Temperature-Induced Fusion of Small Unilamellar Vesicles Formed from Saturated Long-Chain Lecithins and Diheptanoylphosphatidylcholine†

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ABSTRACT: Small unilamellar vesicles which form when gel-state long-chain phosphatidylcholines are mixed with micellar short-chain lecithins undergo an increase in size as the long-chain species melts to its liquid-crystalline form. Analysis of the vesicle population with quasi-elastic light scattering shows that the particle size increases from 90-Å radius to >5000-Å radius. Resonance energy transfer experiments show total mixing of lipid probes with unlabeled vesicles only when the T_m of the long-chain phosphatidylcholine is exceeded. This implies that the large size change represents a fusion process. Aqueous compartments are also mixed during this transition. ^{31}P NMR analysis of the vesicle mixtures above the phase transition shows a great degree of heterogeneity with large unilamellar particles coexisting with oligo- and multilamellar structures. Upon cooling the vesicles below the T_m, the original size distribution (e.g., small unilamellar vesicles) is obtained, as monitored by both quasi-elastic light scattering and ^{31}P NMR spectroscopy. This temperature-induced fusion of unilamellar vesicles is concentration dependent and can be abolished at lower total phospholipid concentrations. It occurs over a wide range of long-chain to short-chain ratios and occurs with 1-palmitoyl-2-stearoylphosphatidylcholine and dimyristoylphosphatidylcholine as well. Characterization of this fusion event is used to understand the anomalous kinetics of water-soluble phospholipases toward these unusual vesicles.

There are only a few documented examples of small unilamellar vesicles forming spontaneously from multilamellar phospholipid structures. This occurs either electrostatically with pH shifts of anionic lipids such as phosphatidic acid (Hauser, 1983) or chemically with the introduction of short-chain phosphatidylcholine (PC)[1] (Gabriel & Roberts, 1984, 1986, 1987; Roberts & Gabriel, 1988) into phosphatidylcholine multibilayers. The latter vesicles, termed SLUVs (short-chain PC/long-chain phospholipid unilamellar vesicles), are small (R_H ~90–100 Å) and relatively homogeneous when the long-chain PC is in a gel phase (e.g., dipalmitoyl-PC, distearoyl-PC). The short-chain PC is asymmetrically distributed in these structures with >80% on the exterior monolayer. Physical studies (NMR) suggest that the short-chain PC is motionally uncoupled or independent from the gellike long-chain species. If the long-chain PC from which SLUVs are formed is in a liquid-crystalline state (e.g., 1-palmitoyl-2-oleoyl-PC, dimyristoyl-PC above 25 °C), then large vesicles form, and the population appears to be much more heterogeneous (Gabriel & Roberts, 1986). If gellike SLUVs are incubated above the T_m of the long-chain PC, there is a large increase in ^1H line width (Roberts & Gabriel, 1988). When the temperature is reduced below the long-chain lipid T_m, the line widths narrow to original values. In these studies, it is unclear whether the vesicles are aggregating or fusing and what the size distribution is for the particles that form above the T_m. The present paper presents data investigating this behavior for diheptanoyl-PC/dipalmitoyl-PC and related lipid systems using quasi-elastic light scattering (QLS), resonance energy transfer (RET) assays, and ^{31}P NMR spectroscopy. We find that there is a sharp thermally induced fusion of small SLUVs from ~90-Å radius to particles >5000-Å radius which appear to be mixtures of large unilamellar and oligo- and multilamellar particles at the T_m of the long-chain component. This size change which occurs upon melting of the long-chain PC matrix is used to interpret anomalous phospholipase kinetics toward the short-chain PC in the SLUVs.

Experimental Procedures

Chemicals. Dipalmitoyl-PC, 1-palmitoyl-2-stearoyl-PC, 1-palmitoyl-2-oleoyl-PC, dimyristoyl-PC, diheptanoyl-PC, *N*-(lissamine rhodamine B sulfonyl)-PE (Rho-PE), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) were obtained from Avanti Polar-Lipids, Inc., in chloroform solutions and used without further purification. 6-Carboxyfluorescein (CF) was purchased from Eastman Kodak Co.

Preparation of Aqueous Lipid Mixtures. The lecithin stock solutions in chloroform were mixed to the desired molar ratio (typically 4:1 long chain to short chain) in a glass vial and dried under N_2 (g). The resultant lipid film was dried under vacuum overnight to remove any remaining traces of organic solvent. Pure water or buffers were added to hydrate the film to a total lipid concentration of 25 mM unless otherwise indicated. Sample solutions were heated briefly (5–10 min) around 40 °C and vortexed for 2–3 min. The samples were allowed to stand at room temperature for 2 h prior to use. For QLS measurements, the aqueous lipid solutions were filtered through Millex-HA filters (Millipore Corp., 0.45-mm pore size) into the scattering cells.

Quasi-Elastic Light Scattering (QLS). The average hydrodynamic radii of SLUVs in aqueous solutions were obtained from the autocorrelation functions (ACFs) of the intensity of scattered light measured in a QLS spectrometer of standard

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[1] Abbreviations: diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; SLUV, *short-chain lecithin/long-chain phospholipid unilamellar vesicle*; cmc, critical micelle concentration; QLS, quasi-elastic light scattering; ACF, autocorrelation function; RET, resonance energy transfer; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE; CF, carboxyfluorescein.

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design. The scattering cell containing the sample solution was placed in a vat of index of refraction matching fluid in which the temperature was controlled by a circulating water bath. The sample was incubated at a given temperature for 10–15 min before QLS data were acquired. On this time scale, all temperature-induced size changes appeared to reach equilibrium, since the R_H as measured by QLS remained constant. Light scattered by the sample from an incident laser beam (argon ion laser, λ = 514.5 nm, power ca. 100 mW) was detected by a photomultiplier tube mounted on a movable arm set at a scattering angle of 90° for these experiments. A pulse amplifier/discriminator transformed the photomultiplier output into standard logic pulses processed by the digital correlator (Langley Ford Instruments, division of Coulter Electronics, Inc.; Model 1096, 256 channels) to compute the photon count ACF. The ACF was approximately an exponentially decaying function as a result of diffusive motion of scatterers in the sample. The average decay rate, Γ, was determined by a second cumulant fit to the correlation function (Koppel, 1972). Γ is related to the diffusion coefficient, D, and the scattering vector, $\mathbf{q}$, by

$$\Gamma = Dq^2 \quad (1)$$

and

$$q = \mathbf{q} = (4\pi n/\lambda) \sin(\theta/2) \quad (2)$$

where n is the refractive index of solvent, λ is the wavelength of incident light in vacuo, and θ is the scattering angle between the incident and scattered light. An average hydrodynamic radius of the particles, R_H, was calculated from the measured diffusion coefficient, D, by using the Stokes–Einstein equation:

$$D = kT/6\pi\eta R_H \quad (3)$$

where k is Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity.

