Short-Chain Lecithin/Long-Chain Phospholipid Unilamellar Vesicles: Asymmetry, Dynamics, and Enzymatic Hydrolysis of the Short-Chain Component†

N. Elise Gabriel and Mary F. Roberts*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received October 7, 1986; Revised Manuscript Received December 24, 1986

ABSTRACT: Asymmetric unilamellar vesicles are produced when short-chain phospholipids (fatty acyl chain lengths of 6–8 carbons) are mixed with long-chain phospholipids (fatty acyl chain lengths of 14 carbons or longer) in ratios of 1:4 short-chain/long-chain component. Short-chain lecithins are preferentially distributed on the outer monolayer, while a short-chain phosphatidylethanolamine derivative appears to localize on the inner monolayer of these spontaneously forming vesicles. Lanthanide NMR shift experiments clearly show a difference in head-group/ion interactions between the short-chain and long-chain species. Two-dimensional ^{1}H NMR studies reveal efficient spin diffusion networks for the short-chain species embedded in the long-chain bilayer matrix. The short-chain lecithin is considerably more mobile than the long-chain component but has hindered motion compared to short-chain lecithin micelles. This differentiation in physical characteristics of the two phospholipid components is critical to understanding the activity of phospholipases toward these binary systems.

The type of aggregate formed when phospholipids are dispersed in aqueous solution depends dramatically on the fatty acyl chain length. Lecithins with 6–8-carbon fatty acyl chains form micelles above a critical micelle concentration, while lecithins with 12 or more carbons in each fatty acyl chain form multilamellar aggregates. A unique class of unilamellar vesicles forms spontaneously upon mixing aqueous suspensions of long-chain phospholipid with small amounts (typically 20 mol %) of micellar short-chain lecithins (Gabriel & Roberts, 1984). Most of these binary mixtures yield unilamellar vesicles with an average diameter of 200–300 Å, although vesicles as large as 1000 Å and as small as 100 Å can be formed (Gabriel & Roberts, 1986). With short-chain lecithin/long-chain phospholipid (1:4) mixtures, neither residual long-chain multibilayers (Gabriel & Roberts, 1986) nor monomer or micellar structures (Gabriel, 1986) are detected. These short-chain lecithin/long-chain phospholipid unilamellar vesicles (SLUVs) are stable and impermeable to ions and fluorescent dyes, even in the presence of erythrocytes and serum (Gabriel, 1986). A number of properties of SLUVs, such as spontaneous assembly and long-term stability, may be attributed to the presence of the short-chain lecithin in the vesicle bilayer.

One of the potential uses of these binary component vesicles is as substrate for water-soluble phospholipases. Lipolytic enzymes such as phospholipase A_2 from *Naja naja naja* and phospholipase C from *Bacillus cereus* show high enzymatic activity toward micelles of short-chain lecithins or detergent-mixed micelles of long-chain lecithins but considerably lower activity toward long-chain lecithins packed in a bilayer vesicle (DeBose & Roberts, 1983; El-Sayed et al., 1985). SLUVs offer a unique opportunity to introduce a good substrate (short-chain lecithin) into a physical state (bilayer) associated with low enzymatic activity. When both components are phosphatidylcholines, the phospholipases have access to substrates with the same head group but different fatty acyl chain lengths. To understand the phospholipase kinetics with SLUVs, we need to know the distribution of short-chain lecithin between the two halves of the bilayer (i.e., any preferential sidedness or asymmetry) and any physical differences between the two components. While both long- and short-chain species have the same polar head group (phosphocholine), the mismatch in chain lengths could lead to differences in molecular packing or motion which are specifically sensed by these lipolytic enzymes. Previous studies (Gabriel & Roberts, 1986) have shown that the acyl chain packing of the long-chain lipids in the presence of short-chain lecithin is similar to that of small unilamellar vesicles of long-chain phospholipids made by using conventional methods. Therefore, the focus of the work is to examine the characteristics of the short-chain lecithin in the bilayer and the interactions between the two lipid components.

Experimental techniques that can be used to study the differences between the two lipid components are limited because of the chemical similarities between short-chain and long-chain lecithins. Nuclear magnetic resonance probes (selective deuteriation or lanthanide shift reagents) were introduced into the system to accentuate spectral features since the resonances of the two components overlap. The pathway of energy transfer studied through two-dimensional NMR spectroscopy suggests that the short-chain lecithin partitioned in the vesicle bilayer is more mobile and isolated from (i.e., noninteracting with) the long-chain phospholipid component. This property along with the asymmetric distribution of the short-chain lecithin gives the vesicle bilayer added stability and flexibility.

Materials and Methods

Chemicals. Dipalmitoylphosphatidylcholine (dipalmitoyl-PC), dipalmitoyl-PC-d_9 [$-N(CD_3)_3$], and diheptanoyl-PC were obtained from Avanti-Polar Lipid, Inc. Dipalmitoyl-PC-d_{62} (fatty acyl chains perdeuteriated) was purchased from Cambridge Isotopes Labs, Inc., while diheptanoyl-PC-d_{26} (fatty acyl chains deuteriated) was synthesized from heptanoic-d_{11} acid (Merck Sharp & Dohme Isotopes) following a procedure outlined by Burns et al. (1983). Phospholipids were checked

† This research was supported by NIH Grant GM-26762. ^{1}H NMR spectra were obtained at the Francis Bitter National Magnet Laboratory, MIT (NIH Grant RR-00995 and NSF Contract C-670).

* Correspondence should be addressed to this author at Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology.

0006-2960/87/0426-2432$01.50/0

for purity by thin-layer chromatography (Burns & Roberts, 1980) and, if pure, were used without any further purification. Total phospholipid was measured by a colorimetric phosphorus assay after conversion of organic phosphates to inorganic phosphate (Eaton & Dennis, 1976). Phospholipase A_2 was purified from cobra snake venom (*Naja naja naja*) (Deems & Dennis, 1981) and phospholipase C from *Bacillus cereus* ATCC 10987 purified from the growth media (Aakre & Little, 1982). Phospholipase A_2 concentrations were determined by the absorbance at 280 nm, assuming $\epsilon^{0.1\%} = 1.8$ (DeBose & Roberts, 1983), while phospholipase C concentrations were determined by assuming $\epsilon = 51\,000$ $(g/L)^{-1}\cdot cm^{-1}$ (Little & Johansen, 1979).

Vesicle Preparation. Vesicles were 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 for NMR studies were hydrated in appropriate solvent containing 0.15 M NaCl. Salt was excluded from vesicles prepared for kinetic assay samples, since high salt concentrations are known to inhibit phospholipase C activity (Aakre & Little, 1982). Hydrated mixtures were bath-sonicated (Electromations Ultrasonic Model 250 cleaner, input power 40 W) for 1 min (this aids in dispersal of material on the side of the tube and does not provide sufficient power to form unilamellar vesicles of pure long-chain phospholipid) and then equilibrated at room temperature for 6–8 h. The pH was adjusted, if necessary, to be within the range of 6.5–7.5.

Phospholipase Kinetics. Enzyme activity toward total phospholipid was assayed at pH 8 by using a Radiometer AA-60 pH-stat by titrating the product monoester phosphoric acid for phospholipase C or the fatty acid for phospholipase A_2; 5 mM $CaCl_2$ was added to assay mixtures for phospholipase A_2 kinetics.

