Poly(ethylene glycol)-Induced Fusion and Rupture of Dipalmitoylphosphatidylcholine Large, Unilamellar Extruded Vesicles†

Donald Massenburg and Barry R. Lentz*

Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7260

Received September 16, 1992; Revised Manuscript Received May 11, 1993®

ABSTRACT: High concentrations (≥20 wt %) of poly(ethylene glycol) (PEG) induce large, unilamellar, dipalmitoylphosphatidylcholine model membrane vesicles to fuse when the bilayers contain small amounts of amphipathic peturbant molecules. In addition to fusion, similar concentrations of PEG induce these vesicles to leak their contents. In this paper, we have asked if fusion could occur independently of leakage or if fusion might be described as local bilayer rupture followed by resealing. By following the release of MW 10 000 fluoresceinated dextran trapped inside vesicles, it was determined that PEG-induced leakage was the result of major membrane disruption and not small-pore formation. Fusion of vesicles containing 0.5 mol % palmitic acid was clearly observed at 20 wt % PEG, while 25 wt % was needed to cause rupture. On the other hand, vesicles containing 0.5 mol % lysophosphatidylcholine ruptured at roughly the same concentration needed to induce rupture. Two methods were developed for removing PEG so that fusion products could be characterized. Quasi-elastic light scattering demonstrated that fusing vesicles grew in size and that nonfusing vesicles did not. Moreover, PEG concentrations that induced rupture led to the appearance of species with mean diameters much larger than those of fused vesicles. High-resolution nuclear magnetic resonance showed that the population of large vesicles that correlated with rupture was composed of multilamellar vesicles while the population resulting from fusion alone remained unilamellar. We conclude that, upon incubation with and subsequent removal of PEG, vesicles were either unaffected, or fused to form larger, unilamellar vesicles, or ruptured to form larger, nonunilamellar species.

Poly(ethylene glycol) (PEG)[1] is used widely to mediate cell–cell fusion in the population of somatic cell hybrids (Davidson & Gerald, 1977) and in fusion injection of macromolecules into cultured cells from erythrocytes (Davidson & Gerald, 1977) or liposomes (Szoka *et al.*, 1981). However, little is known about the mechanism by which PEG induces fusion of cell membranes. We have shown that PEG (MW 8000) induces fusion in phosphatidylcholine model membrane systems that contain small amounts of certain amphipathic compounds (Lentz *et al.*, 1992; Burgess *et al.*, 1991) or various amounts of phosphatidylethanolamine (Burgess *et al.*, 1992), or that have high radii of curvature (Lentz *et al.*, 1992). PEG does not induce fusion in pure dipalmitoylphosphatidylcholine (DPPC)[1] or pure dioleoylphosphatidylcholine large, unilamellar vesicles (Burgess *et al.*, 1991). In both fusing and nonfusing systems, the leakage of encapsulated contents has been observed at high PEG concentrations (Parente & Lentz, 1986; Lentz *et al.*, 1992; Burgess *et al.*, 1991, 1992). In fusing systems, this occurred at or near the PEG concentration at which vesicle contents mixing, and thus fusion, began. Others have raised the possibility that PEG-induced "fusion" might actually reflect rupture and resealing of adjacent membranes (Tilcock & Fisher, 1982), possibly due to detergent-like properties of PEG (Sáez *et al.*, 1982). On the other hand, Aldewinckle *et al.* (1982) have dismissed the possibility that PEG induces lysis or rupture. It is appropriate to ask, then, whether PEG fusion can occur in the absence of vesicle contents loss or whether fusion must be "leaky". Furthermore, what is meant by "leaky"? Does it reflect the formation of small, transient fluctuating pores or the presence of large "holes" in membranes? If the former, then "leaky fusion" might reflect the formation of pores with incidental contents mixing via aligned pores in adjacent vesicles (Aldewinckle *et al.*, 1982). If "leaky" reflects the formation of large holes in membranes, then what has been termed "leaky fusion" should probably be viewed not as fusion but as a more chaotic process, not necessarily yielding a simple, fused-vesicle product. This would not be expected to have many features in common with protein-mediated cell membrane fusion.

To approach these issues, we first characterized leakage events in terms of an associated pore size by measuring the loss of trapped MW 10 000 fluoresceinated dextran after treatment with various concentrations of PEG. The results strongly suggested that PEG-induced leakage of contents resulted from membrane rupture involving pores of at least 45–55-Å diameter. We next identified conditions under which leakage was minimal or nonexistent but fusion could be confirmed by contents mixing, lipid mixing, and vesicle size change. This demonstrated that PEG-induced vesicle fusion was not the same process as PEG-induced vesicle rupture. In addition, we characterized the products of PEG treatment under conditions that led to fusion in the presence or absence of leakage in terms of both vesicle diameter and vesicle morphology (percent exposed lipid) using quasi-electric light scattering (QELS[1]) and high-resolution ^{1}H NMR. High-

† Supported by USPHS Grant GM32707 to B.R.L. This work was completed in partial fulfillment of the requirements for the Doctor of Philosophy degree granted to D.M. by the University of North Carolina at Chapel Hill.

* To whom requests for reprints should be addressed.

® Abstract published in *Advance ACS Abstracts,* August 15, 1993.

[1] Abbreviations: PEG, poly(ethylene glycol); ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *N,N'*-xylylenebis(pyridinium bromide); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, tetrasodium salt; $C_{12}E_8$, dodecyloctaethylene glycol monoether; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DSC, differential scanning calorimetry; LPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; QELS; quasi-elastic light scattering; PA, palmitic acid.

0006-2960/93/0432-9172$04.00/0

resolution ^{1}H NMR spectra of $PrCl_3$-titrated vesicle samples showed that the percentage of phospholipid headgroups present in the exposed outer leaflet of the vesicle membrane after treatment with fusing concentrations of PEG was similar to that of freshly extruded vesicles, while the exposed percentage observed after treament of vesicles under rupturing conditions was much lower.

Thus, PEG-induced vesicle fusion and rupture are separate events which generate two different products. Fusion products appear to be similar in morphology to vesicles not treated with PEG, while the rupture products are large and multilamellar. Also, it seems that the leakage of vesicle contents takes place through major membrane disruption that includes the formation of large pores. Since fusion occurred at or just below PEG concentrations that cause leakage, it may be that both events require membrane disruption but that fusion requires less severe disruption than does rupture.

