Poly(ethylene glycol)-Induced Lipid Mixing but Not Fusion between Synthetic Phosphatidylcholine Large Unilamellar Vesicles†

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ABSTRACT: We have examined the effect of poly(ethylene glycol) (PEG) on stable large unilamellar vesicles formed by a rapid extrusion technique and composed of pure synthetic phosphatidylcholines. The lipid systems studied were the saturated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and the monounsaturated 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). PEG at all concentrations (3.8–40 wt %) induced lipid mixing between large vesicles composed of these phosphatidylcholines. Extensive leakage of internal contents also occurred at high PEG concentrations. However, in contrast to our previous report [Parente, R. A., & Lentz, B. R. (1986) *Biochemistry 25*, 6678], we could detect no mixing of internal contents indicative of fusion. This discrepancy is due to environmental factors that affect the behavior of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), the fluorophore used in the assay for contents mixing and leakage [McIntyre, Parks, Massenburg, & Lentz (1991) (submitted)]. In agreement with the results of the fusion assay, quasielastic light-scattering measurements revealed no increase in vesicle size following treatment with PEG. These results emphasize the importance of using assays for both membrane mixing and contents mixing to demonstrate fusion, since significant lipid mixing occurred in the absence of fusion. We conclude that large vesicles composed of pure phosphatidylcholine do not fuse in the presence of even high concentrations of PEG. However, DOPC vesicles containing a small amount of an amphipathic "impurity" have been shown to fuse in the presence of PEG at 23 °C. These results are discussed in terms of their implications for the mechanism of PEG-induced membrane fusion.

Membrane fusion is essential for many biological processes. Although much effort has been expended trying to establish the requirements for fusion, little is known about the basic mechanism of the fusion process. It is well accepted that aggregation (i.e., bilayer juxtaposition) is a prerequisite for the fusion process (Knutton & Pasternak, 1979; Nir et al., 1983b). Once bilayer juxtaposition is achieved, the fusion process itself is presumably modulated at the molecular level by local perturbations in membrane structure induced by compositional changes or environmental adjustments. The key to understanding cellular membrane fusion is in defining the nature and origin of these local perturbations. While model membranes are a gross oversimplification of biological systems, they are still the best means for unraveling the molecular details of the events that trigger the fusion process.

Overcoming the hydration repulsion between vesicles has been described as a possible initial step in the fusion process (Rand & Parsegian, 1988). In other words, in order for fusion to occur, one must presumably perturb the water structure between membranes. Poly(ethylene glycol) (PEG)[1] is a dehydrating polymer that apparently serves to withdraw water from membrane surfaces, forcing close contact of membranes (Arnold et al., 1982, 1990). Although PEG is used extensively in the preparation of hybridomas (Davidson & Gerald, 1977), the mechanism of its action is poorly understood. We report here the effect of PEG on large unilamellar vesicles (LUV) made by a rapid extrusion technique (LUVET) (Hope et al., 1985; Mayer et al., 1986) and composed of two synthetic phosphatidylcholines. Previous work in this area determined the effects of PEG on highly curved small unilamellar vesicles (SUV) and LUV made by other techniques (e.g., reverse evaporation and ethanol injection) (Morgan et al., 1983; Boni et al., 1984; MacDonald, 1985; Parente & Lentz, 1986). The LUV made by these procedures may contain residual organic impurities that could affect membrane structure. SUV have been shown to be unstable and to fuse rapidly below (Suurkuusk et al., 1976; Schullery et al., 1980) and at a slower rate above their order–disorder phase transition (Lentz et al., 1987). LUVET are stable lipid vesicles that exhibit a well-defined size distribution and contain minimal residual impurities to perturb the bilayer structure. An understanding of the effect of PEG in fusing these well-defined model membranes will aid in defining the role of PEG in hybridoma formation and may suggest general features of the fusion process that will be useful in understanding the fusion of much more complex biological membranes.

Experimental Procedures

Materials. Chloroform stock solutions of DPPC, DOPC, and 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-*sn*-glycero-3-phosphocholine (DPHpPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). 1-Oleoyl-*sn*-glycero-3-phosphocholine (lysoPC) was obtained from Sigma. DPPC stocks were filtered over Norit A neutral activated charcoal to remove trace fluorescent contaminants. Lipids were verified to be greater

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[1] Abbreviations: PEG: poly(ethylene glycol); ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX: *N*,*N*′-*p*-xylylenebis(pyridinium bromide); TES: *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA: ethylenediaminetetraacetic acid, disodium salt; $C_{12}E_8$: dodecyloctaethyleneglycol monoether; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPHpPC: 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-*sn*-glycero-3-phosphocholine; LUV: large unilamellar vesicle; SUV: small unilamellar vesicle; LUVET: large unilamellar vesicles made by a rapid extrusion technique; DSC: differential scanning calorimetry; lysoPC: 1-oleoyl-*sn*-glycero-3-phosphocholine.

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than 98% purity by thin-layer chromatography on Analtech GHL plates. Plates were developed in a 65:25:4 (v/v/v) $CHCl_3/CH_3OH/H_2O$ mixture and were stained with iodine vapors. DPHpPC was also viewed under near-UV light. The fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and its quencher *N,N'-p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Junction City, OR). Both ANTS and DPX solutions were stored at 4 °C. DPX solutions were filtered after preparation through 0.22-μm GS filters (Millipore Corp., Bedford, MA) to remove a small amount of insoluble material. PEG (av mol wt 8000) was obtained from Fisher Scientific (lot #874229). PEG was further purified as described elsewhere (McIntyre et al., 1991). Briefly, PEG was dissolved in doubly distilled water and stirred overnight with sodium borohydride and filtered over a Chelex 100 ion-exchange resin. The water was removed by rotoevaporation and lyophilization. Dried PEG was dissolved in a minimal amount of chloroform and precipitated with peroxide-free diethyl ether. The precipitate was isolated and dried. Purified PEG was stored dessicated in the dark until use. There was no difference in the fusogenic capacity of purified and nonpurified PEG (Parente & Lentz, 1986; McIntyre et al., 1991). Dodecyloctaethyleneglycol monoether ($C_{12}E_8$) and *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) were purchased from Calbiochem. Buffer solutions were filtered prior to use through a Nalgene disposable filter to remove dust, which might interfere with the fluorescence measurements. All other chemicals were of the highest quality available.

