Bilayer Curvature and Certain Amphipaths Promote Poly(ethylene glycol)-Induced Fusion of Dipalmitoylphosphatidylcholine Unilamellar Vesicles†

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ABSTRACT: Unilamellar vesicles of varying and reasonably uniform size were prepared from 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) by the extrusion procedure and sonication. Quasi-elastic light scattering was used to show that different vesicle preparations had mean (*Z*-averaged) diameters of 1340, 900, 770, 630, and 358 Å (sonicated). Bilayer-phase behavior as detected by differential scanning calorimetry was consistent with the existence of essentially uniform vesicle populations of different sizes. The response of these different vesicles to treatment with poly(ethylene glycol) (PEG) was monitored using fluorescence assays for lipid transfer, contents leakage, and contents mixing, as well as quasi-elastic light scattering. No fusion, as judged by vesicle contents mixing and change in vesicle size, was detected for vesicles of diameter greater than 770 Å. The diameters of smaller vesicles increased dramatically when treated with high concentrations of PEG, although mixing of their contents could not be detected both because of their small trapped volumes and because of the extensive leakage induced in small vesicles by high concentrations of PEG. Lipid transfer was detected between vesicles of all sizes. We conclude the high bilayer curvature does encourage fusion of closely juxtaposed membrane bilayers but that highly curved vesicles appear also to rupture and form larger structures when diluted from high PEG concentration, a process that can be confused with fusion. Despite the failure of PEG to induce fusion of large, uncurved vesicles composed of a single phosphatidylcholine, these vesicles can be induced to fuse when they contain small amounts of certain amphiphathic compounds thought to play a role in cellular fusion processes. Thus, vesicles which contained 0.5 mol % L-α-lysopalmitoylphosphatidylcholine, 5 mol % platelet activating factor, or 0.5 mol % palmitic acid fused in the presence of 30%, 25%, and 20% (w/w) PEG, respectively. However, vesicles containing 1,2-dipalmitoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, 1-oleoyl-2-acetyl-*sn*-glycerol, or monooleoyl-*rac*-glycerol at surface concentrations up to 5 mol % did not fuse in the presence or absence of PEG. There was no correlation between the abilities of these amphipaths to induce phase separation or nonlamellar phases and their abilities to support fusion of pure DPPC unilamellar vesicles in the presence of high concentrations of PEG. The results are discussed in terms of the type of disrupted lipid packing that could be expected to favor PEG-mediated fusion.

Poly(ethylene glycol) (PEG)[1] is used widely to mediate cell–cell fusion in the production of somatic cell hybrids including hybridomas (Davidson & Gerald, 1977) and in the fusion injection of macromolecules into cultured cells from erythrocytes (Davidson & Gerald, 1977) or liposomes (Szoka et al., 1981). However, little is known about the mechanism by which PEG induces fusion of cell membranes. In order to understand this mechanism better and perhaps to illuminate the molecular mechanism of in vivo cell fusion processes, several laboratories have examined the effects of PEG on synthetic membrane vesicles. Aggregation of vesicular membranes is widely accepted to be necessary for fusion (Nir et al., 1983; Wilschut & Hoekstra, 1984). However, this may not be sufficient to cause mixing both of bilayer components and of aqueous internal contents, both of which must occur during a fusion event. Our laboratory (Burgess et al., 1991a) has observed that PEG does not induce fusion of large, unilamellar, extruded vesicles composed of a single synthetic phosphatidylcholine species but that it does cause close contact (Burgess et al., 1992) and rapid lipid transfer between these vesicles (Wu & Lentz, 1991; Burgess et al., 1991b). Since PEG serves only to induce contact between vesicles and does not destabilize the lipid bilayer sufficiently to induce fusion, other factors must be involved in PEG–induced cellular fusion.

It has long been accepted that small, unilamellar vesicles (SUV)[1] experience molecular packing defects in their bilayers resulting from their high curvature (Lawaczeck et al., 1976; Chrzeszczyk et al., 1977). The packing inhomogeneities associated with high curvature are believed to account for the unique physical properties of SUV (Sheetz & Chan, 1972; Thompson et al., 1974; Suurkuusk et al., 1976; Lentz et al., 1987). It has been speculated that regions of high curvature may form in cell membranes and impart to a local domain special functional properties, such as the ability to fuse with other membranes (Suurkuusk et al., 1976; Wilschut et al.,

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[1] Abbreviations: PEG, poly(ethylene glycol); LUVET, large unilamellar vesicle(s) made by the rapid extrusion technique; MUVET, medium unilamellar vesicle(s) (900 Å) made by the rapid extrusion technique; MUVET2, medium unilamelar vesicle(s) (770 Å) made by the rapid extrusion technique; SUVET, small unilamellar vesicle(s) made by the rapid extrusion technique; SUV, small unilamellar vesicle(s); DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; DPX, *N*,*N*′-*p*-xylylenebis(pyridinium bromide); QELS, quasi-elastic light scattering; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; $C_{12}E_8$, dodecyl octaethylene glycol monoether; DOG, 1,2-dioleoyl-*sn*-glycerol; LPC, L-α-lysopalmitoylphosphatidylcholine; DPG, 1,2-dipalmitoyl-*sn*-glycerol; MOG, 1-monooleoyl-*rac*-glycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PAF, platelet activating factor; DPH, diphenylhexatriene.

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1981; Lentz et al., 1987) or to provide a special environment for protein function (Wilschut et al., 1978). In this report, we examine the abilities of vesicles of different curvature to fuse when treated with high concentrations of PEG. We find that increased bilayer curvature enhances the ability of PEG to induce vesicle fusion, although, for very highly curved membranes, it is uncertain whether fusion, as opposed to membrane rupture and re-formation, occurs.

Biological membranes also contain a variety of amphipathic compounds, any of which could be important in promoting fusion events within the cell. Ahkong et al. (1973) reported a number of compounds which, when added as a suspension to hen erythrocytes, induced fusion of these cells. Compounds in this list included lysophosphatidylcholine, various diacylglycerols and fatty acids, and 1-monooleoyl-*rac*-glycerol. However, no one has reported screening these compounds in model membrane systems in order to determine if they might play a direct structural role in the fusion process. We report here that small quantities of certain amphipathic compounds favored PEG-induced fusion of synthetic model membranes.

Finally, our results are examined in terms of the type of disrupted lipid packing that could be expected to favor PEG-mediated fusion.

Experimental Procedures

Materials

Chloroform stock solutions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC)[1] and 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC)[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). DPPC stocks were filtered over Norit A neutral activated charcoal in order to remove trace fluorescent contaminants. All lipids were found to be greater than 98% pure by thin-layer chromatography on Analtech (Newark, DE) GHL silica gel plates poured in the presence of 0.01 M dipotassium oxalate. Plates were developed in a 65:25:4 (v/v/v) $CHCL_3/CH_3OH/H_2O$ mixture, and lipids were visualized with iodine vapor. 1-Monooleoyl-*rac*-glycerol (MOG),[1] 1,2-dipalmitoyl-*sn*-glycerol (DPG),[1] 1,2-dioleoyl-*sn*-glycerol (DOG),[1] 1-oleoyl-2-acetyl-*sn*-glycerol (OAG),[1] and L-α-lysopalmitoylphosphatidylcholine (LPC)[1] were purchased from Sigma Chemical Co. (St. Louis, MO). Palmitic acid (PA)[1] was purchased from Nu Chek Prep, Inc. (Elysian, MN). Platelet activating factor (C_{16} species) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] was purchased from Calbiochem (LaJolla, CA). The disodium salt of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)[1] and *N*-*N*'-*p*-xylylenebis(pyridinium bromide) (DPX)[1] were purchased from Molecular Probes (Eugene, OR). Carbowax PEG 8000 (molecular weight 7000–9000) was purchased from Fisher Scientific (Fairlawn, NJ). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)[1] was from Research Organics, Inc. (Cleveland, OH). All other reagents were of the highest quality available.

