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Interaction of Short-Chain Lecithin with Long-Chain Phospholipids: Characterization of Vesicles That Form Spontaneously†

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ABSTRACT: Stable unilamellar vesicles formed spontaneously upon mixing aqueous suspensions of long-chain phospholipid (synthetic, saturated, and naturally occurring phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) with small amounts of short-chain lecithin (fatty acid chain lengths of 6-8 carbons) have been characterized by using NMR spectroscopy, negative staining electron microscopy, differential scanning calorimetry, and Fourier transform infrared (FTIR) spectroscopy. This method of vesicle preparation can produce bilayer vesicles spanning the size range 100 to greater than 1000 Å. The combination of short-chain lecithin and long-chain lecithin in its gel state at room temperature produces relatively small unilamellar vesicles, while using long-chain lecithin in its liquid-crystalline state produces large unilamellar vesicles. The length of the short-chain lecithin does not affect the size distribution of the vesicles as much as the ratio of short-chain to long-chain components. In general, additional short-chain decreases the average vesicle size. Incorporation of cholesterol can affect vesicle size, with the solubility limit of cholesterol in short-chain lecithin micelles governing any size change. If the amount of cholesterol is below the solubility limit of micellar short-chain lecithin, then the addition of cholesterol to the vesicle bilayer has no effect on the vesicle size; if more cholesterol is added, particle growth is observed. Vesicles formed with a saturated long-chain lecithin and short-chain species exhibit similar phase transition behavior and enthalpy values to small unilamellar vesicles of the pure long-chain lecithin prepared by sonication. As the size of the short-chain/long-chain vesicles decreases, the phase transition temperature decreases to temperatures observed for sonicated unilamellar vesicles. FTIR spectroscopy confirms that the incorporation of the short-chain lipid in the vesicle bilayer does not drastically alter the gauche bond conformation of the long-chain lipids (i.e., their transness in the gel state and the presence of multiple gauche bonds in the liquid-crystalline state).

Since the original demonstration by Bangham et al. (1965) that aqueous dispersions of phospholipids were capable of forming closed spherical structures encapsulating aqueous compounds, liposomes have been widely studied as models for cell membranes. Such bilayer vesicles have also been used for functional reconstitution of membrane-bound proteins [for example, see Christianson and Carlsen (1983)], as substrates for a variety of lipolytic enzymes (Kensil & Dennis, 1979; DeBose & Roberts, 1983), as permeability barriers in transport studies (Papahadjopoulos & Watkins, 1967; Paphadjopoulos & Kimelberg, 1973; Racker & Stoeckenius, 1974), and for encapsulation of various agents for assays, drug delivery, etc. (Ryman & Tyrrell, 1980). While multilamellar structures form readily, unilamellar vesicles are more analogous to real cell membranes. Several methods have been developed [for a review, see Gregoriadis (1984)] to form unilamellar vesicles from pure lecithin multibilayers, including (i) sonication, (ii) reverse evaporation from organic solvent, (iii) detergent dialysis, and (iv) pressure/mechanical filtration. All of these methods either require expensive equipment [sonicator, French press (Hamilton et al., 1980)] or depend on the addition and partial removal of nonphospholipid material (organic solvent, detergents) and hence are multistep processes. In some cases, the inclusion of sufficient amounts of phosphatidic acid with the lecithin matrix can be used to induce unilamellar vesicle formation by pH shifts (Hauser, 1983).

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We have previously reported (Gabriel & Roberts, 1984) that unilamellar vesicles form spontaneously upon mixing aqueous suspensions of long-chain lecithins (fatty acid chain lengths of 14 carbons or longer) with small amounts of micellar synthetic short-chain lecithins (fatty acid chain lengths of 6–8 carbons). These short-chain/long-chain unilamellar vesicles (SLUVs)[1] are impermeable to ions and fluorescent dyes and appear to be stable (under some conditions) for several weeks. The present study was undertaken to determine (i) the extent of spontaneous vesicle formation from a wide variety of long-chain phospholipids (differing head groups and unsaturation as well as chain length), (ii) whether the size of the vesicles may be regulated by altering the identity of phospholipid components and/or the ratio of the short-chain and long-chain lipids, and (iii) the motional behavior of the long-chain species in SLUVs. An examination of different vesicle preparations should lead to an understanding of what causes these vesicles to form spontaneously without the addition of exogenous material or severe physical manipulations to break the forces present in the starting multibilayer structures. This knowledge, in turn, may be useful in understanding the dynamics of membrane budding in cells (e.g., endocytosis and exocytosis).

Solubilization of multibilayers by the addition of small amounts of short-chain lecithin can be evaluated by ^{31}P NMR spectroscopy. Multibilayers and large vesicles give rise to characteristically broad asymmetrical line shapes reflecting restricted anisotropic motion of the lipid molecules (Burnell et al., 1980), while smaller vesicles exhibit a narrow symmetrical resonance whose line width is related to vesicle size and the lateral diffusion of the component phospholipids. The presence of vesicles as opposed to micelles can be confirmed by ^{1}H NMR spectroscopy lanthanide shift experiments: lanthanides added to a solution of closed, relatively impermeable bilayer vesicles will shift only those nuclei exposed on the outer monolayer. Many techniques (electron microscopy, light scattering, analytical ultracentrifugation, NMR) can be used to size particles, but most require a homogeneous size distribution, as well as a defined shape. Negative staining electron microscopy was chosen to size these vesicles, since it provides visual evidence for vesicle formation and is particularly effective for heterogeneous samples. Any dramatic temperature-induced size changes can be monitored by ^{31}P NMR spectroscopy. Since the short-chain lipid does not exhibit any phase transition behavior, DSC can be used to examine how the presence of the short-chain lecithin affects the transition temperature and heat of enthalpy of the long-chain component. Finally, FTIR spectroscopy can be used to examine more closely the conformation of the long acyl chain components in these vesicles.

Materials and Methods

Chemicals. Dihexanoyl-PC,[1] diheptanoyl-PC, dioctanoyl-PC, and distearoyl-PC were obtained from Avanti-Polar Lipids, Inc. Dipalmitoyl-PC, dimyristoyl-PC, dipalmitoyl-PE, *N*-methyldipalmitoyl-PE, and *N,N*-dimethyldipalmitoyl-PE were obtained from Calbiochem Behring Corp. Egg-PC and sphingomyelin (bovine) were obtained from Sigma. Phospholipids were checked for purity by thin-layer chromatography (Burns & Roberts, 1980) and, if pure, were used without any further purification. If any lysophospholipids were detected, the phospholipid was chromatographed on silicic acid before use.

Vesicle Preparation. Vesicles were usually prepared by cosolubilizing both long-chain and short-chain phospholipids in $CHCl_3$, removing organic solvent under a stream of N_2, and then evacuating all remaining traces of solvent at low pressure for at least 12 h. Samples were hydrated in the appropriate solvent containing 0.15 M NaCl, bath sonicated (Electromations Ultrasonic Cleaner Model 250; input power, 40 W) for 1 min (this aided in dispersal of material on the side of the tube and did not provide sufficient power to form unilamellar vesicles of pure long-chain phospholipid), and equilibrated at room temperature for 6–8 h. The pH was adjusted if necessary to be within the range of 6.5–7.5. Dioctanoyl-PC micelles phase separate at low concentrations, and this behavior is often suppressed by the addition of 0.2 M KSCN or 0.2 M LiI (Tausk et al., 1974). Therefore, vesicles containing dioctanoyl-PC as the short-chain lipid were hydrated in the presence of 0.2 M KSCN. Vesicles prepared by this cosolubilization method appear equivalent to the vesicles formed by sequentially adding micellar short-chain lecithin to aqueous multibilayers of long-chain phospholipid (Gabriel & Roberts, 1984).

