A Slight Asymmetry in the Transbilayer Distribution of Lysophosphatidylcholine Alters the Surface Properties and Poly(ethylene glycol)-Mediated Fusion of Dipalmitoylphosphatidylcholine Large Unilamellar Vesicles†

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ABSTRACT: Large, unilamellar vesicles (LUV) composed of dipalmitoylphosphatidylcholine (DPPC) were made asymmetric in L-α-lysopalmitoylphosphatidylcholine (LPC) either by adding a small amount (total mole fraction = 0.003) of LPC to their outer leaflets (LUV-LPC$_{out}$) or by extracting a small amount from outer leaflets which already contained 0.0015 mol fraction LPC (LUV-LPC$_{in}$). The slow rate of the transbilayer redistribution of LPC allowed the asymmetric vesicles to be characterized with regard both to their physical properties and to their ability to fuse in the presence of poly(ethylene glycol) (PEG). The fraction of LPC extractable with bovine serum albumin was taken as a measure of LPC transbilayer asymmetry. The ratio of LPC available to that unavailable to BSA extraction was 6 for LUV-LPC$_{out}$ and 0.3 for LUV-LPC$_{in}$. These asymmetries could not be enhanced significantly by increasing the vesicle LPC content. Measurements of the mixing and leakage of vesicle contents showed that LUV-LPC$_{in}$ fused in the presence of 15% (w/w) PEG without loss of contents but that LUV-LPC$_{out}$ did not fuse in the presence of up to 35% PEG. Vesicles prepared from palmitoyloleoylphosphatidylcholine could also be made asymmetric in LPC, but did not fuse even in the presence of 30% PEG. Quasi-elastic light scattering revealed that LUV-LPC$_{in}$ aggregated at 25 °C except when swollen by an osmotic gradient while LUV-LPC$_{out}$ were much less likely to aggregate. Trapped volume determinations suggested that neither type of vesicle was perfectly spherical in shape, but no correlation was found between fusogenicity and vesicle shape. Measurements of the fluorescence properties of TMA-DPH and of C_6-NBD-PC suggested that the interface region of the outer leaflet of LUV-LPC$_{in}$ was slightly less ordered and less well packed than that of LUV-LPC$_{out}$. This slight perturbation of the external vesicle surface correlated with the ability of juxtaposed vesicle bilayers to fuse.

Poly(ethylene glycol) (PEG)[1] is a hydrophilic polymer that causes dehydration of membrane surfaces, forcing close contact between membranes (Arnold *et al.*, 1983, 1990). This dehydrating ability derives from the fact that PEG, as a dynamic polymer with a large hydrated volume (Bailey & Koleske, 1967), is excluded from macromolecular surfaces and thus raises the water chemical potential at a membrane surface (Evans & Needham, 1988). Because of its aggregating ability, PEG is used widely to mediate cell−cell fusion in the production of somatic cell hybrids including hybridomas (Davidson & Gerald, 1977) and in the fusion injection of macromolecules into cultured cells from erythrocytes (Davidson & Gerald, 1977) or liposomes (Szoka *et al.*, 1981). PEG can also be used to induce fusion of phospholipid vesicles as a model for understanding the molecular mechanisms of membrane fusion in biological systems.

Overcoming the hydration repulsion between membrane bilayers is considered to be the initial step in the fusion process (Rand & Parsegian, 1988). PEG does indeed force close apposition between large, unilamellar vesicles (LUV),[1] and the probability of fusion appears to be related to the closeness of approach (Burgess *et al.*, 1992). Nonetheless, PEG-induced close apposition of bilayers is not sufficient to cause fusion (Burgess *et al.*, 1991). However, if membrane bilayers are perturbed by the presence of a small amount of certain amphipaths or by high bilayer curvature, fusion will occur between vesicle bilayers brought into close apposition by PEG (Lentz *et al.*, 1992; Wu & Lentz, 1994). The probability of fusion, then, appears to be related to the probability of molecular contact between apposing bilayers that are perturbed in such a way that formation of a fusion intermediate is favored (Massenburg & Lentz, 1993; Lentz, 1994). The key to gaining insight into the mechanism of membrane fusion is to define the bilayer stuctural changes that favor fusion and then to understand why those particular perturbations are fusogenic.

1-Palmitoyl-3-*sn*-phosphatidylcholine (lysophosphatidylcholine; LPC)[1] was reported to be one of the amphipaths that promote, at very low surface concentrations (0.5 mol %), PEG-mediated fusion of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC)[1] LUV's (Lentz *et al.*, 1992). Surprisingly, however, a subsequent paper reported that addition

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[1] Abbreviations: BSA, bovine serum albumin; C_6-NBD-PC, 1-palmitoyl-2-[[*N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)amino]hexanoyl]phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; LPC, L-α-lysopalmitoylphosphatidylcholine; LUV, large, unilamellar vesicles made by the extrusion technique; LUV-LPC$_{sym}$, LUV prepared from DPPC mixed with a small amount of LPC; LUV-LPC$_{in}$, asymmetric DPPC LUV-LPC with excess LPC in the inner leaflet; LUV-LPC$_{out}$, asymmetric DPPC LUV-LPC with excess LPC in the outer leaflet; PEG, poly(ethylene glycol); QELS, quasi-elastic light scattering; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene.

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of LPC to the external medium prevented fusion between biomembranes in several cellular systems (Chernomordik *et al.*, 1993). The inhibitory effect of LPC was suggested to be explained in terms of the stalk model of membrane fusion (Chernomordik *et al.*, 1987): the "inverted cone" shape of LPC, when present in the outer leaflet of a membrane, should inhibit the formation of a stalk (Chernomordik *et al.*, 1993). In our studies (Lentz *et al.*, 1992), LPC was incorporated into both membrane leaflets. Thus, the disparity between our results and those of Chernomordik *et al.* suggested that the asymmetric perturbation of a bilayer by perturbants such as LPC might be crucial to the fusion mechanism.

In the present paper, we describe methods for preparing and characterizing DPPC LUV's with a small excess of LPC in either their outer leaflet (LUV-LPC$_{out}$) or inner membrane leaflet (LUV-LPC$_{in}$). LUV-LPC$_{in}$ fused in the presence of 15% (w/w) PEG without loss of trapped contents, but LUV-LPC$_{out}$ did not fuse even in the presence of up to 35% PEG, at which point they had ruptured and lost the majority of their trapped contents. Characterization of the surface properties of these vesicles and of vesicles containing randomly incorporated LPC suggested that imperfections in packing of outer leaflet lipids might play a more important role than the shape of the LPC molecule in promoting vesicle fusion in the LUV model system. A preliminary report of these findings has been published in abstract form (Wu & Lentz, 1995).

EXPERIMENTAL PROCEDURES

Materials

Chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC),[1] 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC),[1] 1-palmitoyl-2-[[*N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)amino]hexanoyl]phosphatidylcholine (C_6-NBD-PC),[1] and L-α-lysopalmitoylphosphatidylcholine (LPC)[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and used without further purification. 1,6-Diphenyl-1,3,5-hexatriene (DPH),[1] 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH),[1] the disodium salt of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS),[1] and *N*,*N*′-*p*-xylylenebis(pyridinium bromide) (DPX)[1] were purchased from Molecular Probes (Engene, OR). Carbowax PEG 8000 (molecular weight 7000−9000) was from Fisher Scientific (Fairlane, NJ). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)[1] was from Research Organics, Inc. (Cleveland, OH). 1,2-Di[^{14}C]palmitoyl-3-*sn*-phosphatidylcholine (lot no. 2708-061) and L-α-1-[^{14}C]-lysopalmitoylphosphatidylcholine (lot no. 2708-083) were purchased from DuPont (Wilmington, DE). Bovine serum albumin (BSA)[1] was from Sigma Chemical Co. (St. Louis, MO). Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was from Calbiotech (LaJolla, CA). All other reagents were of the highest purity commercially available.

