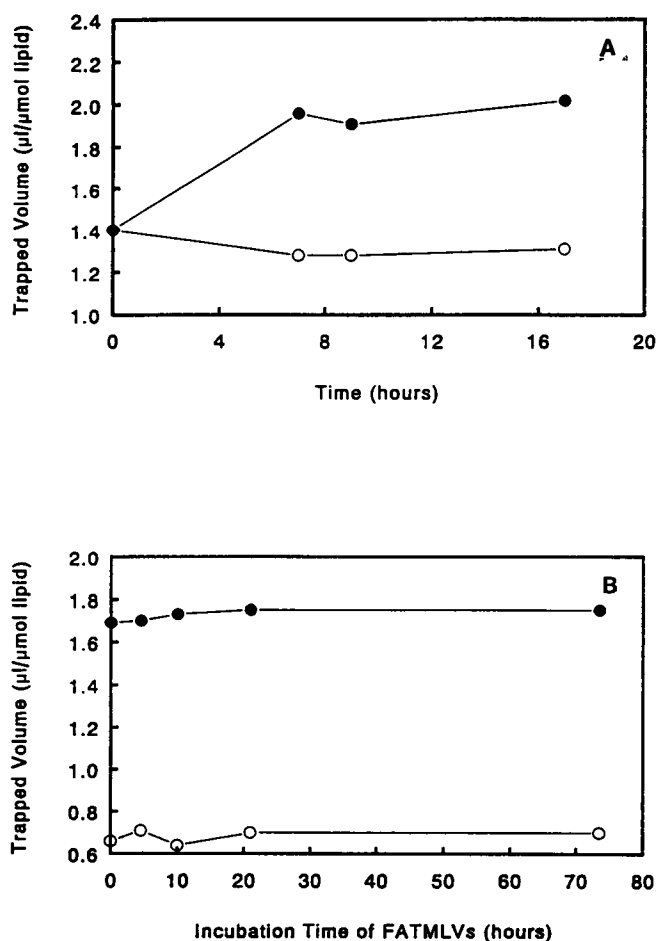


FIGURE 2 Trapped volume characterization of extruded vesicles. (A) Vesicles of EPC:Chol (50 mg ml⁻¹, 190 nm diameter) were made in 10 mM NaCl, 1 mM glucose (1.5 μCi ml⁻¹ [³H]-glucose) and diluted 5-fold with either 1 mM glucose, ●; or 10 mM NaCl, 1 mM glucose, ○, and incubated at 45°C. Trapped volumes were measured at various times as described under Methods. (B) An EPC:Chol FATMLV suspension made in 1 mM glucose (1.5 μCi ml⁻¹ [³H]-glucose) was incubated at 30°C. At various times, aliquots were extruded through 100 nm filters and vesicle trapped volumes measured immediately, ○; or after incubation at 30°C for 75 h, ●.

trapped volume of these vesicles is the same as prior to reextrusion (Table 1). These experiments provide compelling evidence that passage through the filter pores is largely responsible for the non-spherical vesicle morphology observed.

Osmotic lysis of large unilamellar vesicles

Having examined the morphological changes resulting from exposure of extruded LUVs to hypoosmotic solutions, we next sought to characterize osmotic lysis of such systems. In experiments in which the release of a water soluble marker from osmotically-stressed vesicles is measured, however, the first criterion to be established is whether the release represents a lytic event or is due to

permeation. In Fig. 4 is shown the time course of carboxyfluorescein release from EPC:Chol vesicles exposed to an osmotic gradient greatly in excess of that which could be accommodated by "rounding up" of the vesicles (see figure legend for details). It can be seen that most of the resulting loss of fluorophore occurs within about 30 s, with little further release up to 10 min. Control vesicles diluted into an isoosmotic solution, as anticipated, show no release over this time period. It should be noted that the time course shown in Fig. 4 cannot be assumed to represent the rate of lysis *per se*. Dequenching of carboxyfluorescein following vesicle rupture will require probe diffusion which may represent the rate limiting process. In similar experiments in which release of [¹⁴C]-citrate from osmotically-stressed EPC:Chol vesicles was followed for up to 60 min, again essentially all of the loss occurred within the earliest measurable time point (results not shown). These results indicate that the loss of vesicle contents, which occurs when they are subjected to a hypoosmotic gradient, is the result of lysis and is not due to permeation, which would be expected to occur at a fairly constant rate.

Given the observation that, under the conditions described in Fig. 4, an applied osmotic gradient of 1,400 mOsm/kg results in the release of approximately 45% of initially entrapped carboxyfluorescein, two generalized interpretations can be advanced. Either 45% of the vesicles lyse, releasing all of their entrapped fluorophore, or alternatively, all the vesicles lyse but lose only 45% of their contents. To distinguish between these two hypotheses, the experiment described below was performed.