NMR Spectroscopy. ^1H NMR spectra at 500 MHz were obtained on a home-built spectrometer at the Francis Bitter National Magnet Laboratory, MIT. A total of 20 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 6.5–7.0. Two-dimensional COSY and NOESY experiments were run on the same instrument. A total of 16 transients were collected for 512 t_1 values with 512 or 1024 data points over a 3400-Hz spectral width. Each was transformed with a 2.0-Hz exponential and Gaussian weighting function. A 30-μs homospoil pulse was usually applied during the NOESY experiment.

Results

Interaction of Pr^{3+} with SLUVs. NMR spectroscopy has been used extensively in the characterization of SLUVs (Gabriel & Roberts, 1984, 1986). Residual multibilayers in mixtures with less than 20 mol % short-chain lecithin were detected by ^{31}P line shapes. Coexistence of multilamellar structures and unilamellar vesicles implies that a critical ratio of short-chain lecithin to long-chain species is necessary for spontaneous formation of vesicles. Proton NMR studies have shown that for 20 mM long-chain/5 mM short-chain lecithin mixtures, the short-chain lecithin does not exist as a pure micellar aggregate in these mixtures. Finally, lanthanide shift NMR experiments were used to verify the formation of unilamellar vesicles. It has been well documented that Pr^{3+} (or another paramagnetic lanthanide ion) added to a population of impermeable vesicles forms a complex with the accessible phospholipid head groups and shifts resonances from these exterior molecules (Bystrov et al., 1971; Andrews et al., 1973). Only minor perturbations of the resonances from the interior lecithin molecules are observed because the aqueous compartment surrounding these lipid head groups is lanthanide free. Original studies with SLUVs and Pr^{3+} showed that the long-chain lecithin was evenly divided between Pr^{3+}-accessible and Pr^{3+}-free pools (i.e., evidence for a bilayer structure). The short-chain lecithin in SLUVs did not appear to be sensitive to Pr^{3+}. This differential interaction with lanthanides has now been examined in greater detail.

Table I: Maximum Lanthanide-Induced Chemical Shifts of Phosphatidylcholine *N*-Methyl Protons in Different Lipid Aggregates

aggregate[a]	Δ[b] (Hz)
monomer	
dihexanoyl-PC (5 mM)	−4.2 ± 9.1
micelles	
diheptanoyl-PC (5 mM)	−76.8 ± 24.5
Triton X-100 (20 mM)/egg PC (5 mM)	−87.9 ± 24.0
single-component vesicles	
dipalmitoyl-PC (20 mM)	−6050 ± 760
SLUVs	
diheptanoyl-PC (5 mM)	−93.3 ± 45.0
dipalmitoyl-PC (20 mM)	−2650 ± 405

[a] Measurements done at 25 °C, except for dipalmitoyl-PC sonicated vesicles which were carried out at 45 °C. [b] The lanthanide-induced shift at 500 MHz extrapolated for a 1:1 Pr^{3+}/PC interaction based on titrating in Pr^{3+} to a fixed PC concentration and observing the chemical shift change; no correction is made for the fact that in vesicles only the outer leaflet phospholipids can be in contact with Pr^{3+}.

Maximum lanthanide-induced chemical shifts have been estimated for $N(CH_3)_3$ protons of various lipid aggregates. A fixed lecithin solution is examined as Pr^{3+} is titrated into the mixture. Values of the induced shift and the ratio of Pr^{3+}/PC are used to extrapolate the maximum-induced shift for a 1:1 Pr^{3+}/PC complex. Larger chemical shifts are observed for lecithin in vesicles than for short-chain lecithin micelles and monomers or for long-chain lecithins in a detergent matrix (Table I). These data suggest that the lipid head-group packing must be slightly different for long-chain bilayers vs. short-chain micelles in order for the lanthanide to affect the same moiety in these aggregates differentially. ^1H NMR (500 MHz) lanthanide shift experiments with short-chain lecithin/long-chain phospholipid aggregates exhibit intermediate behavior. The resonance from the short-chain lecithin is at best only slightly shifted by the addition of small amounts of Pr^{3+}, while the broader long-chain resonance clearly splits into two peaks. The limiting chemical shift observed for the short-chain lecithin when incorporated into SLUVs is comparable to that observed for micelles while the maximum chemical shift for the long-chain phospholipid/Pr^{3+} interaction is comparable in magnitude to that of sonicated vesicles. Other studies have shown that no short-chain lecithin micelles exist in these SLUV mixtures (Gabriel, 1986). Only a single short-chain lecithin resonance is detected in the ^1H NMR spectrum upon Pr^{3+} addition, although a small amount of short-chain species with a different chemical shift could be obscured by the intense resonances from the long-chain lecithin.

One explanation for differential Pr^{3+} shifts is that the bulk of the short-chain lecithin is on the inner surface of the vesicle and not accessible to Pr^{3+}. Because the Pr^{3+} micelle limiting shifts, where all phosphocholine moieties are exposed to the lanthanide, were so much smaller than for bilayers, this is clearly not the only explanation. The relative lanthanide-induced chemical shifts for the two lecithins will depend on the distance and angular orientation of the Pr^{3+} (presumably

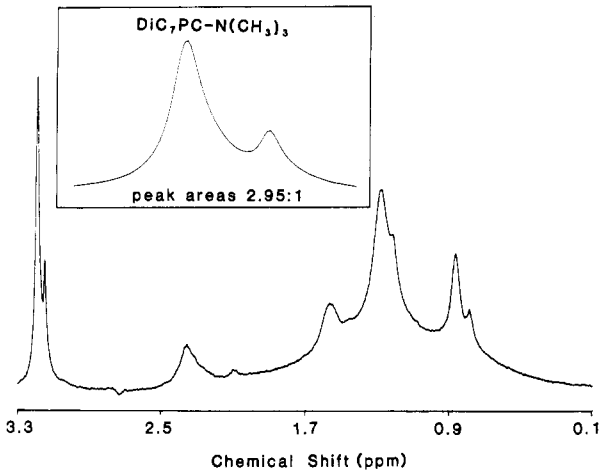

FIGURE 1: 500-MHz ^1H NMR spectrum of diheptanoyl-PC/dipalmitoyl-PC-$N(CD_3)_3$ (5 mM/20 mM) at 25 °C; insert: $N(CH_3)_3$ region.

bound to the phosphate) to the $N(CH_3)_3$ protons and the relative affinity of Pr^{3+} for the two species. Since both molecules have the same phosphocholine head group, Pr^{3+} affinities should be comparable. On the other hand, a difference in mobility or neighboring lecithin head-group interactions of the two species could affect the short-chain lecithin $N(CH_3)_3/Pr^{3+}$ interaction. For example, if the short-chain lecithin choline segment can assume an extended trans conformation more perpendicular to the bilayer surface in the presence of Pr^{3+} rather than the gauche/cis orientation found for bilayers (Seelig et al., 1977; Sundaralingam, 1972) and pure short-chain lecithin micelles (Lin et al., 1986, 1987a,b), the Pr^{3+}–$N(CH_3)_3$ distance would be increased, and the lanthanide-induced chemical shift would decrease. Therefore, the minimal effect of Pr^{3+} on the short-chain lecithin could reflect either (1) an asymmetric distribution of this species with most of the short-chain lecithin on the inner monolayer and not accessible to Pr^{3+} or (2) a difference in phosphocholine packing or conformation between the long- and short-chain species. A choice between these explanations requires another approach to determining short-chain lecithin sidedness in SLUVs.