For DSC experiments small unilamellar vesicles of dipalmitoyl-PC or distearoyl-PC (controls for comparison to SLUVs) were made by sonifying with a Branson Model 350 cell disruptor, power input level 2, for 5 min.

Electron Microscopy. Vesicle samples used for EM were hydrated in 0.15 M NaCl or 0.2 M KSCN phosphate-buffered solutions, pH 7.0. Dilutions were made from a stock of 5 mM short-chain lecithin/20 mM long-chain phospholipid vesicle sample after at least an 8-h equilibration period. In order to obtain electron micrographs of preparations above room temperature, samples were heated in a 45 °C water bath during the equilibration period and subsequently stained with warm staining solution (2% uranyl acetate on carbon-coated 300-mesh grids). The final temperature for such samples was 35–40 °C. T4 bacteriophage, with tail length of 1000 Å, was used as an internal size standard. Pictures were taken at 80 kV on a Jeol 100B transmission electron microscope. For determination of the size distribution in each plate, at least 50 particles were measured on an 8 × 10 in. print.

NMR Spectroscopy. 500-MHz ^{1}H NMR spectra were obtained on a home-built spectrometer at the Francis Bitter National Magnet Laboratory, MIT. Twenty transients with a 90° flip angle (15 μs) and a 5-s repetition time were collected and transformed with a 1.0-Hz exponential weighting function. Lanthanide shift experiments were done by adding microliter amounts of a 10 mM Pr^{3+} stock solution to 0.3-mL samples at pH 6.5–7. 109.3-MHz ^{31}P NMR spectra were obtained on a Bruker 270 spectrometer from 25 to 45 °C. Three hundred or one thousand transients with a 30° flip angle (20 μs) and a 0.5-s repetition time were collected with 4K data points and transformed with a 5.0-Hz exponential weighting function. To detect multilamellar and large structures, ^{31}P NMR spectra were also obtained at 145.8 MHz with a Nicolet/Cryomagnet Systems 360 wide-bore spectrometer. Spectral parameters include a 90° pulse angle (14 μs), 2-s delay between pulses, 20 000-Hz sweep width, ^{1}H noise decoupling, and 40-Hz line broadening. Samples were prepared in 10 mM Mops buffer, pH 8.0, for ^{31}P NMR experiments.

Differential Scanning Calorimetry. Calorimetric studies were carried out on a Perkin-Elmer DSC-2D/TADS SYS-

[1] Abbreviations: SLUVs, short-chain/long-chain unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; diacyl-PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; Mops, 3-(*N*-morpholino)propanesulfonic acid; SPM, sphingomyelin; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; EM, electron microscopy.

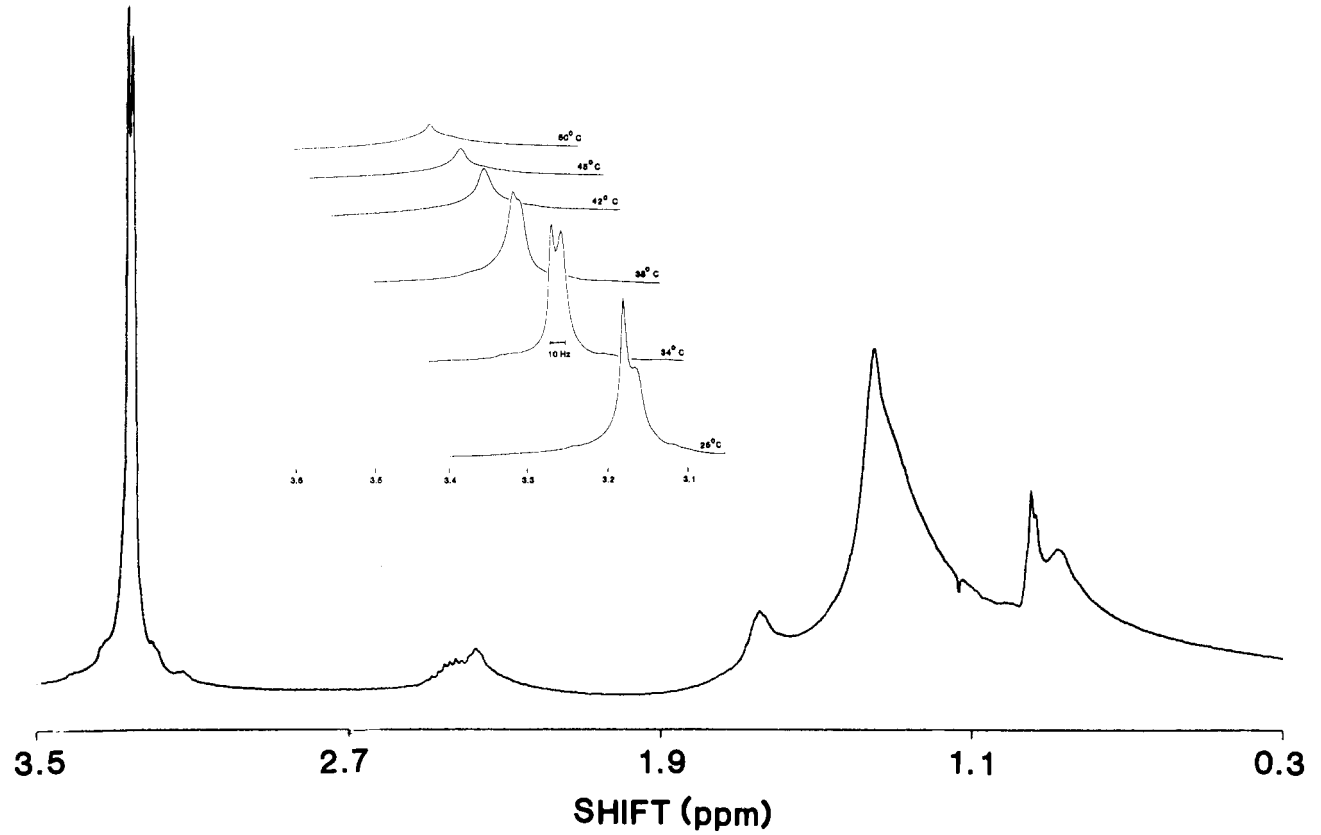

FIGURE 1: 500-MHz ^{1}H NMR spectrum of diheptanoyl-PC/dipalmitoyl-PC (5 mM:20 mM) at 34 °C; (insert) *N*-methylcholine region as a function of temperature.

TEM differential scanning calorimeter with a heating or cooling rate of 1 K/min. The instrument was calibrated with indium standards. Sixty microliters of sample was used along with the appropriate solvent in the reference pan. Three runs were performed on each sample: heating, cooling, and reheating. The transition temperature was considered to be the maximum of the calorimetric curve.

Fourier Transform Infrared Spectroscopy. Samples (25 mM in total phospholipid) were examined in a Harrick cell (path length, 10–25 μm) equipped with CaF_2 windows. Spectra were recorded on a Mattson Instruments Sirius 100 spectrometer equipped with a liquid nitrogen cooled mercury–cadmium–telluride detector. Routinely, 100 interferograms were collected, coadded, apodized with a triangular function, and Fourier transformed to give a resolution of 4 cm^{-1} with data encoded every 2 cm^{-1}. Temperature was controlled with a Haake circulating bath and monitored with a Bailey BAT-12 digital thermometer, whose thermocouple sensor was placed close to the IR windows in the cell.

