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Enzymatic Hydrolysis of Short-Chain Lecithin/Long-Chain Phospholipid Unilamellar Vesicles: Sensitivity of Phospholipases to Matrix Phase State†

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ABSTRACT: Short-chain lecithin/long-chain phospholipid unilamellar vesicles (SLUVs), unlike pure long-chain lecithin vesicles, are excellent substrates for water-soluble phospholipases. Hemolysis assays show that >99.5% of the short-chain lecithin is partitioned in the bilayer. In these binary component vesicles, the short-chain species is the preferred substrate, while the long-chain phospholipid can be treated as an inhibitor (phospholipase C) or poor substrate (phospholipase A_2). For phospholipase C *Bacillus cereus*, apparent K_m and V_{max} values show that bilayer-solubilized diheptanoylphosphatidylcholine (diheptanoyl-PC) is nearly as good a substrate as pure micellar diheptanoyl-PC, although the extent of short-chain lecithin hydrolysis depends on the phase state of the long-chain lipid. For phospholipase A_2 *Naja naja naja*, both K_m and V_{max} values show a greater range: in a gel-state matrix, diheptanoyl-PC is hydrolyzed with micellelike kinetic parameters; in a liquid-crystalline matrix, the short-chain lecithin becomes comparable to the long-chain component. Both enzymes also show an anomalous increase in specific activity toward diheptanoyl-PC around the phase transition temperature of the long-chain phospholipid. Since the short-chain lecithin does not exhibit a phase transition, this must reflect fluctuations in head-group area or vertical motions of the short-chain lecithin caused by surrounding long-chain lecithin molecules. These results are discussed in terms of a specific model for SLUV hydrolysis and a general explanation for the "interfacial activation" observed with water-soluble phospholipases.

Phospholipases are small, water-soluble enzymes that catalyze the hydrolysis of phospholipid ester linkages. Phospholipase A_2 acts specifically on the *sn*-2 fatty acyl bond (Van Deenen & de Haas, 1964) while phospholipase C is specific for the phosphoglycerate bond of phospholipids (Little, 1981). These enzymes exhibit "interfacial activation" or a preference for substrate in aggregated forms (Pieterson et al., 1974; El-Sayed & Roberts, 1985). Furthermore, the type of phospholipid aggregate (e.g., bilayer, vesicle, or micelle) has a dramatic effect on the enzyme specific activity (Dennis, 1983; DeBose & Roberts, 1983). A number of hypotheses have been proposed to explain the observed kinetic trends. Several of these focus on aggregation-induced changes in the substrate (Brockerhoff, 1968; Wells, 1974, 1978; Apitz-Castro et al., 1979; Upreti & Jain, 1980). Other workers have attributed interfacial activation to phospholipid aggregation inducing conformational changes in the enzyme (Verger & de Haas, 1973; Roberts et al., 1977; Pluncknthun & Dennis, 1982). Yet another possibility is that the lipid matrix aggregation state may affect product release (El-Sayed & Roberts, 1985). Part of the problem in testing these different ideas is that different substrate chain lengths as well as physical aggregation states have been used.

To understand the different facets of interfacial activation of phospholipases, we have used a series of novel unilamellar vesicles made with short-chain lecithins and long-chain phospholipids as substrates for phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*). The short-chain species in a micellar matrix are excellent substrates for these enzymes. How its hydrolysis rate will be affected when it is incorporated in a bilayer matrix of long-chain phospholipids, which is normally a poor substrate, should shed light on the reasons for the kinetic differences in micelles vs bilayers. Short-chain lecithin/long-chain phospholipid unilamellar vesicles (SLUVs)[1] have been characterized in considerable detail (Gabriel & Roberts, 1984, 1986, 1987). SLUVs are exceptionally stable and range in size from 150 to 1000 Å in diameter, depending on the phase state of the long-chain component.

Preliminary kinetic studies (Gabriel & Roberts, 1987) have shown that the short-chain lecithin is the preferred substrate for both phospholipase A_2 (~10-fold higher hydrolysis rates for the short-chain species over the long-chain component) and phospholipase C (~100-fold higher rates). In fact, hydrolysis of the short-chain lecithin proceeded at rates comparable to those for short-chain lecithin micelles, while the long-chain lipid in SLUVs was hydrolyzed at rates comparable to those

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[1] Abbreviations: SLUV, short-chain lecithin/long-chain phospholipid unilamellar vesicle; PC, phosphatidylcholine; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; cmc, critical micelle concentration; T_m, midpoint temperature of the gel to liquid-crystalline transition of a long-chain phospholipid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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for unilamellar vesicles of the pure lipid. Therefore, with SLUVs, we have a good substrate as a minor component organized in a bilayer matrix where the matrix molecules are essentially nonsubstrates. This makes these unique vesicles a perfect system for determining (i) apparent V_{max} and K_m values for the short-chain lecithin in bilayer and micelle aggregates, (ii) the extent of short-chain lecithin hydrolysis and the resultant effect on SLUV integrity, (iii) how the phase state of the nonsubstrate long-chain phospholipid matrix affects hydrolysis of the short-chain lecithin, and (iv) if vesicle size affects phospholipase activity.

Materials and Methods

Chemicals. Diheptanoyl-PC, dilauroyl-PC, dimyristoyl-PC, dipalmitoyl-PC, distearoyl-PC, and sphingomyelin (bovine) were obtained from Avanti Polar Lipids (Birmingham, AL). Egg PC was obtained from Sigma Chemical Co. (St. Louis, MO). Dihexadecyl-PC was purchased from Calbiochem Behring Corp. (La Jolla, CA). Phospholipids were checked for purity by thin-layer chromatography (Burns & Roberts, 1980). If any phospholipid degradation products were detected, the phospholipids were purified by chromatography on silicic acid. 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 venom (*Naja naja naja*) (Deems & Dennis, 1981), and phospholipase C was purified from the growth media of *Bacillus cereus* ATCC 10987 (Aakre & Little, 1982).