When carboxyfluorescein is entrapped within EPC:Chol vesicles, fluorescence quenching is seen at fluorophore concentrations above approximately 0.1 mM. This behavior is illustrated in Fig. 5, which indicates a relatively steep concentration-dependent quenching between 0.2–10 mM carboxyfluorescein. We can take advantage of fluorophore quenching to distinguish between the two lysis models described above using a procedure similar to that described by Weinstein et al. (1981). The experimental rationale is that if lysis results in complete loss of intravesicular solute, then any carboxyfluorescein remaining entrapped must be in unlysed vesicles at its original concentration and hence its initial level of quenching. Conversely, if all vesicles lyse, releasing a portion of their contents only, then the level of fluorophore quenching will be reduced in direct proportion to the percentage of entrapped solute released. Vesicles (210 nm, average diameter) were therefore prepared in 7.5 mM carboxyfluorescein, 2.5 M NaCl, 20 mM HEPES, pH 7.4, containing 2 μCi ml⁻¹ [¹⁴C]-citrate. Aliquots of the vesicle preparation were then osmotically shocked by passage down Sephadex G-50 columns preequilibrated with either 1.25 M NaCl, 20 mM HEPES, pH 7.4, or 20 mM HEPES, pH 7.4, only. Vesicles elute in the column void volume and are efficiently separated from any carboxyfluorescein or [¹⁴C]-citrate released. Following iso-

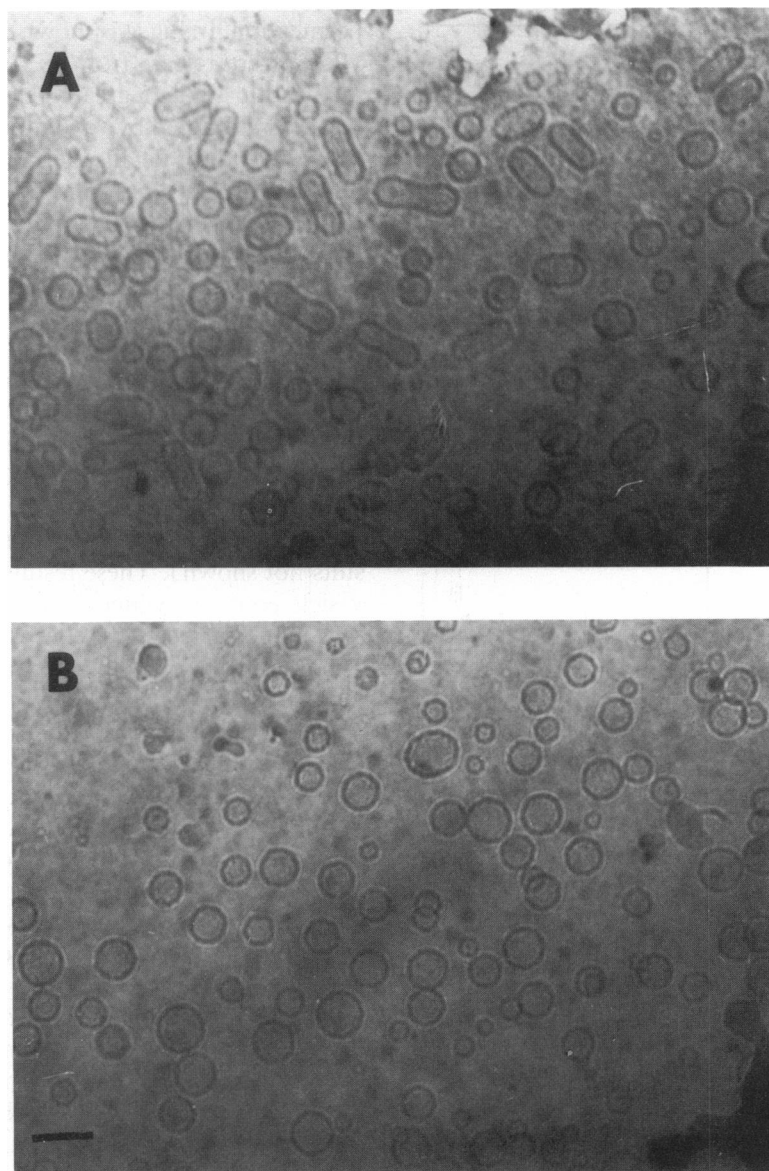


FIGURE 3 Morphology of extruded vesicles revealed by cryo-electron microscopy. Vesicles of EPC:Chol (mean diameter 80 nm) prepared in 10 mM NaCl, 1 mM glucose were diluted into an isoosmotic medium (*A*) or a hypoosmotic solution (2 mM NaCl, 1 mM glucose), (*B*). The lipid concentration is 20 mg ml⁻¹ and the bar represents 100 nm.