EXPERIMENTAL PROCEDURES

Materials

The chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine used in these experiments were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Prior to use, DPPC was filtered through Norit A activated charcoal (Fisher Scientific, Fair Lawn, NJ) to remove trace fluorescent contaminants, checked for purity via thin-layer chromatography, and analyzed to determine lipid concentration through a modification of the phosphate determination of Chen *et al.* (1956). Palmitic acid (PA)[1] was purchased from Nu Chek Prep, Inc. (Elysian, MN). 1-Palmitoyl-3-*sn*-glycerophosphocholine (LPC)[1] along with deuterium oxide (99.9 atom % D) was purchased from Sigma Chemical Co. (St. Louis, MO). The disodium salt of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS),[1] *N*,*N*-*p*-xylylenebis(pyridinium bromide) (DPX),[1] and anionic fluoersceinated dextran (lot 8B) were purchased from Molecular Probes (Eugene, OR). Carbowax PEG 8000 (MW 8000) was purchased from Fisher Scientific and purified as described by Lentz *et al.* (1992). *N*-[Tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES)[1] was purchased from Research Organics, Inc. (Cleveland, OH). Dodecyloctaethylene glycol monoether ($C_{12}E_8$)[1] was purchased from Calbiochem (La Jolla, CA). $PrCl_3$ (lot 102676) was purchased from Alfa Products (Danvers, MA) and stored under argon in a desiccator. [^{14}C]Poly(ethylene glycol) 4000 (batch 38) and 1,2-di[^{14}C]palmitoyl-3-*sn*-phosphatidylcholine (batch 28) were purchased from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest purity commercially available.

Methods

Vesicle Preparation. Phospholipids were stored under argon in chloroform stocks at −20 °C. The phospholipid compositions used were 0.5 mol % PA (PA/DPPC),[1] 0.5 mol % LPC (LPC/DPPC),[1] and pure DPPC. When preparing vesicles, chloroform was removed from measured aliquots of phospholipids by flowing a stream of argon into vials containing the lipid. The dried lipid was resolubilized in cyclohexane, the sample was then frozen in a dry ice/ethanol bath, and the cyclohexane was removed by freeze-drying for at least 6 h. The resultant white powder was suspended to a concentration of 5–40 mM in a 2 mM TES, 100 mM NaCl, and 1 mM EDTA, pH 7.4, buffer at 48 °C. For contents mixing measurements, vesicles were suspended in buffers containing 25 mM ANTS (or 90 mM DPX), 40 mM NaCl, and 10 mM TES, pH 7.4. The ANTS leakage experiments were carried out with 12.5 mM ANTS, 45 mM DPX, 40 mM NaCl, and 10 mM TES, pH 7.4. For dextran leakage measurements, 0.075 mM fluoresceinated dextran, 2 mM TES, 100 mM NaCl, and 1 mM EDTA, pH 7.4, were used. The hydrated samples were incubated above the phospholipid phase transition temperature with frequent vortexing for 30–45 min. The sample was then subjected to 5 cycles of freezing in a dry ice/ethanol bath followed by thawing in a water bath maintained at 40 °C. Care was taken to vortex the sample between each freeze–thaw cycle. Next, the vesicles were warmed above their phase transition and then forced through one or two polycarbonate filters (Nucleopore Corp., Pleasanton, CA) using one of two different extrusion devices. One technique involved forcing 250–500-μL total sample volume through a single 0.1-μm filter approximately 50 times using the two-syringe device developed by MacDonald *et al.* (1991) and then diluting the sample up to the desired concentration. The other technique involved forcing the lipid through one or two filters 7–20 times under 200–500 psi of argon using a stainless-steel apparatus constructed in-house (Burgess *et al.*, 1991; Lentz *et al.*, 1992) after the design of Hope *et al.* (1985) and Mayer *et al.* (1986). To prepare medium, unilamellar vesicles of 620-Å diameter, seven extrusions were made through a 0.05-μm filter stacked onto a 0.03- or 0.05-μm filter (Lentz *et al.*, 1992). For 770-Å vesicles, five extrusions were made through a 0.03-μm filter to yield vesicles of roughly the proper size; this was followed by two extrusions through a 0.05-μm filter to remove large structures. This is a slight deviation from our previously published method (Lentz *et al.*, 1992) but yielded vesicles of the proper size, as judged by QELS.[1] For the NMR measurements, the vesicles were suspended in 50 mM NaCl in deuterium oxide rather than normal aqueous buffer. For the dextran leakage measurements, the buffer contained fluoresceinated dextran.

Contents Mixing and Leakage. The ANTS/DPX contents mixing and leakage assays were carried out as previously described in detail (Lentz *et al.*, 1992). Basically, leakage experiments were carried out by coencapsulating equimolar ANTS and DPX in vesicles, such that DPX quenched at least 85% of ANTS maximum fluorescence. When leakage of contents occurred in the presence of varying concentrations of PEG, the fluorescence intensity increased as ANTS was freed from the DPX; 0% leakage was taken as characterized by the fluorescence intensity resulting from coencapsulated ANTS/DPX plus the intensity contribution of PEG; 100% leakage was indicated by the fluorescence associated with coencapsulated ANTS/DPX vesicles treated with the detergent $C_{12}E_8$ plus a minor contribution from PEG. To carry out contents mixing assays, equal volumes of 0.25 mM ANTS-containing and 0.25 mM DPX-containing vesicles were mixed in a total incubation volume of 0.4 mL of buffer containing a specific concentration of PEG. After incubation, the volume was diluted to 3.7-mL total volume and the fluorescence intensity recorded. Contents mixing was expressed as a percentage of the fluorescence change expected for one ideal round of fusion. An ideal round of fusion is defined as every ANTS vesicle fusing with a DPX vesicle (Lentz *et al.*, 1992). The fluorescence of ANTS-containing vesicles alone was taken as indicative of 0% contents mixing; 100% contents mixing was indicated when the fluorescence intensity reached that of coencapsulated ANTS/DPX vesicles plus PEG. The computations leading to the percent contents mixing properly accounted for the photobleaching of ANTS, for the leakage of contents, and also for the probability that two fusing vesicles

contained ANTS and DPX (Lentz *et al.*, 1992). The only deviation from earlier studies was that measurements were taken after 5-min incubation periods rather than 20-min incubations. Results were independent of incubation times from 2 to 20 min (Burgess *et al.*, 1991; Lentz *et al.*, 1992).

Separation of Vesicles from PEG. For dextran leakage, QELS, and NMR measurements, PEG had to be separated from the vesicles after the 5-min incubation. This separation was carried out either by using Sepharose CL 4B gel filtration chromatography as described previously (Lentz *et al.*, 1992; Burgess *et al.*, 1991) or by using a newly developed centrifugation technique. In the centrifugation procedure, PEG–vesicle samples were diluted to obtain a concentration of 1.67 wt % PEG and then spun at full speed (11 600 rpm) in 1.5-mL Eppendorf tubes (Costar Corp., Cambridge, MA) for a total of 3–10 min at room temperature in a Beckman Microfuge B (Palo Alto, CA). The lipid sedimented to the bottom of the tubes, and the PEG-containing buffer was removed with a Pasteur pipet. The pelleted vesicles were then resuspended in 300–400 μL of buffer. Measurements using [^{14}C]PEG 4000 showed that this technique reduced PEG concentrations to about 0.01 wt %. Quasi-elastic light scattering was used to show that this concentration of PEG did not promote aggregation of vesicles. No indication of fusion or rupture was seen to result from spinning with or without PEG (Burgess *et al.*, 1992).