RESULTS

Solubilization of Phospholipid Multibilayers. As reported previously (Gabriel & Roberts, 1984), vesicle formation occurs spontaneously upon mixing 5 mM short-chain (dihexanoyl-, diheptanoyl-, or dioctanoyl-) and 20 mM long-chain (dipalmitoyl-) phosphatidylcholine. The presence of vesicles is detected by the observation of high-resolution ^{1}H NMR spectra from a mixture of gel-state dipalmitoyl-PC and diheptanoyl-PC as shown in Figure 1. This mixture is only slightly opaque and hence most of the particles must be small- to medium-sized vesicles. If considerably more long-chain lecithin is present, the solution is quite cloudy. To understand how short-chain species cause spontaneous formation of vesicles from multilamellar structures, we need to know if the solubilization process decreases the size of all aggregates to intermediate-size vesicles and if the size depends on the ratio of short-to-long-chain lecithins or if excess multilamellar structures are in equilibrium with smaller vesicles (implying a specific ratio of short-to-long-chain lecithin is necessary to induce vesicle formation).

^{31}P NMR spectroscopy can be used to detect residual multilamellar aggregates in these vesicle preparations. For very large vesicles (>10 000 Å) or multibilayers, the ^{31}P resonance gives rise to an asymmetic pattern whose edge to edge width (chemical shift anisotropy) and shape are indicative of phospholipid phase state or local order around the head group and polymorphism (bilayer vs. hexagonal II) (Seelig, 1978; Cullis & de Kruijff, 1979). Figure 2A,B shows 145.8-MHz ^{31}P NMR spectra for dipalmitoyl-PC/diheptanoyl-PC (40 mM:5 mM); this amount of short-chain lecithin does not completely clarify the solution. At 25 °C (Figure 2A) the ^{31}P NMR spectrum of dipalmitoyl-PC/diheptanoyl-PC (40 mM:5 mM) is easily seen to consist of two slow-exchange components: an isotropic central resonance and a broad pattern whose chemical shift anisotropy is characteristic of gel-state phospholipids (Δ = 65.5 ppm edge to edge). As the temperature of the mixture is raised to 46 °C, the powder pattern narrows dramatically (Δ = 45 ppm edge to edge), indicating that the lipids in multibilayers are now in a liquid-crystalline state. Lecithin mixtures with 20 mM dipalmitoyl-PC/5 mM diheptanoyl-PC exhibit only the isotropic resonance. This behavior suggests that diheptanoyl-PC solubilizes multilamellar aggregates into discrete small particles (presumably vesicles) at a defined ratio of components and that excess long-chain phospholipid is still present in large multibilayer structures which may have some small amount of short-chain lecithin intercalated. The size distribution also depends on the identity and ratio of the two phospholipids. For

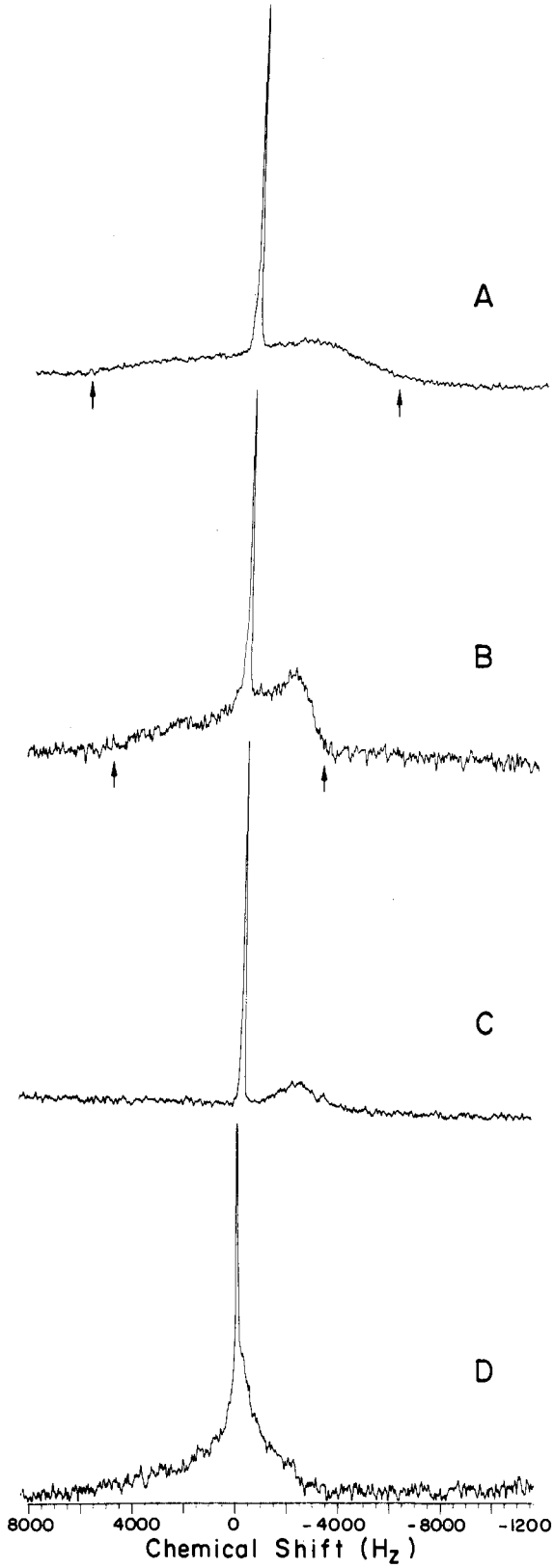

FIGURE 2: 145.8-MHz ^{31}P NMR spectra of diheptanoyl-PC/dipalmitoyl-PC (5 mM:40 mM) at (A) 21 and (B) 46 °C, (C) diheptanoyl-PC/distearoyl-PC (10 mM:20 mM) at 21 °C, and (D) diheptanoyl-PC/sphingomyelin (5 mM:20 mM) at 46 °C.

example, the ^{31}P NMR spectrum of 20 mM distearoyl-PC/10 mM diheptanoyl-PC at 25 °C (Figure 2C) shows an intense narrow component and a shifted broad component whose shape corresponds to intermediate-size vesicles (Burnell et al., 1980).

The isotropic ^{31}P component for many short-chain lecithin/long-chain phospholipid mixtures exhibits a complex temperature profile (Figure 3). With 20 mM dipalmitoyl-PC/5 mM diheptanoyl-PC, two discrete phospholipid resonances are observed at low temperature (this may reflect chemical shift differences for inner vs. outer monolayer phospholipid head groups or a difference in long-chain vs. short-chain lecithin). The observed intensity is consistent with >90% of the total phospholipid observable. As the temperature is increased toward the melting temperature for pure dipalmitoyl-PC, the two peaks coalesce and narrow. With a further increase in temperature (45 °C), the phosphorus resonance broadens dramatically. The inital decrease in ^{31}P line widths presumably reflects increased translational diffusion of dipalmitoyl-PC molecules as they change from a gel-like to a liquid-crystalline state (Cullis, 1976). (Cullis, 1976). The abrupt increase in line width significantly above T_m is very suggestive of vesicle aggregation. This is often detected by increased sample turbidity.