lation, the vesicles were analyzed to determine the amount of carboxyfluorescein and [¹⁴C]-citrate retained, and the level of fluorescent quenching of entrapped carboxyfluorescein. As shown in Table 2, similar losses of both [¹⁴C]-citrate and carboxyfluorescein are observed upon osmotic lysis, the extent of solute release being dependent upon the size of the applied osmotic differential. Interestingly, the level of carboxyfluorescein quenching for osmotically-shocked vesicles is dependent upon the extent of solute release (Table 2). Using the quench curve shown in Fig. 5 we can calculate the average carboxyfluorescein concentration remaining within vesicles exposed to an osmotic gradient (Table 2). Correcting for the volume increase due to "rounding up" (53%, c.f. Fig. 2 *A*) the average fluorophore concen-

tration remaining entrapped is in good agreement with that predicted by a model in which only partial solute release occurs during lysis (Table 2), with the extent of release being dependent on the size of the osmotic gradient. Under lysis conditions producing solute losses of greater than 60%, carboxyfluorescein quenching is actually lower than expected. This likely reflects a shift in the quench curve at lower intravesicular salt concentrations (Mui, B. L-S., unpublished observations).

We next examined in greater detail how the magnitude of the osmotic differential between the vesicle interior and the external solution affected the loss of contents during lysis. Vesicles (100 nm average diameter) composed of EPC:Chol were prepared in a solution of relatively high osmolarity (2.35 M NaCl, 100 mM car-

TABLE 1 Influence of reextrusion on trapped volume of spherical vesicles

	Trapped volumes ($\mu\text{l}/\mu\text{mol lipid}$)*	
	Initial	Final [‡]
First extrusion	1.45 ± 0.04	2.08 ± 0.05
Reextrusion	1.74 ± 0.04	2.04 ± 0.05

* Values given represent the mean and standard deviations of three samples.

[‡] The POPC vesicles were allowed to fully "round up" by incubation at 30°C for 24 h.

boxyfluorescein, 20 mM HEPES, pH 7.4) and then diluted into solutions of lower osmolarity. In Fig. 6 *A* the percentage loss of carboxyfluorescein that occurs is compared to the applied osmotic differential. It can be seen that little carboxyfluorescein release is observed until an osmotic differential of about 2,000 mOsm/kg is applied. At differentials in excess of 2,000 mOsm/kg, progressively more of the entrapped fluorophore is lost. Clearly, however, volume changes associated with vesicles "rounding up" will result in the applied osmotic differential being considerably greater than the actual differential experienced by the vesicle membrane. In Fig. 6 *B*, therefore, we have corrected for both vesicle volume changes and, where applicable, for solute loss during lysis, in order to obtain the theoretical residual osmotic differential following lysis. The predicted relationship between applied and residual osmotic differentials (in the absence of lysis) for extruded LUVs is shown by the dashed line (Fig. 6 *B*). This indicates that as the applied gradient is increased to 1,780 mOsm/kg the vesicles are able to accommodate the resulting influx of water by adopting a more spherical shape, consequently the residual osmotic gradient remains zero. At a differential of

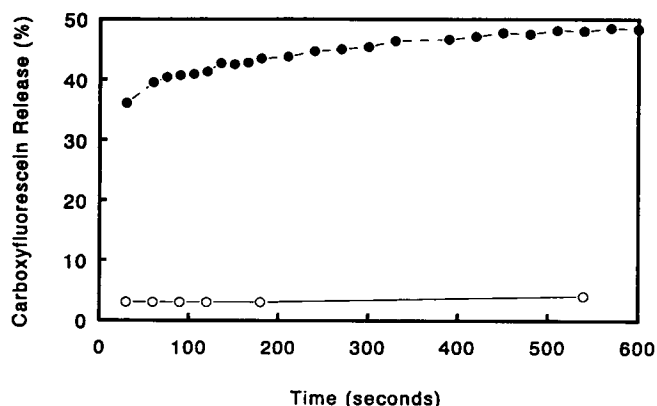


FIGURE 4 Time course of carboxyfluorescein release from osmotically stressed vesicles. EPC:Chol vesicles containing 100 mM carboxyfluorescein, 600 mM NaCl, 60 mM HEPES, pH 7.4, were diluted (1:60) into 10 mM HEPES, pH 7.4 (●), or isoosmotic, 750 mM NaCl, 75 mM HEPES, pH 7.4 (○), at 23°C.