Methods

Vesicle Preparation. Extruded vesicles were prepared by the procedure of Mayer et al. (1986) with some modifications that we found necessary in order to obtain samples suitable for quasi-elastic light scattering (QELS).[1] Lipid components were mixed in chloroform solution, and the solvent was removed under a stream of nitrogen or argon. The lipid residue was redissolved in cyclohexane and frozen in a dry ice/acetone bath, and the solvent was removed under high vacuum to yield a white powder. The dried lipid was hydrated in the appropriate prewarmed buffer at 45 °C for at least 30 min with intermittent agitation to a final concentration of 5 mM. The hydrated sample was then added to a stainless-steel extrusion apparatus (constructed in house) maintained at 45–48 °C in an incubator oven. Samples were extruded through polycarbonate filters and drain disks (Nucleopore Corp., Pleasanton, CA) as follows: LUVET,[1] seven extrusions through one 0.1-μm polycarbonate filter (lot 83AV3D32, reported pore size of 900 Å) and a drain disk at argon pressures between 50 and 100 psi; MUVET,[1] seven extrusions through one 0.05-μm polycarbonate filter (lot 86A9E24, reported pore size of 490 Å) and a drain disk at argon pressures between 100 and 200 psi; MUVET2,[1] five extrusions through two stacked 0.05-μm polycarbonate filters and one drain disk at argon pressures between 300 and 500 psi—filters and drain disk were replaced, and five more extrusions were performed with the last one being into a clean vial to avoid even minute contamination with large multilamellar vesicles; SUVET,[1] five extrusions through two 0.03-μm polycarbonate filters (lot 86B5B17, reported pore size of 290 Å) and one drain disk at argon pressures between 300 and 500 psi—filters and drain disk were replaced, and five more extrusions were performed with the last one being into a clean vial.

Small unilamellar vesicles (SUV)[1] were prepared as described previously by Lentz et al. (1987), with care being taken not to allow the temperature of the vesicles to drop below the gel–liquid-crystalline-phase transition temperature. Pipets and vessels coming into contact with the vesicles were warmed to 50 °C. Vesicles were fractionated by centrifugation for 120 min at or above 45 °C in a Beckmann L5-50E ultracentrifuge using a Ti-75 rotor at 49 000 rpm and were used within 1 h of centrifugation.

For lipid mixing experiments, vesicles were prepared in 100 mM NaCl, 2 mM TES, and 1 mM EDTA,[1] 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. Vesicles eluting from the G-75 gel filtration column were detected by passing the beam from a Spectra Physics Stabilite Model 120 helium–neon laser through the drops as they emerged from the column. An increase in scattered light intensity indicated the location of vesicles in the elution profile. Osmolarities of all buffers were monitored using a μ Osmette microosmometer (Precision Systems, Sudbury, MA). The concentrations of all vesicle samples were determined by phosphate analysis using a modification of the procedure of Chen et al. (1956).

PEG Purification. Carbowax PEG 8000 was purified by a modified combination of the procedures described by Ray and Puvathingal (1985), Washabaugh and Collins (1983), and Honda et al. (1981). PEG was dissolved in doubly distilled water to make a 40% (w/w) solution, and $NaBH_4$ was added to a final concentration of 10 mM in order to reduce aldehydes and peroxides. The PEG solution was allowed to stir at room temperature overnight. The solution was then added to Chelex 100 resin which had been washed with 30% (v/v) methanol followed by doubly distilled water. This step rids Chelex of styrene monomers which catalyze the formation of peroxides. The resin was removed by filtration, and the treated PEG solution was dried using rotary evaporation and then lyophilized. The PEG was then dissolved in the minimum amount

of HPLC-grade $CHCl_3$ and recrystallized from an 8-fold excess of peroxide-free diethyl ether in order to remove organic contaminants such as the antioxidants commonly added to commercial grade PEG (Ray & Puvathingal, 1985). The PEG crystals were collected by filtration and lyophilized. Aldehyde/formaldehyde assays were performed as described by Avigad (1983) to show that these were reduced to 4 nmol/1 nmol per gram of PEG. A peroxide assay was performed as described by Worthington (1972) to show that these were present at less than 2 nmol/g of PEG. The presence of antioxidants in unpurified PEG was detected by the absorbance at 288 nm (Ray & Puvathingal, 1985), which demonstrated that greater than 75% of these were removed, leaving less than 30 nmol/g of PEG.

Lipid Transfer Assay. All fluorescence measurements were made on an SLM 48000 spectrofluorometer (SLM-Aminco, Urbana, IL) equipped with a home-built, three-position, multitemperature cuvette holder (Barrow & Lentz, 1985) and a horizontally mounted, focused 200-W mercury–xenon lamp or 150-W xenon lamp (Photon Technology International, Monmouth Junction, NJ). The temperature of two sample positions was kept at 48 °C for the lipid transfer assay. A detailed description of the DPHpPC fluorescence lifetime lipid mixing assay (Parente & Lentz, 1986a) is given elsewhere (Burgess & Lentz, 1992). In essence, vesicles containing a high surface concentration of DPHpPC were mixed with vesicles free of probe, and the transfer of probe between vesicle populations was monitored through the resulting increase in probe fluorescence lifetime. The data are presented as the observed change in probe surface concentration at a given PEG concentration divided by the change in probe surface concentration expected for complete lipid mixing between all bilayer compartments, times 100. With this definition of the units of lipid transfer, 11% transfer is the value expected for one "ideal" (i.e., uniform) round of fusion (each probe vesicle fusing with a probe-free vesicle). Procedures used here were the same as those described by Burgess and Lentz except that probe-rich vesicles in most of our assays contained lipid and probe in the ratio 25:1 instead of the ratio 10:1 used elsewhere (Burgess et al., 1991a,b). The lower probe concentration had the advantage that there were fewer potential complications due to possible perturbation of the bilayer structure by the probe. However, the lower probe concentration had the disadvantage of producing an assay for which sensitivity was limited at high percentages of lipid transfer (i.e., 45–50% transfer could not be distinguished from 100% due to the inherent error in the lifetime measurements). The assay performed with 10:1 vesicles had a range of 75–80% transfer. In the range of applicability, lipid transfer was independent of lipid:probe ratio between 10:1 and 25:1. The inherent uncertainty in measured fluorescence lifetimes (±0.05 ns) produced standard errors in the percent transfer that increased from ±0.3% at 0% transfer to +10/−6% at the upper limits of the assay.

For lifetime measurements required for the DPHpPC lipid mixing assay, the 366 nm mercury line was used to excite DPHpPC, and emission was monitored through a 3-mm high-pass KV-450 filter (50% transmittance at 450 nm; Schott Optical Glass, Duryea, PA). A Soleil–Babinet compensator (Karl Lambrecht Corp., Chicago, IL) was used in the excitation path and set for half-wave at 366 nm and 17.5° from vertical, which rotated the modulated, linearly polarized light by 35°. Phase-angle shifts were recorded at 30-MHz modulation frequency as described by Barrow and Lentz (1983) using a reference standard of DPH in heptane measured at 23 °C in the third cuvette position (τ = 6.78 ns; 2×10^{-7} M).