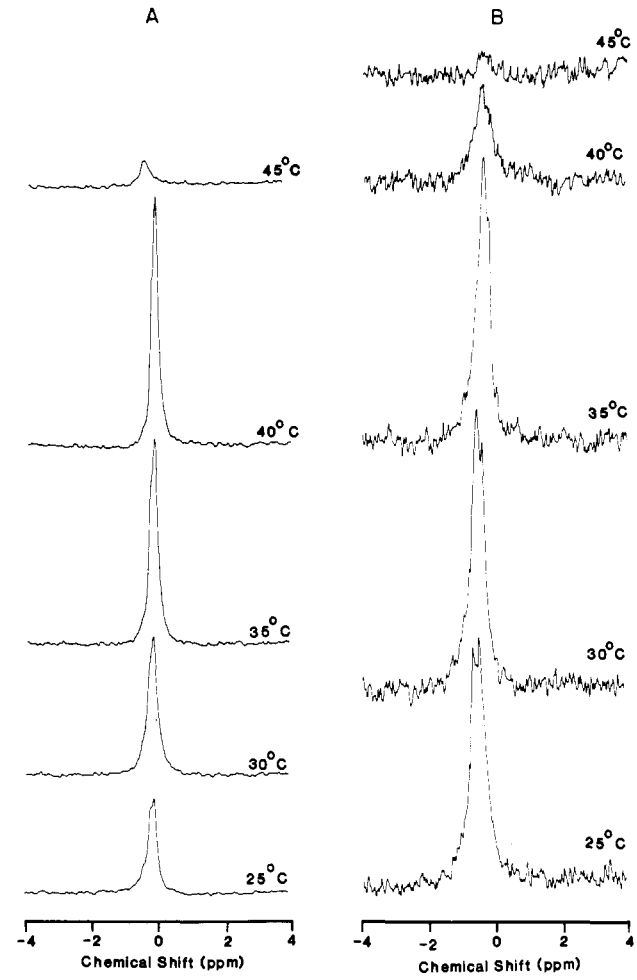

FIGURE 3: 109.3-MHz ^{31}P NMR spectra of (A) diheptanoyl-PC/dipalmitoyl-PC (5 mM:20 mM) and (B) diheptanoyl-PC/sphingomyelin (5 mM:20 mM) as a function of temperature.

Sphingomyelin/diheptanoyl-PC (20 mM:5 mM) mixtures show similar behavior. At low temperature this mixture has only an isotropic component; close inspection shows two well-resolved ^{31}P peaks (one for the short-chain lecithin, the other for the sphingomyelin molecule). Bovine brain sphingomyelin does exhibit a definable phase transition at ~35 °C (Shinitzky & Barenholz, 1974). As the sample temperature is raised from 25 °C, the chemical shift difference of the two ^{31}P resonances decreases and the lines narrow slightly. At 40 °C the phosphorus peak broadens, and a very broad chemically shifted component is distinguishable (Figure 3B). This indicates a size change must occur with the initial sphingo-

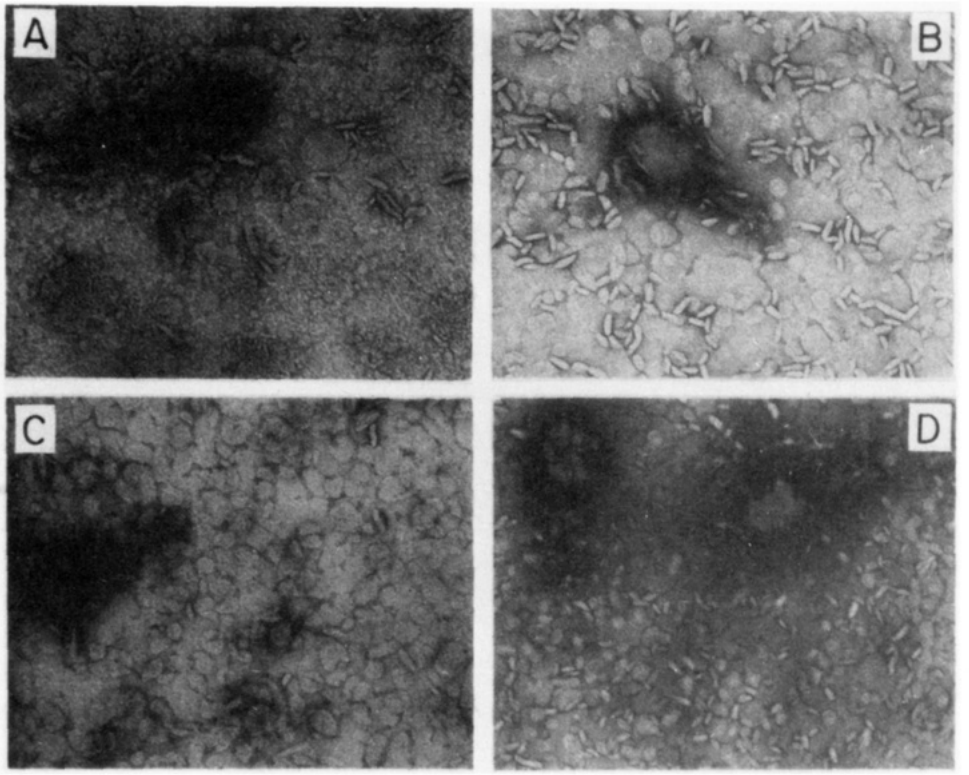

FIGURE 4: Negative stain electron micrographs of vesicles with dipalmitoyl-PC (20 mM) as the long-chain component and various short-chain lecithins (5 mM): (A) dihexanoyl-PC, (B) diheptanoyl-PC, and (C) dioctanoyl-PC with 0.2 M KSCN added, all prepared and equilibrated at 25 °C; and (D) diheptanoyl-PC, prepared and warmed to 35–40 °C. T4 bacteriophage, with a tail length of 1000 Å, was added an internal standard.

myelin/diheptanoyl-PC structures to form large aggregates. The ^{31}P NMR spectrum of this mixture at elevated temperatures is suggestive of large bilayer structures (Figure 2D).

Vesicle Sizing by Electron Microscopy. ^{31}P NMR studies show large multibilayers and relatively small aggregates that give rise to "isotropic" spectra. These could be unilamellar vesicles or micelles. Micelles are not detected as discrete, well-defined particles by electron microscopy; only dust and the phage used as a sizing standard are detected in the electron micrograph of 10 mM diheptanoyl-PC. Therefore, an extensive electron microscopy study of various short- and long-chain lipid combinations was undertaken to prove directly the existence of vesicles and to determine the size and polydispersity of the particles. Results are summarized in Table I. Depending on the identity of the lipids, unilamellar vesicles can be formed with diameters of 150–1000 Å. As is shown in Figure 4, there is no clear trend in size or polydispersity of vesicles formed from a given long-chain component (i.e., dipalmitoyl-PC) as the chain length of the short-chain component is lengthened. The size of SLUVs formed from dipalmitoyl-PC and various short-chain species ranges from diameters of 170 Å to diameters of 400 Å at room temperature and remains essentially unaltered as the mixtures are heated slightly above the phase transition temperature of the long-chain lecithin. When vesicles made from 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC were diluted by factors of 2 and 5, their diameter increased from 170 ± 50 to 320 ± 100 Å and 330 ± 110 Å, respectively, presumably because short-chain lecithin will be depleted from vesicles and equilibrate at the intermicelle concentration in the larger volume. This is of particular relevance for potential in vivo studies, since it is well documented that short-chain lecithin alone exhibits hemolytic properties (Reman et al., 1969; Kitagawa et al., 1977).