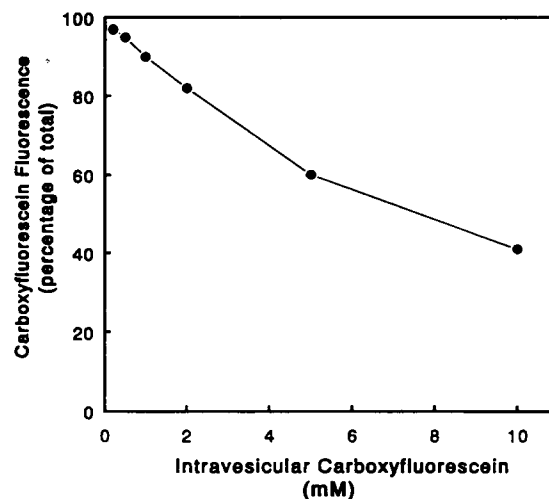


FIGURE 5 Quench curve of carboxyfluorescein entrapped in EPC:Chol vesicles. Carboxyfluorescein fluorescence was determined in EPC:Chol vesicles containing 20 mM HEPES, pH 7.4, 500 mM citrate, and 0.2 to 10 mM carboxyfluorescein. Total carboxyfluorescein fluorescence was obtained by solubilizing the vesicles in 25 mM octylglucopyranoside.

1,780 mOsm/kg, however, the vesicles are fully spherical and any additional increase in the applied gradient produces a corresponding increase in the residual differential. If we now compare the experimental data to this theoretical prediction, a number of observations can be made. At applied differentials between 500–1,500 mOsm/kg the experimental data points lie below the zero line. This results from a slight release of carboxyfluorescein (c.f. Fig. 6 *A*) and is to be expected, given that all vesicles in the population will not accommodate precisely the same volume change upon "rounding up." The fact that this deviation from predicted behavior is relatively small would suggest that extruded LUVs are fairly homogeneous with respect to their volume-to-surface area ratio. At higher applied osmotic differentials, the residual osmotic gradient approaches a limiting value of about 650 mOsm/kg. We would suggest that this value represents the average maximum osmotic gradient the vesicles can withstand. When LUVs are exposed to differentials in excess of this value, lysis (or more likely a series of lytic events) will occur and sufficient intravesicular solute will be lost such that upon membrane resealing the vesicles can withstand the remaining osmotic gradient.

Size of the membrane defect formed during osmotic lysis

Given that osmotic lysis results in only a portion of the intravesicular solute being released such that the internal aqueous solution remains hyperosmotic with respect to the external medium, bilayer resealing must be fairly rapid and the defects generated by lysis may be relatively

TABLE 2 Evaluation of theoretical models of solute release during lysis

Applied osmotic differential (mOsm/kg)	Intravesicular [CF] (mM)					
	% Retained			Theoretical		Measured [§]
	[¹⁴ C]-citrate	CF	% Fluorescence	Total*	Partial [‡]	
0	100	100	42	7.5	7.5	7.5
2400	82	81	67	5.0	4.0	3.9
4800	32	38	87	5.0	1.9	1.3

* Theoretical intravesicular carboxyfluorescein concentration based on a model in which total solute release occurs from vesicles during lysis and hence any retained carboxyfluorescein is in unlysed vesicles. The calculation is based on an initial carboxyfluorescein concentration of 7.5 mM and assumes a 53% maximum increase in vesicular volume due to "rounding up."

‡ Theoretical intravesicular carboxyfluorescein concentration based on a model in which lysis results in partial solute release from all vesicles in the population. Again the calculation is based on an initial carboxyfluorescein concentration of 7.5 mM and assumes a maximum 53% increase in vesicular volume due to "rounding up."

§ Determined from Fig. 5, based on the measured fluorescence quenching.

small. In an attempt to probe the size of the bilayer defects created during osmotic lysis, aqueous markers of differing molecular weights were entrapped within EPC:Chol vesicles (100 nm average diameter) which were then subjected to an osmotic gradient. It can be seen from Table 3 that the percentage release of both glucose (molecular weight 180) and dextran (average molecular weight 70,000) is similar, indicating that the membrane defects occurring during lysis are likely greater than about 12 nm diameter, the approximate average diameter of dextran.

Influence of vesicle size on osmotic lysis

When MLVs are extruded through polycarbonate filters, the mean diameter of the resulting vesicles is determined by the filter pore size. Before examining the influence of vesicle size on osmotic lysis, however, it was necessary to quantitate the volume changes associated with "rounding up" for each system. The experiment described in Fig. 2 was repeated, therefore, to measure trapped volume increases for vesicle preparations of mean diameters 90, 100, 190, and 340 nm (as measured by QELS). Similar volume increases (50, 53, 54, and 50%) were obtained for all four samples. Using conditions similar to those described in Fig. 6, carboxyfluorescein release from these four vesicle systems was determined as a function of the applied osmotic differential. In Fig. 7 *A* are plotted the theoretical residual osmotic differentials for these systems, after correcting for vesicle volume changes and any solute release. Clearly, there is a considerable size dependency with the smaller systems tolerating much greater residual osmotic differentials than the larger vesicles. This observation is consistent with previous studies (Sun et al., 1986) and is predicted by Laplace's Law, which relates the pressure difference across a closed membrane to membrane tension. Laplace's Law for a spherical vesicle can be written as:

$$\tau = \Delta P r / 2$$

where τ is the membrane tension, ΔP is the pressure difference between the inside and outside, and r is the radius. Lysis will occur when the maximal membrane tension is exceeded and so, for a given value of τ , vesicle size and the osmotic pressure required to produce lysis should be inversely related. To confirm that this relationship holds true for the vesicle systems under study, the data presented in Fig. 7 *A* are replotted in 7 *B*. A linear relation between the maximum tolerated osmotic difference and the inverse of vesicle size is obtained, with an intercept close to zero, in agreement with theory. Further, from the slope of the line a value of 40 dyn cm⁻¹ for the membrane tension at lysis can be calculated, in reasonable agreement with values reported by Needham and Nunn (1990) for giant bilayer vesicles composed of stearyloleoylphosphatidylcholine and cholesterol.

While vesicles prepared by extrusion through 100 nm or smaller pore size filters are almost exclusively unilamellar (Hope et al., 1985) systems prepared through larger filters contain some multilamellar structures. Using the ³¹P-NMR technique with manganese as a broadening reagent, the percentage of unilamellar vesicles in systems prepared through 200 and 400 nm pore size filters has been shown to be 90% and 66%, respectively (Mayer et al., 1986). Multilamellar systems are not present, therefore, to an extent where they would compromise the data analysis presented above. An additional consideration, however, concerns the suggestion, as mentioned in the Introduction, that osmotic gradients may play a role in membrane fusion. If fusion accompanied osmotic lysis of extruded LUVs, then values for the mean vesicle diameter would need to be corrected accordingly. However, using a fluorescence assay based on resonance energy transfer between two lipid derivatives, NBD-PE and RHO-PE, no indication of fusion was obtained for vesicles exposed to an osmotic gradient or for control vesicles maintained under isoosmotic conditions.

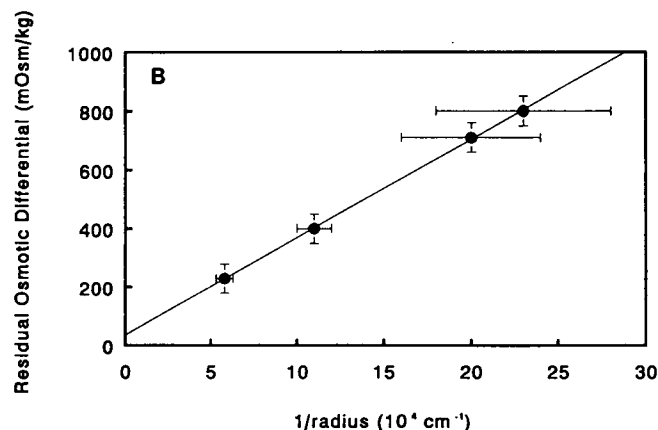
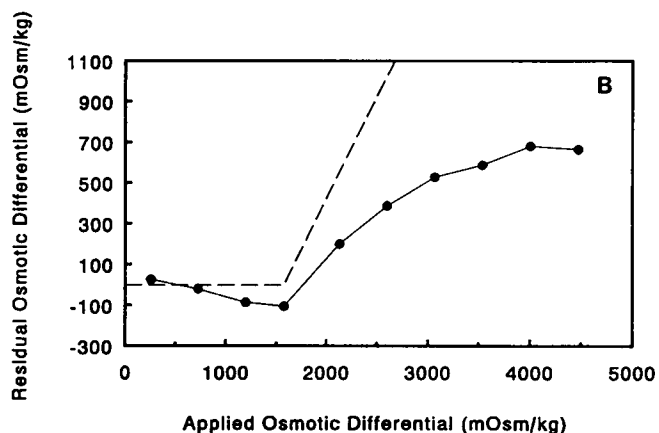
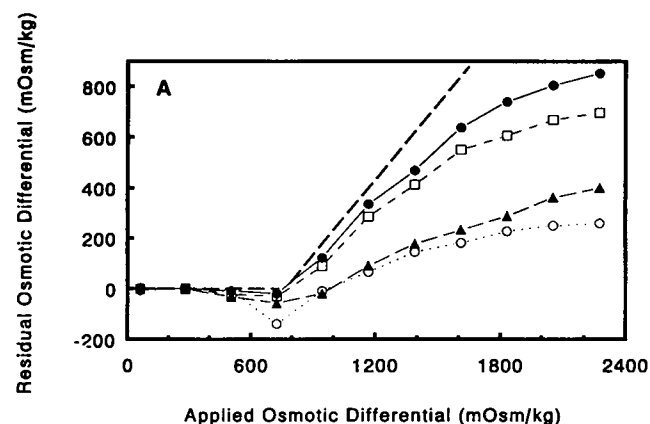
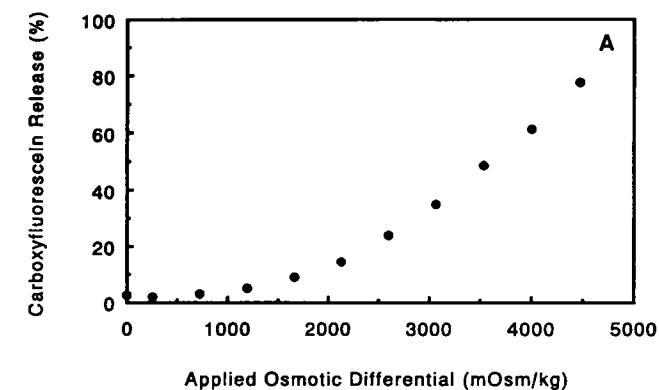