Fluorescence Spectroscopy. Fluorescence emission was monitored with either a Perkin-Elmer 650-10s or a Shimadzu RF5000V spectrofluorometer. High sensitivity settings and 5-nm slit width were used on both instruments. Samples of 2-mL volume and 1-cm path length were used throughout.

Vesicles prepared for resonance energy transfer (RET) assays contained 1.0–0.25 mol % fluorescent labeled lipid. This assays monitors the transfer of energy between the excited dye (NBD-PE) and an acceptor dye (Rho-PE) in spatial proximity. The appropriate amount of dye(s) was cosolubilized with the lipids in $CHCl_3$ before removal of the solvent under N_2 (g). Probe dilution experiments contained both dyes cosolubilized in one vesicle population. This double-labeled (both NBD-PE and Rho-PE in a 1:1 ratio) vesicle preparation was mixed with a 5-fold excess (unless noted otherwise) of unlabeled vesicles. Fluorescence emission was monitored from 470 to 600 nm using an excitation wavelength of 450 nm. Intensities for both Rho-PE and NBD-PE emissions (590 and 460 nm, respectively) were measured and are reported as a ratio of $I_{\text{NBD-PE}}/I_{\text{Rho-PE}}$ or normalized as the fraction of the intensity of NBD-PE fluorescence induced by detergent disruption or freeze–thawing of the vesicles (this represents the value for the probe completely randomized in the dilution assay).

Aqueous component mixing was monitored by using entrapped 6-carboxyfluorescein (CF) dye; 100 mM CF was present in the aqueous solution used to hydrate the lipid film for formation of SLUVs. Nonencapsulated dye was removed by centrifugation at 10000 rpm through a wet-packed 3-mL Sephadex G-25 column. At high concentrations, the CF in the vesicle is self-quenching (excitation 490 nm, emission 520 nm); if the CF is diluted into the external media by disruption of SLUV structure (general leakiness or fusion), the intensity of the emitted peak would be expected to increase (Weinstein et al., 1977).

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were obtained at 121.4 MHz on a Varian XL-300 spectrometer with a 10-mm probe. Spectral parameters include a 60° pulse width of 18 μs, 10000-Hz sweep width, 32000 data points, recycle delay of 1.5 s, and proton noise decoupling. The amount of exponential line broadening varied, depending on particle size and line width. The number of transients accumulated depended on the sample (e.g., around 2000 for SLUV mixtures and 20000 for liquid-crystalline dipalmitoyl-PC multibilayers). Sample temperature was measured by insertion of a thermocouple into the NMR sample tube. The SLUV sample for NMR was prepared as described earlier. Dipalmitoyl-PC powder was hydrated with water and agitated to produce multilamellar particles, while LUVs of the same lipid were prepared by sonication at 45 °C for 5 min, followed by refrigeration overnight. Preparation of these particles paralleled procedures of Schullery et al. (1980). These particles had diameters around 900 Å as determined by QLS [comparable to values found by Wong and Thompson (1982)].

Results

Effect of Temperature on SLUV Size. The initial size distribution of SLUVs at temperatures below the T_m depends on the ratio of long chain to short chain, on the total lipid concentration, and on the history of the sample. The total lipid concentration is quite important because the short-chain PCs have cmc's in the millimolar range (Tausk et al., 1974; Burns et al., 1982; Lin et al., 1987). For example, the cmc of pure diheptanoyl-PC is 1.5 mM. If the concentration of the short-chain lecithin is below its cmc upon rehydration of a cosolubilized mixture or when an aqueous monomeric short-chain PC solution is added to multilamellar vesicles, then very few SLUVs form (Gabriel & Roberts, 1986; Roberts & Gabriel, 1988). At 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC, all the lipid is converted to small unilamellar vesicles with an average R_H of 90 Å. The polydispersity in sizes is very small (less than 0.1) for these particles. If the ratio of the two components is kept constant (1:4) and the total lipid concentration is decreased by hydrating with increasing volumes of water (buffer), then the vesicle size begins to increase at 3.2 mM total lipid, or 0.6 mM diheptanoyl-PC (Figure 1, open circles). The cmc of pure diheptanoyl-PC is 1.5 mM; therefore, this behavior indicates that micellar short-chain PC is necessary to solubilize the DPPC. If, on the other hand, SLUVs are formed in aqueous solution at high concentrations and then diluted, on the scale of 24 h, the SLUVs are stable, and most of the short-chain PC remains in the vesicle even at 100-fold dilutions where the diheptanoyl-PC is 0.05 mM (Figure 1, asterisks). This indicates that SLUVs are relatively stable structures, consistent with other studies (Riedy et al., 1989).

When 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC SLUVs are incubated at increasing temperatures, there is no effect on the average size until 38 °C. By visual inspection of the sample shifted up to 38 °C or above, one detects a cloudy solution immediately, indicating that a large change in particle size has occurred, well within the time necessary for monitoring the scattering by QLS. At these temperatures, the averaged R_H of the SLUVs increases by about 2 orders of magnitude (Figure 2). The polydispersity of the particles is now quite large, suggesting a wide variety of structures coexist. The light scattering will be dominated by large particles—in fact, for particles with a 10-fold difference in R_H,

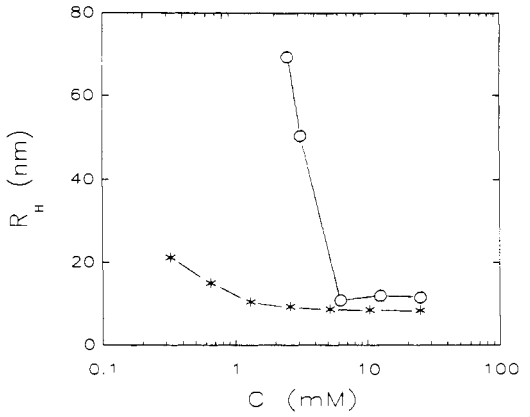

FIGURE 1: Hydrodynamic radius, R_H, of diheptanoyl-PC/DPPC (1:4) SLUVs prepared by (O) cosolubilizing the indicated amount of total lipid at 30 °C or (*) diluting from a 25 mM total PC stock at 20 °C. The higher temperature for the hydration dilution samples appeared necessary to prevent a long-term (on the scale of hours) aggregation of the 25 mM total lipid vesicles to 2–3-fold increases in R_H.