Sidedness of Short-Chain Lecithin in SLUVs. The ambiguity in the lanthanide-induced shift experiments prompted us to examine several other NMR approaches to measuring the sidedness of the short-chain lecithin in SLUVs. It has been shown that the resonances for chain bulk methylene and terminal methyl protons as well as the choline *N*-methyl group of phosphatidylcholines on the inner monolayer are chemically shifted upfield of those on the outer monolayer (Schuh et al., 1982). This has been attributed to differences in lipid packing for both sides of the vesicles (Eigenberg & Chan, 1980). For SLUVs, the short-chain lecithin and long-chain phospholipid chain methylene and methyl resonances overlap. In order to isolate the short-chain lecithin from the resonances of the long-chain phospholipid without altering the vesicle composition, we have used selectively deuteriated phospholipids. There is a slight variation in vesicle size that depends on the phospholipid region deuteriated. Size variations can also occur from one preparation to another. These size fluctuations are reflected in the NMR spectrum or more specifically in the average observed line widths. As an example, in one preparation of 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC-d_9, only one $N(CH_3)_3$ resonance was observed in the 500-MHz ^1H NMR spectrum (Gabriel & Roberts, 1984). In another preparation, two resonances are observed for the short-chain

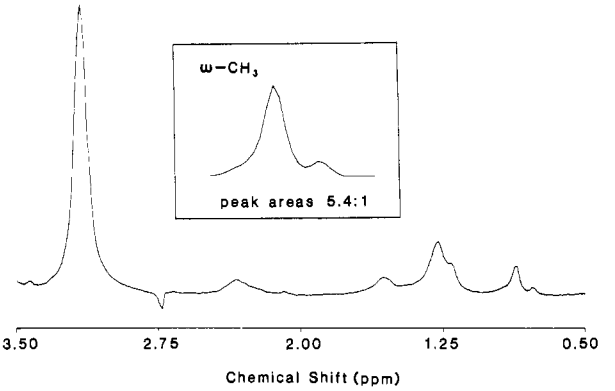

FIGURE 2: 500-MHz ^1H NMR spectrum of diheptanoyl-PC/chain-perdeuteriated dipalmitoyl-PC (5 mM/20 mM) at 25 °C; insert: ω-CH_3 region.

lecithin $N(CH_3)_3$ group (Figure 1). Integration of the two resonances reveals that the short chain is mainly on the outer monolayer with a 3:1 ratio. For another vesicle preparation made with 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC-d_{62} (Figure 2), two resonances are visible for both methyl and methylene groups. Since only the short chain is contributing to the proton spectrum in this region, the two resonances per proton moiety must reflect inner and outer phospholipids. The ratio of the two resolvable terminal CH_3 peaks is 5.4:1. On the basis of the assignment by Eigenberg and Chan (1980), the downfield resonance is from lipids on the outer monolayer. The results of these two experiments imply that the majority of the short-chain lecithin is on the outer monolayer in these SLUVs whose average diameter is 200–400 Å. In light of these results, the original lanthanide-induced shift experiment must be interpreted as a difference in phosphocholine orientation between short-chain lecithin and long-chain lecithins in SLUVs.

This asymmetric distribution of short-chain lecithins in SLUVs could solely depend on the chain length or it could also be affected by head-group identity. While NMR lanthanide shift experiments could not be used to determine the sidedness of the short-chain lecithins, they can be used on vesicles made with a slightly different head group. A diheptanoylphosphatidyl-*N*,*N*-dimethylethanolamine/dipalmitoyl-PC combination gives rise to a ^1H NMR spectrum with the head-group N–CH_3 protons of both lipids clearly separated from each other (Δ = 144 Hz at 45 °C, Figure 3). The N–CH_3 protons of the short-chain *N*,*N*-dimethylphosphatidylethanolamine (*N*,*N*-dimethyl-PE) shift and broaden with the addition of Pr^{3+} (limiting chemical shift of 1606 ± 124 Hz), whereas other short-chain lecithin species do not. This behavior may reflect the greater binding affinity of the tertiary amine in diheptanoylphosphatidyl-*N*,*N*-dimethylethanolamine for the lanthanide as compared to the quaternary amine in PC lipids. The ratio of outer to inner peak areas was measured to be 1:4.7, implying that most of the short-chain PE derivative is on the inner monolayer. This result may reflect a smaller head-group area for the *N*,*N*-dimethyl-PE than for the $N(CH_3)_3$-PC molecule. A recent crystal structure for dilauroylphosphatidyl-*N*,*N*-dimethylethanolamine shows that the head group is not parallel to the bilayer surface, as is seen with phosphatidylcholine and phosphatidylethanolamine lipids, but is rather perpendicular to the surface (Pascher & Sundell, 1986). This head-group orientation is favored by stacking bilayers such that the P–N dipoles of adjacent bilayers can interdigitate (presumably to preserve electroneutrality). While such interdigitation cannot exist with unilamellar vesicles, charge effects/ion layers at the

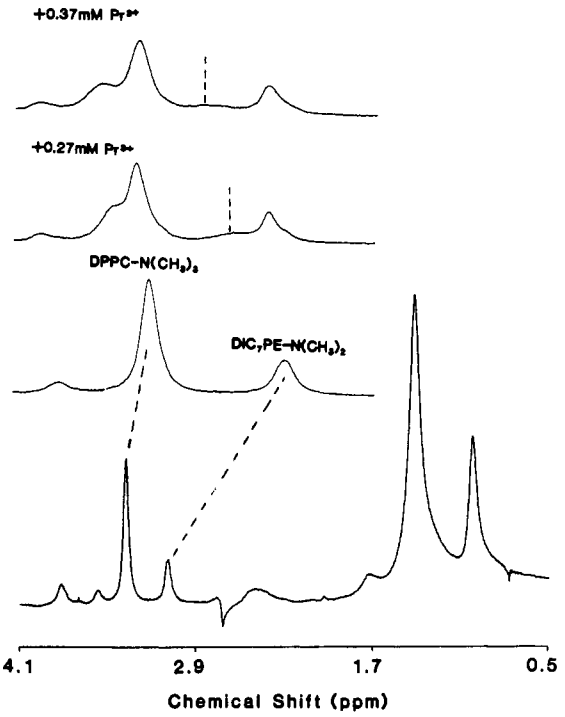

FIGURE 3: 500-MHz ^{1}H NMR lanthanide shift experiment with diheptanoylphosphatidyl-*N,N*-dimethylethanolamine/dipalmitoyl-PC (10 mM/20 mM) at 45 °C; insert: $N(CH_3)$ region with and without the addition of Pr^{3+}. (The dashed lines indicate the Pr^{3+}-shifted peak.)

inner monolayer surface may favor this orientation. If the *N,N*-dimethyl-PE can pack more closely than PC, it may be preferentially used to maintain the high radius of curvature of the inner monolayer.