ANTS/DPX Leakage and Contents Mixing. These assays were performed as described by Ellens et al. (1984) and by Parente and Lentz (1986b) except that a new control was included as described below. For the leakage assay, ANTS and DPX were coencapsulated in one vesicle population (0.5 mM final lipid concentration) such that DPX quenched over 85% of ANTS fluorescence. When leakage occurred, there was an increase in fluorescence due to dilution of ANTS and DPX in solution and loss of quenching efficiency. For the contents mixing assay, equal volumes of 0.25 mM ANTS-containing vesicles and 0.25 mM DPX-containing vesicles were used. For both contents mixing and contents leakage experiments, vesicles were incubated for 2–3 min (curvature studies) or 20 min (amphipath studies) with PEG at 48 °C in a 0.4-mL volume. The shorter incubation time was found to be adequate, since attempts at kinetic studies demonstrated that contents mixing and leakage were essentially complete in under 2 min; the longer incubation time used for the amphipath studies was for convenience only. After incubation, vesicles were diluted to a 3-mL volume, and the fluorescence intensity of ANTS was monitored. The photophysics of ANTS was sensitive to the polarity of its environment, its fluorescence being enhanced somewhat by PEG and apparently also by a seemingly weak interaction with a membrane surface. In the new control, 0.25 mM ANTS vesicles were incubated alone in PEG and otherwise treated like the experimental samples. This control corrected for the fact that ANTS was irreversibly bleached when encapsulated in membrane vesicles treated with PEG, which is also suggestive of a weak ANTS–membrane interaction.

For *leakage experiments*, the value used to represent 0% leakage was the intensity of the ANTS/DPX coencapsulated vesicles plus the small intensity contributed by PEG. The value taken to represent 100% leakage was determined by lysing vesicles with the detergent $C_{12}E_8$. The method used to detect and quantify *mixing of vesicle contents* in the presence of PEG was similar in concept. The fluorescence intensity indicative of 0% contents mixing was the intensity observed for the ANTS vesicles alone in PEG (control). The fluorescence intensity indicative of 100% contents mixing was the intensity of ANTS/DPX coencapsulated vesicles along with the small contribution by PEG. The fluorescence intensities from coencapsulated ANTS/DPX and from mixtures of ANTS plus DPX vesicles were corrected for pipetting errors by normalizing the fluorescence for vesicles lysed by $C_{12}E_8$ in the presence of PEG to the fluorescence obtained with lysed ANTS control vesicles. The difference in normalized fluorescence intensities, before detergent addition, between control and mixed vesicle samples was divided by the difference between control and coencapsulated vesicles to obtain the extent of contents mixing due to fusion. Because leakage of contents affects both the numerator and the denominator of this fraction in the same way, this formulation inherently accounts for the effects of contents leakage on the measurement of contents mixing. This value was divided by the probability that two fusing vesicles would contain ANTS and DPX, respectively (0.5 for 1:1 mixtures of vesicles), and then multiplied by 100 in order to obtain the percentage of contents mixed. This represents the percentage of the fluorescence change expected for one "ideal round of fusion" (i.e., every ANTS vesicle fusing with a single DPX vesicle). It should be noted that an actual experiment may involve a number of fusion events other than pairwise fusion of single ANTS vesicles with single DPX vesicles. Since one "ideal round of

fusion" would result in the largest fluorescence change possible for an experiment, it is not surprising that a value of 100% is never observed and that our measure of contents mixing due to fusion (i.e., percent of one ideal round) represents a lower limit to the number of fusion events that may actually occur. All contents mixing assays were performed in duplicate or triplicate, with results being reproducible to within 1–2% of one round of fusion.

ANTS was excited at a wavelength of 384 nm, and emission was observed through a 2-mm OG-515 filter (50% transmittance at 515 nm; Schott Optical Glass) or through an emission monochromator for spectral measurements.

Quasi-Elastic Light Scattering (QELS).[1] After treatment with PEG, vesicles for light-scattering measurements were separated from the polymer by elution from a Pharmacia Sepharose CL 4B (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) gel filtration column maintained at 48 °C in an incubator oven using a buffer consisting of 100 mM NaCl, 2 mM TES, and 1 mM EDTA, pH 7.4, according to the procedure described by Burgess et al. (1991a).

Light-scattering measurements were made on a multiangle instrument constructed by us. The light source of this instrument was a Spectra-Physics Stabilite Model 120 S helium–neon laser (632.8 nm) with 15-mW maximum power. Two pinhole apertures and one lens focused the light beam onto the center of the cylindrical sample cell. The sample cell was a 6 × 50 mm borosilicate glass disposable culture tube (Baxter Healthcare Corp., McGaw Park, IL) that was cleaned before use by forcing a stream of argon into it for several seconds. The sample cell was held in place by an annodized aluminum cell holder which was hollow so that water could be circulated to maintain a wide variety of constant temperatures. The cell holder was located at the axis of a glass cylinder (0.25-in. walls; Wilmad Glass, Inc., Buena, NJ) which contained silicone oil (Fisher Scientific, Fair Lawn, NJ). The silicone oil provided both a thermal conductor to minimize thermal gradients in the sample cell and a means of preventing incident light from interacting with defects on the surface of the cell. This minimized the formation of local oscillators which produce anomalous scattering results. A Masterflex Model 7553-60 peristaltic pump (Barnant Co., Cole Parmer Instrument Co., Chicago, IL) was used to pass the oil through a 22-μm Millipore filter (catalogue no. GSWP 047 00; Millipore Corp., Bedford, MA) to reduce dust contamination. The scattered light was focused by two pinhole apertures and a glass lens through an adjustable slit onto a Model RF I/S photomultiplier tube (EMI-Gencom, Inc., Plainview, NY) powered by a Model 124 digital photometer (Pacific Instruments, Inc., Concord, CA) high-voltage source. The signal transmitted from the photomultiplier tube was analyzed by a Model 170 computing autocorrelator (Particle Sizing Systems, Inc., Santa Barbara, CA). The data collection was controlled and displayed by software located in an external personal computer. The software supplied by Particle Sizing Systems permitted both a cummulants and a distributional analysis.

Our instrument was tested for multiangle measurements from 30° to 130° by measuring the intensity change of filtered benzene (lot 794140; Fisher Scientific). The intensity deviated only 4% over the entire angle range. In addition, polystyrene beads (lot 90817; Polyscience, Inc.; nominal diameter 1200 Å from electron microscopy measurements) prepared in the presence of 0.06% Triton X-100 (lot 95c-0058; Sigma Chemical Co.) were determined to be 1229 ± 40 Å in diameter. The instrumental precision of measurements performed on a single SUV sample (whether this was kept in the sample cuvette or removed and replaced several times) was ±14 Å. Furthermore, the diameters we obtained for phosphatidylcholine LUVET were identical to those published in earlier reports (Mayer et al., 1986; Hope et al., 1985). All of the measurements performed on this instrument were obtained at an angle of 90°, and only the cumulants analysis was used. The results of the analyses reported here are all intensity-weighted diameters which are calculated from the *Z*-average diffusion coefficients. Controls were done to show that the measured size of the vesicles was not concentration-dependent over the range 0.1–18 mM.

Table I: Summary of Size Distributions and Phase Behavior of Different Vesicle Types

vesicle type	diameter[a] (Å)	distribution[b]	calorimetric behavior: peak positions[c] (°C)	calorimetric behavior: peak widths[d] (°C)
LUVET	1340 (400)	949 (3) 1397 (97)	≃41.1 (98) 41.37 (2)	1.42
MUVET	900 (130)	646 (0.2) 949 (98) 1390 (2)	≃40.7 (92) 40.87 (8)	2.04
MUVET2	767 (110)	646 (44) 949 (56)	40.77 (98) ≃41.3 (2)	2.30
SUVET	630 (130)	440 (17) 646 (82)	40.22 (97) ≃41.5 (3)	2.44
SUV	358 (120)	147 (1) 215 (11) 316 (35) 464 (37) 681 (12) 1000 (2)	38.37 (85) 41.53 (15)	2.84

[a] Mean diameter (distribution width) defined by a cumulant analysis assuming a Gaussian distribution of vesicle sizes. Vesicle diameters of different preparations were reproducible to within ±1.2%. [b] Vesicle size distribution (intensity percentage) defined by the algorithm used by Coulter Instruments (see Methods). [c] Both major and minor endotherms are indicated (enthalpy percentages in parentheses), along with the fractions of total enthalpy (determined by cutting and weighing) associated with both. Uncertainties in resolvable peak positions are ±0.5 °C; the position of less clearly defined peaks is indicated by (≃). [d] Peak width calculated at half-height.