The phase transition temperature of the long-chain lipid component seems to govern whether small unilamellar vesicles

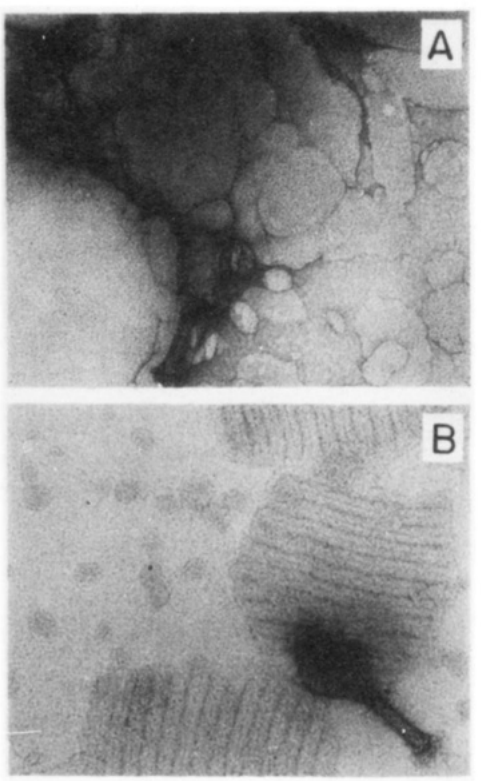

FIGURE 5: Negative stain electron micrographs of vesicles made by using diheptanoyl-PC (5 mM) as the short-chain component and (A) egg-PC (20 mM) or (B) dimyristoyl-PC (20 mM) as the long-chain component.

or large unilamellar vesicles are formed at room temperature. Vesicles made from 20 mM dipalmitoyl-PC or distearoyl-PC (in the gel state) and 5 mM diheptanoyl-PC are relatively small, ~165 Å diameter, as compared to vesicles made from

Table I: Vesicle Sizes Determined from Electron Micrographs of Short-Chain Lecithin/Long-Chain Phospholipid Mixtures

long-chain phospholipid[a]	short-chain lecithin[b]	diameter[c] (Å)
$diC_{16}PC$	diC_6PC	230 ± 100 (400 ± 150)[d]
$diC_{16}PC$	diC_7PC	170 ± 50 (200 ± 60)[d]
$diC_{16}PC$	diC_8PC[e]	360 ± 140 (200 ± 50)[d]
$diC_{14}PC$	diC_7PC	280 ± 50; lge
$diC_{16}PC$	diC_7PC	170 ± 50
$diC_{18}PC$	diC_7PC	160 ± 50
egg-PC	diC_7PC	830 ± 850
$diC_{14}PC$	diC_7PC (5 mM)	280 ± 50; lge
$diC_{14}PC$	diC_7PC (10 mM)	420 ± 340
$diC_{14}PC$	diC_7PC (15 mM)	390 ± 130
$diC_{18}PC$	diC_7PC (2.5 mM)	270 ± 60
$diC_{18}PC$	diC_7PC (5.0 mM)	160 ± 50
$diC_{18}PC$	diC_7PC (7.5 mM)	300 ± 40; 100 ± 30
$diC_{18}PC$	diC_7PC (10 mM)	100 ± 40
$diC_{16}PC$	diC_7PC	170 ± 50
$diC_{16}PE$	diC_7PC	300 ± 70; lge
N-(CH_3)-$diC_{16}PE$	diC_7PC	750 ± 300
N,N-$(CH_3)_2$-$diC_{16}PE$	diC_7PC	320 ± 90
SPM	diC_7PC	150 ± 70
$diC_{16}PC$	diC_7PC	170 ± 50
$diC_{16}PC$ + cholesterol (1 mM)	diC_7PC	310 ± 60
$diC_{16}PC$	diC_8PC (10 mM)[e]	250 ± 60
$diC_{16}PC$ + cholesterol (1 mM)	diC_8PC (10 mM)[e]	260 ± 100

[a] Long-chain phospholipid concentration is 20 mM; abbreviations used include the following: $diC_{16}PC$, dipalmitoyl-PC; $diC_{14}PC$, dimyristoyl-PC; $diC_{18}PC$, distearoyl-PC; $diC_{16}PE$, dipalmitoyl-PE; SPM, sphingomyelin. [b] Short-chain lecithin concentration is 5 mM unless otherwise indicated (value in parentheses); abbreviations: diC_6PC, dihexanoyl-PC; diC_7PC, diheptanoyl-PC; diC_8PC, dioctanoyl-PC. [c] Average diameter was determined by measuring segments of plates with phage tail as size standard; the deviation for each measured vesicle mixture is shown; "lge" denotes large multilamellar structures. [d] Values in parentheses represent average sizes for vesicles in solutions heated to 45 °C and stained with warm fixant. [e] 0.2 M KSCN is included in the hydration buffer to suppress phase separation of dioctanoyl-PC (Tausk et al., 1974).

dimyristoyl-PC or egg-PC (in the liquid-crystalline state) and diheptanoyl-PC, at room temperature (Figure 5). As the percentage of short-chain lipid is increased, the average size of the vesicles remains relatively constant for vesicles made with dimyristoyl-PC, while the polydispersity decreases—perhaps indicating more uniform mixing of the short-chain lipid with long-chain multibilayers. On the other hand, medium-size vesicles made from gel-state distearoyl-PC decrease in diameter from 300- to 100-Å particles as the amount of short-chain is increased while a small polydispersity is maintained. It must be noted that aggregates as small as 100 Å may not be vesicles, and confirmation by another physical method is necessary. If these particles are micellar rather than bilayer in nature, they must have unusual characteristics, since micelles are not usually detected in electron micrographs. If these small particles are bilayers, they are highly curved (even more so than conventional small unilamellar vesicles prepared by sonication or detergent dialysis) with the bulk of the phospholipid on the exterior layer.

Vesicle structures have also been detected by EM with diheptanoyl-PC as the short-chain component and phospholipids with other head groups or backbone structures as the long-chain component (phosphatidylethanolamine, *N*-methylphosphatidylethanolamine, *N,N*-dimethylphosphatidylethanolamine and sphingomyelin). Aggregates formed with ethanolamine derivatives are quite polydisperse with some very large structures and some smaller (presumably)

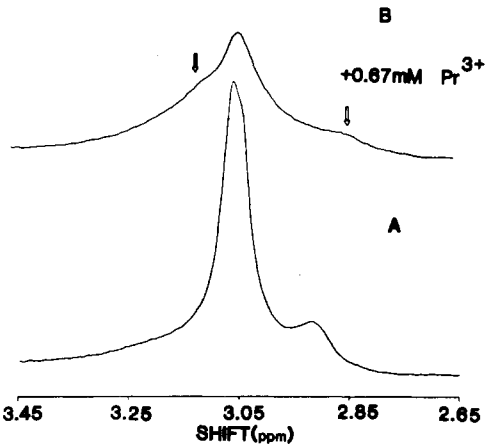

FIGURE 6: 500-MHz ^1H NMR spectra of the *N*-methyl region, illustrating the effect of added lanthanide on distearoyl-PC/diheptanoyl-PC (20 mM:10 mM) at 25 °C: (A) no lanthanide added and (B) 0.67 mM Pr^{3+} added.

unilamellar vesicles of about 300 Å in diameter. Visual examination of these solutions can be used to roughly estimate the size range of vesicles. The clearer solutions on average contain smaller diameter vesicles [in order of decreasing size: egg-PC ~ PE > *N*-(CH_3)-PE > *N,N*-$(CH_3)_2$-PE > PC ~ SPM].

Cholesterol is a component often included in liposomes/vesicles to decrease bilayer permeability. Sonicated vesicles increase in size as the cholesterol content increases (Stockton et al., 1976). In view of this, the influence of cholesterol on the size of short-chain lecithin/dipalmitoyl-PC containing vesicles was examined. Short-chain lecithins alone can form mixed micelles with cholesterol. Dioctanoyl-PC can solubilize up to 18 mol % cholesterol and diheptanoyl-PC up to 10 mol % (Burns & Roberts, 1981). If cholesterol, 10 mol % of the short-chain lipid, is added to a dioctanoyl-PC/dipalmitoyl-PC mixture, the resulting vesicles are unaltered in size from the original SLUVs. This amount of cholesterol is well below the solubilization limit of dioctanoyl-PC micelles. If cholesterol, 20 mol % of the short-chain lipid, is added to diheptanoyl-PC/dipalmitoyl-PC mixtures, significant growth of the vesicle population is observed. This is about twice the amount of cholesterol that diheptanoyl-PC alone can solubilize. It appears that the growth of vesicles containing cholesterol is related to the cholesterol capacity of the short-chain component. If the amount is below the saturation limit of the short-chain lipid, no particle growth is observed, but if the amount of cholesterol is above the solubility limit of the short-chain lipid alone, then vesicle growth is observed.