FIGURE 6 Influence of the osmotic differential on vesicle lysis. (A) EPC:Chol vesicles containing 100 mM carboxyfluorescein, 2.35 M NaCl, 20 mM HEPES, pH 7.4 (4,550 mOsm/kg), were diluted into hypoosmotic NaCl buffers at 23°C and carboxyfluorescein release measured after 3 min. (B) The residual osmotic differential was calculated for the EPC:Chol vesicles shown in (A) after taking into account a 53% increase in vesicular volume. The dotted line represents the expected osmotic differential in the absence of lysis. Results shown have also been corrected for the fluorescence exhibited by the isoosmotic controls (3% of total fluorescence).

FIGURE 7 Influence of vesicle size on osmotic lysis. (A) EPC:Chol vesicles containing 100 mM carboxyfluorescein, 1.15 M NaCl, 20 mM HEPES, pH 7.4, were made by extrusion through 50 nm, 100 nm, 200 nm, or 400 nm pore sized filters and had mean diameters of 90 nm, 100 nm, 190 nm, and 340 nm, respectively, as measured by QELS. Following dilution into various hypoosmotic NaCl buffers, the residual osmotic differential was calculated. Vesicular volume increases of 50, 53, 54, and 50% respectively were also taken into account in the calculations. (B) Relationship between vesicle radius and osmotic tolerance. From the data shown in (A) the maximum osmotic differential tolerated by different sized systems is plotted against the reciprocal of vesicle radius. Data points shown represent the mean \pm standard deviations.

DISCUSSION

Large unilamellar vesicles are widely used as model systems in membrane research. While they can be prepared

TABLE 3 Influence of solute molecular weight on release during vesicle lysis

Osmotic differential (mOsm/kg)	% Release*	
	[¹⁴ C]-glucose	[³ H]-dextran
800	12	12
2200	67	66

* EPC:Chol vesicles were subjected to hypoosmotic lysis as described under Methods.

by a variety of techniques, the extrusion procedure is perhaps the most commonly employed protocol, with advantages of generality, reproducibility, and convenience. The results presented here are frequently counterintuitive and provide important insight into the morphology and osmotic properties of LUVs prepared by extrusion. In addition, the characterization of osmotic lysis in this system has broad implications.

A number of earlier studies have examined the morphology of giant unilamellar vesicles (diameter 20–30 μ m) which can be conveniently visualized using phase contrast microscopy. In addition to characterizing the mechanical and osmotic properties of such systems

(Evans and Waugh, 1977; Evans and Needham, 1987) these studies demonstrated that initially spherical vesicles undergo characteristic shape changes to accommodate any increase in their surface area resulting from thermal expansion of the lipid bilayer (Evans and Rawicz, 1990; Sackmann et al., 1986). Such behavior has also been the subject of theoretical models (Miao et al., 1991; Berndt et al., 1990). In addition to osmotic and thermal factors, the morphology of giant unilamellar vesicles can also be influenced by the relative lipid packing densities of the inner and outer leaflet of the bilayer (Farge and Devaux, 1992). In the studies reported above, the initial, unperturbed vesicle systems were generally spherical and it is not surprising, therefore, that large unilamellar vesicles prepared by extrusion were also assumed to be spheres. Interestingly, it was noted by Hope et al. (1985) that the trapped volume of LUVs prepared through 100 nm pore size filters was appreciably smaller than predicted by theory. We show here that this discrepancy results from the non-spherical morphology of extruded LUVs and that if such systems are allowed to fully "round up," then trapped volumes in good agreement with theory are obtained. Further we have shown that it is passage through the filter pores that is responsible for the shape, and hence trapped volume, of extruded vesicles.

The morphology of LUVs prepared by extrusion has important implications concerning their osmotic properties. The volume changes associated with vesicles "rounding up" has the effect of diluting the intravesicular solute and hence, unless corrected for, leads to an overestimation of the osmotic gradient experienced by the vesicle membrane. In the present work we have quantitated these changes in intravesicular volume, allowing an accurate determination of actual osmotic differentials. When we examine the effect on large unilamellar vesicles, of osmotic gradients of varying magnitude, we observe that vesicles are able to tolerate the osmotic pressure resulting from relatively small differentials. At a characteristic pressure, however, lysis occurs with the amount of intravesicular solute released depending on the magnitude of the osmotic differential. Of considerable interest is the observation that osmotic lysis is not an "all-or-nothing" event, that vesicles release only a portion of their contents during lysis and can in fact reseal while their intravesicular medium remains hyperosmotic with respect to the external solution.