Some measurements were also carried out at 90° on a Coulter Counter Model N4SD (Coulter Electronics, Inc., Hialeah, FL) light-scattering instrument at 48 °C.

Treatment of Vesicles with PEG. PEG was hydrated in the same buffer as the vesicles and was added to the vesicles in varying amounts. The mixture (0.25 mM lipid) was then incubated at 48 °C for varying amounts of time depending on the assay to be performed on the resulting vesicles (2 or 20 min for contents mixing and leakage assays, 30 min for lipid mixing assays, and 30 min for QELS measurements). For contents mixing and leakage assays, the sample was diluted with 7.5 volumes of buffer (100 mM NaCl, 2 mM TES, and 1 mM EDTA, pH 7.4) and placed into a cuvette for fluorescence measurements. For lipid transfer measurements, the vesicle sample was mixed with PEG in the fluorescence cuvette and examined directly without dilution. For QELS measurements, the samples were diluted and separated from PEG as described above.

Results

Characterization of Different Sized Vesicles. The sizes of vesicles prepared by the several procedures described under Methods were determined by QELS. Table I summarizes the intensity-weighted, hydrodynamic diameters of different vesicle preparation. The table also contains information about the distribution of vesicle sizes present in each preparation, either

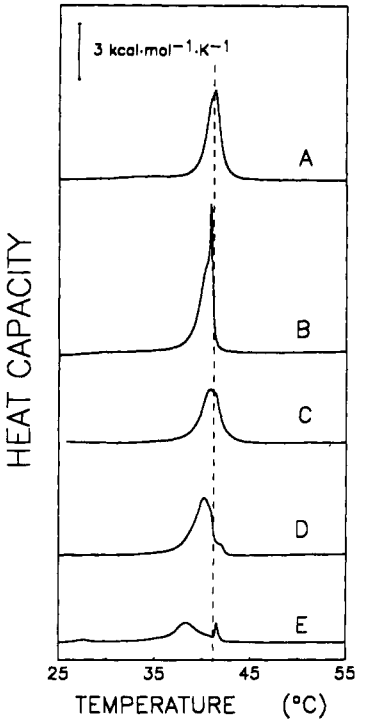

FIGURE 1: Phase behavior of vesicles of different sizes. Methods of vesicle preparation as well as size distribution of vesicles are summarized in Table I. Sample volumes were all 1.2 mL. (A) Large unilamellar extruded vesicles at 1.9 mM; (B) medium unilamellar extruded vesicles of type 1 at 1.7 mM; (C) medium unilamellar extruded vesicles of type 2 at 3.4 mM; (D) small unilamellar extruded vesicles at 3.0 mM; (E) small unilamellar (sonicated) vesicles at 4.6 mM. The dashed line marks 41.2 °C, the phase transition peak temperature of DPPC large multilamellar vesicles (Parente & Lentz, 1984).

in terms of the results of a generalized distributional analysis (Provencher, 1979) or in terms of the width of an assumed unimodal, Gaussian distribution. The two methods of analysis agreed, although the former had the potential advantage of being able to identify samples with considerable size heterogeneity. It was by using this method of analysis that we determined the procedures for preparing reasonably well-defined populations of extrusion vesicles. Note that the diameters in Table I are intensity-weighted or *Z*-average diameters which are expected to be larger than the *N*-average values obtained by electron microscopy or analytical ultracentrifugation. This tendency of light scattering to exaggerate the contribution of larger vesicles in a population is even greater for SUV preparations, as the very small vesicles resulting from sonication scatter much less light than an equal mass of lipid incorporated into LUV.

In order to further characterize our vesicle preparations, we monitored their gel-to-fluid-phase transitions by DSC. It has been shown previously that highly curved vesicles have a greatly broadened phase transition that is shifted to much lower temperature than that of larger vesicles (Suurkuusk et al., 1976; Lichtenberg et al., 1981; Lentz et al., 1987). However, a systematic study of vesicle phase behavior as a function of size has never been reported. Figure 1 reports heat capacity profiles for the five vesicle preparations summarized in Table I. These data were recorded in heating scans. For all samples but the SUV, the prior cooling scans, as well as subsequent heating and cooling scans, were essentially identical to the scans shown. The well-characterized hysteretic phase behavior of DPPC SUV is known to be due to fusion of these vesicles below the phase transition to form larger vesicles (Suurkuusk et al., 1976; Wong et al., 1982). These fusion

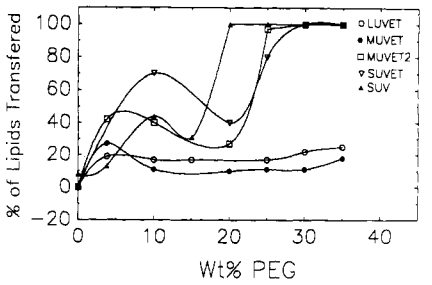

FIGURE 2: Transfer of vesicle probe lipid as a function of the concentration of added PEG. Lipid mixing was monitored at 48 °C using the sensitivity of DPHpPC fluorescence lifetime to the surface concentration of the fluorescent probe (see Methods). Data are shown for five types of DPPC unilamellar vesicles, in order of decreasing radius: LUVET (open circles); MUVET (closed circles); MUVET2 (open squares); SUVET (inverted open triangles); SUV (closed triangles). Data for LUVET and MUVET were obtained with probe vesicles containing unlabeled lipid and probe in the ratio 25:1, while the large extent of transfer observed for the more highly curved vesicles made it necessary to perform these experiments using 10:1 probe-rich vesicles. Data are presented as a percentage of the lipid:probe ratio expected to result from complete mixing of lipids (see Methods).

products show a phase transition approximating that of large, multilamellar vesicles (Suurkuusk et al., 1976; Wong et al., 1982; Parente & Lentz, 1984), the position of which is indicated by the dashed line in Figure 1. Apparently, extruded vesicles that are less severely curved than SUV remain stable below their gel-to-fluid transition.

It is evident from the endotherms shown in Figure 1 that all vesicle samples were slightly contaminated with larger species approximating the phase behavior of large multilamellar vesicles. This was apparently worse for MUVET and SUVET and barley detectable in MUVET2 (see the peak positions and percentages of total enthalpy associated with each peak in Table I). Since this contamination did not result from fusion below the phase transition, it must represent imperfections in the process of forming the extrusion vesicle populations. These results indicate that the vesicles we have prepared do contain some, but not much, contamination by larger vesicles.

Another feature evident in the endotherms of Figure 1 is the shift to lower temperature and broadening of the peak as vesicle size decreased (see also Table I). This behavior is consistent with the limiting behavior of SUV and supports the QELS data by indicating that we have succeeded in preparing populations of vesicles with progressively decreasing diameters. The changes in phase transition properties with decreasing vesicle diameter or increasing curvature (decreasing transition temperature, decreasing enthalpy, decreasing cooperativity, i.e., increasing width at half-height) are consistent with destabilization of the gel or ordered phase due to disrupted lipid packing in more highly curved bilayers.