Dextran Leakage. For these measurements, fluoresceinated dextran was included in the lipid hydration buffer to a final concentration of 0.075 M. After the extrusion process, the vesicles were filtered through a Sephadex G-75 column to remove the untrapped dextran. The vesicle sample was then incubated with varying concentrations of PEG for 5 min. The PEG and any leaked dextran were removed from the sample by sedimentation. The fluorescence of dextran remaining with the vesicles was monitored through a 2-mm OG515 filter (50% transmittance at 515 nm; Schott Optical Glass, Duryea, PA) with an excitation wavelength at 495 nm. The increase of fluorescence with addition of increasing volumes of vesicle samples to the cuvette was compared to the increase of fluorescence with addition of increasing volumes of original dextran buffer. The ratio of these slopes was divided by the sample phospholipid concentration to determine the volume of trapped fluoresceinated dextran per mole of vesicle phospholipid.

Quasi-Elastic Light Scattering. Quasi-elastic light scattering (QELS)[1] measurements were performed as described in detail by Lentz *et al.* (1992). Vesicles were incubated with various concentrations of PEG for 5 min. They were isolated from PEG by gel filtration or sedimentation procedures as described above. Aliquots of these vesicles were then diluted to approximately 5 mM and sized on a light-scattering instrument built around a computer-controlled Nicomp 170 autocorellator (Particle Sizing Systems, Inc., Santa Barbar, CA) (Lentz *et al.*, 1992). The data were analyzed using the software package provided by Particle Sizing Systems, using either the method of cummulants (Koppel, 1972), which assumes a Gaussian distribution, or a more complex distributional analysis based on a simplified version of the Provencher procedure (Provencher, 1979) that assumes multiple Gaussian forms.

Nuclear Magnetic Resonance. High-resolution ^{1}H nuclear magnetic resonance (NMR)[1] was carried out on a Bruker AMX500 instrument (Bruker Instruments, Inc., Billerica, MA). Vesicles suspended in deuterium oxide containing 50 mM NaCl and incubated with PEG dissolved in the same buffer were separated from PEG by spinning. In this case, the vesicles floated to the top of the solution due to the high density of D_2O relative to water. Aliquots (400 μL) were loaded into 5-mm NMR tubes, and measurements were carried out with spinning samples that were held at 48 °C. $PrCl_3$ titrations were carried out by removing the NMR tube and adding 5–10-μL aliquots of a 1.15 mM $PrCl_3$ stock to the tube, vortexing, replacing the tube, and collecting and averaging eight free induction decays per sample. The Fourier-transformed and manually phased spectra were transferred to a personal computer, where peak integrations were carried out using the Lab Calc spectral data analysis package (Galactic Industries Corp., Salem, NH).

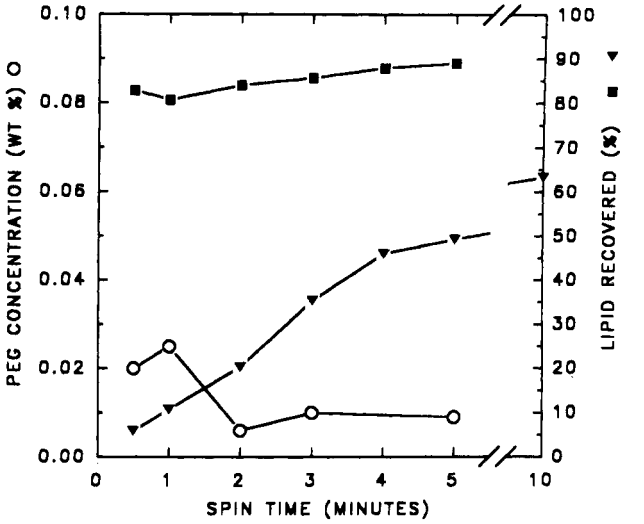

FIGURE 1: Separation of vesicles from PEG by centrigugation. Multilamellar (filled squares) and unilamellar extruded (filled triangles) DPPC vesicles (1.87 mM) were treated with PEG (1.6 wt %) at a total volume of 1275 μL for 2 min at room temperature. After the vesicles were spun in a Beckman Microfuge B, the supernatant was removed, and the pellet was resuspended in 400 μL of buffer. The percentage of starting lipid recovered (filled symbols) and the percentage of [^{14}C]PEG remaining (open circles) are plotted here as a function of spin time.

RESULTS

Isolation of Vesicles from PEG. Two methods were examined for separating PEG from vesicles: Sepharose CL 4B gel chromatography and vesicle sedimentation. Both procedures were found acceptable in terms of their abilities to remove PEG. Not surprisingly, however, neither could recover the entire vesicle sample. Figure 1 shows the characterization of the sedimentation technique. By this method, 65–90% of the original lipid was generally recovered after spinning for 10 min. However, this technique favored the sedimentation of large multilamellar vesicles over large unilamellar vesicles. After spinning for 5 min, 90% of a multilamellar vesicle sample was recovered as compared to 50% of a unilamellar vesicle sample (Figure 1). Figure 1 also shows that typical recovered vesicle samples contained only 0.01 wt % PEG. This concentration did not induce aggregation as determined by QELS (data not shown), or by static light scattering, or by turbidity measurements (Boni *et al.*, 1981; Yamazaki *et al.*, 1989; Tilcock & Fisher, 1982).

The separation of PEG and phospholipid vesicles on a Sepharose CL 4B gel filtration column is illustrated in Figure 2. Frame A shows the separation of 5 wt % PEG from vesicles, while frame B shows the separation of 17.5 wt % PEG. All lipid samples incubated with PEG concentrations greater than

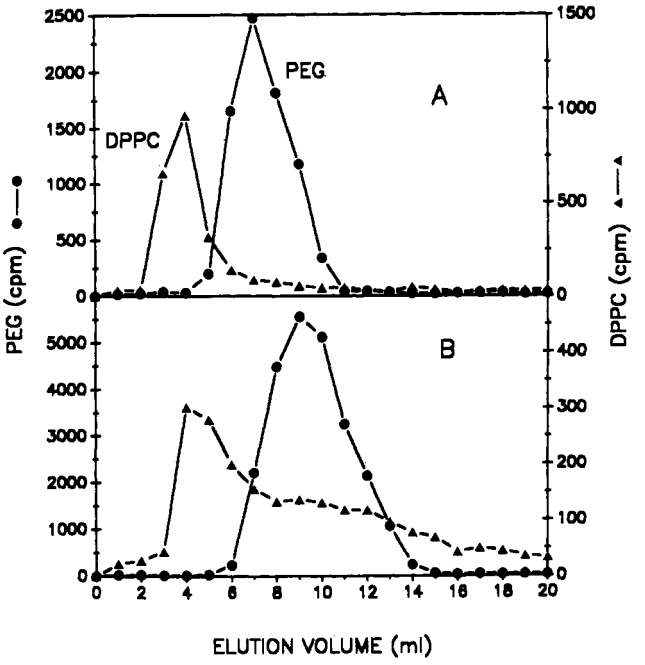

FIGURE 2: Separation of vesicles from PEG by chromatography. Separations were carried out on a Sepharose CL 4B column (1.2 cm in diameter and 12 cm in length) at room temperature. Calibrations were carried out through the separation of [^{14}C]DPPC vesicles (triangles) from unlabeled PEG or of [^{14}C]PEG (circles) from unlabeled DPPC. Panel A is the elution profile for a sample incubated in 5 wt % PEG and panel B for a sample incubated in 17.5 wt % PEG.