Phospholipid Dynamics in SLUVs. The preceding experiments show that the short-chain lecithin is asymmetrically distributed in SLUVs and has different head-group interactions with Pr^{3+} than the long-chain phospholipid component. While these properties of SLUVs may be related to their formation and stability, other information such as the extent of lipid/lipid interactions may also be critical. In an attempt to characterize phospholipid/phospholipid (in particular short-chain lecithin/long-chain lecithin) interactions in SLUVs, we have used two-dimensional (2D) ^{1}H NMR spectroscopic methods to elucidate spin diffusion pathways in this binary system. 2D NOE experiments have been used previously to localize ligands in small vesicles (Ellena et al., 1985). Since ^{1}H NMR spectra of SLUVs are complicated not only by the chemical shift differences of inner and outer leaflet phospholipids but also by shift differences between long- and short-chain lecithins as well, we first carried out several COSY (Aue et al., 1976; Kumar et al., 1980) experiments. Contour plots of the acyl chain region of these are shown in Figure 4. The first is that of diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM). There are two clearly distinguished cross-peaks between the scalar-coupled methylene (region B in Figure 4A) and terminal methyl groups (region A). To discern the source of the COSY cross-peaks, another COSY experiment was performed with diheptanoyl-PC/dipalmitoyl-PC-d_{62} (i.e., chain perdeuteriated). The contour plot of the acyl chain region is shown in Figure 4B. Again, two cross-peaks are observed between the coupled methylene and terminal methyl groups of the short-chain lecithin. On the basis of the work of Eigenberg and Chan (1980) in assigning inside/outside resonances for methylene and methyl groups in gel-phase, small unilamellr vesicles of lecithins, we would assign the more intense and downfield resonance in both the methyl (A) and

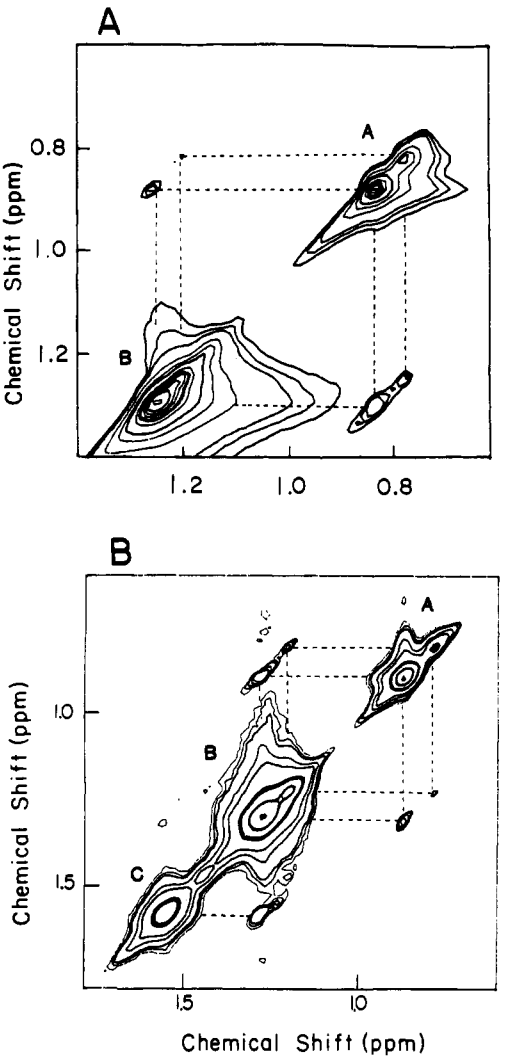

FIGURE 4: 2D-COSY contour plots of diheptanoyl-PC and (A) proteodipalmitoyl-PC; (B) acyl chain deuteriated dipalmitoyl-PC (5 mM/20 mM) at 25 °C.

methylene (B) regions to the short-chain lecithin on the outer monolayer while the upfield, less intense cross-peak is due to short-chain lecithin on the inner monolayer. The differences in cross-peak intensities are consistent with our other results on the asymmetric distribution of this species. Since the same cross-peaks are observed when the long-chain PC is perdeuteriated or in proteo form, inner and outer monolayer phospholipids must have basically the same chemical shift regardless of chain length, or the broader resonances from the dipalmitoyl-PC components give rise to much less intense cross-peaks.

The experiment used to monitor chain dynamics is a two-dimensional cross-relaxation technique, NOESY (Jeener et al., 1979; Macura et al., 1982). Two-dimensional cross-relaxation spectra of a model SLUV mixture, diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM), have been obtained for a series of mixing times ranging from 75 to 800 ms. Contour plots of four experiments are shown in Figure 5. Approximate chemical shifts and peak assignments for the labeled peaks are given in Table II. (In this discussion, the resonances of interest will be identified by chemical group and letter label on the appropriate contour plot.) Spectra were not symmetrized to remove T_1 noise since in our hands this often led to spurious cross-peaks.

At 75-ms mixing time, the major cross-peaks detected occur between the bulk methylene groups (region B in Figure 5A)

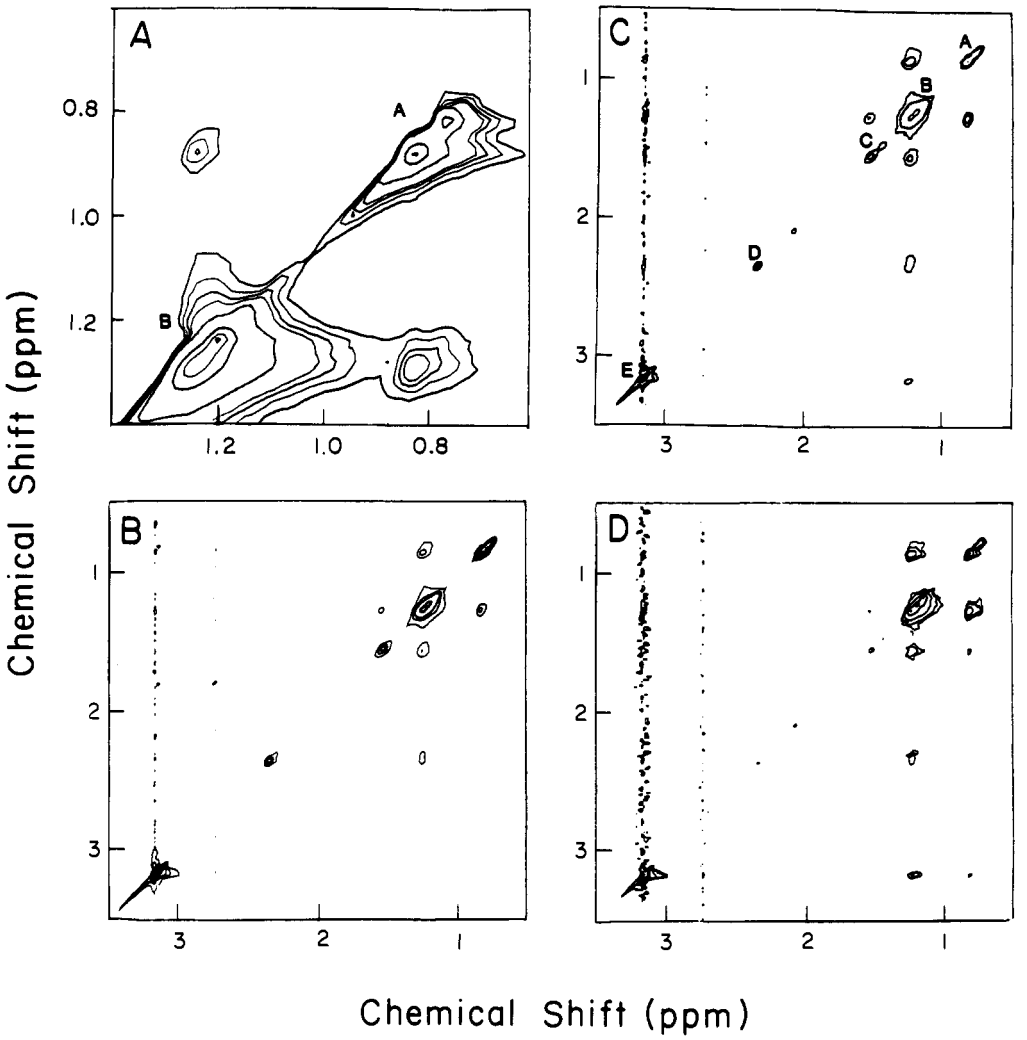

FIGURE 5: 2D-NOESY contours of diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM) at 25 °C with various mixing times: (a) 75 ms with homospoil pulse; (B) 200 ms; (C) 400 ms with homospoil pulse; (D) 800 ms.