Vesicle Preparation. Short-chain lecithin/long-chain phospholipid unilamellar vesicles were prepared for phospholipase assays (Gabriel & Roberts, 1987) by cosolubilizing both lipids in $CHCl_3$, removing organic solvent under a stream of N_2, evacuating the samples at low pressure for at least 12 h, and then hydrating with water. Vesicle samples were often warmed to ~40 °C for a few minutes; this aided in vesiculation of SLUVs formed with saturated chain lecithins. Samples were equilibrated at room temperature 6–8 h prior to pH-stat or TLC assays.

Phospholipase Kinetics. Enzyme activity toward total phospholipid was assayed at pH 8 by pH-stat titration of the monoester phosphoric acid (phospholipase C) or fatty acid (phospholipase A_2) products of lecithin hydrolysis. NaOH (5 mM) was used as the titrant in a Radiometer pH-stat, Model AA-60, with a temperature-controlled water bath. Rates given are initial rates (i.e., within 1–2 min of the reaction profile). Under these conditions, phospholipid aggregates are still vesicular in nature, and product inhibition is minimized (vide infra). All specific activities reported are based on assays of at least two samples. Obtaining all the kinetic measurements spanned 6 months with different batches of SLUVs and enzymes. This leads to some variability in specific activities. In an attempt to normalize this, each assay set (for example, the dependence of activity on substrate concentration, or the dependence of activity toward a specific SLUV mixture on assay temperature) was compared at the same time to enzyme activities toward 5 mM diheptanoyl-PC micelles at 25 °C. Phospholipase A_2 has a specific activity of ~1000 μmol min^{-1} mg^{-1} toward this substrate, while phospholipase C has an activity of ~2500 μmol min^{-1} mg^{-1}.

SLUVs formed with *N*-[^{14}C]methylcholine-labeled dipalmitoyl-PC (Amersham) were used to distinguish the hydrolysis of the long-chain component from total lipid hydrolysis rates measured by pH-stat. Aliquots (100 μL) of the reaction mixture (in 0.1 M Tris buffer, pH 8) were removed as a function of incubation time with the enzymes and quenched with 1 mL of $CHCl_3/CH_3OH$ (2:1), 20 μL of acetic acid, and 0.2 mL of water. The aqueous and organic phases were separated via centrifugation. For phospholipase C kinetics, 50 μL of aqueous and organic phases was counted since unhydrolyzed phospholipid would partition into the organic phase while the radiolabeled phosphocholine would partition into the aqueous phase. The products of phospholipase A_2 hydrolysis of the long-chain lecithin (lyso-PC and fatty acid) will partition into the organic phase with unhydrolyzed substrate and must be separated further by TLC in $CHCl_3/CH_3OH/H_2O$ (65:25:4). After being stained with I_2, equal areas of the silica gel plate containing the long-chain lecithin and the lysolecithin were scraped into scintillation vials and counted. The rate of hydrolysis was calculated from a plot of the normalized cpm of the lyso-PC (indicating product formation compared to total lecithin) vs time.

Hemolysis Assay. Whole blood either was obtained from a blood bank or was freshly drawn from a human volunteer. Erythrocytes were separated from serum by centrifugation in a Dynac centrifuge for 10 min at 2000 rpm. Pelleted erythrocytes were washed 3 times with saline solution (PBS buffer) containing 140 mM NaCl, 5 mM KH_2PO_4, 2.5 mM NaH_2PO_4, and 1 mM $MgSO_4$. After the fourth centrifugation, the pellet was resuspended to 10% pellet volume/total volume in PBS. This preparation was used for analysis of residual monomeric or micellar short-chain lecithin in SLUV solutions. The erythrocyte suspension was diluted 1:1 with pure short-chain lecithin aqueous solution or SLUV solutions and incubated for 10 min at 25 °C or the indicated temperature. The mixture was then centrifuged at 1000 rpm in a clinical centrifuge and the supernatant checked for hemoglobin content by using a Sigma Chemical Co. diagnostic kit (525).

NMR Spectroscopy. ^{31}P NMR spectra at 109.3 MHz were acquired on a Bruker 270 spectrometer. Approximately 300 transients were obtained for samples in 10-mm tubes. Spectral parameters include 60° pulse angle (20 μs), 0.5-s delay between pulses, 4000-Hz sweep width, ^{1}H NMR noise decoupling, and 5.0-Hz exponential weighting function. For phospholipase A_2 activity toward SLUVs composed of sphingomyelin and diheptanoyl-PC, the phosphorus line widths of the long-chain species (40–45 Hz), the short-chain lecithin substrate (10–12 Hz), and the product 1-heptanoyl-PC (5–6 Hz) did not change over the 80-min time course. Therefore, the reaction was monitored by following peak heights of these species.

Results

Erythrocyte Assay for Residual Short-Chain Lecithin Micelles and Monomers. Since the short-chain lecithins have high cmc's compared to long-chain phospholipids, a finite concentration should exist as monomers in SLUV preparations. Micelles of short-chain lecithin or mixed micelles of short- and long-chain species could also coexist with SLUVs. These would not be detectable by electron microscopy and could bias enzyme kinetics since micellar species give rise to high phospholipase A_2 and phospholipase C activities (Pieterson et al., 1974; El-Sayed & Roberts, 1985). Short-chain lecithin monomers and micelles exhibit hemolytic properties (Kitagawa et al., 1977; Reman et al., 1969). The release of hemoglobin from red blood cells as a function of increasing diheptanoyl-PC concentration is shown in Figure 1A. Both monomeric and micellar diheptanoyl-PC readily lyse human erythrocytes. Other micelles, for example, Triton X-100 (5 mM), or mixed micelles of Triton X-100 (20 mM) with dipalmitoyl-PC (5 mM) completely lyse red blood cells in this assay. Thus, if SLUVs contain any residual monomeric or micellar species,