Vesicle Preparation. Large unilamellar extruded vesicles (LUVET) were prepared by the method of Hope et al. (1985). Lipid was dissolved in cyclohexane, and the solvent removed under vacuum. Samples were suspended in a buffer at a temperature above the gel-to-liquid-crystalline phase transition. The large multilamellar vesicles that formed were allowed to equilibrate and fully hydrate above their phase transition for approximately 30 min. These vesicles were then forced seven times through a 0.1-μm polycarbonate filter (Nucleopore) above their phase transition under a pressure of approximately 200 psi of argon. This procedure yielded a fairly homogeneous population of unilamellar vesicles with an average diameter of ≈1300 Å. Vesicle diameters were determined by quasielastic light scattering. The majority of the quasielastic light-scattering measurements were carried out at an angle of 90° and 48 °C on a multiangle light-scattering apparatus constructed by us around a Nicomp Model 170 Computing Autocorrelator (Particle Sizing Systems, Inc., Santa Barbara, CA). This instrument is described elsewhere in detail (Lentz et al., 1991). These vesicles retained their trapped contents for at least 1 week if stored at 4 °C.

The concentrations of all vesicle samples were determined by phosphate analysis with a modification of the procedure of Chen et al. (1956). For lipid-mixing and vesicle-sizing experiments, vesicles were prepared in 100 mM NaCl, 2 mM TES, and 1 mM EDTA, pH 7.4. For the contents-mixing experiments, vesicles were prepared in buffers containing 25 mM ANTS (or 90 mM DPX), 40 mM NaCl, and 10 mM TES, pH 7.4. For contents-leakage experiments, the buffer contained 12.5 mM ANTS, 45 mM DPX, 40 mM NaCl, and 10 mM TES, pH 7.4. Vesicles were eluted from a Sephadex G-75 column (0.7 × 10 cm) with 100 mM NaCl, 2 mM TES, and 1 mM EDTA, pH 7.4, to remove untrapped ANTS or DPX immediately before use. Osmolarities of all buffers were monitored by using a μ Osmette microosmometer (Precision Systems, Sudbury, MA).

Fluorescence. All fluorescence measurements were made on an SLM 48000 spectrofluorometer (Urbana, IL) equipped with a modified, three-position, multitemperature cuvette holder (Barrow & Lentz, 1985) and 200-W Hg-xenon or 150-W xenon arc lamps mounted horizontally in a Photon Technology International (Princeton, NJ) lamp housing. The 366-nm mercury line was used to excite DPHpPC for lifetime measurements, while emission was monitored through a 3-mm high-pass KV-450 filter (50% transmittance at 450 nm; Schott Optical Glass, Duryea, PA). Vertically polarized and modulated light from the Pockel cell was rotated to 35° from vertical by placing a Soliel-Babinet compensator (Karl Lambrecht, Chicago, IL) rotated 17.5° from vertical and set for half-wave at 366-nm in the excitation path. This allowed us to excite with linearly polarized light at the magic angle so as to avoid errors in lifetime calculations without the loss of intensity inherent in the use of polarizers.

Fluorescence-intensity measurements of ANTS were made at an excitation wavelength of 384 nm with the 150-W xenon arc lamp. Emission was observed through a 2-mm OG-515 filter (50% transmittance at 515 nm; Schott Optical Glass, Duryea, PA).

Lipid-Mixing Assay. The mixing of membrane components induced by PEG was demonstrated by using the DPHpPC fluorescent lifetime lipid-mixing assay (Parente & Lentz, 1986). A detailed description of the assay and its intricacies is found in Burgess and Lentz (1991). Briefly, the assay depends on the fact that the fluorescence lifetime of DPHpPC decreases with an increasing surface concentration of this probe in a membrane, so that an increase in lifetime results from lipid mixing between a probe-rich and a probe-free population of vesicles. The standard error (0.05 ns) associated with the measured lifetime of a given lipid-mixing experiment was used to determine the error in the extent of lipid mixing for each PEG concentration.

ANTS/DPX Assay for Contents Mixing and Leakage. We used the ANTS/DPX assay (Ellens et al., 1984, 1985) to determine the extent of internal aqueous contents leakage and mixing. An extensive description of the assay and its modifications for our work is presented in McIntyre et al. (1991).

Calorimetry. Differential scanning calorimetry (DSC) of extruded vesicles was performed with an MC-2 high-sensitivity calorimeter (Microcal, MA). Samples were degassed for at least 45 min prior to loading in the calorimeter. Scans done in the presence of PEG required PEG at the same concentration to balance the heat capacity of the reference cell. The scan in the absence of PEG was done in the buffer described above for lipid-mixing experiments. The scan rate for all concentrations of PEG (except 45 wt %) was 30 °C/h. The scan rate for 45 wt % PEG was 10 °C/h.