^1H NMR Detection of Bilayer Vesicles. Lanthanide shift reagents can be used to distinguish between the types of lipid particles detectable on the NMR time scale (monomers, micelles, and vesicles). It has been well documented that Pr^{3+} added to a population of vesicles complexes with the phospholipid head group and shifts resonances from exterior molecules downfield without appreciably affecting resonances from the interior lecithin molecules. If only monomer or micellar aggregates are present, all the phospholipid head groups will be exposed to lanthanide cations, and all the resonances would shift. ^1H NMR (500-MHz) lanthanide shift titrations were performed with most of the vesicle preparations studied by electron microscopy (especially those that had diameters of <150 Å). For example, Figure 6 shows the $N(CH_3)_3$ region for 10 mM diheptanoyl-PC/20 mM distearoyl-PC (A) without lanthanide at 25 °C and (B) with the addition of Pr^{3+}. As stated earlier, these particles average

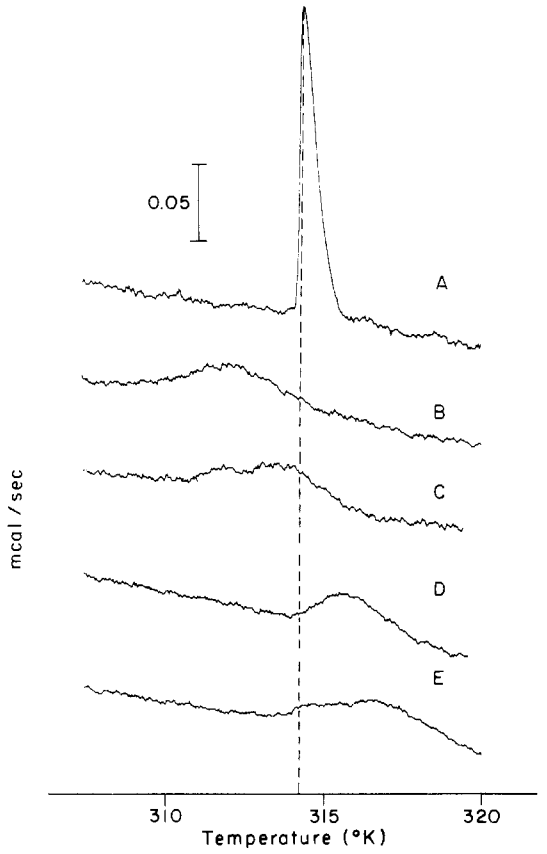

FIGURE 7: Initial-heating DSC thermograms of dipalmitoyl-PC (20 mM) (A) as multibilayers, (B) as sonicated vesicles, (C) in the presence of dioctanoyl-PC (5 mM) and 0.2 M KSCN, (D) with diheptanoyl-PC (5 mM), and (E) with dihexanoyl-PC (5 mM).

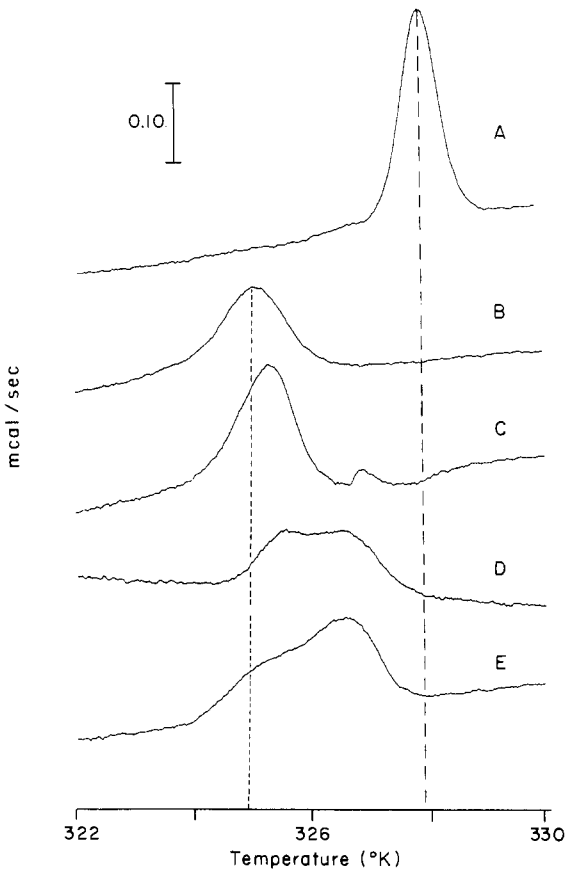

FIGURE 8: Initial-heating DSC thermograms of distearoyl-PC (20 mM) (A) multibilayers and vesicles prepared with decreasing concentrations of diheptanoyl-PC: (B) 10, (C) 7.5, (D) 5, and (E) 2.5 mM.

100 Å in diameter and may not be vesicular, or they may consist of a mixture of vesicle and micelle particles. Without lanthanide there are at least two $N(CH_3)_3$ resonances visible. The sharp component is presumably the short-chain lecithin diheptanoyl-PC, and the broad upfield component is probably distearoyl-PC on the basis of assignments made previously (Gabriel & Roberts, 1984). With the addition of Pr^{3+}, some *N*-methyl intensity shifts downfield and is broadened, while significant intensity at the upfield broad component and sharp short-chain resonance remains unshifted. The broad downfield resonance is from lipid exposed to Pr^{3+} and hence located on the exterior monolayer of the vesicle. The narrower invariant resonance is probably from the short-chain lipid, because (Gabriel & Roberts, 1984) the short-chain component had a narrower line width when vesicles made from proteo short-chain and $N(CD_3)_3$ long-chain lecithin were examined. The *N*-methyl protons of short-chain lecithin micelles are not shifted by these concentrations of Pr^{3+}. Therefore, we cannot use the lack of Pr^{3+}-induced shift as an indication that most of the short-chain lecithin is on the inner monolayer of the vesicle. The third peak is most likely due to distearoyl-PC on the interior monolayer. The observation temperature is considerably less than the T_m of distearoyl-PC. The *N*-methyl line width for this gel-state lecithin is expected to be broad; hence we may only be detecting part of this component (as is the case for ^{31}P NMR spectra focusing on the narrow isotropic component). Similar results were obtained for aggregates made from 5 mM diheptanoyl-PC/20 mM sphingomyelin and other mixtures (data not shown), indicating the presence of a reasonable population of vesicles. Thus, mixing short-chain and long-chain phospholipids forms vesicles covering a wide variety of sizes.

Phase Behavior of SLUVs. Electron microscopy and NMR lanthanide shift experiments have indicated that the phenomenon of spontaneous vesicle formation is universal when short-chain lecithin is mixed with long-chain phospholipids. The question arises as to how the presence of the short-chain component in the bilayer affects the thermodynamic properties of the long-chain component. Whereas in the NMR experiments both short- and long-chain species have distinct spectral features that can often overlap, the DSC experiments follow only the long-chain species.