Methods

Preparation and Characterization of Asymmetric Vesicles. (A) LUV-LPC$_{out}$. The preparation of LUV-LPC$_{out}$ is shown schematically in Figure 1a. DPPC in chloroform was dried under a stream of nitrogen. The lipid residue was redissolved in cyclohexane and methanol (roughly 9:1) and frozen in a dry ice bath. Solvent was removed under high vacuum to yield a white powder. The dried lipid powder was hydrated in prewarmed buffer (100 mM NaCl, 2 mM TES, 1 mM Na_2EDTA, pH 7.4, plus appropriate concentrations of ANTS or DPX for contents mixing or leakage assays, see below) at 48 °C and incubated for at least 30 min with intermittent agitation. The hydrated sample was then added to a custom-built extrusion apparatus maintained at 45−48 °C in an incubator oven and extruded seven times through a 0.1 μm polycarbonate filter (Mayer *et al.*, 1986). This procedure is reported to produce large, unilamellar vesicles (LUV),[1] as demonstrated by broadening of 48% of the NMR signal from choline protons by Mn^{2+} in the external aqueous compartment (Hope *et al.*, 1985). We have shown for our DPPC LUV preparations that 46% of choline proton NMR signal intensity was shifted by externally added Pr^{3+} (Massenburg and Lentz, unpublished), directly confirming that our vesicle preparations were substantially unilamellar.

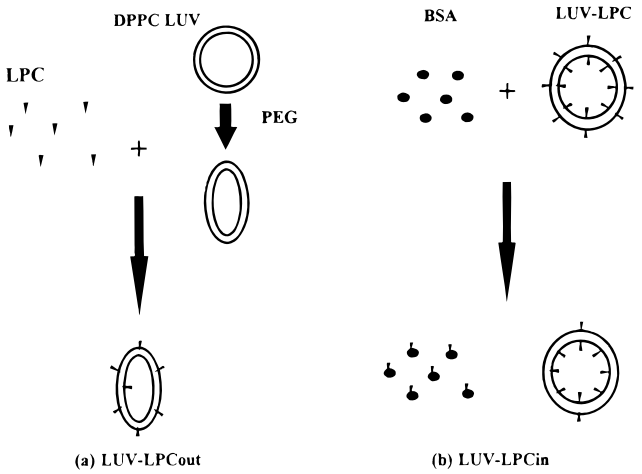

FIGURE 1: Schematic diagram illustrating the preparation of (a) LUV-LPC$_{out}$ and (b) LUV-LPC$_{in}$.

An aliquot of DPPC LUV was added to a PEG solution to a final PEG concentration of around 4% (w/w) and lipid concentration of 20 mM. After incubation for 5 min, LPC containing a small amount of [^{14}C]LPC was added as a methanol stock solution (2 mM) to a final concentration of 0.4 mol %. The resulting LUV-LPC$_{out}$ were separated from PEG and LPC using a Sepharose CL-4B column (1 cm diameter × 20 cm height).

The asymmetry of LUV-LPC$_{out}$ was characterized by incubating them with 4% (w/v) BSA for 15 min, and then fractionating by centrifugation for 8 min at room temperature in a Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor at 40 000 rpm. The concentrations of DPPC and LPC in both the supernatant and pellet were determined by phosphate assay (Chen *et al.*, 1956) and by counting of radioactive [^{14}C]LPC. The asymmetry of LUV-LPC$_{out}$ was expressed as an asymmetry coefficient meant to reflect the ratio of outer-leaflet to inner-leaflet LPC. Derivation of this required two assumptions: (1) that LPC that could not be removed by BSA was located on the inner leaflet, and (2) that transbilayer redistribution of LPC was negligible during BSA treatment and fractionation. The asymmetry coefficient for LUV-LPC$_{out}$ was then calculated as:

$$\mathrm{AC} = \frac{\text{LPC removed by BSA}}{\text{LPC unavailable for BSA}}$$

(B) LUV-LPC$_{in}$. The preparation of LUV-LPC$_{in}$ is illustrated in Figure 1b. DPPC and 0.15 mol % LPC

containing a trace of [^{14}C]LPC were mixed in chloroform solution, and the procedure for preparing LUV was followed. LPC was removed from the outer leaflet of the resulting LUV-LPC$_{sym}$ using BSA, following reports that LPC could be removed from erythrocyte membranes in this way (Mohandas *et al.*, 1982). LUV-LPC$_{sym}$ were incubated with 4% (w/v) BSA for 15 min at room temperature, and the resulting LUV-LPC$_{in}$ were separated from BSA using a Sepharose CL-4B gel filtration column (see above).

The asymmetry of LUV-LPC$_{in}$ was also expressed in terms of an asymmetry coefficient. In order to calculate this, the molar concentrations of DPPC and LPC in a vesicle sample were first determined by phosphate assay and by counting of radioactive [^{14}C]LPC. This allowed us to calculate the mole fraction of LPC in LUV-LPC$_{sym}$ and remaining in LUV-LPC$_{in}$ samples. Two assumptions are needed to calculate an asymmetry coefficient: (1) assume that LPC is evenly distributed between the outer and inner leaflets of LUV-LPC; and (2) assume that the transbilayer LPC redistribution rate in LUV-LPC$_{in}$ is slow (see Results). With these assumptions, the LPC remaining in LUV-LPC$_{in}$ after incubation with BSA for 15 min consists of LPC located on the inner leaflet ($^1/_2$ of the initial LPC content of LUV-LPC) as well as a small amount of outer leaflet LPC that could not be removed by BSA treatment. Recognizing this, the asymmetry coefficient of LUV-LPC$_{in}$ can be calculated as:

$$\mathrm{AC} = [(\text{LPC content of LUV-LPC}_{in}) - {}^1/_2(\text{LPC content of LUV-LPC}_{sym})]/ {}^1/_2(\text{LPC content of LUV-LPC}_{sym})$$

These definitions of asymmetry coefficients are different for the two types of asymmetric vesicles studied, because these two types of vesicles were necessarily prepared in different ways and their asymmetry monitored with different measurements. However, they have in common that they both attempt to document the molar ratio of outer-leaflet to inner-leaflet LPC. As such, their deviation from unity provides a measure of the extent and direction of LPC asymmetry.

The rate of transbilayer redistribution of LPC in asymmetric vesicles (LUV-LPC$_{in}$ or LUV-LPC$_{out}$) was investigated to determine if asymmetry would be maintained for a sufficient time to perform fusion experiments. After storage at 48 °C for different time periods, an aliqout of vesicles was removed and incubated with 4% BSA at 48 °C for 15 min. The LUV−BSA mixture was fractionated by centrifugation for 8 min at room temperature in a Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor at 40 000 rpm to spin down all vesicles. The concentrations of DPPC and LPC in both the supernatant and pellet were determined by phosphate assay and counting of [^{14}C]LPC. Assuming that all removable outer-leaflet LPC would be removed by this 15 min BSA treatment, this allowed the loss of asymmetry with time to be followed.