RESULTS

Time Course of Vesicle Changes Following PEG Treatment. Figure 1 contains representative time courses for the mixing of DPHpPC in vesicles exposed to PEG. For the DPPC in 5 wt % PEG (panel A), it is clear that this lipid mixing was a slow process. By contrast, panel B represents the exchange of lipid for DOPC/0.5 mol % lysoPC vesicles in 35 wt % PEG, where fusion was observed. Under these conditions, lipid mixing was almost complete before the first measurement could be made (~30 s). The release of DPPC vesicle contents was also very rapid following treatment with high concentrations of PEG, as is illustrated in Figure 2. Our manual mixing procedures were too slow for these experiments to yield accurate data on the initial rate of contents leakage, although qualitative information about relative initial rates

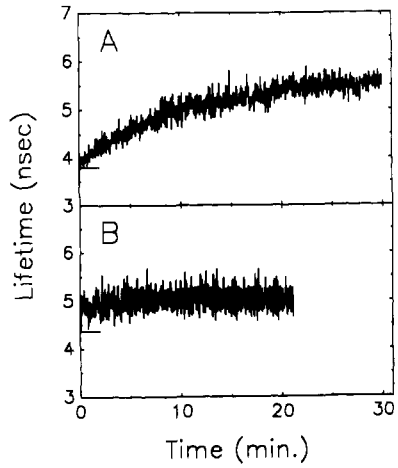

FIGURE 1: Representative time course of lipid-mixing experiments. The fluorescence lifetime of DPHpPC was monitored continuously for 30 min for DPPC LUVET incubated in 5 wt % PEG at 48 °C (panel A) and DOPC/0.5 mol % lysoPC incubated in 35 wt % PEG at 23 °C (panel B). The short horizontal line eminating from the y axis indicates the lifetime of the probe at time $t = 0$. The data show the high-frequency noise associated with the measurements; this was removed by a running average procedure.

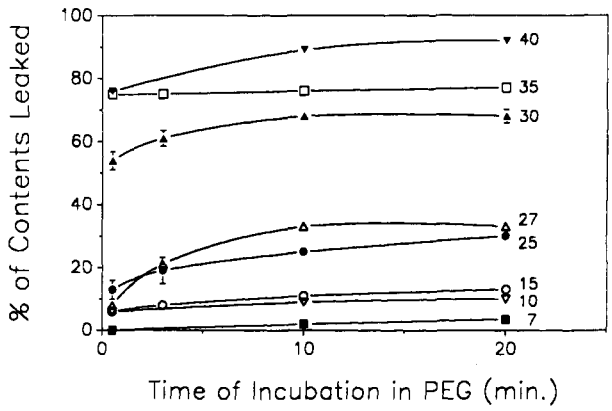

FIGURE 2: Time dependence of the leakage of trapped contents. DPPC LUVET were incubated at 48 °C in various concentrations of PEG. Samples were incubated for the times indicated in various concentrations of PEG before diluting with buffer. Concentrations of PEG used were 7 (■), 10 (▽), 15 (○), 25 (●), 27 (△), 30 (▲), 35 (□), and 40 (▼) wt %. Error bars shown were obtained from three experiments; if not shown, the error bars were smaller than the points.

of leakage can be gleaned from the data in Figure 2. At low PEG concentrations, leakage was minimal and increased very slowly with PEG concentration. However, the data in Figure 2 reveal a rapid increase in both the rate and extent of leakage of DPPC vesicle contents at and above 30 wt % PEG. Mixing of internal contents could not be detected for this lipid system, indicating that contents leakage and contents mixing are separate and independent processes for PEG-induced fusion. However, when contents mixing was observed at high PEG concentration (e.g., in LUVET composed of DOPC and lysoPC, see below), it generally was maximal by 30 s of incubation with PEG.

Effect of PEG on DPPC LUVET. Figure 3 summarizes the response of extruded vesicles composed of DPPC to treatment by different concentrations of PEG. Vesicles were incubated in PEG at 48 °C, which is slightly above the DPPC gel-to-liquid-crystalline phase transition temperature. Lipid mixing was monitored after 30 min of incubation. However, in order to maximize the chance for observing mixing of internal contents, contents mixing and contents leakage were both recorded after only 30 s of incubation. Leakage of internal

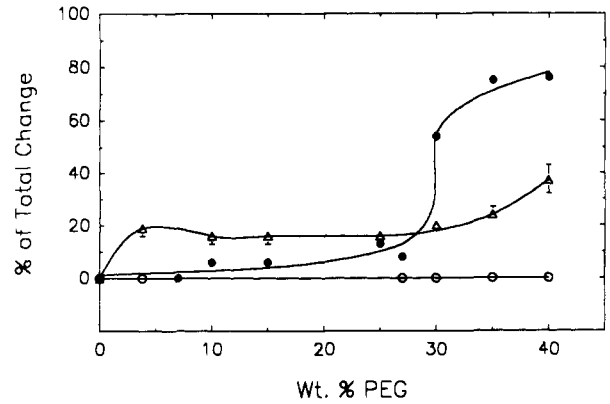

FIGURE 3: Fusion assays for DPPC LUVET. Lipid-mixing (△), contents-mixing (○), and contents-leakage (●) results are shown for DPPC vesicles at 48 °C. In order to maximize the ability to observe fusion, contents leakage and mixing data were obtained by using a 30-s incubation in PEG before diluting with buffer. Lipid mixing was recorded after a 30-min incubation with PEG. Error bars on the lipid-mixing curve were derived from the standard error of lifetime measurements (0.05 ns). Errors bars for contents mixing and leakage are within the size of the data point.