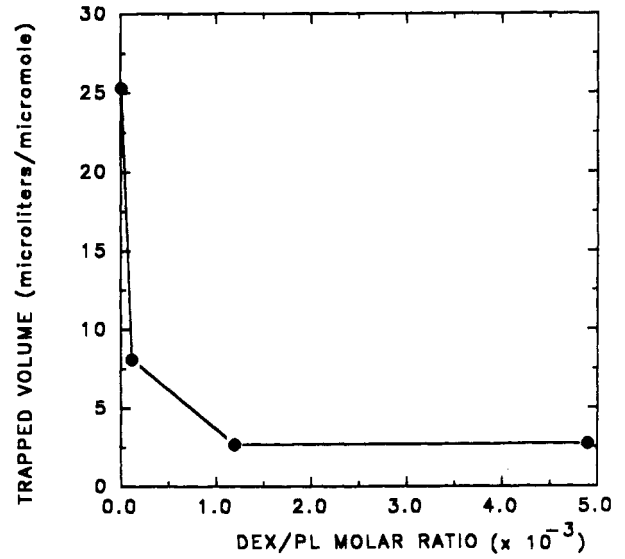

FIGURE 3: Fluoresceinated dextran binding to DPPC large, unilamellar vesicles. Trapped fluoresceinated dextran volume was determined as described under Methods as a function of the dextran to phospholipid ratio in the sample. The large values seen at low dextran concentrations imply adsorption of fluoresceinated dextran to the phospholipid bilayer (Roseman *et al.*, 1978).

17.5 wt % were diluted to between 17.5 and 5 wt % PEG before being loaded as a 400–600-μL aliquot onto the column. Only the lead 1.0–1.5 mL of the eluting vesicles was collected for measurements, suggesting that this method should also favor recovery of larger species but probably not to the extent that the centrifugation method does. The fraction contained approximately 0.01 wt % PEG as judged by ^{14}C-labeled PEG 4000. However, only 1–5% of the original lipid was recovered by this procedure.

PEG-Induced Dextran Leakage. Figure 3 shows that fluoresceinated dextran binds to membranes and that, when

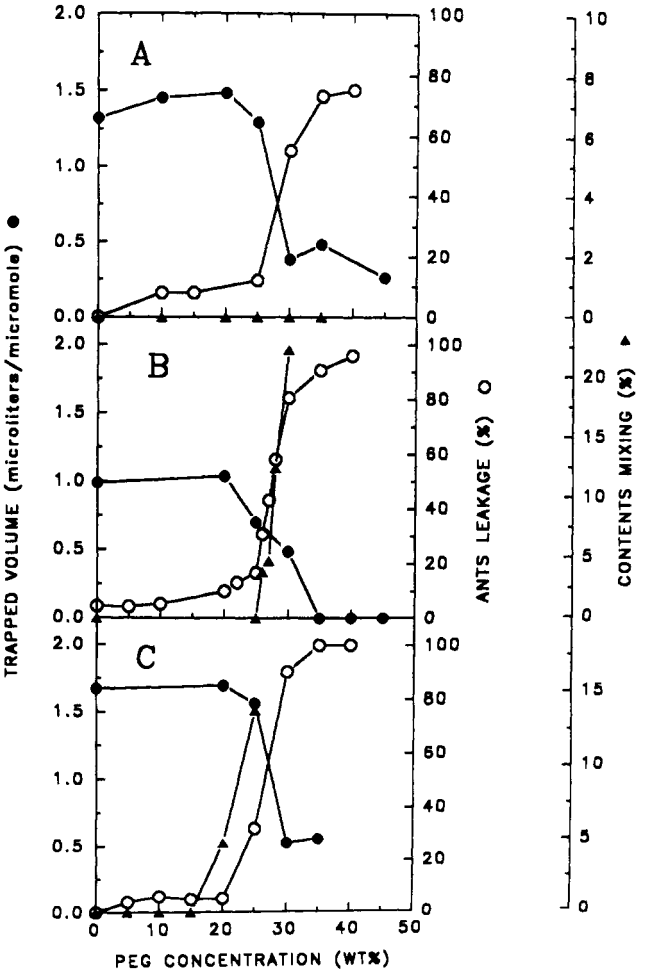

FIGURE 4: Dextran leakage, contents mixing, and ANTS leakage as a function of PEG concentration in (A) DPPC, (B) 0.5 mol % LPC/DPPC, and (C) 0.5 mol % PA/DPPC vesicles. ANTS leakage and contents mixing were determined after 5-min incubations as described under Methods. For dextran trapping experiments, the vesicles were separated from untrapped fluoresceinated dextran on Sephadex G-75 columns, incubated with various concentrations of PEG at 48 °C for 5 min, and separated from PEG by gel filtration on Sepharose CL 4B columns, and the trapped fluoresceinated dextran was measured to determine the trapped volume (microliters per micromoles of phospholipid). Dextran trapped volume (filled circles), ANTS leakage (open circles), and contents mixing (filled triangles) are recorded as a function of PEG concentration. Note that contents mixing at 30 wt % PEG was recorded only for some LPC/DPPC samples (frame B), probably due to the large extent of contents leakage observed at this PEG concentration.

the probe to lipid ratio is below 1.2 × 10^{-3}, the proportion of probe associated with DPPC large, unilamellar vesicle membranes is high compared to the proportion trapped in the vesicle lumen. This condition produces a trapped volume (microliters of probe per micromoles of phospholipid) that is much higher than can be calculated from the vesicle diameter. To minimize distortion due to membrane-associated probe, only probe to lipid concentrations of greater than 1.2 × 10^{-3} were used in the experiments reported here.

Figure 4 contains a summary of our data relating to fluoresceinated dextran trapping, ANTS leakage, and contents mixing for three vesicle systems: (A) DPPC; (B) 0.5 mol % LPC/DPPC; (C) 0.5 mol % PA/DPPC. ANTS, a molecule of 445 molecular weight, began to leak extensively from all three types of vesicles between 25 and 30 wt % PEG, although leakage might have been initiated at a slightly lower PEG content in the two samples containing amphipaths (PA or LPC). Dextran trapping decreased in parallel to the increased