ANTS/DPX Contents Mixing and Leakage Assays. These were carried out as described previously in detail (Lentz *et al.*, 1992). Briefly, for the contents mixing assay, equal volumes of vesicles (5 mM lipid) containing ANTS (trapped concentration of 25 mM) and DPX-containing (90 mM trapped concentration) vesicles (5 mM lipid) were mixed with different final concentrations of PEG at a final lipid concentration of 0.5 mM in each vesicle population in a 0.4 mL volume. For the leakage assay, ANTS and DPX were coencapsulated in one vesicle population (trapped concentration: 12.5 mM ANTS, 45 mM DPX; 0.5 mM final lipid concentration) such that DPX quenched over 85% of ANTS fluorescence. When leakage occurred, there was an increase in fluorescence due to dilution of ANTS and DPX, and therefore dequenching of ANTS occurred. For both assays, vesicles were incubated for 3−5 min with PEG at 48 °C. Results did not change with longer incubation times. After incubation, vesicle suspensions were diluted to a 3.5 mL final volume in a 4 mL fluorescence cuvette, ANTS was excited at 384 nm, and its fluorescence emission was monitored through a 2 mm OG-515 filter (50% transmittance at 515 nm; Schott Glass Technologies, Durea, PA) as described previously (Lentz *et al.*, 1992).

The fluorescence intensity indicative of 0% contents mixing was taken as the intensity observed for ANTS-loaded vesicles alone in PEG. The fluorescence intensity indicative of 100% contents mixing was taken as the intensity observed for ANTS/DPX coencapsulated vesicles added to a small contribution from PEG. For leakage measurements, the value used to represent 0% leakage was the intensity of the ANTS/DPX coencapsulated vesicles plus a small intensity contributed by an appropriate PEG solution. The value taken to represent 100% leakage was determined by lysing vesicles with detergent $C_{12}E_8$. With these definitions, % Leakage and % Contents Mixing were calculated exactly as described previously (Lentz *et al.*, 1992).

Fluorescence Probe Detection of Surface Packing. C_6-NBD-PC (Slater *et al.*, 1994) was added sequentially to preformed vesicles (0.5 mM) from a stock solution in methanol to final concentrations of from 0.3 to 0.9 mol %. The fluorescence intensity was recorded at 530 nm upon excitation at 470 nm using the SLM 48000 MHF spectrofluorometer. Data from five different preparations were normalized relative to a standard solution of C_6-NBD-PC in buffer to obtain average fluorescence intensities as a function of probe concentration.

Vesicle Size Determinations. Quasi-elastic light scattering (QELS)[1] measurements were performed as reported in detail by Lentz *et al*. (1992). Vesicles, prepared in standard buffer (100 mM NaCl, 2 mM TES, 1 mM Na_2EDTA, pH 7.4), were diluted to approximately 0.2 mM into buffers of equal or reduced osmolality and then sized using a light scattering photometer equipped with a computer-controlled Nicomp 170 autocorrelator (Particle Sizing Systems, Inc., Santa Barbara, CA). The data were analyzed by means of a software package provided by Particle Sizing System using a cumulant method, which assumes a Gaussian distribution of particle sizes. Hypoosmolality buffers contained reduced concentrations of NaCl but the same concentrations of TES and Na_2-EDTA as contained in the trapped buffer. Osmolalities of all buffers were monitored directly using a μOsmette freezing point osmometer (Precision Systems, Sudbury, MA).

Measurements of Fluorescence Lifetimes and Time-Resolved Fluorescence Anisotropies. DPH and TMA-DPH, dissolved in acetone and methanol, respectively, were added to vesicle suspensions (roughly 1 μL of probe stock/mL of vesicle suspension), and these mixtures were incubated for 5 min in the dark with continuous stirring. The final probe to phospholipid ratio was 1:500.

Fluorescence measurements were made on an SLM 48000 MHF phase-modulation fluorometer (SLM Instruments Inc., Rochester, NY) operated in either the L (lifetime measurements) or T (dynamic anisotropy measurements) formats. Excitation was with an argon ion laser (Innova 90-4, Coherent Inc., Palo Alto, CA) used in the UV mode (351.1−363.8 nm). Emission was monitored through a 3 mm high-pass KV-450 filter (50% transmittance at 450 nm, Scott Optical Glass, Duryea, PA).

Fluorescence lifetimes were obtained from phase shifts and demodulation ratios of DPH fluorescence measured relative to those of a reference glycogen scattering solution. The excitation polarizer was in the vertical position, and the emission polarizer was set at the magic angle (54.7°) from vertical. This angle was obtained by observing the polarizer position where the scattered light intensity of the glycogen solution was 33% of the maximum intensity that occurs with the polarizer in the vertical position. This orientation of the polarizers eliminates the effects of depolarizing rotation on the observed fluorescence lifetime. Multifrequency data were collected (average = 200) at 30 frequencies from a base frequency of 5 MHz and analyzed using the SLM Spectrum Processor software package (version 1.6 beta12).

Time-resolved anisotropies were determined from the phase angle differences and modulated amplitude ratios of the parallel and the perpendicular components of the fluorescence emission. These quantities were directly measured by rotation of the polarizer in the measurement channel. Control measurements with a horizontal polarizer in the reference channel revealed no time drift of the exciting light. Multifrequency anisotropy data were processed by the SLM Spectrum Processor software package. We used the *hindered cone model* to describe the decay of anisotropy:

$$r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty$$

where r_0 is the anisotropy in the absence of rotational diffusion [held at 0.32 for TMA-DPH in DPPC vesicles and 0.38 for DPH in DPPC vesicles (Lakowicz *et al.*, 1985)], and r_∞ is the anisotropy that would be observed at times much longer than the fluorophore rotational correlation time, ϕ. The values of r_∞ and ϕ were obtained by nonlinear, least-squares fitting.

RESULTS

Characterization of Asymmetric Vesicles: LUV-LPC$_{out}$ and LUV-LPC$_{in}$. Separation of asymmetric vesicles from PEG or BSA was accomplished by Sepharose CL-4B gel filtration chromatography. The separation of [^{14}C]DPPC (triangles) incorporated into LUV-LPC$_{in}$ from BSA (absorbance at 280 nm, circles), illustrated in Figure 2A, was quite complete. Figure 2B illustrates the separation of two populations of [^{14}C]LPC in a LPC-DPPC vesicle population treated with BSA to produce LUV-LPC$_{in}$. The peaks in frame B have the same positions as do the vesicle and BSA peaks in frame A, so the first peak is assigned to LPC remaining in LUV-LPC$_{in}$ and the second peak is LPC binding to BSA. In this experiment, around 30% of total LPC was extracted from LUV-LPC$_{sym}$. Assuming that transbilayer LPC redistribution is negligible on the time scale of this experiment (see below), the ratio of the molar percentage of LPC in outer monolayer to that in the inner monolayer (the asymmetry coefficient, see Methods) is around 0.4 for this experiment.