Table I: Phase Transition of DPPC LUVET in PEG

PEG (wt %)	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kcal/mol)	ΔS (cal/mol/K)
0	41.37	1.58	7.84	24.81
10	41.58	0.85	8.12	25.75
25	41.82	1.00	7.64	24.14
30	42.05	0.93	8.14	25.71
35	42.35	0.85	6.44	20.29
45	42.62	1.00	6.65	20.91

contents was minimal at low PEG concentrations (0–27 wt %), but a dramatic increase in leakage occurred at and above 30 wt % PEG. By 40 wt % PEG, nearly all contents were lost. At high PEG concentrations (≥30 wt %), vesicle dehydration and deformation could account for bilayer rupture or severe destabilization in regions of high curvature, leading to a dramatic increase in leakage. It is worth noting that the concentration range in which PEG induced enhanced leakage from DPPC LUVET is the same concentration range in which PEG is usually seen to induce cell fusion (Blow et al., 1978) or to cause size growth of small unilamellar vesicles (Boni et al., 1984). The detailed mechanism of enhanced leakage is currently being investigated.

In control experiments, vesicles incubated in the absence of PEG were found to leak ~15% of their contents after 20 min (data not shown). This was more leakage than was observed after 20-min incubations in the presence of low concentrations (≤10 wt %) of PEG. At present, we have no definitive explanation for the reduced long-time vesicle leakage observed at low concentrations of PEG. Perhaps the limited dehydration produced by low amounts of PEG reduced the probability of membrane fluctuations that allowed leakage of small solutes in the absence of PEG. It is also possible that the increased viscosity of a PEG solution slowed diffusion of trapped solute sufficiently to slow leakage relative to the rate in the absence of PEG.

Dehydration is known to alter the phase behavior of lamellar-phase lipid (Chapman et al., 1967). Thus, the dramatic effects of high concentrations of PEG might reflect the effects of this polymer on vesicle phase behavior. In order to test for this possibility, the phase behavior of DPPC LUVET was monitored as a function of increasing PEG concentration with differential scanning calorimetry. While the phase behavior was not dramatically affected by PEG, the transition tem-

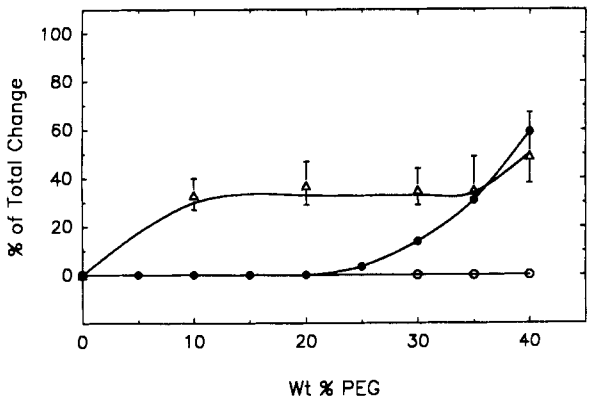

FIGURE 4: Fusion assays for DOPC LUVET. Lipid-mixing (△), contents-mixing (○), and contents-leakage (●) results are shown for DOPC vesicles at 23 °C. Contents leakage and mixing data were obtained following a 2-min incubation in PEG before diluting with buffer. Lipid mixing was recorded after a 30-min incubation with PEG. Error bars were obtained as in Figure 3.

perature increased monotonically as a function of PEG concentration; although it increased at a slightly greater rate around 30 wt % PEG (Table I). The transition peak width at half-height was slightly reduced in the presence of PEG but essentially invariant with varying PEG concentrations (Table I). The enthalpy associated with the main transition remained relatively constant up to 30 wt % PEG but decreased in the presence of more concentrated PEG, consistent with the ordering effect than PEG has on the membrane (Yamazaki et al., 1989).

Another effect of PEG on DPPC LUVET was induction of significant membrane component mixing. As can be seen in Figure 3, approximately 20% of the DPHpPC probe exchanged over a 30-min incubation in the absence of fusion at PEG concentrations below 35 wt %. The fluorescent lipid probe exchanged slowly in the absence of PEG. At PEG concentrations greater than 30 wt %, as much as 40% of the probe exchanged, despite the fact that mixing of vesicle contents could not be detected. The implications of these observations, both for methods of detecting PEG-induced fusion and for the mechanism of PEG-induced fusion, will be dealt with under the Discussion.

Mixing of internal aqueous contents was not observed at any PEG concentration (0–40 wt %).

Effect of PEG on DOPC LUVET. The response of DOPC extruded vesicles to treatment by PEG is shown in Figure 4. Vesicles were incubated in PEG at 23 °C, well above the phase transition of the lipid system. Three types of observations were made. First, leakage of internal contents was monitored but did not shown the same pattern as seen for DPPC LUVET (Figure 3). This could be due to the unsaturated acyl chains of DOPC or to the fact that DOPC was examined at a temperature more than 40 °C above its phase transition, while DPPC was examined at a temperature only about 7 °C above its phase transition. Leakage of contents from DOPC vesicles increased gradually with increasing PEG concentration rather than abruptly as observed for DPPC vesicles, and the extent of leakage at high PEG concentration was much less than that observed for DPPC vesicles (60% for DOPC vs 80% for DPPC). Second, as for DPPC vesicles, mixing of internal contents could not be detected at any concentration of PEG (0–40 wt %). Finally, the lipid-mixing results for DOPC vesicles were qualitatively similar to those observed for DPPC. The extent of lipid mixing was constant at ~30% from 10 to 35 wt % PEG with an increase to 50% mixing by 40 wt % PEG. As with DPPC LUVET, no measurable lipid mixing