Table II: Chemical Shift Assignments of Typical 500-MHz ^1H NMR Spectrum of Diheptanoyl-PC/Dipalmitoyl-PC (5 mM/20 mM)

notation in contour plots	group	chemical shift[a] (ppm)
A 1	ω-CH_3	0.76
2		0.83
B 1	$(CH_2)_n$	1.17
2		1.24
C	β-CH_2	1.54
D 1	α-CH_2	2.29
2		2.34
E	$N(CH_3)_3$	3.17
F	CH_2–N	3.60
G	glyc CH_2OPO	3.93
H	choline CH_2O	4.22
I	CH_2O	4.36
J	HOD	4.67
K	CHO	5.23

[a] Chemical shift with respect to water at 4.67 ppm from external tetramethylsilane.

and the terminal methyl group (region A) of the lipid acyl chains. This is not due to scalar coupling which was removed with a homospoil pulse. Indeed, the relative intensity of these cross-peaks is considerably greater than in the COSY experiment. As the mixing time is increased to 200 ms (Figure 5B) and 400 ms (Figure 5C), additional cross-peaks appear between the bulk methylene group and the β-methylene (peak C in the contour plot), the α-methylene (peak D), and the head-group choline protons (peak E). At 800-ms mixing time (Figure 5D), additional cross-peaks are observed between the terminal methyl group and the α-methylene, and the head-group choline protons. For micellar short-chain lecithins, no comparable cross-peaks are detected at any mixing time. As the mixing times increase with the SLUVs, the intensity of the cross-peaks appears to increase. It is difficult to quantify this trend, because each experiment was run for slightly different times and under different conditions, e.g., with and without a homospoil pulse. These cross-peaks could have arisen from magnetization transferred between spatially close groups, in some cases from spin-coupled protons, and/or from protons undergoing spin diffusion. Magnetization transfer between the terminal methyl group and the head-group methyl protons probably occurs via a spin diffusion pathway, because these protons are not spin coupled to one another nor spatially close, although further data confirming this will be discussed later.

The cross-peaks involving the acyl chain protons could arise via any one of the three possible mechanisms of magnetization transfer. A number of possibilities exist that would result in these protons being close to one another. The acyl chain packing and fluidity could be responsible for interactions between lipids that are adjacent to each other on the same side of the bilayer. The interactions of adjacent lipids would result in numerous cross-peaks between short-chain/short-chain lipids or short-chain/long-chain lipids if the lipids can be within 4 Å of one another. This is possible given that X-ray crystallographic data of dimyristoyl-PC (Pearson & Pascher, 1979) indicated that there is ~2.5 Å between neighboring phos-

pholipid molecules (although these crystals are dehydrated). If such proximity occurs in vesicles, large cross-peaks would arise in the methylene and terminal methyl region. (Interactions between interdigitated short-chain and long-chain lecithins will be extremely difficult to detect since it requires that the terminal methyl resonances be resolvable for both the short-chain and long-chain lecithin as well as their distribution between the monolayers.)

To determine which mechanism (spin diffusion or spatial transfer) is responsible for the observed NOESY cross-peaks, a number of 2D NOESY experiments were performed with deuteriated lipids. Selected spectral features can be highlighted by using deuteriated components. All the deuteriated vesicles were essentially the same size, 250–300 Å in diameter, as determined by electron microscopy, except for SLUVs made from dipalmitoyl-PC-d_{62}. These particles had diameters of 400 Å. This increase in size may result in decreased intensity of the short-chain lecithin due to decreased tumbling motion of the larger particle. One-dimensional spectra are shown in Figure 6 for each SLUV preparation, and contour plots of NOESY experiments for the four SLUV mixtures are shown in Figure 7. The NOESY experiments were carried out at 25 °C with a 400-ms mixing time. The NOESY contour plot for SLUVs with head-group-deuteriated long-chain lecithin [(A) diheptanoyl-PC/dipalmitoyl-PC-d_9] has many cross-peaks. The most striking feature is the cross-peak between the bulk methylene (region B) and the *N*-methylcholine group of the proteo short-chain lecithin (region E). The cross-peak does not contain intensity from spin diffusion along the long-chain phospholipid because the head group of the long-chain phospholipid is deuteriated. The NOESY contour plots in Figure 7B,C were generated from SLUVs with deuteriated acyl chains of one of the lipid components. The SLUVs in Figure 7B have cross-peaks between the *N*-methyl protons and bulk methylenes of the short-chain lecithin only, since long-chain carbons have been perdeuteriated. In Figure 7C, the broad cross-peaks between the bulk methylene and head-group choline protons in the mixture made with acyl chain deuteriated short-chain lecithin show that both long- and short-chain lecithins are involved in transferring magnetization. The intensity of the cross-peaks in this contour plot (Figure 7C) is from the long-chain phospholipid since the acyl chains of the short-chain lecithin are deuteriated.

Finally, the contour plot (Figure 7D) obtained from SLUVs made with acyl chain deuteriated short-chain lecithin and head-group-deuteriated long-chain phospholipid confirms that the terminal methyl group is not spatially close to the head group of the lipid. If the two groups were close to one another (within the 4-Å detection limit), there should be cross-peaks between the bulk methylene protons of the long-chain lipid and the head-group choline protons of the short-chain lecithin. No such cross-peaks were detected, even when the sample was heated above the phase transition temperature of the long-chain phospholipid. For other SLUV mixtures, the intensity of the cross-peaks usually increased above the T_m of the long-chain phospholipid because these resonances narrow considerably, reflecting the increased lateral diffusion of the long-chain component. Therefore, the NOESY cross-peaks (especially at 25 °C) reflect spin diffusion along the short-chain lecithin molecule and not spatial or intermolecular transfer of magnetization.