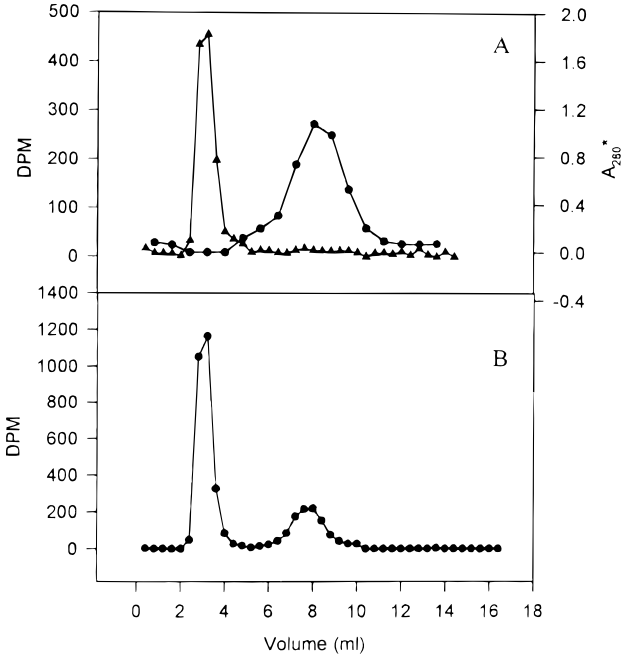

FIGURE 2: Separation of vesicles from BSA by Sepharose CL-4B size-exclusion chromatography carried out at room temperature. (A) Column profile illustrating separation of [^{14}C]DPPC (triangles, disintegrations per minute) and BSA (circles, absorbance at 280 nm). (B) Column profile illustrating association of [^{14}C]LPC (circles, disintegrations per minute) with LUV-LPC$_{in}$ (peak at 3 mL) and with BSA (peak at 8 mL) used to remove LPC from LUV-LPC.

Table 1: Effects of Different Starting Concentrations of LPC on the Asymmetry of DPPC Vesicles

	mol %		
	starting LPC	final LPC	asymmetry
LUV-LPC$_{out}$	0.4	0.16	7.2
	0.8	0.33	10.0
	1.6	0.86	18.9
	2.4	1.50	11.7
	3.2	2.36	3.8
LUV-LPC$_{in}$	0.15	0.095	0.27
	0.30	0.277	0.85
	0.60	0.556	0.85

The procedure for preparing asymmetric vesicles was established by systematically varying the initial LPC content of DPPC LUV. Table 1 summarizes the effect of initial LPC content on the asymmetry coefficient of vesicles. For both LUV-LPC$_{out}$ and LUV-LPC$_{in}$, an increase of initial molar content of LPC led to an increase in the final LPC molar content in DPPC vesicles, but the asymmetry of both types of vesicles became worse after reaching an optimum value at very low initial LPC content. For LUV-LPC$_{out}$, increasing LPC content apparently induced some LPC transbilayer redistribution from the outer leaflet to the inner leaflet. For LUV-LPC$_{in}$, the same increase in initial LPC content apparently prevented more LPC from being extracted from the outer monolayer by BSA, again resulting in a decrease in asymmetry coefficient.

The effects of BSA concentration and incubation period on LUV with different distributions of LPC was also investigated, with the results being used to define conditions for preparation and characterization of asymmetric DPPC LUV. First, we noted that 1% BSA could extract only 20% of total LPC from LUV-LPC$_{sym}$. By increasing the BSA

Table 2: Asymmetry Coefficient of LUV-LPC$_{out}$ and LUV-LPC$_{in}$ under Optimal Conditions[a]

vesicle type	asymmetric coeff	final mol % of LPC
LUV-LPC$_{out}$	6.30 ± 1.37[b]	0.300 ± 0.050[b]
LUV-LPC$_{in}$	0.29 ± 0.05[b]	0.098 ± 0.004[b]

[a] For LUV-LPC$_{out}$, 0.4 mol % of LPC was added to DPPC LUV incubated for 30 min at room temperature. For LUV-LPC$_{in}$, 0.15 mol % LPC was added to a DPPC stock solution from which LUV-LPC$_{sym}$ were prepared. Incubating LUV-LPC$_{sym}$ with 4% BSA for 15 min at room temprature led to LUV-LPC$_{in}$. [b] Average values and nominal standard deviations are based on four independently characterized preparations.

concentration to 4%, over 30% of LPC was removed; 8% BSA produced almost no further LPC removal. Second, incubation of LUV-LPC$_{in}$ (circles) with 1−8% BSA extracted almost no LPC; while nearly 95% of LPC was extracted from LUV-LPC$_{out}$. Third, incubation of LUV-LPC$_{sym}$ with 4% BSA beyond 5 min had no significant effect on the asymmetry of LUV-LPC$_{in}$ produced, nor did extended incubation of LUV-LPC$_{out}$ with BSA alter the asymmetry coefficient measured by this BSA incubation.

Based on the preceding experiments, standard conditions for preparing and characterizing aymmetric LPC/DPPC LUV were established. For LUV-LPC$_{out}$, the LPC content was set at 0.4 mol %, which was added to the external aqueous compartment of previously formed LUV. For LUV-LPC$_{in}$, the starting LPC content was chosen as 0.15 mol %, and DPPC LUV-LPC$_{sym}$ of this composition were incubated with 4% BSA for 15 min. All incubations were performed at room temperature (below the phase transition of DPPC LUV) in order to achieve maximum asymmetry. Table 2 contains average asymmetry coefficients of LUV-LPC$_{out}$ and LUV-LPC$_{in}$ prepared under these standard conditions.

In order to be assured that the measured asymmetry coefficients summarized in Table 2 actually characterize the vesicles at the point of a fusion or other experiment, we characterized asymmetry at various times after LUV were first prepared and characterized. The asymmetric LPC distribution produced by our methods was invarient for at least 1−2 h at room temperature, *i.e.*, below the order−diorder phase transition of DPPC (data not shown). However, the stability of an asymmetric LPC distribution at the temperature needed for fusion and other experiments (48 °C) was much more crucial to our studies. As shown in Figure 3, both LUV-LPC$_{out}$ and LUV-LPC$_{in}$ that were transferred to 48 °C (*i.e.*, to above their phase transition) maintained for up to 2 h at least 85−90% of their original asymmetry. This is consistent with a recent paper reporting that LPC was removed exclusively from the outer leaflet of small, unilamellar egg phosphatidylcholine vesicles containing 5 mol % LPC and that the resulting asymmetric distribution of LPC was maintained for a period of several hours after BSA treatment (Bhamidipati & Hamilton, 1995). Even after 48 h, our asymmetric DPPC LUV were still asymmetric, although they had reverted considerably to a symmetric LPC distribution (Figure 3). It should be mentioned that all surface characterization and fusion assays reported here were performed within 2 h of preparation of asymmetric vesicles.

Fusion of Asymmetric LPC/DPPC Vesicles. Asymmetric vesicles, LUV-LPC$_{out}$ and LUV-LPC$_{in}$, were incubated with various concentrations of PEG, and their responses were monitored using the contents mixing and leakage assays

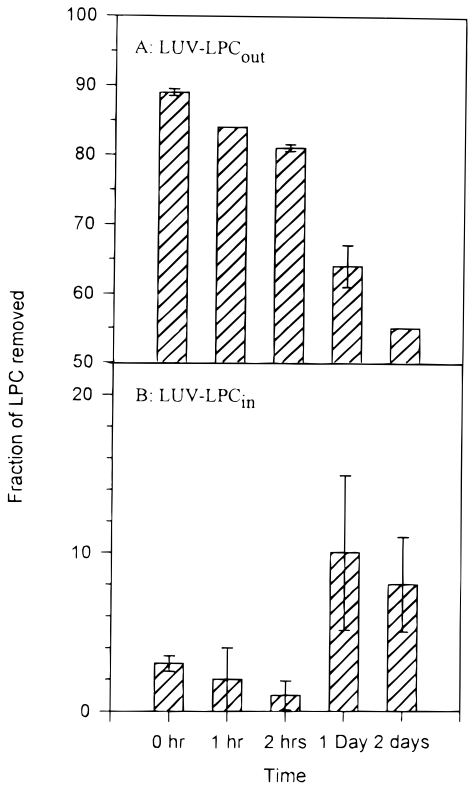

FIGURE 3: Transbilayer redistribution of LPC in (A) LUV-LPC$_{out}$ and (B) LUV-LPC$_{in}$. The data are expressed as the mol % of LPC that can be extracted by 4% BSA from the outer leaflet as a function of time of incubation at 48 °C after vesicle preparation at room temperature.