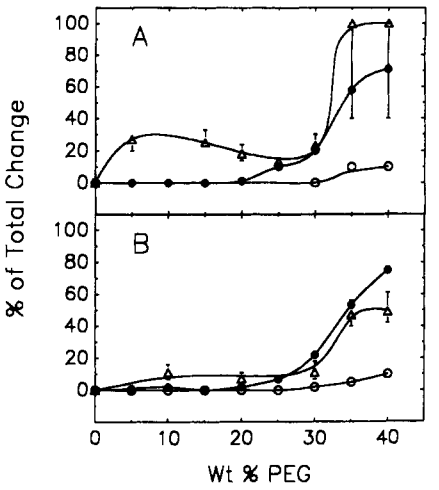

FIGURE 5: Fusion assays for anomalous DOPC LUVET. Lipid-mixing (△), contents-mixing (○), and contents-leakage (●) are shown for DOPC vesicles at 23 °C containing (A) unknown impurity, or (B) 0.5 mol % monooleoylphosphatidylcholine. Contents leakage and mixing experiments were done by using a 2-min incubation in PEG before dilution with buffer. Lipid mixing was recorded after a 30-min incubation with PEG. Error bars on the lipid-mixing curve represent the standard error of lifetime measurements (0.05 ns). Error bars for contents mixing and leakage are within the size of the data point.

was observed on the time scale of our experiment in the absence of PEG.

Effect of Amphipathic "Impurities" on the Response of DOPC Vesicles to PEG. Preliminary experiments with DOPC vesicles indicated that these vesicles mixed their contents in the presence of high concentrations of PEG (see Figure 5A). Further experiments with a different lipid batch and with a different stock tube of the same batch failed to reproduce these results. Other work from this laboratory has indicated that certain amphipathic compounds impart a capacity for fusion to otherwise nonfusogenic DPPC LUVET (McIntyre et al., 1991). One of these compounds was monoacylphosphatidylcholine (lysoPC). McIntyre et al. (1991) showed that addition of a small amount (0.5 mol %) of lysoPC to DPPC LUVET induced fusion in the presence of high concentrations (≥30 wt %) of PEG. We added 0.5 mol % monooleoyl PC to a nonfusogenic DOPC stock and tested the response to PEG of vesicles prepared from this stock. The results (Figure 5B) were similar to those obtained by McIntyre et al. (1991) and to those obtained with the anomalous DOPC stock (Figure 5A). The small amount of contents mixing (<10%) observed in these systems is significant in view of the extensive leakage observed and demonstrates fusion of these vesicles. The apparent difference in lipid mixing at high PEG concentrations is due to the use of a lower initial probe concentration in the lipid-mixing assay used to obtain the data in Figure 5B, limiting the useful range of the assay to low-percentage exchange (Burgess & Lentz, 1991). A similar experiment incorporating 0.1 mol % of the lysoPC did not result in detectable vesicle contents mixing in the presence of PEG (data not shown). We extracted the DOPC samples containing lysoPC and were able to detect by thin-layer chromatography 0.5 mol % of this component. However, thin-layer chromatographic analysis detected no lipid impurities in the anomalous DOPC stock. We conclude from this that the behavior of the anomalous DOPC stock could be explained as due to a small contamination by some amphipathic compound similar to but not necessarily identical with lysoPC. This result is significant to the present paper first because it demonstrates that fusion can be detected by the assays used here. Second, these results confirm and extend

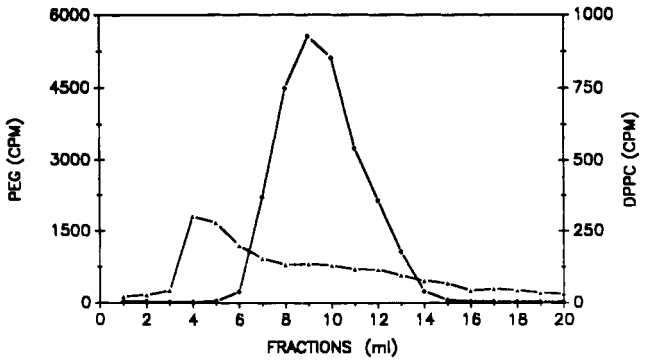

FIGURE 6: Separation of LUVET from PEG. Separation was accomplished on a Sepharose CL 4B gel-filtration column (1.2-cm diameter, 12-cm length) at room temperature. Calibration of the column was carried out through the separation of [^{14}C]DPPC-labeled DPPC LUVET (▲) from 17.5 wt % PEG and the separation of [^{14}C]PEG 4000 (●) from unlabeled DPPC LUVET.

to unsaturated DOPC vesicles the importance of bilayer perturbants on the fusion process, as discussed in more detail by McIntyre et al. (1991).

When contents mixing was observed in the presence of lysoPC, its rate was also too rapid to quantitate by our methods, and only final extents of contents mixing can be reported. In addition, extents of contents mixing after 1- and 2-min incubations were the same. This observation and the fact that lipid mixing was almost complete in the first 30 s indicates that fusion occurs rapidly at and above 30 wt % PEG.

QELS Vesicle Size Measurements. In order to confirm that pure DOPC vesicle fusion did not take place even in the presence of high concentrations of PEG, the distribution of vesicle sizes was determined before and after treatment with PEG.

Separation of vesicles from PEG was obtained by eluting 400-μL vesicle aliquots containing varying concentrations of PEG (maximum 17.5 wt %) through Sepharose CL 4B columns at room temperature. Samples containing PEG at greater than 17.5 wt % concentration were diluted to this concentration before application to the column. Complete separation of vesicles and PEG was not possible through size-exclusion chromatography alone (Figure 6), consequently only the leading edge of the broad vesicle band was substantially free (<0.5 wt %) of PEG. For this reason, only the leading 1.0 to 1.5 mL of the band was routinely collected and used for size determinations. Approximately 24% of the phospholipid was recovered prior to PEG elution when a sample containing 17.5 wt % PEG was used, with increasing recovery observed with lower starting concentrations of PEG.