SLUV Hydrolysis by Phospholipases. Both phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) will hydrolyze lecithin in small unilamellar vesicles (DeBose & Roberts, 1983; El-Sayed et al., 1985), but the specific

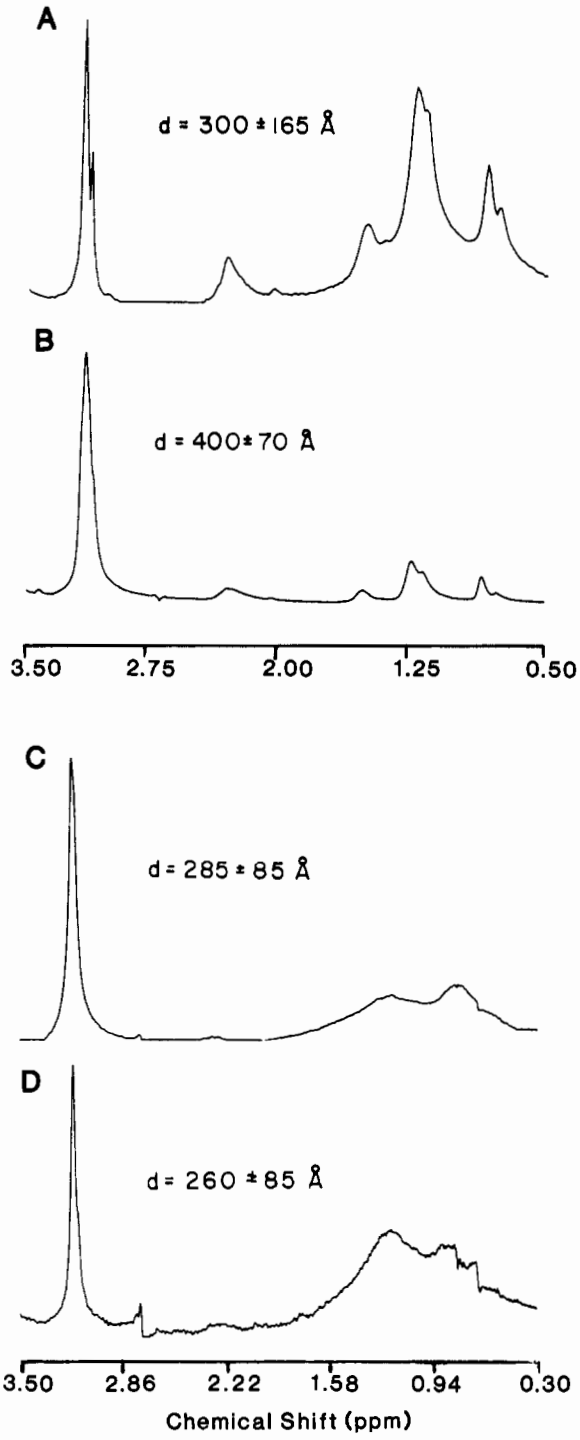

FIGURE 6: 500-MHz ^{1}H NMR spectra of diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM) SLUVs with different species deuteriated: (A) dipalmitoyl-PC-d_9[-N$(CD_3)_3$]; (B) dipalmitoyl-PC-d_{62} (acyl chain perdeuteriated); (C) diheptanoyl-PC-d_{26}; and (D) dipalmitoyl-PC-d_9 and diheptanoyl-PC-d_{26}. Average vesicle diameter is listed above each spectrum.

activities are usually low (<50 μmol min^{-1} mg^{-1}) compared to micellar substrates (typically 1000–2000 μmol min^{-1} mg^{-1}). In contrast, as shown in Table III, these enzymes hydrolyze SLUVs at rates comparable to those observed for pure short-chain lecithin micelles or detergent mixed micelles (Roberts et al., 1978; El-Sayed et al., 1985). Several different hydrolysis reactions can occur in these lipid mixtures: (1) both lipids are hydrolyzed, perhaps at different rates; (2) the short-chain lecithin is selectively hydrolyzed at rates comparable to those of micellar aggregates; or (3) the long-chain phospholipid is preferentially hydrolyzed at rates greatly ac-

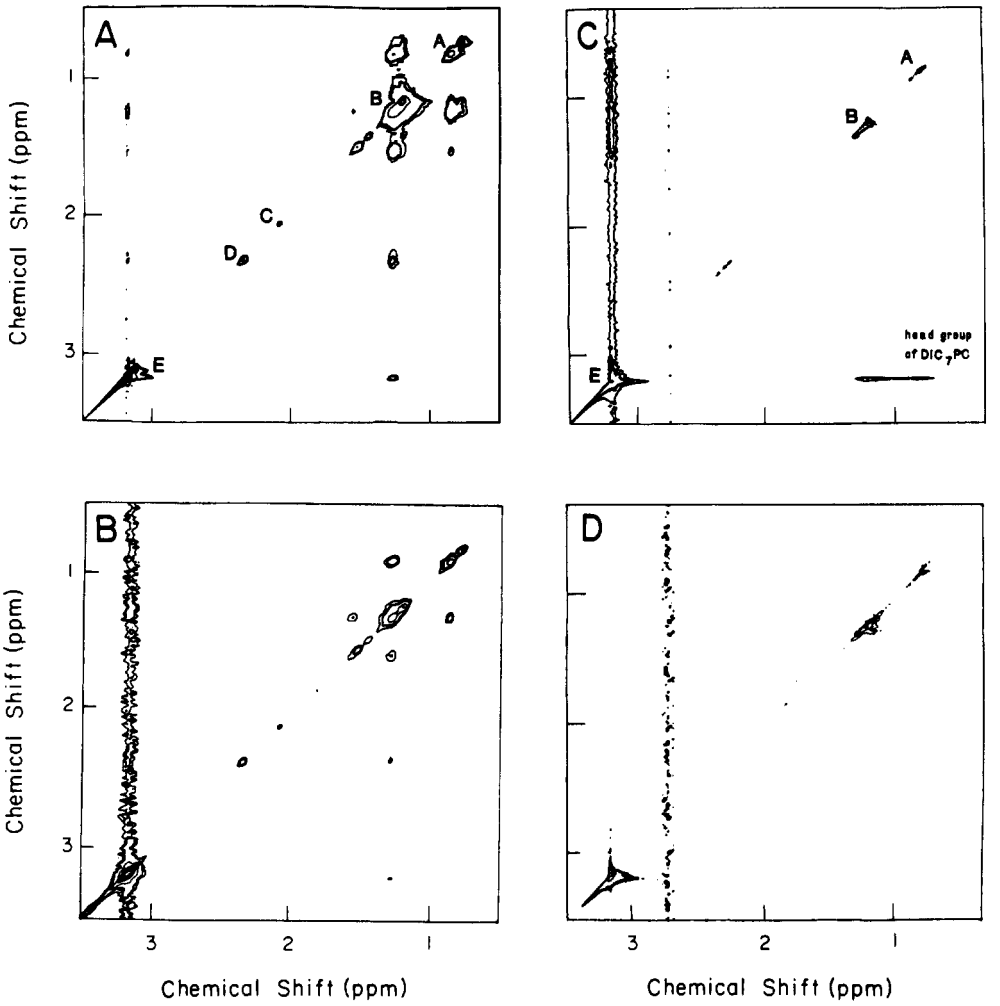

FIGURE 7: Contour plots of 400-ms NOESYs (with homospoil pulse) at 25 °C for diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM) SLUVs with different species deuteriated: (A) dipalmitoyl-PC-d_9; (B) dipalmitoyl-PC-d_{62}; (C) diheptanoyl-PC-d_{26}; and (D) diheptanoyl-PC-d_{26}/dipalmitoyl-PC-d_9. Resonances are identified in Table III.