Fusion of Different Sized Vesicles. In order to test for vesicle fusion, we monitored the transfer of the lipid probe DPHpPC between DPPC vesicle populations in the presence of increasing concentrations of PEG. This assay reports lipid transfer regardless of whether it results from PEG-induced, intervesicle lipid exchange (Burgess et al., 1991a; Wu & Lentz, 1991) or from lipid mixing due to fusion. The results (Figure 2) reveal that PEG induced only a minor and roughly constant degree of probe transfer between the largest vesicles studied, MUVET and LUVET up to 30 wt % PEG, with a very small increase at 35 wt % PEG. This is consistent with our previous studies (Burgess et al., 1991a) which showed that pure phosphatidylcholine vesicles did not fuse but did experience enhanced lipid exchange in the presence of PEG. Extensive

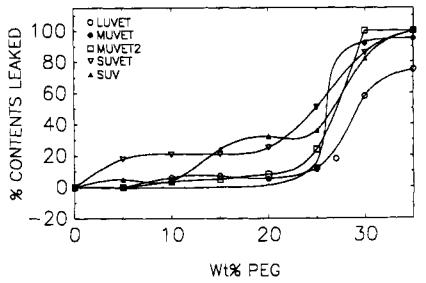

FIGURE 3: Leakage of vesicle contents as a function of the concentration of added PEG. Leakage was monitored at 48 °C by the increase in fluorescence associated with the release of ANTS coencapsulated with DPX, which serves to quench ANTS fluorescence (see Methods). Data are presented as a percentage of maximum leakage possible for five types of vesicles; symbols as in Figure 2.

probe transfer was observed, however, in all three of the smaller, more highly curved vesicle preparations. This lipid transfer was of two types: the first occurred at low PEG concentrations, with a maximum at 5–10 wt % PEG; the second occurred at high PEG concentrations following a minimum in transfer centered at roughly 15–20 wt % PEG. This pattern was independent of the probe:lipid ratio used in the probe-rich vesicle population of our lipid transfer assay. Since these highly curved vesicles remained the same size after treatment with low PEG concentrations (see below), this low-PEG lipid transfer must be related to other than the fusion process. Low-PEG-induced lipid transfer occurred most readily in SUV and SUVET, slightly less readily in MUVET2, perceptibly in MUVET, and barely perceptibly in the uncurved LUVET, indicating that this phenomenon was enhanced by bilayer curvature. We can speculate that the maximum in the extent of transfer might result from a playoff between an increase in the rate of transfer between aggregated vesicles and a decrease in the rate or extent of transfer due to the large viscosity of PEG solutions. We have found (Wu & Lentz, 1991; Burgess et al., 1991b) that PEG-induced vesicle aggregation does increase the rate of lipid transfer. Vesicle curvature might enhance the rate of lipid transfer between vesicles even further, thus overcoming to some extent the effect of increased viscosity. Direct measurements of the rate of lipid transfer as a function of vesicle curvature will be needed to test this hypothesis.

Lipid transfer also occurred at high PEG concentration, with a dramatic increase being observed between 20 and 25 wt % for MUVET2 and SUVET and between 15 and 20 wt % for the highly curved SUV.

In order to determine whether high-PEG-mediated transfer might reflect vesicle fusion, we performed assays designed to detect the mixing of trapped vesicle contents. The leakage of vesicle contents was monitored using vesicles containing a mixture of ANTS and its quencher DPX, as recorded in Figure 3. Except for SUV and SUVET, leakage was minimal up to 25 wt % PEG; however, all vesicles rapidly lost their contents between 25 and 30 wt % PEG. Even for SUV and SUVET, leakage was incomplete below 30 wt % PEG, offering hope that fusion could be demonstrated by contents mixing.

We attempted to measure the mixing of vesicle contents induced by PEG treatment using the ANTS/DPX assay, as described under Methods. However, contents mixing could not be detected in SUV, despite several attempts, and although it could be sporadically measured for SUVET, the extent of mixing was very small and not reproducibly measurable. Only for MUVET2 could contents mixing be reproducibly measured, as shown in Figure 4, demonstrating clearly that these vesicles fused. It is not surprising that the contents of MUVET

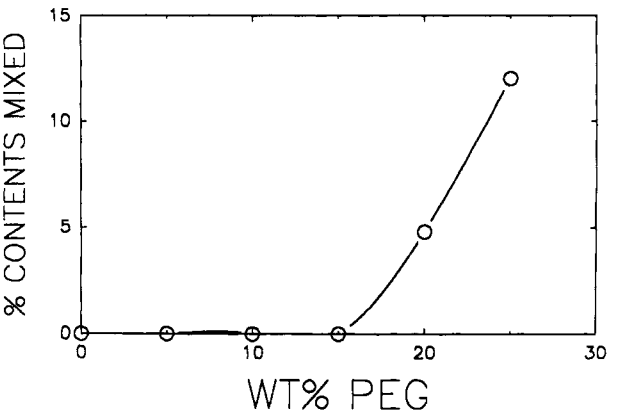

FIGURE 4: Mixing of vesicle contents as a function of PEG added to MUVET2. Contents mixing was monitored at 48 °C by following the drop in fluorescence intensity associated with mixing of the contents of two vesicle populations: one containing ANTS and one containing its quencher, DPX (see Methods). Data are presented as a percentage of fluorescence intensity expected for one ideal around of fusion (see Methods).

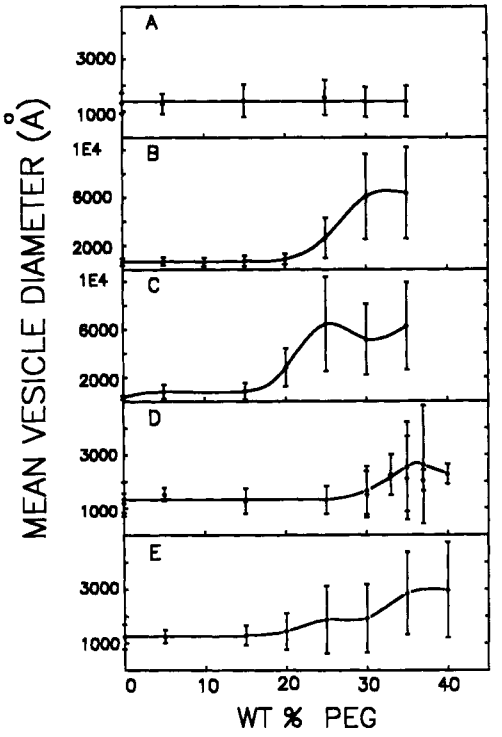

FIGURE 5: Increase in vesicle size as a function of increasing concentrations of added PEG. Data for vesicles of three sizes [(A) LUVET; (B) MUVET2; (C) SUV] are shown along with data for LUVET prepared from DPPC with either 0.5 mol % LPC (D) or 0.5 mol % PA (E). Vesicles (3–5 mM) were incubated at 48 °C with 0–40 wt % PEG, isolated as described under Methods, and sized using QELS. Vertical bars represent Gaussian distribution widths, not error bars. Measurements of the vesicle diameters were reproducible to within ±1–2% for samples that were never treated with PEG and to within ±3–4% for samples treated with ≤35 wt % PEG.

or of LUVET did not mix in the presence of PEG, as lipids were not transferred between these vesicles above a constant background amount up to 30 wt % PEG (see Figure 2). The lack of contents mixing between SUV or SUVET treated with PEG could mean that these do not fuse or that fusion was too leaky in this system to be detected, especially considering the low trapping volume of these smaller vesicles. Small trapping volumes decrease the sensitivity of contents mixing measurements.

Because of the difficulty of confirming fusion in the highly curved vesicles by contents mixing assays, we used QELS to

measure the change in size of three types of vesicles treated with PEG. The results, shown in Figure 5, demonstrate that both SUV and MUVET2 increased in size when treated with high concentrations of PEG. Similar results have been reported for chicken egg yolk phosphatidylcholine sonicated vesicles treated with PEG6000 (Boni et al., 1984a), although the PEG threshold for vesicle size growth was not well-defined. In agreement with our results for lipid transfer and contents mixing, the LUVET did not increase in size even when incubated with 35 wt % PEG (Figure 5). It is also clear from the results in Figure 5 that a substantial increase in vesicle population heterogeneity (indicated by the width of bars associated with data points) accompanied the increase in size seen at the highest PEG concentrations. The PEG concentration at which MUVET2 size increase first occurred (20–25 wt %) corresponded with the concentration at which the extent of PEG-induced lipid transfer increased and also matched roughly the PEG concentration range over which mixing of vesicle contents occurred (15–25 wt %).