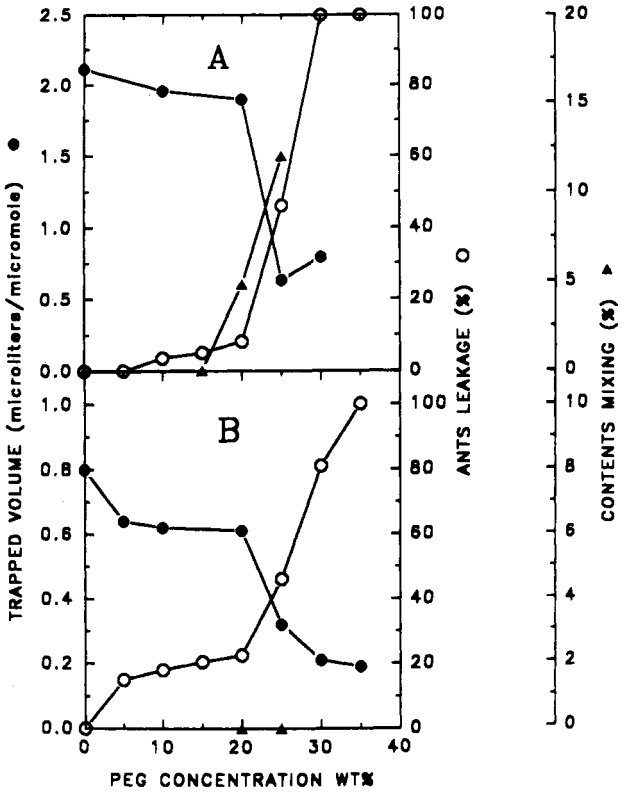

FIGURE 5: Dextran leakage, contents mixing, and ANTS leakage as a function of PEG concentration in DPPC (A) unilamellar extrusion vesicles of 770-Å diameter and (B) small extrusion vesicles of 620-Å diameter. Symbols and methods are as described in Figure 4.

leakage of ANTS in all these systems: a slight drop at 25 wt % PEG was followed by a significant decrease at 30 wt % and above. The correlation between dextran release and ANTS release is obvious and suggests that these two trapped materials leak through similar mechanisms.

Contents mixing, on the other hand, showed a different pattern in each of the vesicle systems and, consequently, did not correlate exactly with leakage. In DPPC vesicles, contents mixing did not occur, although leakage was extensive at and above 30 wt % PEG. The onset of contents mixing in the LPC/DPPC system took place at 26 wt % PEG in correlation with leakage of trapped contents, while the onset of contents mixing in the PA/DPPC system occurred at 20 wt % PEG, a lower PEG concentration than that required for extensive leakage. Thus, ANTS and dextran leakage correlated with the mixing of contents only in the LPC/DPPC vesicle system. These data indicate clearly that leakage (rupture) and contents mixing (fusion) are separate events.

In order to address the generality of the relationship between rupture and fusion, we examined vesicles induced to fuse by bilayer curvature rather than by the presence of amphipaths in the bilayer. Figure 5 shows ANTS leakage, dextran trapping, and contents mixing data for pure DPPC unilamellar extrusion vesicles with average diameters of (A) 770 Å and (B) 620 Å. As published earlier (Lentz *et al.*, 1992), these highly curved vesicles are more likely to fuse or rupture, probably due to greater packing strain in the bilayer (Sheetz & Chan, 1972). For both vesicle systems, as for the large, unilamellar vesicles described above, the correlation between dextran loss and ANTS leakage was remarkable, even though contents mixing was recorded in only the 770-Å vesicles. For these vesicles, fusion was first detected at a PEG concentration (20 wt %) lower than required for significant ANTS or dextran leakage (25 wt %; Figure 5). These results once more suggest

Table I: Diameters (Å) of Vesicles Isolated by Centrifugation[a]

sample	PEG (wt %)	peak 1 (Å)[b]	peak 2 (Å)[c]
DPPC	0	1300	
	20	1300	
	25	1280	
	30	1070	2300 + larger vesicles
LPC/DPPC	0	1400	
	20	1400	
	25	1410	
	30	1520	larger vesicles
	35	1130	5000 + larger vesicles
PA/DPPC	0	1140	
	10	1150	
	20	1330	
	22.5	1310	20000
	25	1340	20000
	30	1290	5000 + larger vesicles

[a] Data were analyzed by a Provencher-type method as described under Methods. Diameters were reproducible to within 1% for samples having single peak distributions. Otherwise, peak 1 values were reproducible to within about 3%, while peak 2 diameters are only approximate. [b] "Peak 1" refers to the peak in a multimodal vesicle size distribution with the smallest mean diameter. [c] "Peak 2" refers to the peaks in a multimodal distribution with larger mean diameters.

that contents mixing (fusion) and leakage (rupture) are separable events. It should also be noted that dextran binding to 770-Å vesicles was a more severe problem than with any of the other systems in this report. After filtration on a Sepharose CL 4B column to remove vesicles from PEG, released dextran had surprisingly become concentrated in the PEG-free fractions. This untrapped dextran could be separated from the vesicles by gel filtration on a Sepharose G-75 column (0.7 × 10 cm; Pharmacia, Piscataway, NJ). The reason for this unusual behavior is not known, but it was observed only with curvature-stressed vesicles.

The data presented in Figure 5B for 620-Å vesicles also show considerable dextran loss at low PEG concentrations, when compared to the data in Figures 4 or 5A. This indicates that more highly curved vesicles are more sensitive to rupture by PEG, as previously suggested (Lentz *et al.*, 1992). Rupture and loss of contents, in addition to low trapping volume, probably explain why mixing of vesicle contents becomes impossible to detect in more highly curved vesicles (Lentz *et al.*, 1992).

Changes in Vesicle Size Detected by Quasi-Elastic Light-Scattering Measurements. In order to obtain further insight into the effects of PEG on DPPC extrusion vesicles, we used QELS to measure the size of vesicles before and after treatment with different concentrations of PEG. Since neither the sedimentation nor the gel filtration method could recover the entire vesicle sample after treatment with PEG, we compare QELS results obtained on both types of samples. Table I summarizes the results of experiments performed with vesicles separated from PEG by the sedimentation procedure described under Methods. This procedure recovered a large fraction of the original lipid, with larger structures being preferentially recovered. In Table I, vesicle diameters collected under the heading "peak 1" represent the mean diameter of the smaller vesicle population detectable in a sample; diameters summarized under "peak 2" represent populations with larger measurable diameters plus any contribution from vesicles with diameters too large ("larger vesicles") to be resolved with autocorrelator channel widths needed to size the smaller vesicles.

Pure DPPC extrusion vesicles showed only one vesicle population, even when recovered from PEG concentrations as high as 28 wt %. However, a larger vesicle population (peak

Table II: Gaussian Mean Diameters (Å) and Half-Height Widths (Å) of Vesicles Isolated by Chromatography[a]

sample	PEG (wt %)	mean diameter	half-height width
DPPC	0	1360	330
	5	1350	380
	15	1390	470
	25	1450	510
	30	1380	520
LPC/DPPC	0	1350	640
	25	1320	520
	30	1670	910
	35	2600	1700
PA/DPPC	0	1250	460
	15	1290	360
	20	1450	670
	25	1880	1250
	30	1940	1260

[a] Data were all well fit by a single Gaussian distribution and were analyzed by the method of cumulants. Measurements were reproducible to within ±1.2% for samples that were never treated with PEG and to within 3.3% for samples treated with ≤35 wt % PEG.