Table III: Phospholipase A_2 (*Naja naja naja*) and Phospholipase C (*Bacillus cereus*) Activity toward Phospholipid Vesicles

	specific activity (μmol min^{-1} mg^{-1})	
sample	phospholipase A_2[a]	phospholipase C
sonicated vesicles		
DPPC[b]	50[c]	43 ± 5[d]
DMPC		15 ± 2[e]
DHPC	0	3 ± 3[d]
SPM	0	<2[e]
SLUVs		
diC$_7$PC/DPPC	550 ± 50	1590 ± 160
diC$_7$PC/DMPC	1100 ± 100	3000 ± 300
diC$_7$PC/DHPC	270 ± 60	1550 ± 100
diC$_7$PC/SPM	1100 ± 30	1300 ± 80
e-diC$_7$PC/DPPC	45 ± 5	14 ± 5
e-diC$_7$PC/DMPC	65 ± 10	50 ± 10

[a] Assay mixture contains 1 mM Ca^{2+}. [b] Abbreviations: DPPC, dipalmitoyl-PC; DMPC, dimyristoyl-PC; DHPC, dihexadecyl-PC; SPM, sphingomyelin; diC$_7$PC, diheptanoyl-PC; e-diC$_7$PC, diheptyl-PC. [c] For 5 mM phospholipid, 41 °C assay temperature [from DeBose and Roberts (1983)]. [d] From El-Sayed et al. (1985). [e] M. Y. El-Sayed (1984), Ph.D. Thesis, MIT, Cambridge, MA.

celerated over those for unilamellar vesicles of pure long-chain lecithin. This last possibility is unlikely given that high activities were measured with SLUVs made from dihexadecyl-PC, an ether-linked analogue of dipalmitoyl-PC that acts as a competitive inhibitor of phospholipase A_2 and a nonsubstrate for phospholipase C (El-Sayed, 1984), or sphingomyelin, a nonsubstrate for both enzymes, as the long-chain component. These kinetic results strongly suggest that phospholipases A_2 and C preferentially hydrolyze the short-chain lecithin in SLUVs. To verify this, SLUVs were prepared with an ether-linked short-chain lecithin, diheptyl-PC, whose physical properties mimic the ester-linked compound diheptanoyl-PC (Burns et al., 1981), and dipalmitoyl-PC. Both enzymatic activities were low toward the remaining long-chain phospholipid in SLUVs. In most of the SLUV mixtures represented in Table III, the long-chain phospholipid is in a gellike state (Gabriel & Roberts, 1986). If SLUVs made from dimyristoyl-PC (which will be liquid crystalline at the assay temperature) and diheptanoyl-PC or diheptyl-PC are examined, the same preferential hydrolysis of short-chain lecithin occurs. Thus, the short-chain lecithin is the major substrate in binary component SLUVs.

Discussion

The unilamellar vesicles formed by mixing small amounts of short-chain lecithin with long-chain phospholipid are unique not only for their stability and spontaneous assembly but also for the asymmetric distribution of the short-chain lecithin in the vesicle bilayer. The mechanism of formation probably involves the initiation of areas of curvature as the short-chain lecithin inserts itself into long-chain multilamellar structures. This curvature or budding of the long-chain lamellae is most likely caused by the mismatch of fatty acyl chain lengths between the two lipids. Unilamellar vesicles have been shown to assemble spontaneously only with the addition of short-chain

lecithin (Gabriel & Roberts, 1984) or with a shift to fairly basic pH if phosphatidic acid is present in the multibilayer (Hauser et al., 1983). One assumes that the first method relies on differences in chain packing to drive vesicle formation, while the second exploits titration of a monophosphate to yield a more repulsive dianion. Both utilize more wedge-shaped phospholipids to stabilize small, curved bilayer vesicles. In SLUVs made with short- and long-chain lecithins, even though all polar head groups are the same chemical species (phosphocholine), head-group differences could occur due to chain packing. Experiments using lanthanide NMR shift reagents suggest that the mobility or head-group packing of the short-chain lecithin is different than the long-chain component. Two-dimensional NMR studies confirm that the short-chain lecithin has more mobility than the long-chain phospholipid in the particles and is "isolated", i.e., not strongly interacting, from the long-chain species. The mobile short-chain lecithin is partitioned in the vesicle bilayer and does not easily dissociate, even when SLUVs are diluted 100-fold (Gabriel, 1986).

These binary vesicles are excellent for probing the "interfacial activation" of water-soluble lipolytic enzymes such as phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*). Even though SLUVs are asymmetric with most of the short-chain lecithin on the outer monolayer (for 5 mM diheptanoyl-PC, the average partitioning is 4 mM outside and 1 mM inside), more than 70% of the total phospholipid in this leaflet is the long-chain species (for 1:4 short-chain/long-chain vesicles). SLUVs are excellent substrates for phospholipases, but only the short-chain component is hydrolyzed at high rates. Previous work using erythrocyte hemolysis to detect monomers or micelles of short-chain lecithins (Gabriel, 1986) clearly shows that free short-chain lecithin concentrations must be less than 0.1 mM. Neither phospholipase shows much activity toward this concentration of short-chain lecithin. Therefore, the high specific activities of the phospholipases toward SLUVs are for the short-chain lecithin embedded in the long-chain phospholipid bilayer matrix. Conventional single-component lecithin vesicles are poor substrates for these enzymes, but the individual phospholipid molecules become good substrates if detergents such as Triton X-100 (Dennis, 1972) or bile salts (Stark et al., 1985) are added to transform the lipid bilayer into mixed micelles. With SLUVs, we have the first example of substrate in a zwitterionic bilayer whose enzymatic hydrolysis rate is comparable to what occurs with the same molecule as micelles.

We have measured two physical properties of SLUV components which show clear-cut differences between short-chain and long-chain lecithins and which may help to explain the kinetic preference of water-soluble phospholipases for short-chain species. The small Pr^{3+}-induced chemical shift for short-chain lecithins in SLUVs or in micelles vs. the large effect for long-chain lecithins in vesicles (SLUVs or sonicated vesicles of a single component) implies that the phosphocholine moiety of the diheptanoyl-PC molecule can assume a different orientation in the presence of Pr^{3+} than the phosphocholine groups of long-chain lecithins. Without lanthanides, the phosphocholine group has a gauche conformation and is oriented parallel to the interface in both bilayers and short-chain lecithin micelles (Lin et al., 1986, 1987a,b). Pr^{3+} binding to the phosphodiester group will neutralize the negative charge. The choline moiety is also positively charged and will want to maximize its distance from the Pr^{3+}. If phosphocholine groups of many molecules are involved in a hydrogen-bonding or electrostatic network, it will be difficult for a given phosphocholine group to assume a more extended conformation. In fact, Raman studies of the influence of metal ions on the choline conformation in dipalmitoyl-PC bilayers show that lanthanides do not induce a conformational change of the O–C–C–N segment from gauche to trans either in the gel or in the liquid-crystalline state (Akutsu et al., 1986). This would certainly be expected for the long-chain lecithins in SLUVs since DSC studies show they exhibit phase behavior comparable to single-component unilamellar vesicles and multilamellar structures. A short-chain lecithin phosphocholine group may be more mobile and have weaker head-group interactions with long-chain neighbors because of weaker fatty acyl chain interactions with the long-chain lecithins. Hence, it may assume an extended conformation where the *N*-methyl groups are further from the phosphate when Pr^{3+} is added. Precedence for such a conformation exists in studies of the effect of Pr^{3+} on lysolecithin head-group conformation (Hauser et al., 1978). A difference between the head-group orientation of short-chain and long-chain species could be exploited by the water-soluble phospholipases. Perhaps these enzymes need a certain phosphocholine conformation or need a molecule uncoupled from neighbors in order for efficient substrate binding to occur. The head-group area per molecule for short-chain vs. long-chain lecithin in SLUVs is unlikely to be very different and by itself is unlikely to explain the observed phospholipase kinetics. Recents SANS studies of short-chain lecithin micelles (Lin et al., 1986, 1987a,b) have confirmed that these micelles can be modeled as long spherocylinders where the area per molecule is 70–75 $Å^2$ in the cylinder and 95–100 $Å^2$ in the end caps. In lecithin bilayers, the head-group area per molecule is typically 65 $Å^2$. Molecules in the cylindrical section of the micelle are most analogous to lecithins packed in a vesicle; hence, differences in short-chain and long-chain lecithin head-group areas per molecules would be small.