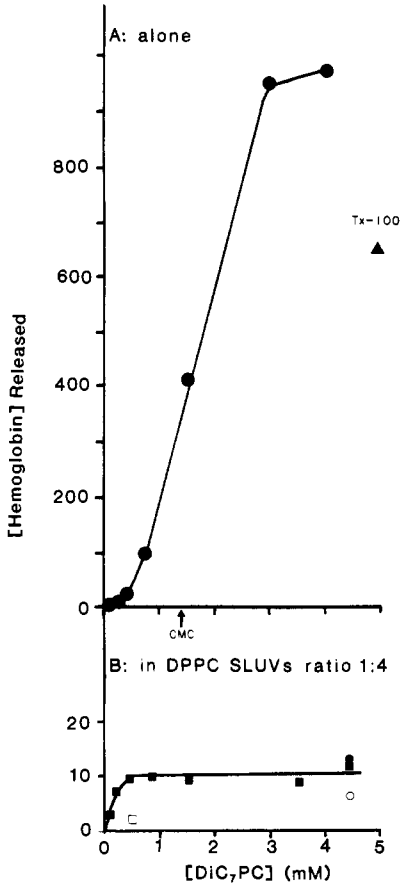

FIGURE 1: Release of hemoglobin from human erythrocytes as a function of diheptanoyl-PC concentration: (A) (●) Monomeric and micellar diheptanoyl-PC (the lecithin cmc is indicated by an arrow); (▲) micellar Triton X-100 (5 mM) at 25 °C. (B) Dipalmitoyl-PC/diheptanoyl-PC (4:1) SLUVs at 25 °C (■) and at 37 °C (●) (open symbols indicate blanks at the two different temperatures).

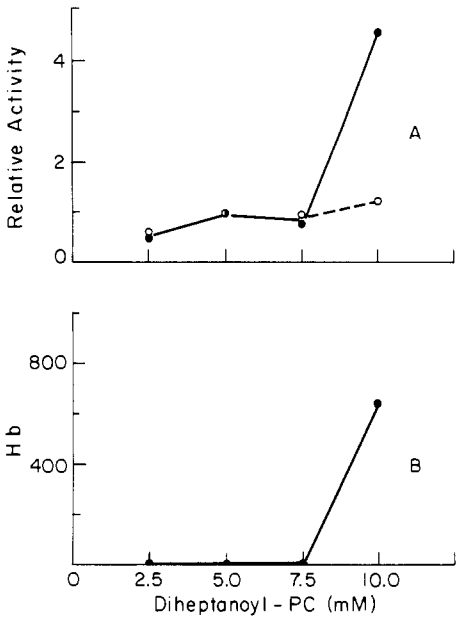

FIGURE 2: (A) Relative enzymatic activity of phospholipase A_2 (●) and phospholipase C (○) toward SLUV preparations with 20 mM distearoyl-PC and variable concentrations of diheptanoyl-PC. The values of the pH-stat assay for the 20 mM distearoyl-PC/5 mM diheptanoyl-PC SLUV sample are used to normalize the data. (B) Hemoglobin released from human erythrocytes after incubation with the same SLUV preparations used in (A).

they will be detected by lysis of erythrocytes and release of hemoglobin into the supernatant. As shown in Figure 1B, SLUVs made with diheptanoyl-PC/dipalmitoyl-PC (1:4) do not lyse erythrocytes. In this experiment, the ratio of long-chain lecithin to short-chain lecithin is kept constant at 4, while the total amount of lecithin is varied from 0.25 to 25 mM. This assay confirms that the short-chain phospholipid stays partitioned in the SLUV bilayer (>98% in the bilayer) even if the vesicles are diluted by factors up to 100. The very small level of hemolysis detected is barely above the blank background (where the erythrocytes are incubated with buffer instead of SLUVs) and is considerably below levels observed for the addition of monomeric short-chain lecithin. Nonbilayer short-chain lecithin must be less than 0.02 mM in the 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC SLUV mixture. This amount of monomeric diheptanoyl-PC is well below the monomer K_m observed for either phospholipase A_2 or phospholipase C (El-Sayed & Roberts, 1985). Therefore, the high enzymatic activity observed toward SLUVs, 550 μmol min^{-1} mg^{-1} for phospholipase A_2 and 1590 μmol min^{-1} mg^{-1} for phospholipase C (Gabriel & Roberts, 1987), must represent activity toward the phospholipid species situated in the bilayer. The ability of the 5 mM diheptanoyl-PC/20 mM dipalmitoyl-PC SLUVs to induce hemolysis was also examined as a function of temperature (Figure 1B). No increase in hemolysis was detected at or above the phase transition temperature (T_m) of these vesicles (Gabriel & Roberts, 1986). In general, SLUVs made of 5 mM short-chain lecithin and 20 mM long-chain phospholipid showed no lysis at 25 °C in this assay. Therefore, observed enzyme activities reflect hydrolysis of bilayer and not micellar or monomeric short-chain species.

Diheptanoyl-PC/distearoyl-PC SLUVs prepared with increasing ratios of short-chain lecithin yield particles whose average diameter decreases from 300 to 100 Å (Gabriel & Roberts, 1986). Both phospholipase A_2 and phospholipase C activities show a dependence on the ratio of long-chain (20 mM) to short-chain (variable) lecithin. Phospholipase A_2 activity is roughly constant for 2.5–7.5 mM diheptanoyl-PC and then dramatically increases for 10 mM diheptanoyl-PC in the SLUV mixture. Phospholipase C shows about a 20% increase in activity over the same range of increase for short-chain lecithin/long-chain lipid. If the enzyme specific activities are compared with the hemolytic abililty of the same SLUV mixtures (Figure 2), one notes that the sudden increase in enzyme activities occurs when micellar/monomer species are detected. Other SLUV mixtures which yield small particles as detected in electron micrographs, such as those prepared from sphingomyelin (20 mM) and 5 mM diheptanoyl-PC, do not lyse erythrocytes; hence, they have very little micellar or monomeric short-chain species.