It is clear from our measurements that highly curved model membrane vesicles increased dramatically in size and mixed their bilayer lipids when treated with high concentrations of PEG. Intermediate sized vesicles (MUVET2) also mixed their trapped contents at 20 and 25 wt % PEG, indicating that at least some of these vesicles fused in the presence of PEG. Larger vesicles (MUVET and LUVET) clearly did not fuse even in the presence of higher PEG concentrations. The bulk of our data suggest, then, that increased membrane curvature (i.e., lower radius) promotes fusion of vesicles treated with the dehydrating polymer PEG. Promotion of fusion commences at a vesicle diameter of roughly 700–800 Å, the diameter at which the bilayer packing anomalies associated with increased membrane curvature are predicted to begin (Sheetz & Chan, 1972). The extent to which vesicle rupture may accompany vesicle fusion in more highly curved vesicles will be addressed under Discussion.

Effect of Amphipathic Compounds on PEG-Induced Fusion. We show above that PEG does not induce fusion of pure DPPC LUVET but does induce leakage of vesicle contents and rapid transfer of lipids between vesicles (Figures 2 and 3). However, small amounts of certain amphipathic, surface-active compounds present in the vesicle bilayer make even these uncurved vesicles susceptible to fusion induced by PEG. For example, Figure 6 summarizes the results of lipid transfer (closed circles), contents leakage (open circles), and contents mixing (open triangles) assays performed on DPPC LUVET containing (A) 0.5 mol % LPC, (B) 5 mol % PAF, or (C) 0.5 mol % PA in the presence of varying concentrations of PEG. Note in Figure 6 that a small increase in lipid transfer always accompanied the observation of mixing of vesicle contents. The observation of contents mixing in conjunction with lipid transfer between vesicles is reasonable proof of vesicle fusion. Note, too, that the extent of lipid transfer increased more dramatically as vesicle contents were lost due to treatment with even higher PEG concentrations. This suggests that vesicle rupture and resealing may occur at high PEG concentrations, perhaps simultaneously with fusion. The relationship between contents loss, vesicle rupture, and fusion will be examined further under Discussion.

The abilities of these several amphipaths to encourage fusion in the presence of PEG were not equal. Consideration of the data in Figure 6 shows that the presence of 0.5 mol % PA allowed fusion to occur at 15–20 wt % PEG and 5 mol % PAF helped promote fusion at 20–25 wt % PEG while 0.5 mol % LPC promoted fusion at 25–30 wt % PEG. Contents mixing

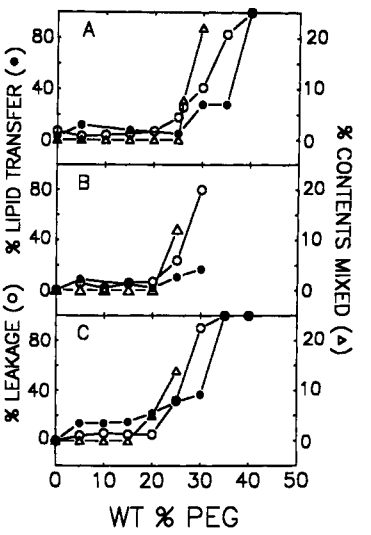

FIGURE 6: PEG-induced contents mixing, lipid mixing, and leakage for DPPC LUVET containing amphipathic fusogens. The results of assays, as described under Methods, for leakage (open circles), lipid mixing (closed circles), and contents mixing (open triangles) were obtained at 48 °C for DPPC LUVET containing (A) 0.5 mol % LPC, (B) 5 mol % PAF, and (C) 0.5 mol % PA. The meanings of the percentage units are described under Methods.

of DPPC vesicles containing these amphiphiles did not occur with lower concentrations of PEG.

QELS measurements of the size of LUVET composed of pure DPPC or of DPPC containing different fusogens were made after treatment with varying concentrations of PEG (Figure 5). As noted above, the results showed that pure DPPC LUVET did not increase significantly in size when treated with PEG up to a concentration of 40 wt %. In addition, there was not a significant increase in the half-width of the DPPC vesicle size distribution following treatment with PEG (vertical bars in Figure 5A). The presence of 0.5 mol % LPC or 0.5 mol % PA in DPPC LUVET resulted in PEG causing an increase in average vesicle diameter (Figure 5B,C respectively). LPC-containing vesicles grew in size from 1400 Å at low [PEG] to 2250 Å at 33 wt % PEG. PA-containing vesicles increased in diameter from 1265 to 1450 Å at 20 wt % PEG and to nearly 2000 Å at 30 wt % PEG. The ratio of the diameters of these vesicles after PEG treatment to their diameters at low [PEG] was about 1.6. A value of 1.4 would have been expected if, on average, each vesicle had fused with only one other and a ratio of 1.7 if each vesicle had fused with two others. It should be noted that vesicle size was observed to increase at the same PEG concentrations for which mixing of vesicle contents and a small increase in lipid transfer were first observed (25–30 wt % for LPC, 20–25 wt % for PAF, and 15–20 wt % for PA).

In addition to the several compounds that we have found to foster PEG-mediated fusion of DPPC LUVET, several other compounds thought to be fusogens in vivo were tested and found not to favor fusion in our model membrane system at experimentally obtainable PEG concentrations. Figure 7 contains a summary of our results for lipid transfer (closed circles), contents mixing (open triangles), and contents leakage (open circles) assays performed with vesicles containing (A) 0.5 mol % OAG, (B) 0.5 mol % DOG, (C) 5 mol % DOG, and (D) 5 mol % DPG. Vesicle contents mixing was not observed at these or even higher amphipath surface concentrations (e.g., 2 mol % OAG and 20 mol % MOG). DPPC LUVET containing 0.5 mol % OAG (and other amphipaths of this class) demonstrated no contents mixing even when the experiment was conducted with short incubation times of 3

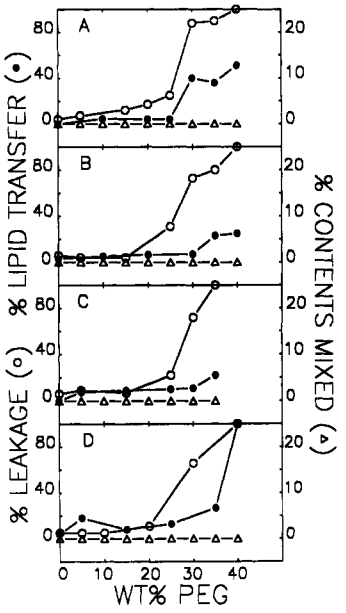

FIGURE 7: PEG-induced contents mixing, lipid mixing, and leakage for DPPC LUVET containing nonfusogenic amphipaths. Assays for leakage (open circles), lipid mixing (closed circles), and contents mixing (open triangles) were performed at 48 °C for DPPC LUVET containing (A) 0.5 mol % OAG, (B) 0.5 mol % DOG, (C) 5 mol % MOG, and (D) 5 mol % DPG. The meanings of the percentage units are described under Methods.

min so as to minimize contents leakage (≤30%). Despite the failure to observe PEG-induced contents mixing, lipid transfer was enhanced in these vesicles at high PEG concentrations. However, this enhanced lipid transfer correlated roughly with extensive loss of vesicle contents, a phenomenon also seen with vesicles containing "fusogenic" amphipaths (Figure 6), and possibly reflective of vesicle rupture. In order to test further for fusion, the size of certain vesicles (DPPC LUVET containing 0.5 mol % DOG) was also examined by quasi-elastic light scattering. The vesicles remained unchanged in size (1220 Å) when treated with up to 35 wt % PEG. The failure to observe mixing of vesicle contents correlated with an increase in lipid transfer and vesicle size, all of which were observed for the "fusogenic" amphipaths (Figure 6), makes it highly unlikely that fusion occurred between DPPC vesicles containing the "non-fusogenic" amphipaths OAG, DOG, MOG, or DPG.