The ^1H NMR studies of SLUVs also show motional differences between the short-chain and long-chain lecithin components. The short-chain fatty acyl chains in SLUVs are more immobile than in pure short-chain lecithin micelles, since spin diffusion along the fatty acyl chains is not observed in micelles but is clearly seen in SLUVs. In NOESY experiments, the short-chain lecithins do not show cross-peaks with long-chain components. Therefore, short-chain lecithin/long-chain lecithin interactions are probably small and not cooperative. The greater mobility and lack of interlipid interactions may make the short-chain lecithin more "accessible" to phospholipases. Following localization of phospholipase at the surface via initial binding, the enzyme must interact with the glycerol backbone and the fatty acyl linkages in order for phospholipid hydrolysis to occur. [While phospholipase C removes the phosphocholine segment from lecithin, the enzyme from *Bacillus cereus* requires at least one fatty acyl linkage for a phospholipid to be productively bound and hydrolyzed (El-Sayed et al., 1985).] If the short-chain lecithin in SLUVs is dynamically "uncoupled" or "isolated" from its long-chain neighbors, this is now easier. This accessibility may involve easier partial extraction/removal of the short-chain lecithin from the bilayer onto the enzyme for hydrolysis or better binding of the short-chain lecithin to the enzyme, since no additional energy is required to disrupt phospholipid/phospholipid packing interactions. If product release is the rate-limiting step, as has been suggested for phospholipase C from *B. cereus* (El-Sayed & Roberts, 1985), the hydrolysis products must be reinserted or reabsorbed into the aggregate matrix, and this may be more facile in micelles or for short-chain products from SLUVs.

This leads to the following view of phospholipase activity toward different aggregates. Small unilamellar vesicles (or multibilayers) of a single lecithin are poor substrates for these enzymes because of strong intermolecular phospholipid interactions which must be disrupted prior to enzymatic hydrolysis (and possibly for release of product from the enzyme). Short-chain lecithin micelles or detergent mixed micelles with long-chain lecithin are good substrates because phospholipid/phospholipid interactions are weakened or replaced by less effective detergent/phospholipid interactions. SLUVs have both a good substrate (short-chain lecithin) and a poor substrate (long-chain lecithin) in a bilayer arrangement. The more numerous long-chain molecules have strong lateral interactions, while the short-chain species are uncoupled to some extent from these interactions.

ACKNOWLEDGMENTS

We thank Dr. J. N. S. Evans for synthesis of diheptanoylphosphatidyl-*N*,*N*-dimethylethanolamine, Drs. C. D. DeBose and M. Y. El-Sayed for purification of phospholipases A_2 and C, Nike Agman for carrying out several of the phospholipase assays, and Dr. E. Hartweig of the MIT Biology Department for performing the electron microscopy.

REFERENCES

Aakre, S. E., & Little, C. (1982) *Biochem. J. 203*, 799–801.
Akutsu, H., Suezaki, Y., Yoshikawa, W., & Kyogoku, Y. (1986) *Biochim. Biophys. Acta 854*, 231–238.
Andrew, S. B., Faller, J. W., Gilliam, J. M., & Barrnett, R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1814–1818.
Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229–2246.
Burns, R. A., Jr., & Roberts, M. F. (1980) *Biochemistry 19*, 3100–3106.
Burns, R. A., Jr., Friedman, J., & Roberts, M. F. (1981) *Biochemistry 20*, 5945–5950.
Burns, R. A., Jr., Donovan, J. M., & Roberts, M. F. (1983) *Biochemistry 22*, 964–973.
Bystrov, V. F., Dubrovia, N. I., Barsukov, L. I., & Bergelson, L. D. (1971) *Chem. Phys. Lipids 6*, 343–350.
DeBose, C. D., & Roberts, M. F. (1983) *J. Biol. Chem. 258*, 6327–6334.
Deems, R. A., & Dennis, E. A. (1981) *Methods Enzymol. 71*, 703–711.
Dennis, E. A. (1973) *J. Lipid Res. 14*, 152–159.
Eaton, B. R., & Dennis, E. A. (1976) *Arch. Biochem. Biophys. 176*, 604–609.
Eigenberg, K. E., & Chan, S. I. (1980) *Biochim. Biophys. Acta 559*, 330–335.
Ellena, J. F., Hutton, W. C., & Cafiso, J. S. (1985) *J. Am. Chem. Soc. 107*, 1530–1537.
El-Sayed, M. Y. (1984) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.
El-Sayed, M. Y., & Roberts, M. F. (1985) *Biochim. Biophys. Acta 831*, 133–141.
El-Sayed, M. Y., DeBose, C. D., Coury, L. A., & Roberts, M. F. (1985) *Biochim Biophys. Acta 837*, 325–335.
Gabriel, N. E. (1986) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.
Gabriel, N. E., & Roberts, M. F. (1984) *Biochemistry 23*, 4011–4015.
Gabriel, N. E., & Roberts, M. F. (1986) *Biochemistry 25*, 2812–2821.
Hauser, H., Guyer, W., Levine, B., Skrabal, P., & Williams, R. J. P. (1978) *Biochim. Biophys. Acta 508*, 450–463.
Hauser, H., Gains, N., & Mueller, M. (1983) *Biochemistry 22*, 4775–4781.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.
Kumar, A., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 96*, 1156–1163.
Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. (1986) *J. Am. Chem. Soc. 108*, 3499–3507.
Lin, T.-L., Chen, S.-H., Gabriel, N. E., & Roberts, M. F. (1987a) *J. Phys. Chem. 91*, 406–413.
Lin, T.-L., Chen, S.-H., & Roberts, M. F. (1987b) *J. Am. Chem. Soc.* (in press).
Little, C., & Johansen, S. (1979) *Biochem. J. 197*, 509–514.
Macura, S., Wuthrich, K., & Ernt, R. R. (1982) *J. Magn. Reson. 47*, 351–357.
Pascher, I., & Sundell, S. (1986) *Biochim. Biophys. Acta 855*, 68–78.
Pearson, R. H., & Pascher, I. (1979) *Nature (London) 281*, 499–501.
Roberts, M. F., Otnaess, A.-B., Kensil, C. A., & Dennis, E. A. (1978) *J. Biol. Chem. 253*, 1252–1257.
Schuh, J. R., Banerjee, U., Mueller, L., & Chan, S. I. (1982) *Biochim. Biophys. Acta 687*, 219–225.
Seelig, J., Gally, H.-U., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–119.
Stark, R. E., Gosselin, G. J., Donovan, J. M., Carey, M. C., & Roberts, M. F. (1985) *Biochemistry 24*, 5599–5605.
Sundaralingam, M. (1972) *Ann. N.Y. Acad. Sci. 195*, 324–355.