Particle Integrity upon Enzymatic Hydrolysis. ^{31}P NMR spectroscopy was used to check the integrity of SLUV particles during enzymatic hydrolysis of the short-chain lecithin. Diheptanoyl-PC (5 mM)/sphingomyelin (20 mM) was incubated with phospholipase A_2 (0.1 μg/mL) and 2 mM Ca^{2+} or phospholipase C (0.05 μg/mL), and spectra were acquired (at 30 °C) as a function of time after addition of enzyme. This SLUV mixture was used since the phosphorus resonances for sphingomyelin and lecithin are resolvable (0.37 ppm separation) and distinct from either hydrolysis product, lysolecithin or phosphocholine. During phospholipase A_2 hydrolysis, the intensity of the sphingomyelin resonance remains constant while the diheptanoyl-PC resonance decreases in intensity and a new resonance (lyso-PC) appears (Figure 3). The amount of enzyme used was 0.25 of that used in pH-stat assays. Basic line shapes and line widths of the three resonances stay constant up to 80 min (at which point about 30% of the diheptanoyl-PC has been hydrolyzed), indicating that the vesicles

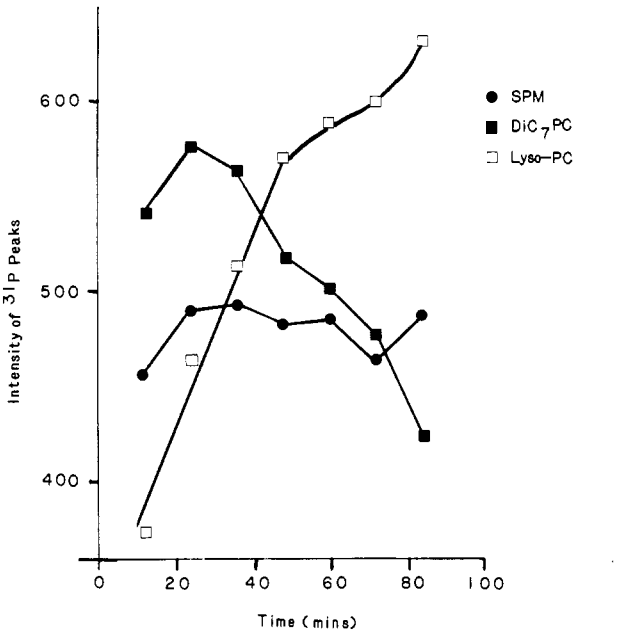

FIGURE 3: Phospholipase A_2 catalyzed hydrolysis of sphingomyelin/diheptanoyl-PC (20 mM:5 mM) followed by ^{31}P (109.3 MHz) NMR spectroscopy at 30 °C. Enzyme concentration was 0.1 μg/mL. Since resonance line widths remained constant over the time course of the incubation, peak height, rather than integrated area, is plotted as a function of time: (■) diheptanoyl-PC; (●) sphingomyelin; (□) 1-heptanoyl-PC (lysolecithin).

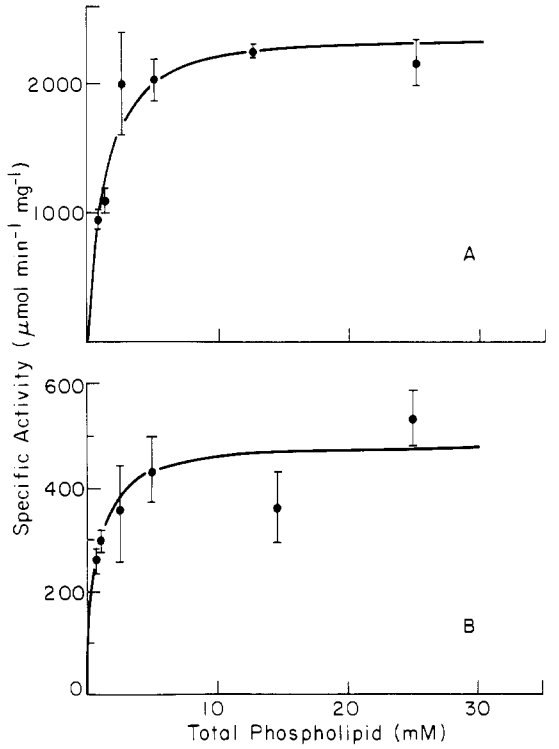

FIGURE 4: Phospholipase specific activity at 25 °C (determined by pH-stat) toward dipalmitoyl-PC/diheptanoyl-PC (4:1) SLUVs as a function of total lecithin concentration: (A) phospholipase C (*B. cereus*); (B) phospholipase A_2 (*N. naja naja*).

do not undergo any drastic morphological change with the phospholipase A_2 catalyzed hydrolysis of the diheptanoyl-PC. Enzyme specific activity was estimated as 200 μmol min^{-1} mg^{-1} under these conditions. A similar study was done following the hydrolysis of SLUVs by phospholipase C (data not shown). Phosphorus line shapes are initially constant, but after 15–20% hydrolysis of diheptanoyl-PC to diglyceride by phospholipase C, both sphingomyelin and lecithin resonances broaden dramatically. The resonance for water-soluble phosphocholine remains sharp during this time course. This indicates that a large change in vesicle structure occurs when 15–20% diglyceride is produced. Before this point, in particular for the first few minutes of the pH-stat assay when <5% of the short-chain lecithin is hydrolyzed, the vesicles remain intact.