Discussion

Relationship of Fusion to Leakage. Our results show that the most highly curved vesicles did not mix their contents under circumstances that resulted in lipid mixing and size growth. It may be that these vesicles did not fuse in the presence of PEG but rather ruptured and resealed, leading to the formation of larger vesicles, but not through a fusion process. The very large diameters attained by vesicles treated with concentrations of PEG (Figure 5) might support this interpretation, as do electron micrographs showing that the product of treating SUV with PEG6000 is multilamellar vesicles (Boni et al., 1984a). Another possibility is that vesicle fusion does occur under these conditions but that the small internal space of the highly curved vesicles makes contents mixing very difficult to measure and, consequently, vesicle fusion impossible to confirm. At present, our data support the hypothesis that highly curved vesicles are induced to rupture and extensively transfer their lipids at 25–30 wt % PEG but that they may undergo some leakage of contents and limited lipid transfer accompanied by some size growth at even lower PEG concentrations (15–25 wt %). Apparently, the tendency for PEG-induced rupture increases with bilayer curvature as does the tendency for fusion. It may be that highly curved bilayers are unable to accommodate the increased curvature associated with loss of trapped water in the presence of highly dehydrating concentrations of PEG, thus leading to rupture. Less highly curved vesicles (MUVET2) may be less destabilized at high PEG concentrations, allowing fusion to occur just prior to rupture.

In contrast to membrane curvature, "fusogenic" amphipaths appeared not to have enhanced leakage at the same time that they enhanced fusion. The PEG-induced leakage profile of DPPC LUVET was not affected by the addition of 5 mol % PAF or of 0.5 mol % LPC (compare Figures 3 and 6). By contrast, PEG-induced leakage of contents from DPPC LUVET was increased in the presence of, for example, 0.5 mol % DOG (compare Figures 3 and 7B). This is not surprising, since 5 mol % DOG has been reported to disrupt DPPC bilayer packing and cause lipid immiscibilities (Ortiz et al., 1988). PAF and LPC both enhanced PEG-mediated fusion of DPPC LUVET while DOG did not. These results suggest that, in contrast to the highly curved vesicles described above, enhanced leakage did not enhance fusion of DPPC LUVET. PEG-induced leakage of vesicle contents may reflect bilayer structural events different from those associated wtih fusion. However, a more precise and detailed comparison of the fusion and leakage processes will be necessary to confirm this suggestion.

A somewhat related issue is whether fusion occurs during incubation in the concentrated PEG solution or whether dilution of PEG is necessary to observe fusion. It is possible to imagine that PEG does not induce fusion at all but that the osmotic stress associated with addition and subsequent removal of PEG causes rupture of vesicles that then reseal to form larger species. Our two most reliable indications of fusion were mixing of vesicle contents and increase in vesicle size. Since both these measurements required dilution or removal of PEG, it has been impossible to obtain an unambiguous resolution of this issue. Several observations, however, argue against this scenario. First, lipid transfer, which can be detected in concentrated PEG solutions, always showed a small increase at exactly the same PEG concentration at which contents mixing (Figure 6) and vesicle size (Figure 5) first increased. Second, a small amount of contents mixing, a small increase in lipid mixing (Figure 6C), and a very small increase in vesicle diameter (Figure 5E) occurred in PA-containing vesicles at 20 wt % PEG, a concentration at which no detectable contents leakage occurred. Thus, fusion can apparently occur in the absence of vesicle contents loss. Third, we cannot envision how the contents of ANTS- and DPX-containing vesicles could remain trapped and mix as a result of a process involving vesicle rupture. Finally, it is difficult to imagine how a catastrophic event such as osmotically induced rupture could occur in DPPC LUVET and not lead to the same result as in DPPC LUVET containing a very small amount (0.5 mol %) of amphipathic fusogen such as LPC or PA.

Structural Basis of PEG-Mediated Fusion. The exact structural role that amphipaths such as PA, PAF, and LPC may play in the PEG-mediated fusion process is not known. There are at least four possible destabilizing effects that these amphipaths could have upon the membrane which would promote model membrane fusion. First, they could introduce lipid-phase separations into the bilayer. Second, they may be responsible for introducing nonbilayer structures, such as hexagonal phases, into the membrane. Third, and related to the second, because of their inverted conical structure, these

amphipaths may destabilize the membrane by introducing regions of curvature into the bilayer. Fourth, they could alter lipid packing order within the bilayer.

Lateral lipid-phase separations have long been thought to be the bilayer destablizing event in calcium-induced membrane fusion (Papahadjopoulos et al., 1977) and have been offered as well as a mechanism for PEG-induced fusion of DPPC vesicles (Tilcock & Fisher, 1979). In our studies, however, there was no clear correlation between the ability of amphipaths to induce phase separations under our conditions and their abilities to promote fusion mediated by PEG. Published phase behavior studies for mixtures of saturated phosphatidylcholines with LPC, PA, and PAF demonstrate that these compounds do not induce lateral lamellar phase separation under the conditions that we investigated (Bratton et al., 1988b; Koynova et al., 1988). By contrast, the diacylglycerols that failed to support fusion in the presence of high levels of PEG have been reported to induce solid-phase or even fluid-phase immiscibility at the low surface concentrations that we examined (Ortiz et al., 1988; deBoeck & Zidovetski, 1989). *The ability of amphipaths to induce lateral-phase separations, then, does not seem to offer a likely explanation for our results.*

It is also possible that PA, PAF, and LPC might destabilize the bilayer and promote fusion by favoring *nonbilayer structures* in the membrane. The potential importance of structures related to cubic or inverted hexagonal phases as intermediates in membrane fusion has been widely discussed [e.g., see Ellens et al. (1989], even with regard to PEG-induced fusion (Boni et al., 1984b). Lipids that form inverted hexagonal or cubic phases are thought to predispose a membrane bilayer to local fluctuations away from a purely lamellar structure of one containing "inverted micellar" fusion intermediates (Callis et al., 1986; Siegel, 1986). However, LPC (and presumably the very similar PAF) raises the lamellar-to hexagonal-phase transition temperature and, thus, stabilizes the bilayer structure (Epand, 1985). On the other hand, diacylglycerols decrease the lamellar-to-hexagonal transition temperature (Epand, 1985) and, therefore, should encourage formation of nonlamellar structures. In our system, diacylglycerols did not promote fusion whereas LPC and PAF did, indicating that *the ability of an amphipathic compound to induce local inverted hexagonal-like or cubic-like structures does not correlate with the ability to promote PEG-mediated vesicle fusion.*

Our results with different sized vesicles show that *bilayer curvature* promotes PEG-mediated fusion. Different amphipathic molecules, because of their different dynamic shapes when incorporated into mesomorphic structures, are said to have different "intrinsic curvatures" (Gruner, 1985). Even though small quantities of amphipaths are not expected to induce actual curved regions in a bilayer, the presence of such compounds results in a tendency toward curvature and in a strain in lipid packing. It is conceivable that either positive or negative intrinsic membrane curvature might favor the fusion process. PAF has been suggested to preferentially partition into regions of high surface curvature within membranes (Huang et al., 1986), and LPC is structurally similar to PAF. Because of their inverted conical shapes, LPC and PAF should promote positive curvature within a membrane monolayer. Because it is also expected to have an inverted conical shape (a large headgroup cross section relative to the hydrophobic region cross section) in the charged state, PA might be expected to behave in a similar manner. Compounds such as diacylglycerols, which favor the formation of inverted hexagonal phase, have a negative intrinsic curvature (Gruner, 1985). The fact that OAG, which has an acyl chain composition just like PAF, did not induce fusion in our system emphasizes the importance of positive intrinsic curvature brought about by a bulky headgroup to the fusogenic capability of the amphipath. Thus, *molecules with positive intrinsic curvature promoted PEG-mediated vesicle fusion.*