Some typical calorimetric traces of multibilayers and spontaneous vesicles are shown in Figures 7 and 8. In general, no pretransition temperatures were detected for any of the aggregates examined. The transitions that occurred upon cooling a heated sample were at least 1 K lower than the T_m values observed in the initial heating runs, while the second heating curves were usually identical with the first run. This behavior has been observed for multibilayer samples (Ruocco et al., 1985). As shown in Figure 7A,B, transforming multibilayers into small unilamellar vesicles by sonication lowers the enthalpy, indicated by the area under the transition curve (Mabrey & Sturtevant, 1978). Traces from a dipalmitoyl-PC/short-chain lecithin series are shown in Figure 7. The phase transition temperature is similar to that for unilamellar vesicles prepared by sonication. The width of the transition at base line and enthalpy are also comparable to that observed for vesicles prepared by sonication for 5 min. Sometimes two transitions are observed, which indicates that there are two environments for long-chain lipid in the aggregates. Several explanations are possible: (i) one transition could be due to the dipalmitoyl-PC on the interior monolayer of the vesicle and another from the exterior dipalmitoyl-PC, (ii) the di-

Table II: Effect of Short-Chain Lecithin on Phase Behavior of Saturated Long-Chain Lecithins As Monitored by DSC

long-chain PC[a]	short-chain PC[b]	T_m[c] (K)	T_m at base line (K)	$T_m - T_m^0$[d] (K)
$diC_{14}PC$		297.0	1.8	
$diC_{14}PC$	diC_7PC	–[e]		
$diC_{16}PC$		315.2	1.7	
$diC_{16}PC$ (sonicated)		311.9	5.2	–3.3
$diC_{16}PC$	diC_6PC	314.8, 316.9	3.8	–0.4, 1.7
$diC_{16}PC$	diC_7PC	315.9	3.4	0.7
$diC_{16}PC$	diC_8PC[f]	312.4, 313.7	4.8	–2.8, –1.5
$diC_{18}PC$		328.0	2.0	
$diC_{18}PC$	diC_7PC (2.5 mM)	325.6, 326.6	3.9	–2.4, –1.4
$diC_{18}PC$	diC_7PC (5.0 mM)	325.6, 326.4	3.7	–2.4, –1.3
$diC_{18}PC$	diC_7PC (7.5 mM)	325.2, 327.4	4.1	–2.8, –0.6
$diC_{18}PC$	diC_7PC (10.0 mM)	324.4	3.9	–3.1

[a] Concentration of long-chain lecithin is 20 mM; abbreviations are as in Table I. [b] Short-chain lecithin concentration is 5 mM, unless otherwise noted. [c] Average T_m calculated from heating times only. [d] Difference between T_m observed for short-chain/long-chain vesicles and for multibilayers of the pure long-chain lecithin. [e] No transition observed. [f] 0.2 M KSCN is used in rehydrating samples with dioctanoyl-PC.

palmitoyl-PC may not be uniformly mixed with diheptanoyl-PC, and the lipid next to diheptanoyl-PC may exhibit a slightly different (lower) transition than that in pure dipalmitoyl-PC patches, or (iii) two discrete vesicle sizes with distinct melting profiles are formed, and the ratio of these varies with the amount of added short-chain lecithin. In any case, it may be concluded that the packing in these spontaneous vesicles resembles that of sonicated unilamellar vesicles with less of a temperature drop in the T_m.

Figure 8 depicts the traces for a series of distearoyl-PC mixtures with increasing amounts of diheptanoyl-PC. Again, two transitions are often observed for the long-chain component; both T_m values are below that for pure distearoyl-PC multibilayers (Table II). As the concentration of diheptanoyl-PC increases, the ratio of the area of the two transitions changes. The transition at the lower temperature increases in area while the area under the higher transition decreases. Concurrently, the peak at the lower T_m shifts to lower temperatures. Eventually, a single transition well below that of the pure long-chain lecithin is observed. It must also be noted that no residual multibilayer distearoyl-PC is present in these samples since no peak occurs at the T_m of pure distearoyl-PC. These data agree with the results of electron microscopy and ^{31}P NMR spectroscopy, where no multilamellar material was detected and the average size of the vesicles decreased as more short-chain lecithin was incorporated into the vesicle bilayer. As the radius of curvature of the vesicle becomes greater, the vesicles melt at lower temperatures.

Conformation of Long-Chain Phospholipid. FTIR spectroscopy was used to study the nature of the acyl chain and head group packing in these spontaneously formed vesicles. The frequency of the CH_2 stretching modes is an indication of the acyl chain conformational disorder (Snyder et al., 1978). An increase in frequency of the CH_2 stretches indicates an increase in the gaucheness of the acyl chains (Mantsch, 1984; Dluhy et al., 1983). Often, line broadening is observed due to an increase in the motion of the methylene groups (Snyder et al., 1978). FTIR spectra were obtained for short-chain lecithin/long-chain lecithin aggregates at temperatures above and below the T_m of the long-chain component. Since 4 times as much long-chain phospholipid is present as short-chain lecithin, the FTIR spectra are dominated by the long-chain component. The antisymmetric C–H stretching modes of the acyl chain methylene group occur between 2918.9 and 2921.9 cm^{-1} for short-chain lecithin/dipalmitoyl-PC vesicles at 25 °C and between 2924.3 and 2925.9 cm^{-1} at 45 °C. The asymmetric C–H stretching modes have a wider range, 2853.2–2852.2 cm^{-1} at 25 °C and 2854.4–2858.1 cm^{-1} at 45 °C. A shift of at least 3 cm^{-1} in both C–H stretches was observed for each lipid combination as the vesicles were warmed through the phase transition temperature. Cameron et al. (1980) report a 4.3-cm^{-1} shift for the antisymmetric methyl stretching as fully hydrated dipalmitoyl-PC multibilayers are warmed from 25 to 45 °C, while Urbaneja et al. (1985) have reported a 2.7-cm^{-1} shift for the antisymmetric C–H stretching under the same conditions. Thus, it would appear that the acyl chains of the long-chain species in these unique vesicles have a similar conformation to that found in normal long-chain phospholipid multibilayers. The incorporation of the short-chain lipid does not drastically increase the gauche bond conformations of the long-chain phospholipid in these vesicles.

It has been demonstrated that the frequencies of the C–O stretch in the ester linkage and the phosphate stretches (symmetric and antisymmetric) shift to lower wavenumbers as the backbone region of the lipid is hydrated (Fringeli & Gunthard, 1976; Davenport & Fisher, 1975). In addition, Chapman et al. (1967) have shown that the phosphate vibrations are invariant with temperature. Thus, the hydration of the head groups in these novel vesicles can be examined. For short-chain/long-chain mixtures all of these vibrations varied with

Table III: FTIR Vibrational Frequencies for Short-Chain/Long-Chain Phospholipid Mixtures as a Function of Temperature

long-chain phospholipid[a]	short-chain PC[b]	T (°C)	sym CH_2 (cm^{-1})	antisym CH_2 (cm^{-1})	C–O stretch (cm^{-1})	sym P–O (cm^{-1})	asym P–O (cm^{-1})
$diC_{16}PC$	diC_6PC	45	2855.1	2925.2	1070.9	1087.0	1232.0
$diC_{16}PC$	diC_7PC	25	2853.2	2921.3			
		45	2858.1	2925.5			
$diC_{16}PC$	diC_8PC[c]	25	2851.2	2918.9	1073.6	1087.8	1232.4
		45	2854.6	2924.5	1069.9	1085.3	1233.7
$diC_{16}PC$/cholesterol[d]	diC_7PC	25	2851.4	2919.7	1071.1	1087.8	1224.8
		45	2854.4	2924.3	1071.1	1084.6	1234.1
$diC_{14}PC$	diC_7PC	20	2852.5	2920.0	1070.1	1086.1	1232.3
		30	2854.7	2922.6	1069.9	1082.6	1233.6
$diC_{18}PC$	diC_7PC	35	2850.9	2918.8	1069.2	1087.1	1235.0
		55	2854.4	2924.4	1067.0	1085.9	1233.5
$diC_{14}PC$	diC_7PC	40	2851.8	2920.8		1081.5	1224.5

[a] Concentration of long-chain lecithin is 20 mM; abbreviations are as in Table I. [b] Short-chain lecithin concentration is 5 mM. [c] 0.2 M KSCN is included in hydration buffer. [d] Cholesterol concentration is 0.5 mM.

temperature (Table III). The C–O vibrational band and the symmetric P–O mode shifted to lower frequency over a 20 °C temperature rise. The asymmetric P–O mode showed no trend. It appears that upon heating these vesicles above the phase transition temperature of the long-chain phospholipid, the head groups become less hydrated around the backbone of the lipid moiety.