Effect of Substrate Concentration. Phospholipase specific activity was measured as a function of substrate concentration at 25 °C. While the pH-stat assay does not distinguish which lecithin is hydrolyzed, we have already established that the short-chain species are the major substrate for phospholipase A_2 and essentially the sole substrate for phospholipase C (Gabriel & Roberts, 1987). Figure 4 shows specific activities of both phospholipases C and A_2 as a function of increasing total phospholipid concentration at a fixed ratio of diheptanoyl-PC to dipalmitoyl-PC (1:4). Eadie–Hofstee plots of the data yield an apparent K_m of 1.1 mM total lecithin (0.2 mM diheptanoyl-PC) and a V_{max} of 2400 μmol min^{-1} mg^{-1} for phospholipase C, and an apparent K_m of 0.81 mM total lecithin (0.16 mM diheptanoyl-PC) and a V_{max} of 500 μmol min^{-1} mg^{-1} for phospholipase A_2. Comparable activities have been observed for detergent-mixed micelles of long-chain lecithins (Roberts et al., 1978). While the SLUV V_{max} values are 0.5–0.8 of those of pure diheptanoyl-PC micelles, the apparent K_m values are considerably higher, even if only the short-chain lecithin is considered.

Apparent K_m and V_{max} values have been obtained for other SLUV mixtures (Table I). V_{max} values for phospholipase C hydrolysis of diheptanoyl-PC in the different long-chain matrices are all similar. Apparent K_m values, on the other hand, range from 0.03 to 0.6 mM diheptanoyl-PC. The lowest apparent K_m, which is comparable to the value obtained for pure diheptanoyl-PC micelles, is for diheptanoyl-PC in sphingomyelin, which is neither a substrate nor an effective inhibitor for phospholipase C (*B. cereus*). Dihexadecyl-PC is also a poor substrate, but it does affect the hydrolysis of dipalmitoyl-PC in mixed vesicles (El-Sayed et al., 1985). This suggests that the differences in the apparent K_m for diheptanoyl-PC in various SLUV mixtures might be rationalized by treating the long-chain species as a competitive inhibitor and that the identity of the long-chain matrix has no specific effect on the rate-limiting step in catalysis. An apparent "K_i" for the long-chain species can be estimated from the relationship

Table I: Kinetic Parameters for Phospholipase-Catalyzed Hydrolysis of Diheptanoyl-PC in SLUVs (4:1, Long-Chain Phospholipid/Short-Chain Lecithin) at 25 °C

enzyme	long-chain phospholipid[a]	V_{max} (μmol min^{-1} mg^{-1})	K_m[b] (mM)	K_m-(diC$_7$PC)[c] (mM)	K_i(long-chain species)[d] (mM)
PLC	DPPC	2450	1.1	0.2	1.3
	DHPC	1660	1.0	0.2	1.0
	egg PC	1675	3.1	0.6	0.5
	SPM	1246	0.16	0.03	
	e	2650		0.02	
PLA_2	DPPC	490	0.8	0.14	
	egg PC	90	3.2	0.6	
	SPM	1160	1.8	0.36	
	e	1000			

[a] Abbreviations: PLC, phospholipase C; PLA_2, phospholipase A_2; DPPC, dipalmitoyl-PC; DHPC, dihexadecyl-PC; SPM, sphingomyelin; diC$_7$PC, diheptanoyl-PC. [b] Apparent K_m in terms of total phospholipid. [c] Apparent K_m for diheptanoyl-PC. [d] Apparent K_i extrapolated for the long-chain phospholipid acting as a competitive inhibitor of phospholipase-C. [e] No long-chain lecithin present; hence, these samples are pure lecithin micelles [K_m values for phospholipase C taken from El-Sayed et al. (1985)].

$V_{obsd} = V_{max}S_0/[K_m(1 + I/K_i) + S_0]$ using 0.03 mM (obtained from diheptanoyl-PC/sphingomyelin SLUVs) as the uninhibited K_m for diheptanoyl-PC. Because SLUVs as the sole aggregate only form within a narrow range of ratios of short-chain lecithin to long-chain phospholipid (Gabriel & Roberts, 1986), we cannot vary the ratio of these two compounds without introducing the significant complication of other phospholipid aggregates. For a short-chain lecithin: long-chain lipid ratio less than 0.25, we will have excess multilamellar structures coexisting with SLUVs; for a short-chain lecithin:long-chain lecithin ratio greater than 0.3, micellar species can coexist with the SLUV bilayers. Our apparent K_i values, which cannot be obtained in the usual kinetic fashion, allow us to compare how different long-chain phospholipids interact with phospholipase C. The "K_i" values (1.3 mM for dipalmitoyl-PC, 1.0 mM for its ether-linked analogue dihexadecyl-PC, and 0.5 mM for egg PC) obtained in this fashion are less than the apparent K_m for phospholipase C hydrolysis of dimyristoyl-PC sonicated vesicles (~6.7 mM; Burns et al., 1982) and the K_m for dipalmitoyl-PC (~6 mM) in mixed sonicated unilamellar vesicles with dihexadecyl-PC (which has an apparent K_i of 3.5 mM) (El-Sayed et al., 1985). Therefore, in these binary component unilamellar vesicles, both long-chain and short-chain species bind with reasonable affinity to phospholipase C.

Phospholipase A_2 kinetics on different SLUV mixtures are considerably more complex (Table I). Both apparent V_{max} and K_m values vary about 5-fold. Hydrolysis of the long-chain lecithin in SLUVs is approximately one-tenth that of diheptanoyl-PC (Gabriel & Roberts, 1987). To see the extent to which this might affect our parameters for diheptanoyl-PC, we can treat the dilution kinetics as reflecting enzyme activity toward two competitive substrates, each characterized by its own V_{max} and K_m. On the basis of the work of Kensil and Dennis (1979) with egg lecithin vesicles, these parameters for the long-chain species in SLUVs are estimated as V_{max} = 100 μmol min^{-1} mg^{-1} and K_m = 5 mM. For diheptanoyl-PC in the gel phase dipalmitoyl-PC matrix, the apparent K_m becomes 0.19 mM, while V_{max} is 560 μmol min^{-1} mg^{-1}. Diheptanoyl-PC in the egg PC matrix has dramatically different values: K_m = 1.3 mM and V_{max} = 80 μmol min^{-1} mg^{-1}. Diheptanoyl-PC in sphingomyelin, which is an inhibitor but not a substrate, shows both a higher V_{max} and a higher apparent K_m. Both dipalmitoyl-PC and sphingomyelin are in a gel phase at 25 °C and form small, highly curved SLUVs, while egg PC is liquid crystalline at that temperature and forms very large SLUVs (~1000 Å; Gabriel & Roberts, 1986). This suggests that phospholipase A_2 prefers either diheptanoyl-PC in a gel-state matrix or smaller SLUVs.