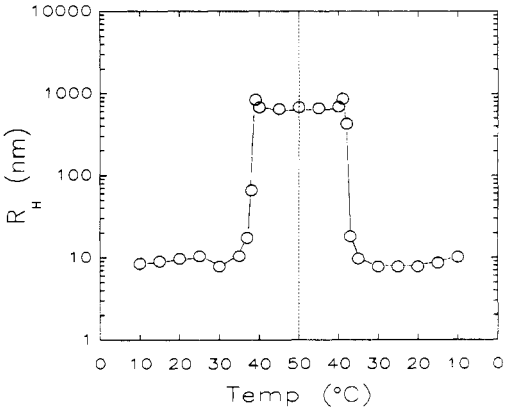

FIGURE 2: Hydrodynamic radius, R_H, of diheptanoyl-PC/DPPC (5 mM:20 mM) SLUVs as a function of temperature. Samples were allowed to equilibrate at a given temperature for 10–15 min before QLS data were acquired.

one can only detect the smaller structures (using a bimodal fit) if they represent >90% of the total species (Eum and Roberts, unpublished results). Therefore, QLS is detecting a large size change for some fraction of the SLUVs. QLS alone cannot distinguish between massive vesicle aggregation and vesicle fusion nor can it provide information on the extent of vesicle aggregation/fusion (i.e., how many of the SLUVs are involved in this process). Above 40 °C, there is a slight and reproducible decrease in R_H, but it is still very large compared to the initial R_H below 37 °C (90 Å). The dramatic growth at 38 °C correlates with the gel to liquid-crystalline phase transition of the dipalmitoyl-PC long-chain lipid matrix. The T_m is 41 °C in multilamellar structures (Mason & Huang, 1981) and 37 °C in small unilamellar vesicles prepared by sonication. This large change in R_H is, however, reversible: the SLUV population returns to an average size of 90-Å R_H as the solution is cooled down below 38 °C, and no hysteresis is observed upon cooling (Figure 2). Increasing the ionic strength of the medium by comparing R_H/temperature profiles for SLUVs in H_2O, 20 mM NaCl, and PBS (phosphate-buffered saline) has no detectable effect on either the temperature at which growth is initiated (38 °C) or the final particle size (R_H ~5000–6000 Å). Increasing NaCl concentration to 500 mM causes a slight decrease in particle growth with a maximum R_H of 3000 Å. [If the SLUV mixture is "old" (i.e., has been at room temperature for several days), a slight aggregation is detected when the vesicles are incubated

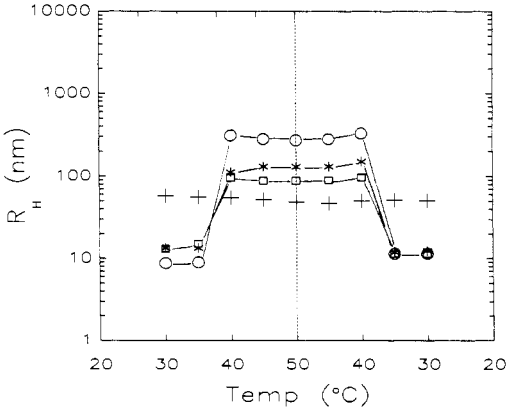

FIGURE 3: Temperature dependence of R_H for diheptanoyl-PC/DPPC (1:4) SLUVs in solutions of different phospholipid concentrations (prepared by cosolubilizing the indicated amounts of lipid). Total phospholipid concentrations are (O) 25 mM, (*) 12.5 mM, (□) 6.25 mM, and (+) 3.1 mM.

below 25 °C, but not at all comparable to the size change upon melting of the long-chain PC. The vesicles increase a factor of 2 or so in R_H. This is somewhat reminiscent of the fusion of small unilamellar vesicles of pure dipalmitoyl-PC incubated below T_m, but unlike the pure dipalmitoyl-PC case at comparable concentrations, for which a distinct fusion event occurs (Schullery et al., 1980; Lichtenberg et al., 1981), increasing the incubation temperature to 30 °C, which is not above the T_m of the long-chain PC, brings the SLUV R_H back to 90 Å. SLUV-encapsulated carboxyfluorescein is also not released in this aggregation or when the sample is heated to 30 °C.]

In contrast to SLUVs prepared with a gel-state long-chain matrix, SLUVs formed with liquid-crystalline long-chain PCs such as 1-palmitoyl-2-oleoyl-PC are found to be initially very large (R_H ~4000–5000 Å) and polydisperse by QLS. Furthermore, they do not show a large size change (either increase or decrease) over the temperature range 20–50 °C. This is consistent with EM studies of such mixtures where a wide range of sizes was detected (Gabriel & Roberts, 1986).

The large size change at 38 °C persists for SLUVs prepared with the same 1:4 ratio of components and greater than 3.1 mM total lipid, although the weight-averaged large size reached decreases in the more dilute samples (Figure 3). Dramatic growth in R_H with increasing temperature is not observed for particles that are initially 500–600 Å in radius (3.1 and 2.5 mM total lipid upon rehydration); otherwise, the R_H/temperature profile parallels that for the 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC mixture. Because of the concentration dependence of the size change, the growth process requires collision of vesicles, i.e., bi or higher order molecular reaction. QLS studies of vesicles prepared by diluting a concentrated SLUV stock (25 mM) to 2.5 mM and examined either immediately or after 2 h do not detect the transition to very large structures (Figure 4). These unilamellar vesicles are 300–400 Å in radius from 30 to 50 °C. In cases where the total phospholipid concentration is low, a hysteresis is observed. Upon cooling, the R_H increases to the value that would have been observed if the same concentration of lipids had been cosolubilized and hydrated—in this case, 500–600 Å (Figure 4).

Variation in the Ratio of Short-Chain PC to Long-Chain PC. When the long-chain PC (20 mM) is in the gel state, the SLUV sizes are 90–100 Å with little deviation for dipalmitoyl-PC:diheptanoyl-PC ratios in the range 2–7. If an excess of dipalmitoyl-PC is present (20:1), medium-size vesicles of 800 Å dominate the scattering—any small SLUVs that form

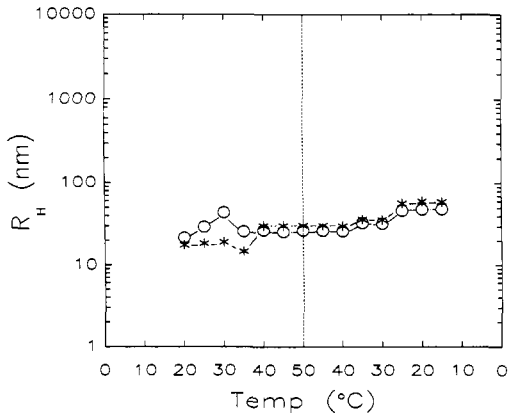

FIGURE 4: Temperature dependence of R_H for diheptanoyl-PC/DPPC (0.5 mM:2.0 mM) SLUVs prepared by dilution from a 25 mM phospholipid sample: (*) examined immediately upon dilution; (O) after 2-h incubation at the dilute concentration.