Their inverted conical structure (or, equivalently, their positive intrinsic curvature) should allow the fusogenic amphipaths to disrupt in a similar fashion the normal lamellar *lipid packing* within their immediate environment. Indeed, LPC, PAF, and PA have been reported to affect the physical properties of DPPC bilayers in similar ways. Both PAF and LPC reduce membrane order associated with calcium-induced lateral-phase separations (Bratton et al., 1988a). Interestingly, PAF has been reported to reduce calcium-induced membrane order within the glycerol region and not within the hydrophobic region of the bilayer (Bratton et al., 1988b), suggesting that PAF exerts an influence on the interfacial region of the membrane. Others (Allegrini et al., 1983; Killian et al., 1986) have reported a greater motional freedom of the headgroup and a conformational change within the headgroup region with bilayers containing a mixture of DPPC and LPC, suggesting that LPC, like PAF, exerts its influence upon the headgroup or interfacial region of the membrane. At high concentrations in DPPC multilayers, PA caused a reduction in the electron density in the glycerol backbone region of the bilayer (Katsaras & Stinson, 1990), also consistent with a reduction of order in this portion of the bilayer. *It may be that the ability of these amphipaths, even at low surface concentrations, to disrupt packing in the interface or the headgroup regions of the bilayer is the essential feature that makes them fusogenic.* Further and more systematic studies will be needed to test this possibility.

It is easy to envision the effect of curvature on a bilayer as being similar to that suggested here for fusogens, i.e., creating imperfect or disrupted packing in the outer leaflet of the membrane bilayer (Chrzeszczyk et al., 1977; Lawaczeck et al., 1976). One can imagine that such imperfect packing would expose hydrophobic regions of the bilayer to water and thereby raise the free energy of the bilayer, explaining the observed higher surface tension of SUV (Schindler, 1980). This is consistent with the observation that highly curved vesicles spontaneously fuse to form less curved, and thermodynamically more stable, vesicles, even at temperatures above their phase transition (Lentz et al., 1987). The free energy of the intermediate fusion state, which must involve exposure of the membrane interior to water, must be greater than that of the unperturbed bilayer. For bilayers that have been brought into near-molecular contact by PEG, the probability of formation of a fusion intermediate state will be proportional to the exponential of the free energy difference between the normal bilayer and the intermediate state. Both increased membrane curvature and the amphipathic compounds we have identified may enhance fusion by raising the free energy (i.e., destabilizing) of the "normal" bilayer state.

Possible Relationship of Results to Fusion of Cellular Membranes. It is appropriate to ask whether the concentrations of amphipaths examined in our model membrane studies are comparable to concentrations expected in biological membranes. In contrast to a previous study of fusogens (Ahkong et al., 1973), the quantities of fusogenic amphipaths used in our experimental system are comparable to those reported within cells. Creutz (1981) has reported that addition of arachidonic acid to synexin-aggregated chromaffin granule

membranes (at 4–8% of total granule lipids) stimulated their fusion. Marcus (1978) has estimated that the amount of free arachidonic acid formed during stimulation of the human platelet may be as high as 8% of total platelet lipid. We have used 0.5 mol % PA to stimulate fusion of PEG-aggregated DPPC LUV. PAF is predominantly found in blood cells (Hanahan, 1986), while lysolipids can be found in all cells (Ansell et al., 1973). The inner membrane of chromaffin granules contains 17% LPC (Winkler & Carmichael, 1982), while our experiments employed only 0.5 mol % LPC. Stimulated human neutrophils contain 16 μM 18:0 PAF (Oda et al., 1985). Our experiments demonstrated that 5 mol % PAF (corresponding to 12.5 μM) was sufficient to induce fusion. Phagosomal membrane fractions prepared from stimulated neutrophils contain 0.5–1 mol % PAF (Riches et al., 1985). This amount of PAF did not induce fusion in our system. However, the global amounts of PAF or of LPC may not be as important as the local concentrations in promoting cellular fusion. In addition, the lipid composition of a phagosomal membrane may be more conducive to fusion induced by PAF than was the pure DPPC used in our experiments. The amount of diacylglycerols transiently produced in normal rat kidney cells is 0.61 mol % (Preiss et al., 1986). The addition of 0.5 mol % or 5 mol % DOG to DPPC vesicles did not result in fusion.

In a cell, many factors other than the presence of amphipathic fusogens are likely to contribute to the ability of a membrane to fuse. For example, the presence of other lipids (e.g., phosphatidylethanolamine or cholesterol) or of proteins (e.g., synexin, spectrin, or other skeletal proteins) will likely play roles in cellular fusion. Amphipaths identified in this study as "non-fusogenic" in pure phosphatidylcholine model membranes may serve as fusogens in other membranes, including certain biological membranes. For example, Siegel et al. (1989) have shown that diacylglycerols stabilize nonlamellar phases and encourage fusion in membranes composed of monomethylphosphatidylethanolamine. Although this is not a physiological lipid, the contrast between this result and ours illustrates that the effects of amphipaths on membranes can be expected to vary with the membrane composition. The main conclusion to be drawn from our results is that a simple lipid bilayer composed of well-packed lipids cannot be induced to fuse by the molecular contact forced by PEG. Other factors seem necessary to cause bilayer defects that allow fusion. Perhaps the structural effects of these factors can be better defined using the model system and particular fusogens identified here.

Acknowledgments

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Modulation of Poly(ethylene glycol)-Induced Fusion by Membrane Hydration: Importance of Interbilayer Separation†

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ABSTRACT: Large unilamellar vesicles composed of lipids with different hydration properties were prepared by the extrusion technique. Vesicles were composed of dioleoylphosphatidylcholine in combination with either 0.5 mol % monooleoylphosphatidylcholine or different molar ratios of dilauroylphosphatidylethanolamine. Fusion was revealed via a fluorescence assay for contents mixing and leakage, a fluorescent lipid probe assay for membrane mixing, and quasi-elastic light scattering to detect vesicle size growth. As the percentage of poorly hydrating phosphatidylethanolamine increased, the concentration of poly(ethylene glycol) (PEG) required to induce fusion decreased. From differential scanning calorimetry studies of membrane-phase behavior and X-ray diffraction monitoring of phase structure in PEG, it was concluded that PEG did not induce a hexagonal-phase transition or lamellar-phase separation. Electron density profiles derived from X-ray diffraction studies of multi- and unilamellar vesicles indicated that the water layer between vesicles had a thickness of approximately 5 Å at PEG concentrations at which vesicles were first induced to fuse. At this distance of separation, the choline headgroups from apposing bilayers are in near-molecular contact. Since pure phosphatidylcholine vesicles did not fuse at this interbilayer spacing, a reduction in the interbilayer water layer to a critical width of ~2 water molecules may contribute to but is not sufficient to produce PEG-mediated fusion of phospholipid membranes. Comparison of these results with other results from this laboratory also indicates that, while close contact between bilayers promotes fusion, near-molecular contact is apparently not absolutely necessary to bring about fusion. A tentative model is presented to account for these results.

It is well recognized that membrane fusion is important to numerous biological processes. In order to understand the mechanism behind these cellular processes, researchers have used model membrane systems to probe the detailed mechanism of the fusion event. While model membranes may not perfectly mimic their cellular counterparts, they should still provide insight into basic structural events necessary for membrane fusion. Among the structural events thought to contribute to fusion, bilayer dehydration (Wilschut et al., 1985b), local alterations in bilayer curvature (Nir et al., 1982; Lentz et al., 1987), changes in membrane "fluidity" (Wilschut

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