2) was clearly evident after treatment with 30 wt % PEG. For LPC/DPPC LUVET, the diameter of the peak 1 population increased perceptibly at 30 wt % PEG, where fusion occurred (see Figure 4B). This increase in peak 1 diameter was accompanied by the appearance of a very large vesicle population (peak 2). The mean diameter of PA/DPPC vesicles increased measurably at 20 wt % PEG. For these vesicles, the appearance of larger vesicles in peak 2 did not occur until 22.5 wt % PEG. In all three samples, the appearance of larger vesicles as a peak 2 population occurred at PEG concentrations at which vesicles ruptured and leaked their contents (see Figure 4). In the same samples, the increase in diameter of the peak 1 population occurred at PEG concentrations where contents mixing was recorded (see Figure 4). The data from peak 1 and peak 2 of Table I reinforce the distinction between the leakage and fusion events by suggesting that different vesicle products result from these two events.

Only in fusing systems were there measurable increases in vesicle diameter, and then only at PEG concentrations at which fusion occurred. The size increases evident in the peak 1 population of LPC/DPPC and PA/DPPC vesicles correspond to 41% and 21%, respectively, of the size increases that might be expected from one round of fusion. One round of fusion is defined as each vesicle in the sample fusing with one other vesicle.

For DPPC and LPC/DPPC vesicles, there was a decrease in the peak 1 diameter when these vesicles were treated with 30 and 35 wt % PEG, respectively. We can suggest two possible reasons for this observation. It may reflect the rupture of vesicles with subsequent re-formation of smaller as well as larger vesicles, as we have reported for some phosphatidylethanolamine-containing vesicles (Burgess *et al.*, 1992). Alternatively, and more likely, this could reflect the presence of a small amount of larger species at PEG concentrations below 30 and 35 wt %. If there were too few larger vesicles to be resolved by the Laplace deconvolution procedure, the monomodal size distribution that produced the best fit to the data would be shifted toward slightly larger diameters. The presence of larger vesicles in greater numbers apparently allows resolution of a large vesicle population by the multimodal distributional analysis, thus shifting the main peak to a smaller mean diameter.

Table II reports the mean diameters and Gaussian distribution widths obtained with vesicles separated from PEG by Sepharose CL 4B chromatography (see Methods). This

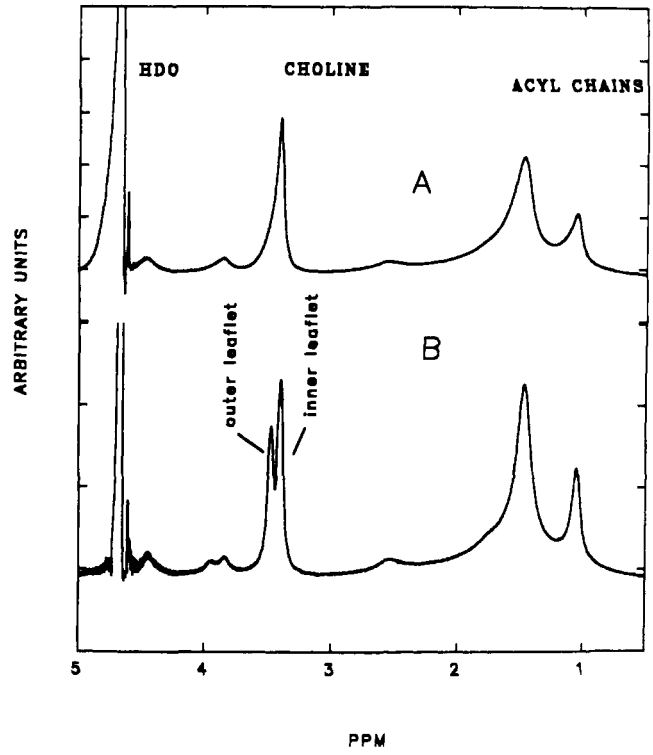

FIGURE 6: ^{1}H NMR spectra of DPPC unilamellar vesicles (A) before and (B) after $PrCl_3$ addition. In spectrum A, a single choline headgroup peak is evident. Spectrum B shows two such peaks; since Pr^{3+} shifts to lower field strength, and the resonance of choline headgroups is present in the outer leaflet of the vesicle bilayer.

separation technique allows recovery of only a small fraction of the vesicles but is probably less skewed toward recovery of larger species than is the centrifugation method (see Methods). For this reason, the chromatography separation technique probably allows a somewhat better assessment of how extrusion vesicles grow due to fusion by PEG. DPPC vesicles isolated in this way showed no significant change in mean vesicle diameter except after incubation with up to 30 wt % PEG. For LPC/DPPC and PA/DPPC vesicles, the onset of vesicle size increase was first detected at 30 and 20 wt % PEG, respectively, with increasing PEG concentrations. It is especially important to note that the essential results obtained with vesicles isolated from PEG by this separation method were in agreement with the results summarized in Table I for vesicles recovered by the sedimentation method; namely, fusion occurred in PA/DPPC extrusion vesicles at 20 wt % PEG, *i.e.*, at a concentration below that at which rupture was first observed. Since the two methods recover very different vesicle subpopulations, this agreement means that our results are not likely to be significantly distorted by sampling artifacts.

PEG-Induced Change in Vesicle Morphology. Pure DPPC and PA/DPPC extruded vesicles prepared in 50 mM NaCl in D_2O were isolated from PEG by sedimentation, as described under Methods. ^{1}H NMR spectra were then collected during titration of the vesicle suspension with the shift reagent $PrCl_3$. As seen in Figure 6, $PrCl_3$ caused the downfield shift of the choline headgroups located on the outer surface of the vesicle (Shaw *et al.*, 1977; Chrzeszczyk *et al.*, 1981; Kaszuba *et al.*, 1990). Once the choline headgroup peak was sufficiently resolved into two peaks as shown in Figure 6B, the peaks were integrated, and the area of the shifted peak was compared to the total area of the choline peaks. Figure 7 shows a titration profile as the fraction of total choline signal due to shifted choline groups *versus* the Pr^{3+}/phopsholipid ratio. The addition of too much $PrCl_3$ caused the shifted peak to broaden

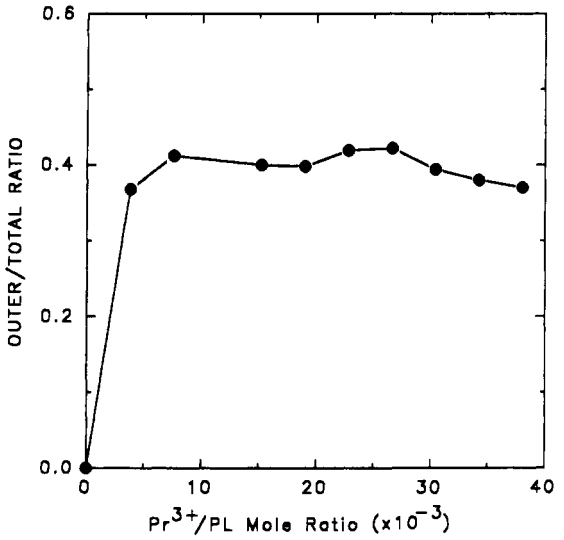

FIGURE 7: $PrCl_3$ titration of DPPC choline headgroups in large, unilamellar vesicles. The ratio of outer leaflet to total choline headgroup signals varied with $PrCl_3$ concentration until a stable value was reached. At least three stable points were averaged to obtain the ratios recorded in Table III.