Discussion

We have developed a technique for spontaneously forming unilamellar vesicles from short-chain lecithin (fatty acyl chain lengths of 6–8 carbons) and long-chain phospholipids with a variety of head groups. The method of formation is easy and rapid and requires no elaborate equipment. There is no need to use detergents or organic solvents that might denature material to be encapsulated or that might remain with the vesicles in small but toxic amounts. A wide range of vesicle sizes can be obtained (egg-PC ~850 Å; sphingomyelin, ~150 Å), but most mixtures form vesicles in the 200–300-Å range. The average size for a particular mixture is governed by the phase transition temperature of the long-chain component. Vesicles made from lecithin with T_m values below room temperature tend to form large unilamellar structures (5 mM diheptanoyl-PC/20 mM egg-PC, diameter ~850 Å), while those made from gel-state lecithins tend to form small unilamellar vesicles (e.g., 5 mM diheptanoyl-PC/20 mM distearoyl-PC, diameter ~150 Å). The major lecithin species in egg-PC is 1-palmitoyl-2-oleoyl-PC. At room temperature the conformation of this lecithin differs from distearoyl-PC primarily by the number of gauche bonds. The acyl chains of the unsaturated species adopt conformations with multiple gauche bonds that increase the chain cross-sectional area. In a gel-state bilayer (distearoyl-PC at room temperature) the acyl chains are nearly all trans and packed closer together; upon melting, their cross-sectional area also increases due to the introduction of many gauche bonds. A liquid-crystalline bilayer of long acyl chains can accommodate the addition of short-chain lecithin molecules by altering the position of gauche kinks with little change in surface curvature or interchain interactions. Unilamellar vesicles formed would not be small in this case. A short-chain molecule added to a gel-state matrix is not so easily accommodated. Because its acyl chains are half or less the length of the long chain component, its insertion into the gel-state matrix will disrupt the cooperative packing of the long chains. This might initiate local curvature leading to the production of small highly curved vesicles with all trans long-chains and spacer short-chain molecules whose motional properties would be uncoupled to a large extent from long-chain neighbors. The exact size distribution of the vesicles and whether or not multilamellar structures remain are dictated by the ratio of short-chain to long-chain species. Increasing the amount of short-chain lecithin in a vesicle mixture tends to decrease the amount of larger structures and increase the amount of smaller vesicles as well as sometimes decreasing their average size.

The long-chain phospholipids in these mixed lipid vesicles behave spectroscopically much like pure long-chain phospholipid in sonicated unilamellar vesicles. Observation of two discrete T_m values in DSC traces for some ratios of diheptanoyl-PC/distearoyl-PC correlates with the bimodal size distribution suggested by ^{31}P NMR spectra. The larger species dominate at small amounts of diheptanoyl-PC, while more smaller species are observed with higher amounts of the short-chain lecithin. This suggests strongly that in this system the observed T_m is related to vesicle size and (as a reflection of particle curvature) polydispersity. The presence of short-chain lecithin serves to decrease the size of the cooperative unit but has little effect on the overall conformation of the long-chain phospholipid. Many amphiphilic additives have a more dramatic effect on phase behavior (Blume, et al., 1976; Alonso & Goni, 1983; Eliasz et al., 1976). The addition of short-chain lecithin tends to stabilize the long-chain lipids in a bilayer conformation, as illustrated by the electron micrographs of diheptanoyl-PC/dipalmitoyl-PE (5 mM:20 mM). Phosphatidylethanolamine tends to form a hexagonal II phase unless mixed with other bilayer-forming lipids such as phosphatidylcholine or sphingomyelin (Cullis & deKruijff, 1979). In the PE system, the short-chain lecithin behaves in a fashion similar to a long-chain counterpart.

The ease of formation of these vesicles may provide some insight into the mechanisms involved in membrane biogenesis. We can envision the short-chain lecithin as providing a way of initiating curvature and/or minimizing the edge energy necessary to close a large two-dimensional array into a spherical particle. This induction by small amounts of short-chain lecithins of unilamellar vesicle formation from multibilayers cannot be mimicked by single-chain detergents; therefore, specific head group interactions must also be critical. In real cells, similar short-chain phospholipids, amphiphilic proteins, or small amounts of other detergent-like material may have this same role in order for such functions as endocytosis or exocytosis to occur. Since we have demonstrated that vesicles form spontaneously upon mixing certain micellar and multibilayer aggregates, it is conceivable that other naturally occurring molecules (perhaps even a lipid like platelet-activating factor) are capable of insertion into membranes initiating local curvature and eventually membrane budding.

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Registry No. (*R*)-diC$_{16}$PC, 63-89-8; (*R*)-diC$_{14}$PC, 18194-24-6; (*R*)-diC$_{18}$PC, 4539-70-2; (*R*)-diC$_{16}$PE, 923-61-5; (*R*)-*N*-CH$_3$-diC$_{16}$PE, 3930-13-0; (*R*)-*N*,*N*-(CH$_3$)$_2$-diC$_{16}$PE, 3922-61-0; diC$_6$PC, 53892-41-4; diC$_7$PC, 35387-75-8; diC$_8$PC, 41017-85-0; cholesterol, 57-88-5.

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Conformation and Stability of the Constant Fragment of the Immunoglobulin Light Chain Containing an Intramolecular Mercury Bridge

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ABSTRACT: The constant fragment of the immunoglobulin light chain in which the intramolecular disulfide bond is reduced (reduced C_L fragment) assumes a conformation very similar to that of the intact C_L fragment and contains two sulfhydryl groups buried in the interior of the molecule [Goto, Y., & Hamaguchi, K. (1979) *J. Biochem. (Tokyo) 86*, 1433–1441]. In order to understand the role of the disulfide bond, a derivative in which the disulfide bond is replaced by an S–Hg–S bond was prepared and its conformation and stability were studied. The derivative was prepared by reacting the reduced C_L fragment with mercuric chloride. Kinetic studies showed that the reaction is rate-limited by the unfolding process of the reduced C_L fragment. The mercury derivative was as compact as the intact C_L or reduced C_L fragment, and a tryptophyl residue was found to be buried near the S–Hg–S bond in the interior of the protein molecule. Judging from the circular dichroic spectrum, however, the β-structure characteristic of the immunoglobulin fold was disturbed. The stability of the derivative to guanidine hydrochloride was lower than that of the intact C_L fragment, but the unfolding transition was reversible and cooperative. Decreased stability of the mercury derivative is due to its folded conformation being distorted by introduction of the S–Hg–S bond.

The protein molecule forms a unique three-dimensional structure determined by its primary structure. Elucidation of the pathway by which the three-dimensional structure of a protein molecule is built up is essential for understanding the structure and function of the protein. We have studied the pathway of protein folding experimentally using the isolated domains of immunoglobulin (Goto et al., 1979; Goto & Hamaguchi, 1979, 1981, 1982a,b; Sumi & Hamaguchi, 1982; Ashikari et al., 1985). Each domain of the immunoglobulin molecule consists of about 110 amino acid residues and has only one intrachain disulfide bond buried in the interior hydrophobic region between two β-sheets (Davies et al., 1975; Amzel & Poljak, 1979). The loop formed by the disulfide bond consists of 60 amino acid residues. The conformations of the domains are retained intact even when they are isolated as fragments. Intrachain disulfide bonds play a decisive role in determining the three-dimensional structures of many proteins. Therefore, the immunoglobulin domain may be

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