Our observation that dextran and glucose are released to the same extent during lysis would suggest that for LUVs of 100 nm diameter a lower limit of about 12 nm diameter can be set for the bilayer defect created, based on the average hydrodynamic diameter of dextran. We can also derive a size estimate for this lysis-induced defect from theoretical considerations. When spherical vesicles are exposed to an osmotic differential, the resulting

hydrostatic pressure produces swelling and hence a small increase in membrane surface area. If we consider that at a critical pressure lysis occurs, the hydrostatic pressure is relieved, and the membrane returns to its original surface area, then the area of the defect created will equal the area difference between the initial and fully swollen membrane surface areas. Based on a value for the critical areal strain (α_c) of 0.03 (Needham and Nunn, 1990; SOPC:Chol vesicles) we can calculate that for 100 nm diameter systems this area difference equals 9.42×10^{-12} cm² corresponding to a spherical hole of about 17 nm diameter. While this calculation is instructive and yields a hole size consistent with our experimental data we should caution that additional considerations such as the energy factor relating to exposure of the hydrophobic bilayer interior may constrain hole growth.

Based on the observations reported herein it is our hypothesis that osmotic lysis involves the following series of events. Upon exposure of LUVs to a relatively large osmotic differential, water influx generates an osmotic pressure resulting in bilayer rupture. The hydrostatic pressure is thereby released, a fraction of the intravesicular solute lost, and the bilayer then reseals. The intravesicular solution remains hyperosmotic, however, resulting in further water influx, subsequent membrane rupture, additional solute loss, followed again by membrane resealing. This cycle continues until sufficient intravesicular solute has been released such that the lipid bilayer is able to withstand the osmotic pressure resulting from the residual osmotic differential. Based on this model, the residual osmotic differential would closely approximate the maximum osmotic gradient the vesicle bilayer could withstand without rupturing. We have measured the maximum residual osmotic differential for vesicle systems of different sizes and observe a linear relationship between vesicle diameter and lysis pressure, as predicted by Laplace's Law. Further, the slope of this line yields a value for the membrane tension at lysis (40 dyn cm⁻¹) that is in reasonably good agreement with values obtained, using the micropipette technique, for giant unilamellar vesicles composed of stearylcholesterolphosphatidylcholine and cholesterol (Needham and Nunn, 1990). In passing, it should be noted that large unilamellar vesicles prepared by extrusion have previously been employed to determine a membrane Young's modulus using photon correlation spectroscopy to follow osmotically induced changes in vesicle diameter (Rutkowski et al., 1991). Our results would indicate that caution should be exercised in the interpretation of such data given the morphological changes that precede true vesicle swelling.