Table III: Ratio of the Shifted Signal to the Total Choline Headgroup Signal[a]

	PEG (wt %)	outside total ratio
DPPC	0	0.45
	10	0.42
	20	0.44
	25	0.44
	30	0.12
PA/DPPC	0	0.43
	10	0.44
	20	0.44
	22.5	0.29
	25	0.23
	30	0.21

[a] The standard deviation in measurements was 3–4%.

significantly, so that this fraction actually decreased under these conditions. The recorded values in Table III are the average of three or more points on the plateau region of such profiles, with care taken to avoid values taken at very high $PrCl_3$/phospholipid ratios. As seen from Table III, DPPC vesicles experienced no change in the shifted fraction of total choline group signal from 0 through 25 wt % PEG. At concentrations higher than this, the exposed choline decreased. The exposed choline fraction for PA/DPPC vesicles remained invariant through 20 wt % PEG, above which it decreased. It is significant that treatment of PA/DPPC vesicles with 20 wt % PEG resulted in contents mixing (Figure 4) and vesicle size growth (Table I and II), but no rupture (Figure 4), and, at the same time, did not alter the unilamellar nature of the vesicles (Table III). However, treatment of either DPPC or PA/DPPC vesicles with concentrations of PEG that resulted in leakage also resulted in a decrease in the exposed choline fraction, suggesting the formation of multilamellar species or of smaller vesicles entrapped in larger ones.

DISCUSSION

PEG-Induced Vesicle Rupture. Our results demonstrate a strong correlation between the leakage of dextran (MW 10 000) and ANTS. The difference between the pores needed for ANTS leakage and the pores needed for dextran leakage is that ANTS leakage pores may be only a few angstroms in diameter while dextran would likely leak only through openings present during major membrane disruptions, *i.e.*, rupture or lysis. In every case examined here, decreases in dextran trapping paralleled increases in ANTS leakage. This strong correlation suggests that both ANTS leakage and dextran escape occur through the formation of large areas of membrane disruption at least 45–55 Å in diameter (Peters, 1984; Paine *et al.*, 1975), and not through small fluctuating pores (Blok *et al.*, 1975; Phillips *et al.*, 1969). The observations of leakage in this work are consistent with observations made by Aldwinckle *et al.* (1982), who reported that the leakage of Arsenazo III and PEG 4000 from PEG-6000-treated multilamellar vesicles occurred at similar PEG concentrations. However, these authors concluded that PEG did not induce membrane lysis but rather pore formation similar to that thought to occur at the order–disorder phase transition temperature. This conclusion seems unlikely, since a pore of the required dimensions would require displacing 20–30 lipid molecules.

The source of these large disruptions is uncertain, but they could originate with membrane packing defects produced by phase separation or by regions of high local curvature, both of which could be induced by dehydration. Phase separation, the coexistence of ordered and disordered lipid domains, has been suggested to be the mechanism of Ca^{2+}-induced fusion of phosphatidylserine vesicles (Papahadjopoulos *et al.*, 1977) and of PEG-induced fusion of phosphatidylcholine vesicles (Surewicz, 1983). PEG has been shown to increase acyl chain order in membranes (Tilcock & Fisher, 1979; Burgess *et al.*, 1991) probably due to its dehydrating effect (Phillips *et al.*, 1969; Bryant & Wolfe, 1989). However, PEG apparently does not induce extensive phase separation in DPPC extrusion vesicles under fusing or rupturing conditions (Burgess *et al.*, 1991). Nonetheless, the existence of microdomains of phospholipid ordered by dehydration coexisting with disordered regions cannot be ruled out completely.

A more likely explanation for vesicle rupture derives from the dehydrating ability of PEG, which induces vesicle aggregation through an excluded volume and osmotic compression based mechanism (Tilcock & Fisher, 1982; Arnold *et al.*, 1985, 1990; Evans & Needham, 1988a,b). When the vesicles are aggregated at higher PEG concentrations, they are apparently flattened and stacked due to dehydration (Burgess *et al.*, 1992). In this stacked state, vesicle edges must form regions of high curvature where rupture may occur due to high surface tension (Schindler, 1980) and packing defects (Lawaczeck *et al.*, 1976; Chrzeszczyk *et al.*, 1981). A reviewer has pointed out that the energy of adhesion between two vesicles in 25 wt % PEG [*ca.* 1.7 erg/cm; extrapolated from the data of Evans and Needham (1988b)] can be estimated to be sufficient to produce a tensile stress in the curved vesicle edges equal to the tensile strength of a fluid phase membrane (5.7 dyn/cm; Needham & Nunn, 1990). Although this is a crude estimate, because the exact geometry of our aggregated vesicle complexes cannot be determined, it demonstrates the feasibility of this mechanism. The feasibility of this mechanism for leakage is also supported by the dextran trapping results obtained with the highly curved 770- and 620-Å vesicles, in which rupture is observed at lower PEG concentrations (20–25 wt %; Figure 5) than required (25–30 wt %) with larger extruded vesicles of saturated or unsaturated phosphatidylcholines or phosphatidylethanolamine mixtures (Figure 4; Burgess *et al.*, 1991, 1992). However, in order to prove this mechanism for rupture, significant further experimentation would be required.

PEG-Induced Change in Vesicle Structure. The results presented here suggest that the products of fusion and rupture are different. The diameters of PA/DPPC and LPC/DPPC vesicles increased slightly (but measurably; see the error limits in the table legends) at PEG concentrations where contents mixing began (peak 1 in Table I and Table II), while the appearance of distinct populations of larger vesicle occurred at PEG concentrations where rupture was observed (peak 2 in Table I). These results indicate that the product of fusion of large, unilamellar vesicles is a vesicle slightly larger in diameter than the original, while the product of rupture is a species with a much larger diameter.

The results obtained with high-resolution ^{1}H NMR provide further insight into the nature of products resulting from fusion *versus* rupture. In both DPPC and PA/DPPC vesicles treated with PEG, there was no change in choline group exposure as measured by $PrCl_3$ titration until the vesicles were incubated with PEG concentrations that caused rupture. At these concentrations, the products of PEG treatment took on an oligolamellar character. This oligolamellar character is likely due to the presence of smaller vesicles encapsulated in larger ones, but not to many layered hydrated sheets. This conclusion derives from the observation that no sharp X-ray diffraction patterns characteristic of multilamellar hydrated sheets were observed when 1,2-dioleoyl-3-*sn*-phosphatidylcholine vesicles, with and without 0.5 mol % lysooleoyl-PC, were treated with 0–35 wt % PEG and then separated from the PEG before observation (D. Massenberg and T. McIntosh, unpublished data).