described in Methods. Figure 4 records the results of these experiments. Figure 4B shows that, for both LUV-LPC$_{out}$ (squares) and LUV-LPC$_{in}$ (circles), leakage of vesicle contents was minimal up to 20% (w/w) PEG. Untreated DPPC LUV and LUV-LPC, from which the asymmetric vesicles were prepared, have previously been shown (Massenburg & Lentz, 1993) to experience a constant and minimal level of leakage up to 20% PEG. However, all four types of vesicles lost some contents at 25% PEG and then demonstrated a dramatic increase in the loss of their contents between 25% and 30% PEG (Figure 4B; see also Massenburg & Lentz, 1993). Contents mixing occurred for LUV-LPC$_{in}$ at PEG concentrations greater than or equal to 15%, while their parent vesicles (DPPC LUV-LPC) fused only at 30% PEG (Lentz *et al.*, 1992; Massenburg & Lentz, 1993). By contrast, LUV-LPC$_{out}$, like their parent untreated DPPC LUV (Lentz *et al.*, 1992; Massenburg & Lentz, 1993), did not experience contents mixing at any PEG concentration, as revealed by the ANTS/DPX assay (Figure 4A). Apparently, LPC is not inherently fusogenic; the fusogenicity of LUV-LPC$_{in}$ must result either from the asymmetric excess of LPC in the inner leaflet or from its removal from the outer leaflet.

The error bars on LUV-LPC$_{in}$ data in Figure 4A (circles) represent the standard deviation of values observed during five separate experiments on five separate vesicle samples. While it is clear that PEG-mediated fusion was considerably enhanced in LUV-LPC$_{in}$, it is also clear that the extent of contents mixing of LUV-LPC$_{in}$ was not very reproducibly measurable. The possible reason for this is that the asymmetry of LUV-LPC$_{in}$ was not reproduced identically between various preparations of asymmetric vesicles (see Table 2).

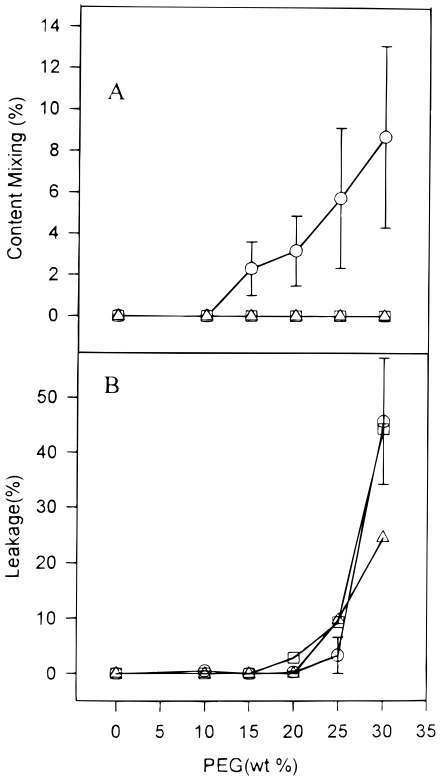

FIGURE 4: PEG-induced (A) contents mixing and (B) leakage of DPPC LUV-LPC$_{in}$ (circles), LUV-LPC$_{out}$ (squares), and POPC vesicles analogous to DPPC LUV-LPC$_{in}$ (triangles). All assays, as described in Methods, were performed on vesicles incubated for 3 min at 48 °C in the presence of the indicated amounts of PEG before being diluted in buffer. LUV-LPC$_{in}$ contents mixing and leakage values represent averages and nominal standard deviations (bars) of five determinations performed on separate occassions on different samples.

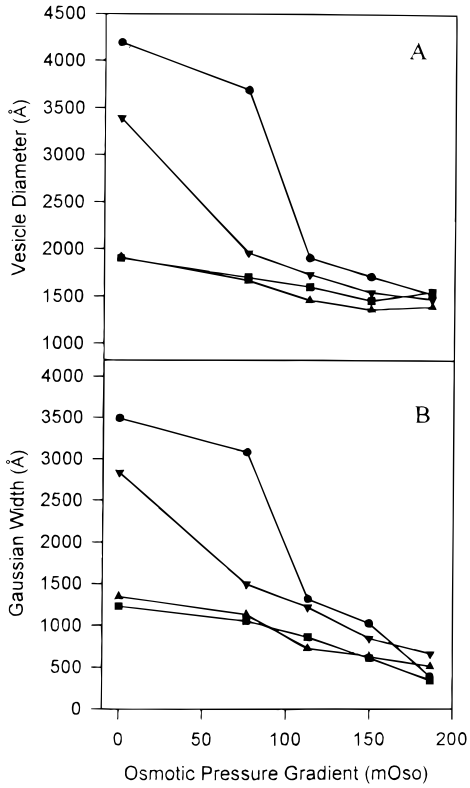

FIGURE 5: Mean DPPC vesicle diameters (A) and Gaussian diameter distribution widths (B) under various osmotic pressure gradients ($\Pi_{in} - \Pi_{out}$). LUV-LPC$_{out}$ (squares), LUV-LPC$_{sym}$ (inverted triangles), LUV (triangles), and LUV-LPC$_{in}$ (circles) were diluted to approximately 0.2 mM before measurement by QELS at room temperature.

Dependence of Fusion Results on Lipid Species. All of the experiments described so far have utilized vesicles whose major lipid component is DPPC. While DPPC is commonly used in model membrane studies, its two saturated, 16-carbon acyl chains are expected to be more ordered and more closely and critically packed (smaller area per molecule), even at 48 °C, than are lipids containing unsaturated chains of similar length at room temperature (Seelig & Seelig, 1977; Rand & Parsegian, 1989; Ho *et al.*, 1995). To test whether a less well packed lipid bilayer might respond differently to the introduction of LPC asymmetry than did DPPC bilayers, we produced POPC LUV asymmetric in LPC. To prepare these POPC LUV-LPC$_{in}$, we began with POPC LUV containing 0.5 mol % LPC and used the same procedures outlined above for LUV-LPC$_{in}$. After removal of LPC from the outer leaflet of these vesicles by treatment with BSA, the final LPC content and asymmetry coefficient of these vesicles were $0.31 \pm 0.05\%$ and 0.25 ± 0.03, respectively. These vesicles maintained this asymmetry for the 2−3 h required to perform fusion experiments. The results of these experiments are also shown in Figure 4 (triangles). The results clearly demonstrated that asymmetric POPC LUV with LPC removed from their outer leaflets did not fuse even in the presence of 30% PEG. We have shown previously that POPC LUV containing 0.5 mol % 1-oleoyl-*sn*-glycero-3-phosphocholine experienced clearly measurable PEG-mediated fusion only in the presence of 35% or higher percent PEG (Burgess *et al.*, 1991). Thus, the asymmetric distribution of amphipaths seemed to have very little effect on the fusion of POPC vesicles. This is in contrast to the results obtained with DPPC LUV-LPC$_{sym}$ *vs* LUV-LPC$_{in}$. Apparently, the effect of the manipulations we performed to create LUV asymmetric in LPC depends on the properties of the phospholipid matrix to or from which amphipath is added or subtracted.