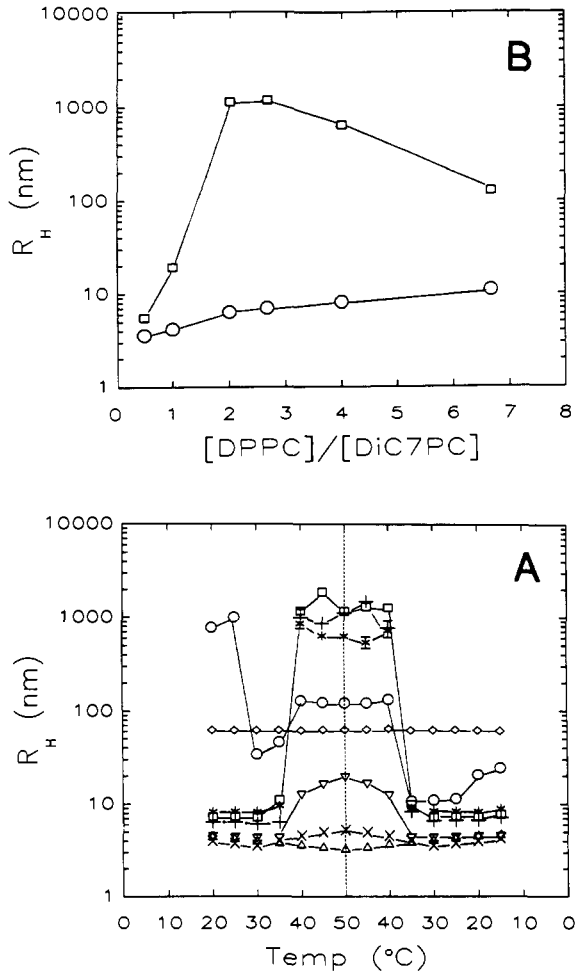

FIGURE 5: (A) Temperature dependence of R_H for SLUVs prepared by cosolubilizing 20 mM DPPC with the indicated amount of diheptanoyl-PC: (◇) 1 mM; (O) 3 mM; (*) 5 mM; (□) 7.5 mM; (+) 10 mM; (▽) 20 mM; (×) 40 mM; for comparison, 20 mM diheptanoyl-PC alone is shown (△). (B) log R_H (in nanometers) is plotted as a function of the ratio of DPPC to diheptanoyl-PC for SLUVs at 30 °C (O) and 50 °C (□).

are overshadowed by the scattering of the larger particles—and no dramatic growth is observed as the dipalmitoyl-PC melts. For 7:1 to 2:1 ratios of dipalmitoyl-PC to diheptanoyl-PC, no micelles coexist with the SLUVs (Roberts & Gabriel, 1988), and the vesicles grow dramatically around 38 °C (Figure 5A). If proportionately more diheptanoyl-PC is added to the fixed concentration of dipalmitoyl-PC (such that [dipalmitoyl-

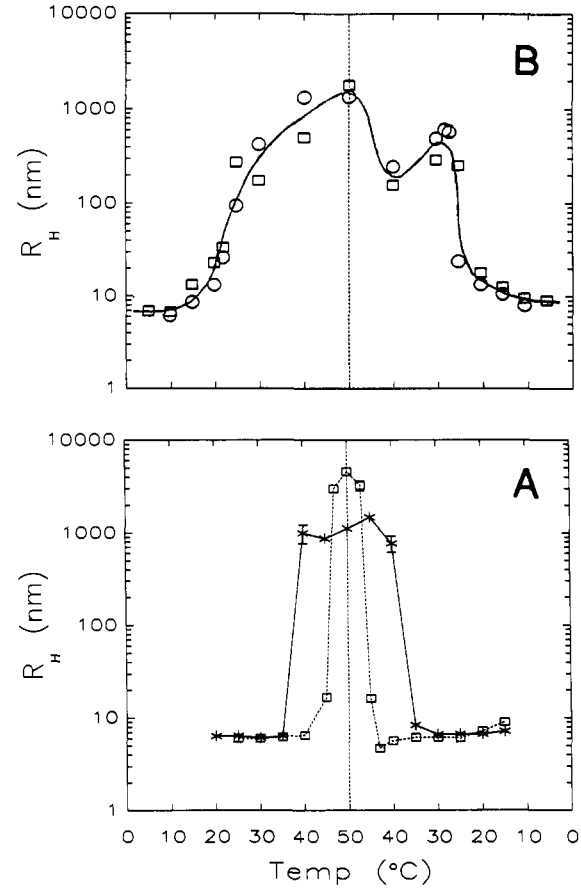

FIGURE 6: Temperatue dependence of R_H for SLUVs (1:2, 10 mM diheptanoyl-PC/20 mM saturated long-chain PC) of (A) (*) dipalmitoyl-PC and (□) 1-palmitoyl-2-stearoyl-PC and (B) dimyristoyl-PC [two separate sample runs (O and □)].

PC]/[diheptanoyl-PC] <1.0 in Figure 5), the particle size decreases to 50–60 Å at both gel and liquid-crystalline temperatures and no large size change occurs around T_m. Such a small size is unlikely to reflect vesicles and is probably due to a population of dipalmitoyl-PC/diheptanoyl-PC mixed micelles. In fact, hemolysis assays indicate that micelles exist for [dipalmitoyl-PC]/[diheptanoyl-PC] ≤1.0 (Riedy et al., 1989). A comparison of R_H as a function of the ratio of dipalmitoyl-PC/diheptanoyl-PC for gel and liquid-crystalline temperatures is shown in Figure 5B. Gel-state SLUVs are small (~90-Å radius) as long as sufficient diheptanoyl-PC is added to convert dipalmitoyl-PC multi- or oligolamellar structures to small stable vesicles. As more short-chain PC is added, smaller mixed micelles form. Liquid-crystalline SLUVs are large and polydisperse, and increasing the amount of the short-chain species initially increases R_H (from 1300- to 15 000-Å radius) until the ratio of the two components is 1:1. If the short-chain PC in SLUVs is an integral part of the large increase in vesicle size, then samples with excess dipalmitoyl-PC will show less particle growth than those with a 4:1 dipalmitoyl-PC/diheptanoyl-PC ratio. At 1:1, micelles definitely exist, and one measures an R_H which reflects an average of a few remaining 5000–6000-Å vesicles with the small 50–60-Å mixed micelles (Figure 5B).