Separate Fates for PEG-Treated Membranes: Rupture, Fusion, No Change. Our purpose in this work was 2-fold: first, to define better the nature of the contents leakage that accompanies PEG-mediated vesicle fusion; second, to determine if contents leakage was inexorably associated with and therefore an intrinsic part of the fusion process. As summarized above, we have shown that the contents leakage observed for large extruded vesicles in 25–30 wt % PEG is due to a dramatic loss of bilayer integrity, *i.e.*, to rupture. With regard to the second issue, our data indicate that rupture and fusion are separate events with different products. Nonetheless, they may also be related in that they both occur at high PEG concentrations (≥20 wt %). Figure 8 presents a model which summarizes the findings of this work. It shows that unilamellar vesicles treated with PEG form primarily small aggregates (Wu and Lentz, unpublished observations) shown here as stacked, flattened disks, based on the sharp X-ray diffraction patterns observed by Burgess *et al.* (1992). The aggregates appear to be stable at low PEG concentrations (<20 wt %). High PEG concentration (≥25 wt %) is hypothesized to produce such stress in the highly curved vesicle ends that they rupture and release the vesicle contents. Fusion was observed at PEG concentrations (20–25 wt % PEG for PA/DPPC; 25–30 wt % for LPC/DPPC) close to but not necessarily equal to those required for rupture. Fusion is presumed to occur as a separate process between closely apposed regions of stressed bilayers at the highly curved ends. It might be viewed as an alternative process (to rupture) for relieving the stress associated with high curvature induced by dehydration. Upon removal of PEG, the vesicles can be seen from our data to have experienced one of three possible fates, depending on the vesicle species and the PEG concentration: no change, rupture to form large, oligolamellar vesicles, or fusion to form large, unilamellar vesicles. Why certain amphipaths, certain mixtures of phospholipids, membrane curvature, *etc.* allow fusion to compete with rupture is not clear. Resolution of these issues promises to provide new insights into the general mechanism of membrane fusion and constitutes the focus of future research efforts.

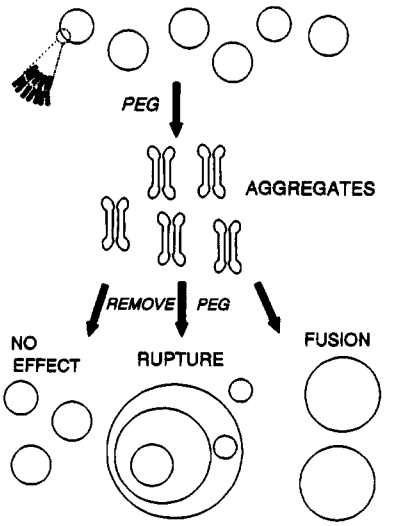

FIGURE 8: Summary of effect of PEG on large, unilamellar extruded vesicles. When large, unilamellar vesicles were treated with PEG, they aggregated into small, stacked clusters (Burgess *et al.*, 1992; Wu & Lentz, 1993). Upon removal of PEG, these small aggregates had either separated into the original unaltered vesicles, had ruptured and formed multilamellar vesicles, or had fused to form larger unilamellar vesicles.

ACKNOWLEDGMENT

We express our appreciation to an anonymous reviewer who pointed out that rupture at the highly curved edges of flattened vesicles was consistent with the expected tensile strength of the bilayer and also made useful suggestions for experiments to define the rupture mechanism.

REFERENCES

Aldwinckle, T. J., Ahkong, Q. F., Bangham, A. D., Fisher, D., & Lucy, J. A. (1982) *Biochim. Biophys. Acta 689*, 548–560.

Arnold, K., Herrman, A., Gawrisch, K., & Pratsch, L. (1985) *Stud. Biophys. 110*, 135–141.

Arnold, K., Zschoernig, O., Barthel, D., & Herold, W. (1990) *Biochim. Biophys. Acta 1022*, 303–310.

Boni, L. T., Stewart, T. P. Alderfer, J. L., & Hui, S. W. (1981) *J. Membr. Biol. 62*, 65–70.

Bryant, G., & Wolfe, J. (1989) *Eur. Biophys. J. 16*, 369–374.

Burgess, S. W., Massenburg, D., Yates, J., & Lentz, B. R. (1991) *Biochemistry 30*, 4193–4200.

Burgess, S. W., McIntosh, T. J., & Lentz, B. R. (1992) *Biochemistry 31*, 2653–2661.

Chen, P. S., Jr., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Chrzeszczyk, A., Wishnia, A., & Springer, C. S., Jr. (1981) *Biochim. Biophys. Acta 648*, 28–48.

Davidson, R. L., & Gerald, P. S. (1977) in *Methods in Cell Biology* (Prescott, D., Ed.) pp 325–338, Academic Press, New York.

Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.

Evans, E., & Needham, D. (1988a) *Macromolecules 21*, 1822–1831.

Evans, E., & Needham, D. (1988b) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Ed.) pp 83–99 Plenum Publishing Corp., New York.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Kaszuba, M., & Hunt, G. R. A. (1990) *J. Inorg. Biochem. 40*, 217–225.

Koppel, D. E. (1972) *J. Chem. Phys. 57*, 4814–4820.

Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313–330.

Lentz, B. R., McIntyre, G. F., Parks, D. J., Yates, J. C., & Massenburg, D. (1992) *Biochemistry 31*, 2643–2653.

MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K., & Hu, L. (1991) *Biochim. Biophys. Acta 1061*, 297–303.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161–168.

Needham, D., & Nunn, R. S. (1990) *Biophys. J. 58*, 997–1009.

Paine, P. L., Moore, L. C., & Horowitz, S. B. (1975) *Nature 254*, 109–114.

Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Parente, R. A., & Lentz, B. R. (1986) *Biochemistry 25*, 6678–6688.

Peters, R. (1984) *EMBO J. 3*, 1831–1836.

Phillips, M. C., Williams, R. M., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 234–244.

Provencher, S. W. (1979) *Makromol. Chem. 180*, 201–209.

Roseman, M. A., Lentz, B. R., Sears, B., Gibbes, D., & Thompson, T. E. (1978) *Chem. Phys. Lipids 21*, 205–222.

Sàez, R., Alonso, A., Villena, A., & Goñi, F. M. (1982) *FEBS Lett. 137*, 323–326.

Schindler, H. (1980) *FEBS Lett. 122*, 71–79.

Shaw, J. M., Hutton, W. C., Lentz, B. R., & Thompson, T. E. (1977) *Biochemistry 16*, 4156–4163.

Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.

Surewicz, W. K. (1983) *FEBS Lett. 151*, 228–232.

Szoka, F. E., Magnusson, K. E., Wojcieszyn, J. W., Hou, Y., Derzko, Z., & Jacobson, K. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1685–1689.

Tilcock, C. P. S., & Fisher, D. (1979) *Biochim. Biophys. Acta 577*, 53–61.

Tilcock, C. P. S., & Fisher, D. (1982) *Biochim. Biophys. Acta 688*, 645–652.

Yamazaki, M., Ohnishi, S., & Ito, T. (1989) *Biochemistry 28*, 3710–3715.