Extent of Short-Chain Lecithin Hydrolysis. The total amount of lecithin in SLUVs that is hydrolyzed by phospholipase C and phospholipase A_2 was measured for several vesicle mixtures (Table II). At 25 °C, phospholipase C hydrolyzes 19% ± 2% of the total phospholipid in SLUVs made with gel phase long-chain components (dipalmitoyl-PC, sphingomyelin). Since dipalmitoyl-PC is not appreciably hydrolyzed in these vesicles and since sphingomyelin is not a substrate, this corresponds to 95% of the diheptanoyl-PC. In other words, phospholipase C can hydrolyze all of the short-chain lecithin. Since 20–30% of the diheptanoyl-PC is on the inner monolayer (Gabriel & Roberts, 1987), as phospholipase C hydrolyzes some of the short-chain lecithin to diglyceride, the SLUV particles must undergo increased transbilayer flip-flop, fusion, or inner–outer exchange to make all short-chain lecithins accessible. This is consistent with the ^{31}P NMR data suggesting extensive aggregation or fusion upon phospholipase C hydrolysis of sphingomyelin/diheptanoyl-PC SLUVs. As the assay temperature is increased for the 10 mM diheptanoyl-PC/40 mM dipalmitoyl-PC sample, the extent of short-chain lecithin hydrolyzed by phospholipase C decreases (54% at 35 °C, 21% at 45 °C). In SLUVs made with egg PC, only 36% of the short-chain lecithin is converted to diglyceride at 25 °C. This implies that the phase state of neighboring long-chain molecules affects the total availability of short-chain lecithin. In a fluid long-chain matrix, less diheptanoyl-PC is accessible for phospholipase C hydrolysis at near-micellar rates.

Table II: Total Phospholipid in SLUVs (4:1, Long-Chain Phospholipid/Diheptanoyl-PC) Hydrolyzed by Phospholipases[a]

long-chain phospholipid	total phospholipid (μmol)	temp (°C)	phospholipid hydrolyzed (μmol) PLA$_2$	PLC
dipalmitoyl-PC	10	25	1.25	1.82
	5	25	0.46	0.97
	2.5	25	0.38	0.58
	1.25	25	0.19	
	50	35		5.44
	50	45		2.12
dipalmitoyl-PC/cholesterol (0.2 mM)	10	25	1.31	2.00
sphingomyelin	50	25		9.32
	25	25		3.81
	10	25	1.73	1.96
	5	25	0.84	0.74
	2.5	25		0.49
	1.25	25		0.22
egg PC	10	25		0.73

[a] Determined from the amount of base added in the pH-stat assay for phospholipase activity.

Phospholipase A_2 exhibits slightly different behavior. Only the short-chain lecithin is susceptible to phospholipase hydrolysis in sphingomyelin/diheptanoyl-PC SLUVs. The amount of product formation corresponds to 85% hydrolysis of short-chain lecithin. SLUVs made with dipalmitoyl-PC have two competing substrates. At 25 °C, 13% ± 2% of the total phospholipid is hydrolyzed by phospholipase A_2 at the same rate (i.e., no curvature visible in enzyme progress curves) before an apparent cessation in activity. If diheptanoyl-PC/[*methylcholine*-^{14}C]dipalmitoyl-PC (5 μmol:20 μmol) is used as the substrate in buffer and the extent of dipalmitoyl-PC hydrolysis measured by TLC, one finds that less than 0.2 μmol of dipalmitoyl-PC has been hydrolyzed. By pH-stat, 3.25 μmol of total PC would be hydrolyzed. Therefore, 3.05 μmol of this represents diheptanoyl-PC. Phospholipase A_2 hydrolyzes greater than 60% of the total short-chain species. These SLUVs are small, asymmetric vesicles with 70–80% of the total short-chain lecithin on the outer monolayer as detected by NMR experiments (Gabriel & Roberts, 1987). Hence, phospholipase A_2 hydrolyzes nearly all exterior short-chain lecithin and produces a modified vesicle (now three components) which is stable to rapid transbilayer flip-flop of interior diheptanoyl-PC. SLUVs made with sphingomyelin are slightly smaller (150 Å) than those made with dipalmitoyl-PC (170 Å) and would be expected to have more short-chain lecithin on the exterior monolayer.

Effect of Temperature on Enzymatic Activity. The preference of phospholipase A_2 for diheptanoyl-PC in SLUVs where the long-chain phospholipid is in a gel-state matrix suggests that the phase state of the long-chain phospholipid may be important. For several of the SLUV mixtures, the long-chain species forming the nonsubstrate (or poor substrate) matrix undergoes a transition from gel phase to a liquid-