Physical Properties of Fusogenic Membranes. We have noted previously that PEG promotes LUV fusion in part by bringing apposing bilayers into near molecular contact (Burgess *et al.*, 1992). However, PEG is not sufficient to induce fusion without some bilayer-perturbing component (amphipathic impurities) or condition (high curvature) (Burgess *et al.*, 1991; Lentz *et al.*, 1992). As shown above, the distribution of amphipath (in this case, LPC) between the two bilayer leaflets is critical in determining whether the amphipath causes changes in bilayer structure that encourage rather than discourage fusion. In this section, we report several measurements that collectively suggest that the key feature of LUV-LPC$_{in}$ that favors fusion is a decrease in the packing density of lipids in the outer leaflet of these asymmetric vesicles. We suggest that this would result in increased exposure of water to hydrocarbon and thereby create an unfavorable free energy state that would drive the formation of a fusion intermediate.

Vesicle Diameters. Using QELS, we tried to determine whether manipulation of the LPC content of DPPC LUV caused alteration in the shape of vesicles. Measurements were made on vesicles diluted into buffer of decreasing osmotic strength relative to the buffer used to prepare LUV (100 mM NaCl, 2 mM TES, 1 mM EDTA). Figure 5 shows that the apparent average diameters of the four different types

Table 3: Effect of Osmotic Pressure on the Entrapped Volume of DPPC Vesicles[a]

	entrapped vol (μL/μmol of lipid)[b]			
	LUV	LUV-LPC$_{out}$	LUV-LPC	LUV-LPC$_{in}$
isoosmotic medium	1.52 ± 0.10 (3)	1.49 ± 0.23 (3)	1.37 ± 0.21 (3)	1.50 ± 0.32 (3)
hypoosmotic medium	1.86 ± 0.31 (3)	2.23 ± 0.36 (3)	1.61 ± 0.18 (3)	1.97 ± 0.41 (3)

[a] Vesicles were prepared in 100 mM NaCl, 2 mM TES, 1 mM EDTA, and 1 mM glucose (containing a trace of [^{14}C]glucose) and diluted 5-fold either with the same buffer (isoosmotic medium) or with 1 mM glucose (containing [^{14}C]glucose) only (hypoosmotic medium). Samples were incubated at room temperature for 1 h to allow reequilibration of glucose between the trapped and external compartments. Vesicle entrapped volumes were determined after rapid removal of external [^{14}C]glucose using a Sephadex G-75 column in a buffer containing nonradioactive glucose. [b] Values given represent the mean and nominal standard deviations of three determinations.

of vesicles were different in a nearly isosmotic medium at room temperature. LUV-LPC$_{in}$ displayed the largest apparent diameter (circles in Figure 5A), although the data for these vesicles indicated such a degree of polydispersity (Gaussian widths shown in Figure 5B) that a unique mean diameter was difficult to define for this experiment at zero osmotic gradient. The data for LUV-LPC$_{sym}$ (squares), LUV (triangles), and LUV-LPC$_{out}$ (inverted triangles) were such that mean diameters could be estimated. The apparent diameter and Gaussian width of LUV-LPC$_{sym}$ were nearly as large as for LUV-LPC$_{in}$, while the other types of vesicles displayed much smaller apparent diameters and distribution widths. As the osmotic pressure gradient ($\Pi_{in} - \Pi_{out}$) increased, the data were better fit by a Gaussian distribution and mean vesicle diameters obtained from these fits approached a common limiting value. Repeating these experiments with different LUV-LPC$_{in}$ and LUV-LPC$_{out}$ preparations gave variable estimates of mean diameters at low osmotic gradients but always the same trend and very similar limiting values at high osmotic gradient. The sensitivity of these results to osmotic gradient suggests that swelling of the vesicles reversed whatever effect led to the apparent difference in size of fusogenic (LUV-LPC$_{in}$ and LUV-LPC$_{sym}$) *versus* nonfusogenic (LUV-LPC$_{out}$ and LUV) DPPC vesicles. These results seemed likely to reflect vesicle aggregation and a difference in the aggregation state of vesicles with different LPC asymmetries or possibly differences in the shapes of vesicles with different asymmetries.

The possibility that different vesicle types had significantly different shapes was addressed by the entrapped volume experiments summarized in Table 3. If bathed at room temperature in a [^{14}C]glucose solution at the same osmolality as the trapped buffer (*i.e.*, osmotic pressure across the vesicle membrane equal to zero), all types of DPPC vesicles had almost the same entrapped volumes, roughly 1.5 μL/μmol of phospholipid. Had the different vesicle types had very different shapes under isoosmotic conditions, their entrapped volumes should have been quite different. This value is smaller than the calculated entrapped volume of a spherical vesicle with diameter of 1500 Å (4.2 μL/μmol). LUV trapped volumes generally are reported to be smaller than expected from measured diameters (Mayer *et al.*, 1986; Mui *et al.*, 1993). This anomaly is not due to a deviation from unilamellar character, as extruded LUV have been shown by others (Mayer *et al.*, 1986) and in our laboratory (Massenburg and Lentz, unpublished; see Methods) to have a nearly equal distribution of lipids between inner and outer membrane leaflets. After being swollen in a hypoosmotic medium ($\Pi_{in} - \Pi_{out} = 190$ mOsm) containing [^{14}C]glucose, the entrapped volumes of all vesicles increased to the same extent to roughly 1.9 μL/μmol. This small increase in entrapped volume associated with osmotic swelling must indicate that all four types of vesicles studied here were slightly nonspherical in shape, in agreement with the suggestion of Mui *et al.* (1993). The fact that measured entrapped volumes of osmotically swollen LUV are still lower than the theoretical value may be due to: (a) inaccuracies in the methods for measuring vesicle diameters; (b) nonspherical shape of even the swollen LUV (a prolate ellipse with minor axis of 1000 Å and a major axis of 1500 Å would have roughly the observed trapped volume); (c) partial solute release during swelling (Mui *et al.*, 1993); or (d) inhomogeneous solvent properties of trapped water. For our purposes, it is not necessary to resolve these issues but only to note that all four types of vesicles were similarly nonspherical and behaved similarly under the influence of osmotic stress. Thus, we conclude that the large apparent diameter of LUV-LPC$_{in}$ relative to other vesicle types is not due to a grossly distorted vesicle shape.

Since the large apparent diameter of LUV-LPC$_{in}$ cannot be explained as due to a large difference in vesicle shape, we explored whether it might reflect vesicle aggregation. DPPC small, unilamellar vesicles are known to aggregate and fuse below their phase transition temperature (Wong *et al.*, 1982). DPPC LUV, on the other hand, have nearly identical main phase transitions when stored below or above their phase transition (Parente & Lentz, 1984; Lentz *et al.*, 1992), which suggests that they do not fuse when stored in the gel phase but does not prove that they do not aggregate. To test whether aggregation below the phase transition temperature might account for the apparent large size of LUV-LPC$_{in}$, we measured the diameters of the same LUV preparations below and above the phase transition temperature under conditions of zero osmotic gradient. The results, shown in Figure 6, demonstrate that all four types of vesicles aggregate below their phase transition but that aggregation is reversible, with all four vesicle types having essentially identical diameters and Gaussian widths (bars on each data point) at 48 °C. Thus, the large apparent diameter of LUV-LPC$_{in}$ at room temperature is seen to reflect a significantly greater tendency to aggregate than displayed by DPPC LUV or LUV-LPC$_{out}$, with LUV-LPC$_{sym}$ showing an intermediate tendency to aggregate. We see that the tendency to aggregate parallels the tendency to fuse. Also, swelling the vesicles by means of a transbilayer osmotic gradient eliminated the tendency to aggregate at room temperature in all four types of vesicles.