QELS measurements of the size of DOPC LUVET were made after separation of PEG from the vesicles. The results are shown in Figure 7A as a function of the starting concentration of PEG and demonstrate that pure DOPC LUVET did not increase in size even when treated with 40 wt % PEG. In addition, there was no significant increase in the distribution half-width (indicated by vertical bars in Figure 7) following treatment with PEG. The addition of 0.5 mol % lysoPC to pure DOPC LUVET resulted in increases in the average vesicle diameter at 30 and 35 wt % PEG (Figure 7B). These vesicles increased in size from roughly 1400 Å at low PEG to 1850 Å at 35 wt % PEG. The ratio of the diameter of the vesicles treated with 35 wt % PEG to that of vesicles treated with low concentrations of PEG was 1.3. A value of 1.4 would have been expected for one round of fusion, if spherical vesicles are assumed. The decrease in average hydrodynamic vesicle diameter following treatment with >35 wt % PEG is a reproducible phenomenon that we do not fully understand but

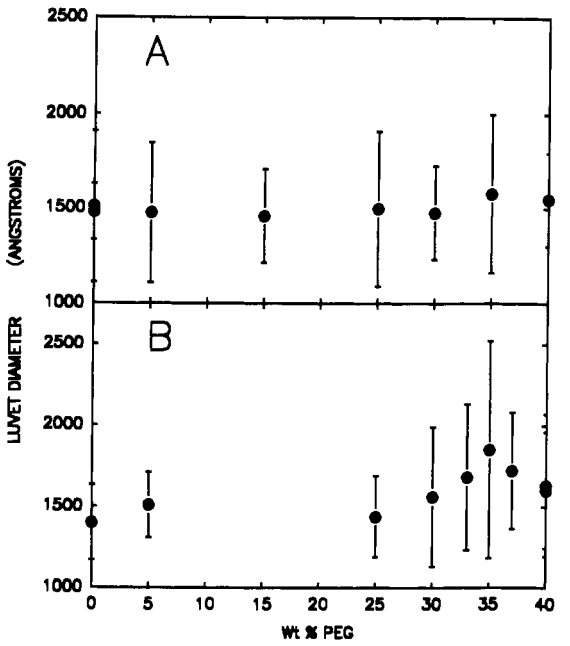

FIGURE 7: Measurement of vesicle size changes associated with PEG treatment. LUVET prepared from (A) "anomalous" DOPC or (B) DOPC plus 0.5 mol % lysoPC were incubated with 0–40 wt % PEG and isolated from PEG as illustrated in Figure 6. The vesicles were sized in a quasielastic light-scattering photometer at 23 °C, as described under Experimental Procedures. Vesicle concentrations in the cuvette were 0.2–1.0 mM phospholipid. The vertical bars represent Gaussian distribution half-widths derived from a cumulants analysis. Measurements of vesicle diameters for different vesicle preparations 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.

that may reflect the disruption of membrane integrity due to complete dehydration by PEG (Blow et al., 1978; Arnold et al., 1983).

Discussion

Proper Interpretation of Assays. During the course of our investigations, we discovered anomalies associated with our assays of which neither we nor others had been aware. The anomalies uncovered with the ANTS/DPX assay are described in detail elsewhere (McIntyre et al., 1991). A less severe subtlety associated with the lipid-mixing assay is documented here. As for most fluorescent molecules (Lakowicz, 1983), the fluorescence properties of the probe DPHpPC are sensitive to the environment. For this reason, the presence of PEG in the system affected the lifetime of the probe. Consequently the lifetime standard curve for each lipid system had to be determined at each PEG concentration used in the study. Comparison of the lifetime after PEG-induced lipid mixing with a standard curve generated without PEG present led to overestimation of the extent of lipid mixing.

It is also evident from our results that lipid mixing was not necessarily coincident with fusion (Figures 3 and 4). The failure of high concentrations of PEG to fuse DOPC LUVET was confirmed by vesicle size determinations using quasielastic light scattering (Figure 7A). This observation requires one to reevaluate much of the past work in membrane fusion that relied on membrane mixing assays and turbidity measurements to monitor fusion. Several studies on membrane fusion have used the NBD-PE/Lis-rhodamine-PE lipid-mixing assay (Struck et al., 1981). Although we used a different lipid probe, the mechanism of probe transfer in the absence of fusion should be the same. Other lipid-mixing assays, then, would be subject to the same misinterpretation. Without supporting information, e.g., observation of contents mixing or mea-

surement of vesicle size, it is easy to draw incorrect conclusions from lipid-mixing results, especially under conditions where extensive membrane aggregation occurs.

Fusion of Large Unilamellar Vesicles. Recent studies have reported that LUV are induced to fuse in the presence of high concentrations of PEG. Because these studies are in disagreement with the results reported here, it is appropriate to comment in detail on the reasons for this disagreement. Both MacDonald (1985) and Parente and Lentz (1986) reported intervesicle mixing of lipids in the presence of PEG, in agreement with our results. In addition, Parente and Lentz used as well an assay for vesicle contents mixing to confirm fusion induced by PEG. However, more recent work in our laboratory (McIntyre et al., 1991) has shown that our apparent observation of contents mixing in the presence of PEG (Parente & Lentz, 1986) was due to a subtle anomaly associated with photosensitivity of the ANTS probe only when it is encapsulated in vesicles that have been subjected to the dehydrating influence of PEG. Our failure to detect this anomalous behavior caused us to conclude incorrecty that LUV were induced to fuse by concentrations of PEG as low as 3.8 wt %. When this anomaly is taken into account, we can detect no fusion of DPPC LUV (Figure 3) or of DOPC LUV (Figure 4) in the presence of any concentration of PEG, in agreement with our studies of vesicle size (Figure 7).