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REFERENCES

- Alhanaty, E., and A. Livne. 1974. Osmotic fragility of liposomes as affected by antihemolytic compounds. *Biochim. Biophys. Acta*. 339:146–155.
- Bangham, A. D., J. De Gier, and G. D. Greville. 1967. Osmotic properties and water permeability of phospholipid liquid crystals. *Chem. Phys. Lipids*. 1:225–246.
- Berndl, K., J. Kas, R. Lipowsky, E. Sackmann, and U. Seifert. 1990. Shape transformations of giant vesicles: extreme sensitivity to bilayer asymmetry. *Europhys. Lett.* 13:659–664.
- Blok, M. C., L. L. M. van Deenen, and J. De Gier. 1976. Effect of the gel to liquid crystalline phase transition on the osmotic behaviour of phosphatidylcholine liposomes. *Biochim. Biophys. Acta*. 433:1–12.
- Cohen, F. S., M. H. Akabas, and A. Finkelstein. 1980. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science (Wash. DC)*. 217:458–460.
- Evans, E. A., and R. M. Hochmuth. 1978. Mechanochemical properties of membranes. *Curr. Top. Membr. Transp.* 10:1–62.
- Evans, E. A., and D. Needham. 1987. Physical properties of surfactant bilayer membranes: thermal transitions, elasticity, rigidity, cohesion, and colloidal interactions. *J. Phys. Chem.* 91:4219–4228.
- Evans, E., and W. Rawicz. 1990. Entropy-driven tension and bending elasticity in condensed-fluid membranes. *Phys. Rev. Lett.* 64:2094–2097.
- Evans, E. A., and R. Waugh. 1977. Osmotic correction to elastic area compressibility measurements on red cell membrane. *Biophys. J.* 20:307–313.
- Farge, E., and P. F. Devaux. 1992. Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. *Biophys. J.* 61:347–357.
- Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.
- Gruner, S. M., R. P. Lenk, A. S. Janoff, and M. J. Ostro. 1985. Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. *Biochemistry*. 24:2833–2842.
- Haines, T. H., W. Li, M. Green, and H. Z. Cummins. 1987. The elasticity of uniform, unilamellar vesicles of acidic phospholipids during osmotic swelling is dominated by the ionic strength of the media. *Biochemistry*. 26:5439–5447.
- Hampton, R. Y., and R. W. Holz. 1983. Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *J. Cell Biol.* 96:1082–1088.
- Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta*. 812:55–65.
- Johnson, S. M., and N. Buttress. 1973. The osmotic insensitivity of sonicated liposomes and the density of phospholipid-cholesterol mixtures. *Biochim. Biophys. Acta*. 307:20–26.
- Li, W., S. Aurora, T. H. Haines, and H. Z. Cummins. 1986. Elasticity of synthetic phospholipid vesicles and submitochondrial particles during osmotic swelling. *Biochemistry*. 25:8220–8229.
- Li, W., and T. H. Haines. 1986. Uniform preparations of large unilamellar vesicles containing anionic lipids. *Biochemistry*. 25:7477–7483.
- Martinac, B., M. Buechner, A. H. Delcour, J. Adler, and C. Kung. 1987. Pressure-sensitive ion channel in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. 84:2297–2301.
- Mayer, L. D., M. J. Hope, P. R. Cullis, and A. S. Janoff. 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta*. 817:193–196.
- Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta*. 858:161–168.
- Miao, L., B. Fourcade, M. Rao, M. Wortis, and K. P. Zia. 1991. Equilibrium budding and vesiculation in the curvature model of fluid lipid vesicles. *Physical Rev. A* 43:6843–6856.
- Miyamoto, S., T. Maeda, and S. Fujime. 1988. Change in membrane elastic modulus on activation of glucose transport system of brush border membrane vesicles studied by osmotic swelling and dynamic light scattering. *Biophys. J.* 53:505–512.
- Morris, C. 1990. Mechanosensitive ion channels. *J. Membr. Biol.* 113:93–107.
- Needham, D., and R. S. Nunn. 1990. Elastic deformation and failure of lipid bilayer membranes containing cholesterol. *Biophys. J.* 58:997–1009.
- Olson, F., C. A. Hunt, F. C. Szoka, W. Vail, and D. Paphadjopoulos. 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochim. Biophys. Acta*. 587:522–539.
- Pollard, H. B., C. J. Pazoles, C. E. Creutz, J. H. Scott, O. Zinder, and A. Hotchkiss. 1984. An osmotic mechanism for exocytosis from dissociated chromaffin cells. *J. Biol. Chem.* 259:1114–1121.
- Rutkowski, C. A., L. M. Williams, T. H. Haines, and H. Z. Cummins. 1991. The elasticity of synthetic phospholipid vesicles obtained by photon correlation spectroscopy. *Biochemistry*. 30:5688–5696.
- Sackmann, E., H.-P. Duwe, and H. Engelhardt. 1986. Membrane bending elasticity and its role for shape fluctuations and shape transformations of cells and vesicles. *Faraday Discuss. Chem. Soc.* 81:281–290.
- Sun, S.-T., A. Milon, T. Tanaka, G. Ourisson, and Y. Nakatani. 1986. Osmotic swelling of unilamellar vesicles by the stopped-flow light scattering method. Elastic properties of vesicles. *Biochim. Biophys. Acta*. 860:525–530.
- Szoka, F. C., F. Olson, T. Heath, W. Vail, E. Mayhew, and D. Paphadjopoulos. 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 microns) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. *Biochim. Biophys. Acta*. 601:559–571.
- Talmon, Y., J. L. Burns, M. H. Chestnut, and D. P. Siegel. 1990. Time-resolved cryotransmission electron microscopy. *J. Electron Microsc. Tech.* 14:6–12.
- Uster, P. S., and D. W. Deamer. 1981. Fusion competence of phosphatidylserine-containing liposomes quantitatively measured by fluorescence resonance energy transfer assay. *Arch. Biochem. Biophys.* 209:385–395.
- Weinstein, J. N., R. D. Klausner, T. Innerarity, E. Ralston, and R. Blumenthal. 1981. Phase transition release, a new approach to the interaction of proteins with lipid vesicles. Application to lipoproteins. *Biochim. Biophys. Acta*. 647:270–284.
- Weinstein, J. N., E. Ralston, L. D. Leserman, R. D. Klausner, P. Dragsten, P. Henkart, and R. Blumenthal. 1984. Self-quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome-cell interaction. In *CRC Liposome Technology* 3. 183–204.
- Yang, X.-C., and F. Sachs. 1989. Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions. *Science (Wash. DC)*. 243:1068–1071.