Sizes of Other Diheptanoyl-PC/Long-Chain PC SLUVs. SLUVs prepared from other long-chain PCs with saturated fatty acids (e.g., dimyristoyl-PC, 1-palmitoyl-2-stearoyl-PC) in a 2:1 ratio with diheptanoyl-PC are similar in size (R_H ~80–90 Å) to dipalmitoyl-PC/diheptanoyl-PC SLUVs when examined at temperatures below their T_m (Figure 6A,B). When these mixtures are examined as a function of temper-

ature, they also show a dramatic increase in R_H upon melting of the long-chain PC. The change is reversible since incubating the mixtures below T_m regenerates the original size distribution of the particles. SLUVs made from 1-palmitoyl-2-stearoyl-PC show growth around 45 °C to 50000-Å particles. The T_m of multibilayers of this pure lipid is 49 °C (Chen & Sturtevant, 1981). For comparison, the 2:1 dipalmitoyl-PC/diheptanoyl-PC SLUVs grow to about 10000 Å in radius upon melting of the DPPC. Dimyristoyl-PC SLUVs grow from 150 to about 15000 Å around 25 °C, where the dimyristoyl-PC chains melt. The T_m for pure dimyristoyl-PC is 24 °C (Mason & Huang, 1981). A reproducible decrease (R_H ~3000 Å) followed by an increase (6000–7000 Å) in size occurs upon cooling these SLUVs from 50 to 40 to 30 °C. This behavior is only observed with dimyristoyl-PC SLUVs. Furthermore, if one compares the temperature range over which the size change upon heating occurs, it is larger for dimyristoyl-PC (10–15 °C) than for the other two systems (2–3 °C).

Fluorescence RET Assays of Lipid Mixing. The QLS experiments show that a reversible size change occurs when the long-chain PC undergoes its gel-to-liquid transition in SLUVs. This could be due to an aggregation of the vesicles or to fusion to a large unilamellar vesicle or multilamellar structure. While the evidence could be more consistent with fusion than aggregation (e.g., high concentrations of NaCl do not affect the size increase with temperature as would be expected for an aggregation process), more direct evidence is necessary to determine which of these options is occurring. Resonance energy transfer techniques have often been used to investigate fusion (Struck et al., 1981). The donor and acceptor either are comixed and incubated with unlabeled vesicles (probe dilution) or are mixed from separate populations (probe mixing). Since the probe mixing method appears less suited to the delineation of aggregation versus fusion (Duzgunes et al., 1987), it was not used in this investigation.

In a population of diheptanoyl-PC/dipalmitoyl-PC (1:4, 1.5 mM total lipid) SLUVs cosolubilized with 0.5 mol % each NBD-PE and Rho-PE and prepared by dilution of a 25 mM total lipid SLUV solution at 25 °C, pronounced quenching of the NBD-PE by the Rho-PE is observed (compared to what would be expected for that amount of NBD-PE alone in the vesicles). The dyes should partition on both sides of the SLUV bilayer, and in these small vesicles, there should be twice as much dye in the outer leaflet as in the inner leaflet. The NBD-PE quenching by Rho-PE can be quantified by comparing the fluorescence intensity of the NBD-PE peak to that of the Rho-PE peak. When a 5-fold excess of unlabeled SLUVs of the same diheptanoyl-PC:dipalmitoyl-PC ratio is added to this mixture at 25 °C, the fluorescence intensity of the NBD-PE increases gradually over a 60-min period and then levels off, indicating physical separation of the two dyes. The extent of total dye mixing in this dilution experiment can be estimated by comparing the NBD-PE fluorescence intensity observed after 80-min incubation with unlabeled SLUVs to that from the same mixed vesicles which have been frozen and thawed or disrupted by the addition of detergents (e.g., Triton X-100). In the latter case, all the probes are randomized over the total vesicle population, and the NBD-PE fluorescence intensity represents 100% dye mixing and dilution. From this comparison, one finds that when the increase in NBD-PE fluorescence levels off at room temperature, only ~70% of the total NBD-PE has been separated from the Rho-PE (Figure 7). Since the vesicles show no alteration of R_H under these conditions, the fluorescent probes must be exchanging and equilibrating between the two vesicle populations: those

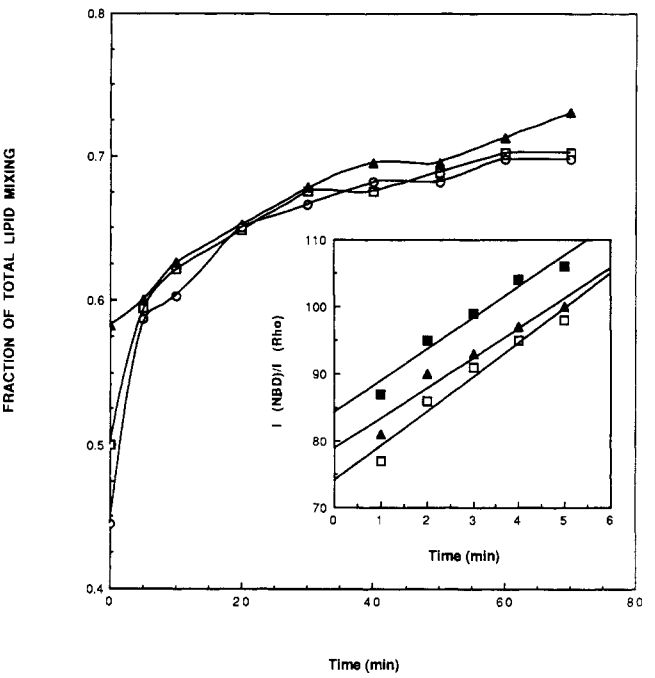

FIGURE 7: Fraction of dye mixing as a function of time (at 25 °C) for 0.3 mM diheptanoyl-PC/1.2 mM dipalmitoyl-PC SLUVs containing equimolar NBD-PE and Rho-PE at (○) 1.0 mol % each, (□) 0.50 mol % each, and (▲) 0.25 mol % each. Inset: Dye mixing in the probe dilution assay as a function of increasing amount of short-chain lecithin and a total PC of 1.5 mM [diheptanoyl-PC:dipalmitoyl-PC ratio of (□) 1:4, (▲) 1:3.5, and (■) 1:3] as monitored by the ratio (×100) of NBD-PE fluorescence intensity to Rho-PE intensity over short time periods.