MacDonald (1985) used only lipid mixing and turbidity to monitor fusion of LUV. The author argued that if lipid mixing were due only to lipid monomers diffusing through the aqueous phase, no more than 50% of lipid should exchange, since only the lipid in the outer monolayer should have been exchangeable on the time scale of fusion experiments. However, under the dehydrating conditions and osmotic stress of PEG, vesicles might deform so as to enhance lipid flip-flop and allow more probe to exchange. In addition, MacDonald recognized the ambiguities associated with turbity measurements, but maintained that the results reflected fusion since samples were diluted sufficiently to reverse aggregation. However, on the basis of MacDonald's reported protocol, a sample incubated in 40 wt % PEG would still be suspended in 4 wt % PEG after dilution. This concentration of PEG is sufficient to aggregate vesicles (Yamazaki et al., 1989). Therefore, the observed increase in turbity could simply have been due to aggregation. Finally, MacDonald reported that electron microscopy results supported the conclusion that fusion occurred. However, MacDonald presented only micrographs for sonicated vesicles, which we believe behave differently in PEG than do LUV (Lentz et al., 1991).

As a control, we present the data in Figure 5 to show that the lipid-mixing and contents-mixing assays, when used in conjunction and with appropriate controls, are capable of revealing fusion events. Mixing of trapped contents, and therefore fusion, was observed at and above 35 wt % PEG for vesicles composed of both the "anomalous" DOPC and DOPC plus a small amount of lysoPC. Vesicle size determinations by quasielastic light scattering for the DOPC/lysoPC system confirmed an increase in mean vesicle diameter from 1400 to 1850 Å that paralleled the mixing of trapped vesicle contents (Figure 7B). The capacity for certain amphipathic molecules to induce fusion in otherwise fusion-resistant systems has been documented in more detail elsewhere (McIntyre et al., 1991).

Effect of Unsaturation on Membrane Fusion. Recent studies by Roos and co-workers on PEG-fusion-resistant cell lines determined that fusion resistance was associated with an increase in the proportion of lipids containing saturated fatty acid chains (Roos & Choppin, 1985a,b; Roos, 1988). Other systems have been described that exhibit some dependence on unsaturation. For example, Helmkamp (1983) described several phospholipid-transfer proteins whose activities were directly related to the amount of unsaturated phospholipid present in the system. While Roos and co-workers indicated that fusion resistance did not correlate with the membrane acyl-chain order parameter, the results described by Helmkamp appeared to depend on membrane order. Our assay results showed that vesicles containing pure phosphatidylcholines, and no lysoPC, did not fuse in the presence of PEG, regardless of their acyl-chain composition (see Figures 3 and 4). The unsaturated system did, however, display a greater extent of lipid mixing in the presence of PEG. This indicates that neither the degree of unsaturation nor the membrane order alone are sufficient to allow fusion of vesicles aggregated by PEG. It does not rule out the possibility of the rate of fusion of an already fusogenic multicomponent system, such as a cell, being sensitive to the degree of unsaturation. To examine this possibility, kinetic experiments will be required so that rates of fusion can be compared for different lipid systems.

Bilayer Dehydration, Lipid Transfer, and Fusion. Our aim has been to obtain information about the mechanism of PEG-induced fusion and, hopefully, of membrane fusion in general. Our results have resolved some questions and established a baseline of information regarding the ability of PEG to induce fusion of pure phosphatidylcholine vesicles. At the same time, they have generated many new questions concerning the mechanism of lipid transfer and fusion between vesicles that have been aggregated and dehydrated by the presence of PEG. Significant amounts of lipid mixing occurred at all concentrations of PEG examined. Whether exchange occurred through the reduced aqueous layer at membrane contact points or through the bulk phase of the aqueous medium is not known. Jones and Thompson (1989) studied the transfer of lipids between vesicles and found that, in addition to the normal slow mechanism of transfer through the bulk aqueous phase, there was a component of lipid transfer that depended on the collision of vesicles. Their interpretation was that lipids transferred more rapidly through the reduced aqueous layer between colliding vesicles. By aggregating the vesicles with a polymer, we have effectively increased the number of contacts between vesicles in solution and, therefore, should have increased the rate of lipid mixing between vesicles. However, if this mechanism is correct, it is difficult to explain why lipid mixing ceased before 50% of the probe had exchanged. On the other hand, lipid mixing might not require aggregation but might occur at an enhanced rate through the bulk aqueous phase in the presence of PEG. The dielectric constant of the bulk aqueous phase is dramatically lowered by high concentrations of PEG (Arnold et al., 1985). In this scenario, exchange would occur between aggregates, with aggregation serving to limit the extent of lipid mixing, since the water on the interior of an aggregate would not have the same dielectric constant as the water exposed to the polymer. More work is needed to establish the mechanism of lipid transfer in the PEG-induced aggregated state.

In addition to high background lipid mixing observed at low PEG concentrations, the extent of lipid transfer increased above a critical PEG concentration. In both single component systems studied, lipid mixing increased significantly between 35 and 40 wt % PEG. There are several possible explanations for the increase in membrane mixing at this PEG concentration. First, coexistence of fluid and solid phases could destabilize the bilayer and enhance exchange. Although these high concentrations of PEG produced a slight increase in the

temperature of the order–disorder phase transition (Table I), our data indicate that DPPC membranes were still in a fluid phase at the temperature of our experiments (48 °C). Second, extreme dehydration might alter the dielectric properties of the water layer between vesicles such that lipid mixing between contacting vesicles would be more favorable. A third possibility is that the tremendous osmotic stress caused by high PEG concentrations might induce rupture of vesicles, leading to exposure of the vesicle interior to PEG. Leakage of vesicle contents became extensive at these PEG concentrations (see Figures 3 and 4), and polydispersity increased in light-scattering measurements (Figure 7), possibly indicating severe loss of membrane integrity. Above 40 wt % PEG, vesicle size increased dramatically. Since loss of vesicle contents was almost complete by this concentration of PEG, this may indicate vesicle rupture. We are currently studying the mechanism of contents leakage at high concentrations of PEG to determine if rupture occurs. These studies as well as studies of the fast kinetics of lipid mixing should help frame the mechanism by which somewhat lower concentrations of PEG induce vesicle fusion.