Bilayer Packing Reported by Fluorescent Probes. The enhanced tendency of LUV-LPC$_{in}$ to aggregate in the gel phase suggested to us that they might have a less well packed lipid−water interface, *i.e.*, a more "hydrophobic surface", than the other vesicle types examined. To test further for

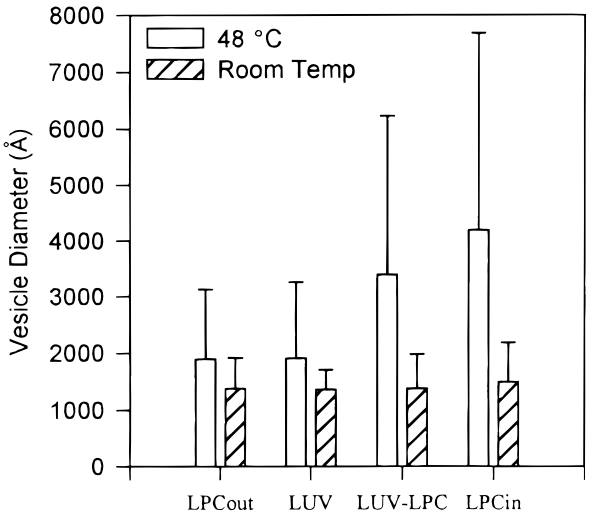

FIGURE 6: The effect of measurement temperature on the mean (open and striped bars) and Gaussian distribution widths (line bars) of DPPC vesicle diameters. The different types of vesicles (indicated on the abscissa) were incubated under zero osmotic pressure gradient and diluted in the same buffer to around 0.2 mM for QELS measurements.

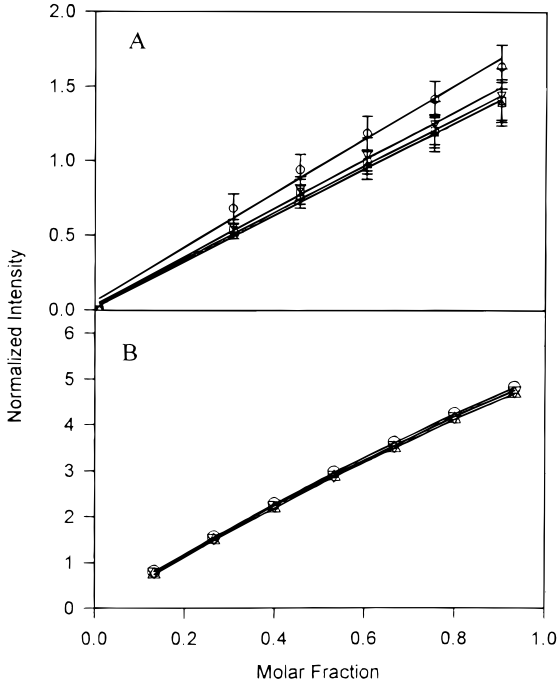

FIGURE 7: Fluorescence intensity of C_6-NBD-PC absorbed to (A) DPPC and (B) POPC vesicles. Vesicle samples (0.35 mmol total lipid) were titrated at 48 °C in a cuvette with various amounts of C_6-NBD-PC (mol % of C_6-NBD-PC in the sample is shown on the abscissa). For DPPC vesicles in frame A, each point represents the average of determinations made on separate occassions on three different samples, with the bars being the nominal standard deviations of these measurements. Intensities were normalized between experiments using a standard solution of C_6-NBD-PC. For POPC vesicles (B), only one set of determinations was made. In both cases, lines through the data were obtained by least-squares regression analysis. Data are shown for LUV-LPC$_{out}$ (squares), LUV-LPC$_{sym}$ (inverted triangles), LUV (triangles), and LUV-LPC$_{in}$ (circles).

this possibility, we used a fluorescent probe, C_6-NBD-PC, reported to incorporate into the upper regions of the bilayer (Chattopahyay & London, 1987) and whose fluorescence signal has previously been shown to be sensitive to bilayer headgroup spacing (Slater *et al.*, 1994). Figure 7A shows that probe fluorescence intensity increased roughly linearly with the concentration of probe added to suspensions of all four types of vesicles. However, the slope of this increase was significantly greater for LUV-LPC$_{in}$ (circles) than for any other vesicle type. DPPC LUV-LPC$_{sym}$ (inverted triangles) showed a slightly greater slope than did either DPPC LUV or LUV-LPC$_{out}$, which were indistinguishable. Slater *et al.* (1994) showed that C_6-NBD-PC fluorescence intensity varied in phosphatidylcholine/phosphatidylethanolamine bilayers roughly in proportion to the "curvature index", which is related to phospholipid headgroup spacing (Keller *et al.*, 1993). Applying the arguments of Slater *et al.*, we would conclude from our data that there is a correlation between altered headgroup spacing in LUV-LPC$_{in}$ and the enhanced ability of these vesicles to fuse.

We sought also to determine whether POPC membranes made asymmetric in LPC might display the packing defects detected by C_6-NBD-PC in LUV-LPC$_{in}$. Figure 7B shows the fluorescence intensity of this probe added to POPC LUV (triangles), POPC LUV containing 0.5% LPC (inverted triangles), and POPC LUV-LPC$_{in}$ (circles). It is evident that all three types of vesicles interacted with C_6-NBD-PC in a nearly identical fashion. Apparently, the unsaturated POPC bilayer is able to accommodate some loss of LPC without experiencing a packing disruption detectable by C_6-NBD-PC. It may be that the chain disorder associated with an unsaturated phospholipid is sufficient to allow headgroup packing to adjust to the loss of a small amount of LPC so that C_6-NBD-PC does not partition more favorably into DOPC LUV-LPC$_{in}$. Since DOPC LUV-LPC$_{in}$ displayed no enhanced ability to fuse in the presence of PEG (Figure 4), this observation reinforces our conclusion that altered lipid packing correlates with the ability to fuse in the presence of PEG. This result also indicates that it is not simply the presence or absence of LPC from vesicles that results in fusogenicity but rather the bilayer structural changes, revealed in this case by C_6-NBD-PC, that are essential to fusion. Apparently, outer leaflet packing disruption that is supportive of fusion can be produced by removal of LPC from tightly packed DPPC LUV-LPC$_{sym}$ but not by removal of LPC from the less well packed DOPC LUV-LPC$_{sym}$.

We have also examined the properties, in our four types of DPPC vesicles, of a class of probes commonly used to report bilayer dynamics, namely, two probes containing the DPH moiety. Table 4 shows that the fluorescence lifetime and dynamic properties of the hydrophobic probe DPH were indistinguishable among these types of vesicles. The amphipathic probe TMA-DPH showed the same lifetime in LUV, LUV-LPC$_{sym}$, and LUV-LPC$_{out}$, but a marginally lower lifetime in LUV-LPC$_{in}$, consistent with a slightly more polar or dynamic environment for this probe in the LUV-LPC$_{in}$. The dynamics of this probe were indistinguishable among vesicle types except for a marginally slower wobbling motion in LUV-LPC$_{out}$ (Table 4).