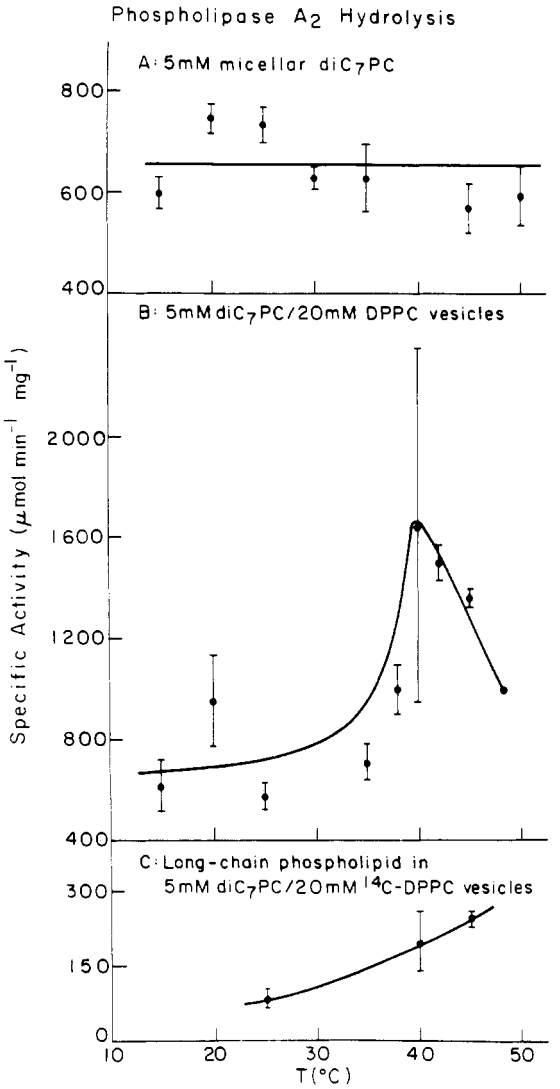

FIGURE 5: Specific activity (determined by pH-stat) of phospholipase A_2 (*N. naja naja*) toward (A) diheptanoyl-PC micelles (5 mM) and (B) dipalmitoyl-PC/diheptanoyl-PC (20 mM:5 mM) SLUVs as a function of temperature. In (C), the enzyme activity toward the dipalmitoyl-PC component is shown in SLUVs (determined by using ^{14}C-labeled dipalmitoyl-PC and a TLC assay for product production).

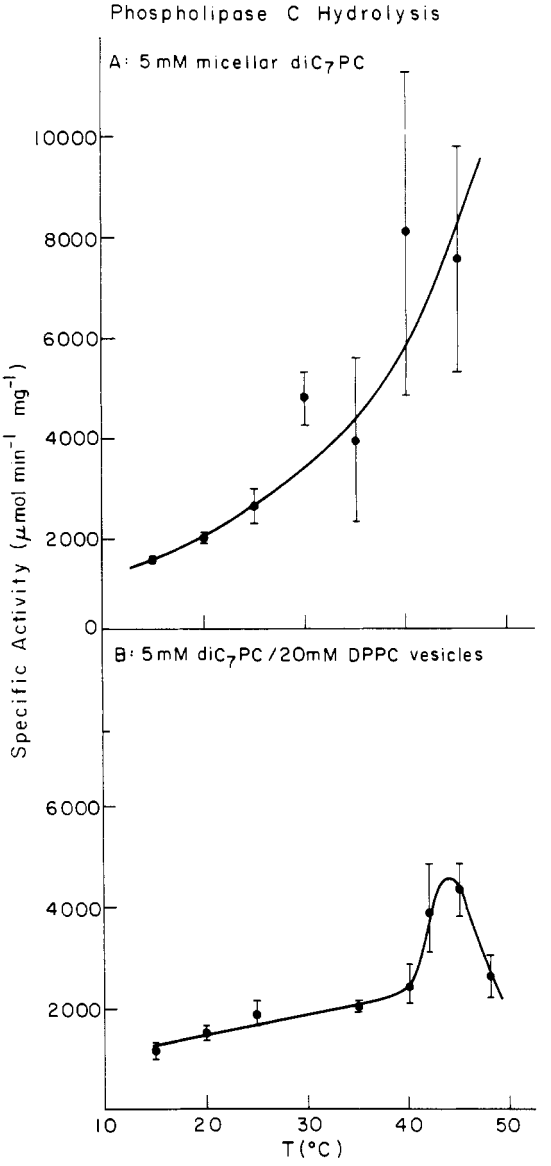

FIGURE 6: Specific activity (determined by pH-stat) of phospholipase C (*B. cereus*) toward (A) diheptanoyl-PC micelles (5 mM) and (B) dipalmitoyl-PC/diheptanoyl-PC (20 mM:5 mM) SLUVs as a function of assay temperature.

crystalline phase without a large perturbation in bilayer size, at least immediately above the T_m (Gabriel & Roberts, 1986). This change in the motional properties of the matrix may affect enzymatic hydrolysis of the short-chain species. Both phospholipase A_2 and phospholipase C activities toward micellar diheptanoyl-PC (5 mM) have been determined as a function of temperature. Diheptanoyl-PC micelles exhibit no marked morphological transitions from 20 to 50 °C (Burns et al., 1983); hence, enzyme kinetics toward these rod-shaped micelles are assumed to reflect the intrinsic temperature properties of the enzyme. Phospholipase A_2 specific activity is basically constant in this series of assays (640 ± 70 μmol min^{-1} mg^{-1}) over that temperature range (Figure 5A), while phospholipase C activity increases significantly (Figure 6A). An activation energy (E_a) of 10.6 kcal/mol is calculated from an Arrhenius plot of micellar diheptanoyl-PC hydrolysis by the latter enzyme. E_a for phospholipase A_2 hydrolysis of diheptanoyl-PC is estimated to be less than 2.5 kcal/mol. For comparison, Kensil and Dennis (1979) report an E_a of 6.9 kcal/mol for cobra venom phospholipase A_2 hydrolysis of mixed micelles of Triton X-100 and egg PC, and Wells (1974) obtained an E_a of 9.6 kcal/mol for dioctanoyl-PC hydrolysis by a rattlesnake venom phospholipase A_2.