Finally, the much different effects that we have observed at high and low PEG concentrations (see Figures 3, 4, and 5) suggest that there may be different mechanisms of PEG action on a bilayer, depending on the extent of dehydration induced by PEG. Our current hypothesis is that an initial mild dehydration serves to aggregate the vesicles and reduce the aqueous space between bilayers. During this phase of dehydration, the bilayer is progressively destabilized but remains intact, and lipids exchange to a constant extent. At high concentrations of polymer, vesicles come into "critical contact". Lipid mixing is enhanced through this close association, perhaps through membrane rupture or, if the appropriate bilayer perturbant is present, through membrane fusion. This indicates that intimate membrane contact through bilayer dehydration alone is not sufficient to induce the fusion process. We are currently trying to define the extent of hydration and the interbilayer distance critical to the occurrence of high-PEG effects, including fusion.

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Phosphatidylethanolamine Enhances the Concentration-Dependent Exchange of Phospholipids between Bilayers†

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ABSTRACT: It has previously been demonstrated that lipid exchange between phosphatidylcholine vesicles, at higher concentrations, is characterized by a second-order concentration-dependent exchange process in addition to the first-order process operative at lower concentrations (Jones, J. D., & Thompson, T. E. (1989) *Biochemistry 28*, 129-134). Furthermore, it was demonstrated that the second-order process occurs as a result of an enhancement of the first-order desorption process, possibly resulting from attractive interactions between a potentially desorbing lipid molecule and a transiently apposed bilayer (Jones, J. D., & Thompson, T. E. (1990) *Biochemistry 29*, 1593-1600). In this work we have studied the exchange of [^{3}H]dimyristoylphosphatidylcholine (DMPC) between large vesicles of the compositions 100% DMPC, 70/30 (mol/mol) DMPC/dimyristoylphosphatidylethanolamine (DMPE), and 68.25/30/1.75 (mol/mol/mol) DMPC/DMPE/dimyristoylphosphatidylglycerol (DMPG). The second-order exchange process is enhanced by 100-fold or more in vesicles containing 30 mol % DMPE relative to 100% DMPC and is reduced or eliminated by the addition of 1.75% of the anionic lipid DMPG. These effects can be achieved by alterations in the equilibrium bilayer separation of 5 Å or less. The results are in accord with the model of Jones and Thompson and indicate that relatively low concentrations of PE in a PC bilayer can have significant effects on bilayer surface properties and on potential interactions between bilayers.

It is well established that lipid molecules exchange between bilayers via a spontaneous first-order process that involves desorption of lipid molecules from the donor bilayer surface followed by rapid diffusion through the aqueous phase to an acceptor bilayer (Martin & MacDonald, 1976; Roseman & Thompson, 1980; Doody et al., 1980; Nichols & Pagano, 1981, 1982, Massey et al., 1982; De Cuyper et al., 1983; Arvinte & Hildenbrand, 1984). Recently, however, an additional second-order concentration-dependent process was observed at lipid concentrations above about 2 mM (Jones & Thompson, 1989). This second-order process was found to be an enhancement of the first-order process, probably resulting from attractive interactions between transiently apposed bilayers and some fraction of the potentially desorbing lipid molecules (Jones & Thompson, 1990).

This model suggests that factors that influence vesicle–vesicle interactions, such as surface hydration or charge, will have significant effects on the efficiency of the concentration-dependent exchange process. For instance, phosphatidylethanolamine-containing (PE-containing) bilayers tend to be less well hydrated than phosphatidylcholine (PC) and as a result have a smaller equilibrium bilayer separation and a deeper interaction energy "well" (McIntosh & Simon, 1986; Rand & Parsegian, 1989). As a result, one would expect PE-containing bilayers to have a more efficient concentration-dependent exchange compared to PC bilayers. In the work presented here this idea was tested by studying the concentration-dependent exchange process in vesicles of pure dimyristoylphosphatidylcholine (DMPC),[1] of 70/30 (mol/mol) DMPC/dimyristoylphosphatidylethanolamine (DMPE) and of 68.25/30/1.75 (mol/mol/mol) DMPC/DMPE/dimyristoylphosphatidylglycerol (DMPG). The results indicate that the presence of 30 mol % PE in a liquid-crystalline PC bilayer has large effects on the concentration-dependent exchange of lipids between bilayers, probably through its effects on bilayer surface properties and the potential interactions between bilayers. A small amount of charged lipid in the bilayers reduces or eliminates the effects of PE.

Experimental Procedures

Vesicle Preparation. All lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Purity was periodically confirmed by thin-layer chromatography. [^{3}H]DMPC (66 Ci/mol) was prepared by the method of Jones and Thompson (1989), and [^{14}C]cholesteryl oleate (56.6 Ci/mol) was obtained from New England Nuclear (Boston, MA).

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; MLV, multilamellar vesicles; LUV, large (100-nm diameter) unilamellar vesicles prepared by extrusion; OLV, oligolamellar (350-700-nm diameter) vesicles; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

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