DISCUSSION

The work presented here was inspired by the apparent disparity between our observations (Lentz *et al.*, 1992) and those of Chernomordik *et al.* (1993) regarding the ability of LPC incorporated into membranes to promote fusion. Our current results make it clear that LPC is not inherently fusogenic when incorporated into bilayers at low surface

Table 4: Analysis of Fluorescence Lifetimes and Time-Resolved Fluorescence Anisotropy for TMA-DPH and DPH in Various DPPC Vesicles[a]

		LUV	LUV-LPC	LUV-LPC$_{out}$	LUV-LPC$_{in}$
TMA-DPH	τ_{av} (ns)	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.5 ± 0.1
	r_∞	0.14 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
	ϕ (ns)	1.25 ± 0.11	1.25 ± 0.13	1.48 ± 0.17	1.23 ± 0.07
DPH	τ_{av} (ns)	8.4 ± 0.1	8.3 ± 0.1	8.5 ± 0.1	8.4 ± 0.1
	r_∞	0.057 ± 0.004	0.050 ± 0.004	0.059 ± 0.004	0.058 ± 0.005
	ϕ (ns)	0.92 ± 0.07	0.87 ± 0.04	0.96 ± 0.06	0.93 ± 0.04

[a] Averages and nominal standard deviations are based on four independent measurements on the same sample. All experiments were performed at 48 °C.

concentrations, but that its effects on fusion will depend on its location in the bilayer. In the experiments of Chernomordik *et al.* (1993), LPC was added to membranes from the outer or extracellular compartment. Our results argue that the asymmetric distribution of LPC produced by this procedure might be maintained for some reasonable period of time. Chernomordik *et al.* (1993) argued that LPC distributed in this way would, because of its molecular shape, inhibit formation of a "single-bilayer stalk" structure that these authors see as an intermediate leading to fusion. Our observation, that DPPC LUV-LPC$_{out}$ do not fuse even though DPPC LUV-LPC$_{sym}$ do, is consistent with this view of Chernomordik *et al.* (1993). However, arguments related to LPC molecular shape also support the proposition that LPC located on the inner leaflet of a bilayer might favor fusion due to a tendency to favor breakdown of the "stalk" to a fusion pore (Kozlov *et al.*, 1989). Indeed, a paper that appeared after this work was completed (Chernomordik *et al.*, 1995) reported that LPC added to the *cis* compartment of a black lipid film (exposed to the noncontacting leaflet) encouraged fusion with vesicles added to the *trans* compartment. While it is difficult to know either exactly how much LPC entered the *cis* leaflet of the black lipid film or whether it remained asymmetrically disposed, we can speculate that the bilayer content of LPC in the work of Chernomordik *et al.* (1995) must have been 1 or 2 orders of magnitude greater than in our studies. At such high surface concentrations, it may be that LPC does favor fusion by altering the curvature of the nonopposed leaflets, as suggested by Chernomordik *et al.* (1995). However, at the very low concentrations we use, we favor a different mechanism for the effects of LPC on the fusogenicity of DPPC LUV, as suggested by our observations of the surface properties of the asymmetric vesicles described here.

Our data support the hypothesis that the main factor dictating the fusogenicity of our vesicles is not their asymmetry in LPC but rather the changes in outer leaflet packing produced by the manipulations we performed in order to create this asymmetry. We note that this hypothesis is consistent with the correlation noted by Ohki (1989) between enhanced membrane surface tension and fusion of phosphatidylserine-containing membranes by calcium. Three observations indicate that removal of LPC from the outer leaflet of DPPC LUV-LPC$_{sym}$ creates a high free energy surface. First, LUV-LPC$_{in}$, and to some extent LUV-LPC$_{sym}$, showed a marked tendency to aggregate in the gel phase. This tendency was reversed when vesicles were swollen under the influence of a transbilayer osmotic gradient or when the temperature was raised above the lamellar phase transition. A likely explanation of these observations might be that removal of outer leaflet LPC creates a packing defect in this leaflet. This packing disruption might localize at the edges of polyhedral faces visualized in freeze−fracture micrographs of gel phase LUV (Parente *et al.*, 1985). Aggregates would be stabilized by contact between these hydrophobic edges. Faceted vesicles containing edge defects are not evident in freeze−fracture micrographs of fluid phase vesicles (Parente *et al.*, 1985), and swelling might also be expected to eliminate these structures. Whether or not this exact scenario obtains, it is clear from our results that removal of LPC from the outer leaflet of DPPC LUV-LPC$_{sym}$ creates a perturbed vesicle with enhanced tendencies both to fuse and to aggregate below the phase transition.

The second observation in support of our hypothesis is that the fluorescence observed on adding C_6-NBD-PC to vesicles increased in the same order as did vesicle aggregating ability: LUV-LPC$_{in}$ > LUV-LPC$_{sym}$ > LUV ∼ LUV-LPC$_{out}$. Since C_6-NBD-PC fluorescence has been suggested to correlate with polar headgroup spacing (Slater *et al.*, 1994), the correlation between this measurement and aggregating ability was anticipated. Both properties correlated with the ease of fusion by PEG. Finally, the measurably shorter fluorescence lifetime of TMA-DPH when this probe was incorporated into LUV-LPC$_{in}$ indicates a slightly more polar or more dynamic interfacial region as compared to other membranes.

All three observations support our interpretation that it is disrupted lipid packing in the outer leaflet of DPPC LUV-LPC$_{in}$ that accounts for the fusogenicity of these vesicles. The results obtained with POPC vesicles provide strong corroboration of this interpretation. According to the arguments of Chernomordik *et al.* (1993) and Kozlov *et al.* (1989), the distribution of LPC in POPC LUV-LPC$_{in}$ is expected to favor, first, formation (low outer leaflet LPC) and, then, breakdown (high inner-leaflet LPC) of the "stalk" intermediate. However, our observation that POPC LUV-LPC$_{in}$ and POPC LUV-LPC$_{sym}$ did not fuse in the presence of even 30% PEG and also did not show different abilities to enhance C_6-NBD-PC fluorescence supports our hypothesis that it is packing disruption and not LPC asymmetry that is critical to fusion. We propose that the packing disruption we created within apposing membrane leaflets created an unstable state that relaxed through formation of a fusion intermediate, probably the "stalk" proposed by Kozlov *et al.* (1989). We cannot dispute that inner leaflet LPC might favor breakdown of the stalk to form a pore, but our data argue that the key to obtaining fusion in our system resides in disrupted outer leaflet packing leading to formation of an intermediate.

In the context of this hypothesis, we can understand the apparent disparity between the observations of Lentz *et al.* (1992) and of Chernomordik *et al.* (1993) regarding the ability of LPC to induce fusion. Chernomordik *et al.* (1993) added LPC to the external compartments of membrane

systems. Partitioning of LPC into membrane outer leaflets is expected to enhance membrane packing, thus lowering surface free energy and thereby inhibiting fusion, as was observed by Chernomordik *et al.* (1993) and in this study. On the basis of both their tendency to aggregate and their ability to enhance C_6-NBD-PC fluorescence, we suspect that the outer leaflet of DPPC LUV-LPC$_{sym}$ possesses somewhat disrupted lipid packing, and that this accounts for our previous report that these membranes are induced to fuse by 30% PEG (Lentz *et al.*, 1992). Further disruption of lipid packing in the outer leaflet of these vesicles by removal of a very small amount of LPC apparently creates a highly fusogenic vesicle.

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