originally containing both dyes and those that are unlabeled. In comparable experiments where pure 1-palmitoyl-2-oleoyl-PC sonicated vesicles containing 0.5 mol % of each dye are incubated with unlabeled 1-palmitoyl-2-oleoyl-PC vesicles, no such exchange or dilution of the fluorescent probes between labeled and unlabeled pools occurs over the same time scale. Diheptanoyl-PC/POPC SLUVs containing both dyes, however, do show an increase in NBD-PE fluorescence intensity when diluted with unlabeled SLUVs. Thus, it is the short-chain PC in the vesicles that facilitates separation of NBD-PE and Rho-PE between bilayers. At a fixed concentration of labeled and unlabeled vesicles, this rate of dye exchange is independent of probe concentration. SLUVs prepared with various dye concentrations (1.0, 0.50, 0.25 mol %) and mixed with a 5-fold excess of unlabeled SLUVs show similar rates of dye dilution, with about 70% of the probes mixing in 2 h (Figure 7). Residual quenching of some of the NBD-PE by Rho-PE occurs (unless these gel-state vesicles are completely disrupted by detergent addition etc.) and is stable to further prolonged incubation of the two pools. In these small vesicles, about 70% of the total lipid (and presumably 70% of both dyes) would be in the outer leaflet. The fact that about 30% of the NBD-PE is still quenched by the Rho-PE implies that only dye in the outer monolayer of the gel-state SLUVs is accessible to room temperature dilution into unlabeled vesicles. Dye molecules in the interior leaflet are not mixed in this probe dilution assay, and removal of that remaining 30% quenching of NBD-PE can be used as a marker for fusion as opposed to aggregation of SLUVs.

Samples prepared with varying amounts of short chain-PC in the gel-state SLUVs (constant dye of 0.5 mol % total lipid, 20–25% diheptanoyl-PC) show similar rates of probe dilution (Figure 7, inset). The relative amount of short-chain PC is limited to this range where we know that SLUVs form as a

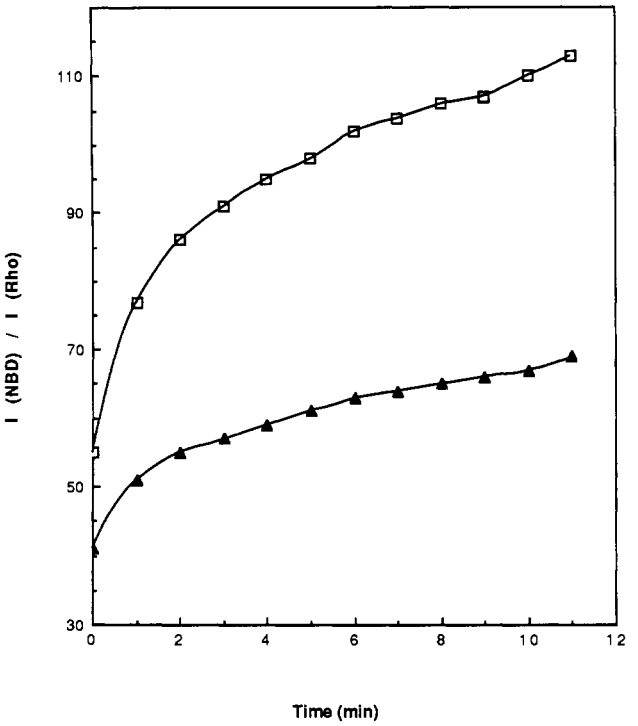

FIGURE 8: Ratio (×100) of NBD-PE fluorescence intensity compared to Rho-PE upon mixing diheptanoyl-PC/dipalmitoyl-PC (1:4) SLUVs containing a 1:1 mixture of the two dyes with a 5-fold excess of unlabeled vesicles at total lecithin concentrations of 7.5 mM (▲) and 1.5 mM (□). The dye concentration was constant at 0.50 mol % each NBD-PE and Rho-PE.

single phase (i.e., where micelles and/or multilamellar structures do not coexist). Decreasing the amount of unlabeled SLUVs added (2-fold excess), and maintaining the same concentration of total lipid, has little effect on the rate of dye exchange at room temperature (data not shown) or the extent of exchange (no more than 70%). The one quantity that does alter the rate of fluorescent probe dilution between labeled and unlabeled SLUV populations is the total PC concentration. Figure 8 compares dye dilution for SLUVs at 7.5 and 1.5 mM total phospholipid. Higher lipid concentrations (e.g., 25 mM total phospholipid) decrease the rate of dye exchange even further. Thus, the rate of external leaflet dye equilibration between cosolubilized labeled SLUVs and unlabeled SLUVs is independent of increasing the relative amount of short-chain PC and depends solely on total lipid. This suggests that exchange of the fluorescent probe between outer leaflets of the SLUVs is facilitated by a "sub-micelle" species. Previous studies have found these fluorescent probes to be nonexchangeable (Struck et al., 1981; Hoekstra, 1982; Kumar et al., 1982). However, none of the systems investigated contained a species with significant (i.e., greater than 1 μM) monomer concentrations or the tendency to form micelles. SLUVs do not contain much monomer; estimates are <100 μM in a 25 mM total phospholipid mixture in the temperature range 20–45 °C as detected by hemolysis assays (Roberts & Gabriel, 1988; Riedy et al., 1989), but the limits are well above what would coexist with long-chain phospholipid vesicles at the same concentration. It is probable that the short-chain PC in gel-state SLUVs is responsible for dye dilution in the RET assays by forming a transient complex with the dye molecule, dissociating into the aqueous phase, and reinserting into another SLUV. At higher total lipid concentrations, there is less monomeric short-chain PC compared to the amount of other lipid species in the SLUV bilayer. These observations are also consistent with the fact that SLUVs solubilize crystalline cholesterol in amounts and at rates similar to what is observed with the pure short-chain PC micelles (Roberts & Gabriel, 1988; Burns & Roberts, 1981).

If fluorescent labeled diheptanoyl-PC/dipalmitoyl-PC (5 mM:20 mM) SLUVs are mixed with unlabeled vesicles, immediately heated to 45 °C for a few minutes, and then cooled back down to 25 °C, the NBD-PE fluorescence intensity relative to Rho-PE increases to a value representing 100% separation of the two probes. Simple aggregation would be expected to involve only the probes in the outer monolayer as observed in the short-chain PC-mediated probe exchange at 25 °C (which only reaches 70% maximum). The same experiment with diheptanoyl-PC/POPC SLUVs shows at most 70% maximum dye dilution after heating to 45 °C and allowing the sample to remain at room temperature for 1–2 h. Heating the SLUVs above the T_m of the long chain-PC results in a major reorganization of the vesicles such that the probes on the inner leaflet of the vesicle are exchanged. Therefore, the increase in temperature does not merely increase the rate of probe exchange but must induce fusion of the vesicles. This 100% probe randomization at 45 °C is not observed for diheptanoyl-PC/dipalmitoyl-PC SLUVs at low concentrations (2.5 mM) and is consistent with the QLS results, indicating that the fusion process is lipid concentration dependent.

Fluorescence of Aqueous Components. During the T_m-induced particle growth of SLUVs, phospholipids in the inner leaflet of the bilayer are randomized over the entire vesicle population, implying that the gel-state SLUVs fuse when the long-chain PC melts. If fusion occurs, the internal aqueous components may leak out. Previous experiments with encapsulated carboxyfluorescein were always carried out with SLUVs made and observed at a given temperature (Gabriel & Roberts, 1984; Roberts & Gabriel, 1988). Therefore, no changes were detected in the fluorescence intensity of that water-soluble dye. If diheptanoyl-PC (5 mM)/dipalmitoyl-PC (20 mM) SLUVs containing carboxyfluorescein (100 mM) are incubated for 5 min at 45 °C and then cooled to 25 °C, there is a 5.1-fold increase in the dye fluorescence intensity, indicating that during the T_m-induced size change entrapped aqueous material leaks out and equilibrates with the buffer. A repeat of this experiment at lower total PC concentration (2.5 mM) where the vesicles were prepared by dilution of the concentrated SLUVs with the CF already entrapped results in no dye leakage when the vesicles are heated above T_m. Under these conditions, the vesicle size and bilayer composition are the same as the original 10-fold concentrated 4:1 mixture. While the loss of CF could be caused by membrane destabilization in an aggregate of many vesicles, in light of the RET results, it is consistent with SLUV fusion as the long-chain PC enters a liquid-crystalline phase.

^{31}P NMR Investigations of SLUVs. In previous work (at 40.5 MHz), it was shown that gel-state SLUVs give rise to a phosphorus resonance with a line width consistent with small vesicles (Roberts & Gabriel, 1988). If SLUVs are observed at a higher field (121.4 MHz), two separate overlapping resonances separated by 0.26 ppm at 25 °C are detected in the ^{31}P NMR spectrum. The sharper downfield resonance (80-Hz width at half-height) has a chemical shift consistent with an environment similar to micellar short-chain PC and is assigned to the short-chain PC. The broader component (470-Hz line width) presumably belongs to the gellike long-chain phospholipid (Figure 9A and insert). (These two distinct resonances do not belong to interior versus exterior leaflet phospholipid, since the ratio of the downfield component depends on the amount of short-chain PC added.) Above the

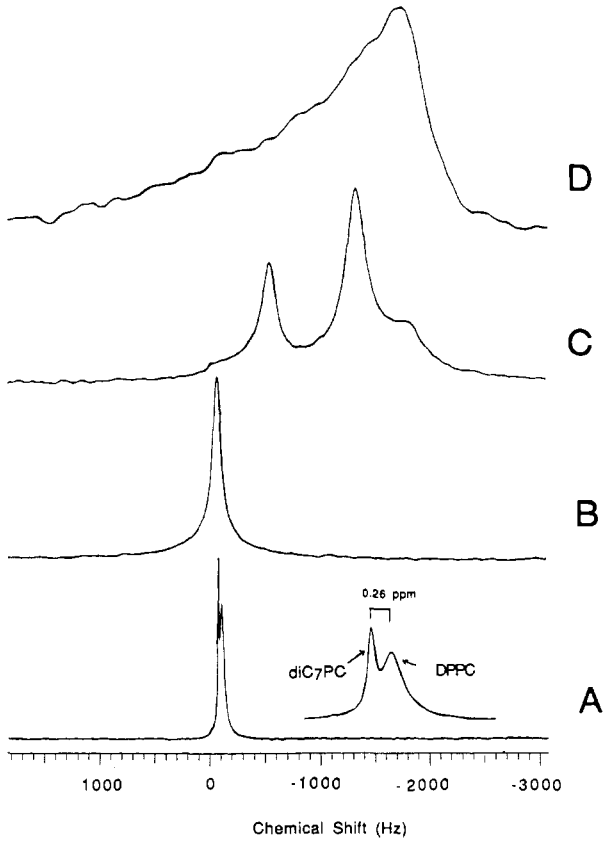

FIGURE 9: ^{31}P NMR spectra at 121.4 MHz of (A) 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC SLUVs at 27 °C, (B) large unilamellar vesicles of dipalmitoyl-PC (20 mM) at 43 °C, (C) the SLUVs in (A) incubated at 43 °C, and (D) multilamellar suspensions of dipalmitoyl-PC at 43 °C. Spectral parameters are described in the text with 2850 transients for (A) and a line broadening of 4 Hz, 6277 transients for (B) and 8358 transients for (C), both of which were processed with a line broadening of 40 Hz, and 20000 transients for (D), which was processed with a line broadening of 100 Hz. The inset in (A) shows the two distinct phosphorus resonances in gel-state SLUVs and assignments.

dipalmitoyl-PC T_m, the SLUV ^{31}P NMR spectrum (5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC at 43 °C) is dramatically different, showing considerable heterogeneity in structures (Figure 9C). For comparison, the spectrum observed for large unilamellar vesicles (R_H ~1000 Å) of dipalmitoyl-PC at 43 °C is shown (Figure 9B). This species has a line width of about 100 Hz. The typical powder pattern for liquid-crystalline multilamellar dipalmitoyl-PC (an axially symmetric phosphate with an edge-to-edge splitting of 45 ppm) is also shown (Figure 9D). In the liquid-crystalline SLUVs at 43 °C, a component consistent with multilamellar material is observed, along with broad resonances whose chemical shifts (upfield from the LUV or gel-phase SLUV peaks) are consistent with large unilamellar or oligolamellar vesicles (Burnell et al., 1980). There are few, if any, small unilamellar vesicles left in solution at this point. At higher SLUV temperatures (>45 °C) and prolonged incubations, most of the phospholipid phase separates as multilamellar material and floats to the top of the NMR tube. Very little residual lipid is detected in the aqueous phase. Therefore, when $T > T_m$, SLUVs fuse into a variety of large aggregates which eventually collapse into multilamellar structures.

Discussion

Thermal Instability of Vesicles. Using QLS and fluorescence techniques, we have shown that short-chain lecithin/long-chain phospholipid vesicles (SLUVs) undergo a temperature-induced size change when the long-chain component melts from a gel state to a liquid-crystalline matrix. Vesicles grow by up to 2 orders of magnitude under this perturbation and form multilamellar as well as large oligo- and unilamellar structures as monitored by ^{31}P NMR. RET assays show that all dye-labeled lipids are rapidly mixed when SLUVs are incubated above T_m. Hence, the process represents vesicle fusion. If the mixture is cooled below the T_m of the long-chain component, the original size distribution of vesicles (R_H ~90 Å) is obtained. In this sense, it represents a "reversible" fusion process. With pure dipalmitoyl-PC small unilamellar vesicles incubated below T_m, aggregation and subsequent fusion to 700–900-Å vesicles do occur (Wong & Thompson, 1982; Lichtenberg et al., 1981; Schullery et al., 1980). The small, curved, pure dipalmitoyl-PC vesicles (<400 Å) are stable when the lipids are in a liquid-crystalline state but become unstable when the lecithin is incubated below T_m. This is the reverse of the SLUV process where the small (180-Å diameter) gel-state SLUV is the stable structure and the melting of the long-chain component destabilizes the small, highly curved vesicle so that very large structures are formed by vesicle fusion. Therefore, the thermal instability shown by SLUVs is novel in that (i) it is the liquid-crystalline state of the lipids that is unstable and (ii) the fusion size change is reversible.

SLUV Fusion Mechanism. The transition to large polydisperse structures is dependent on the phospholipid concentration and appears to be a property of any saturated gel-state PC used to form SLUVs. What property of the melted long-chain PC molecules drives that fusion? In particular, two distinct changes can occur when a long-chain PC such as dipalmitoyl-PC melts in a SLUV structure. (i) The extended (all-trans) gel-state dipalmitoyl-PC chain decreases in average length upon melting due to increased gauche isomerizations of chain segments. If a large chain mismatch is necessary for spontaneous formation and stabilization of small SLUV vesicles, then one would expect that a T_m-induced fusion would not occur with shorter long-chain species and that such gel-state SLUVs would be relatively large. The diheptanoyl-PC/dimyristoyl-PC mixture forms small vesicles below T_m and still shows fusion to large structures when the temperature is raised above T_m. Conversely, a greater chain mismatch occurs with 1-palmitoyl-2-oleoyl-PC and dihexanoyl-PC, but these components (which are both in a liquid-crystalline state) form very large vesicles (Gabriel & Roberts, 1986). This makes the chain mismatch and changes in those lengths unlikely causes of SLUV fusion. (ii) There could be a partial phase separation of diheptanoyl-PC in gel-state dipalmitoyl-PC bilayers. This "patching" of the short-chain species may help to stabilize the small, highly curved SLUVs. It may drive formation of SLUVs from multilamellar structures. When the temperature is increased above T_m, it is assumed that any partial phase separation or patching would be destroyed. The two PC species should move about randomly in the plane of the liquid-crystalline bilayer. In such a situation, the short-chain PC would be expected to have increased interactions with its long-chain neighbors and no longer serve as a "plug" for stabilizing high-curvature structures.

Physical studies have shown that the short-chain PC in the gel-state matrix has motional and conformational properties closer to a micellar species than to other pure long-chain PC bilayers (Gabriel & Roberts, 1987; Roberts & Gabriel, 1988). Furthermore, the phase characteristics of the long-chain component (as measured by DSC) are minimally altered by the presence of 20 mol % short-chain PC (Gabriel & Roberts, 1987; Roberts & Gabriel, 1988). These observations are

certainly consistent with partial phase separation of the short-chain PC in gel-state SLUVs. Because of the dramatic particle growth which occurs when the long-chain PC enters the liquid-crystalline phase, comparable physical studies to characterize the short-chain PC motion and dynamics have thus far been unsuccessful. Of the SLUV mixtures examined here, the dimyristoyl-PC/diheptanyl-PC system exhibits QLS behavior that may be related to the idea of patching of the short-chain PC. Upon heating these SLUVs, the large T_m-induced increase in size occurs over a much wider temperature range (10–15 °C) than for the other longer chain PCs (typically 2–3 °C). A much less dramatic increase in SLUV size also occurs between 10 and 20 °C (from 80–90 to 200 Å) in gel-state diheptanoyl-PC/dimyristoyl-PC SLUVs. None of the other lipids examined showed this behavior. It may reflect the fact that the 14-carbon myristoyl chains are closer in size to the 7-carbonyl heptanoyl chains. Perhaps there is less phase separation of short chain from long chain in these vesicles below their T_m. This might mean some population of the dimyristoyl-PC would melt slightly lower than 25 °C. Consistent with this is the observation that no T_m was observed in DSC studies of dimyristoyl-PC/diheptanoyl-PC SLUVs (Gabriel & Roberts, 1986). This suggests that short-chain PC patching in the gel-state matrix and then completely mixing randomly with long-chain molecules in a liquid-crystalline matrix are the driving force for the T_m-induced fusion.

SLUV Fusion and Phospholipase Kinetics. SLUVs as substrates for lipolytic enzymes have the advantageous feature of solubilizing in a bilayer state a substrate molecule which normally exists in a micellar state and is an excellent substrate (e.g., diheptanoyl-PC). This allows direct comparisons between micellar and bilayer substrate. Phospholipids oriented in bilayers are usually very poor substrates for water-soluble phospholipases. In contrast, the short-chain PC is an excellent substrate in SLUVs, yielding specific activities similar to what is observed for pure short-chain PC micelles (Gabriel & Roberts, 1987; Gabriel et al., 1987). In analyzing these kinetics, two interesting kinetic observations were made for the SLUV/water-soluble phospholipase A_2 and C systems: (i) the specific activity of both phospholipases toward the short-chain PC increases in an anomalous fashion around the T_m of the long-chain phospholipid; (ii) the total fraction of short-chain PC hydrolyzed at T_m and higher temperatures decreases (to 25–30% of the total PC with PLC). Both of these can now be understood with a detailed knowledge of the bilayer structure in this temperature regime. Around the T_m, oligo- and multilamellar structures coexist with large unilamellar vesicles. First of all, this implies that vesicle size is not relevant to enzyme action on the short-chain component of SLUVs. It suggests that the anomalous increase in enzyme specific activity at this temperature most likely reflects increased motion and accessibility of the substrate, perhaps due to the dynamics of the fusing bilayers (one can imagine the vesicles fusing, breaking apart, refusing, etc.). The second observation, the decrease in the extent of short-chain PC hydrolyzed by both phospholipases A_2 and C above T_m, is now understood as reflecting the fact that the enzymes are presented with some multilamellar structures and only a portion of the total short-chain PC is accessible. It is certainly possible that these multilamellar particles are asymmetric in the distribution of short-chain PC and preferentially solubilize this lipid in smaller layers in the multilamellar structures—this might be an effective way of sequestering them from PLC (or PLA_2) action, which hydrolyzes all of the short-chain PC in gel-state SLUVs (Gabriel et al., 1987). This correlation of SLUV physical state with lipolytic enzyme action illustrates the necessity for detailed physical characterization of lipid aggregates.

Registry No. Diheptanoyl-PC, 35387-75-8; dipalmitoyl-PC, 2644-64-6; 1-palmitoyl-2-stearoyl-PC, 10589-47-6; dimyristoyl-PC, 13699-48-4; phospholipase A_2, 9001-84-7; phospholipase C, 9001-86-9.

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