In Figures 5B and 6B are shown the temperature profiles for phospholipase A_2 and phospholipase C hydrolysis of SLUVs made with diheptanoyl-PC/dipalmitoyl-PC (5 mM:20 mM). The profiles do not resemble those obtained with the micellar substrate but are similar to one another. A pronounced increase in specific activity occurs as the phase transition temperature of dipalmitoyl-PC is approached, followed by a decrease in activity above T_m. The specific activities observed at all temperatures are less than micellar rates for phospholipase C but can be considerably greater than specific activities of phospholipase A_2 toward diheptanoyl-PC. An E_a of 4.5 kcal/mol is estimated for phospholipase C catalyzed hydrolysis of the short-chain lecithins in SLUVs at temperatures below the phase transition temperature of the long-chain lipid. This is less than the E_a for pure diheptanoyl-PC micelles. Therefore, even though only the short-chain lecithin is hydrolyzed, the bilayer matrix dramatically affects its rate. The rate of dipalmitoyl-PC hydrolysis was followed separately by using diheptanoyl-PC/[*methylcholine*-^{14}C]dipalmitoyl-PC SLUVs. Phospholipase A_2 activities toward this species (Figure 6C) are similar to values for sonicated vesicles, except that no break/peak is observed at the T_m of the long-chain lecithin.

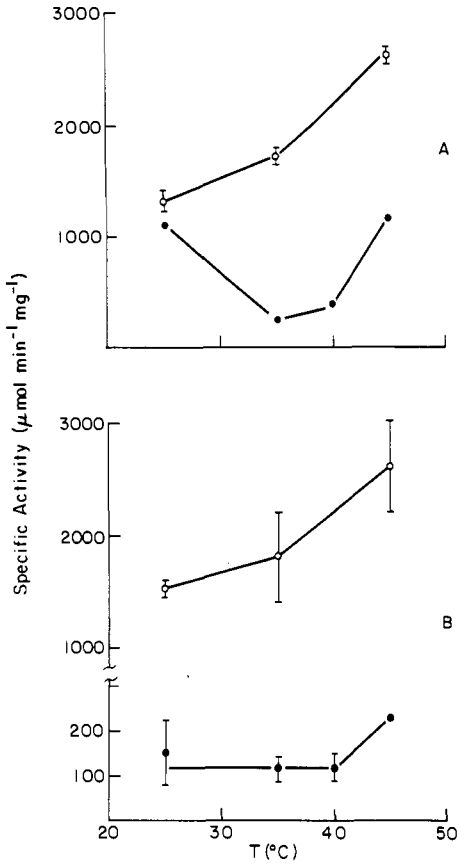

FIGURE 7: Specific activities of phospholipase A_2 (●) and phospholipase C (○) toward SLUVs prepared from (A) sphingomyelin/diheptanoyl-PC (20 mM:5 mM) and (B) egg lecithin/diheptanoyl-PC (20 mM:5 mM) as a function of assay temperature.

No detectable hydrolysis (<10 μmol min^{-1} mg^{-1}) of the long-chain lipid is observed with phospholipase C at elevated temperatures. Similar temperature profiles (i.e., an anomalous increase in specific activity for short-chain lecithin hydrolysis around the T_m of the long-chain lipid) have been obtained for phospholipase A_2 hydrolysis of diheptanoyl-PC in SLUVs made with dimyristoyl-PC or dihexadecyl-PC as the long-chain matrix.

Dramatically different temperature profiles are obtained for phospholipase A_2 hydrolysis of SLUVs made with 5 mM diheptanoyl-PC and 20 mM sphingomyelin or 20 mM egg PC (Figure 7). Bovine brain sphingomyelin has a broad gel-to-liquid-crystalline phase transition centered approximately at 35 °C. Instead of reaching maximum enzymatic activity toward diheptanoyl-PC around this temperature, a minimum in phospholipase A_2 activity is observed with higher activities on either side of the T_m (Figure 7A). Hemolysis assays show evidence of a small amount of monomeric or micellar material at 25 °C. A comparison of the amount of hemoglobin released by pure SLUV mixture at 25 °C to the amount released by diheptanoyl-PC suggests ~0.1 mM of the short-chain lecithin may be free (i.e., not in the bilayer). This concentration of monomers would yield a specific activity of <50 μmol min^{-1} mg^{-1} for phospholipase A_2 (Pieterson et al., 1974; Allgyer & Wells, 1979). The high activity at 45 °C is on a SLUV preparation with little, if any, free diheptanoyl-PC (none above background is detected with the hemolysis assay). Therefore, this represents hydrolysis of the short-chain lecithin in the bilayer. SLUVs made with egg PC (Figure 7B) exhibit a constant low specific activity with temperature. It is comparable to that for pure egg PC vesicles and significantly lower than that observed with other SLUVs.

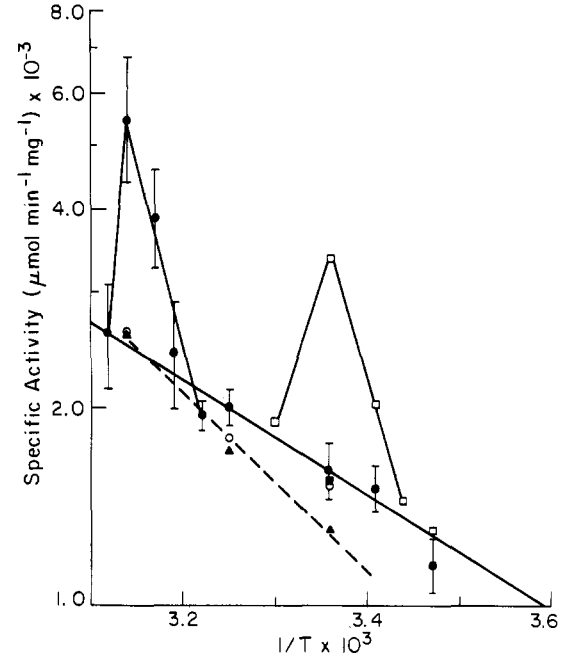

FIGURE 8: Arrhenius plot for phospholipase C (*B. cereus*) activity toward diheptanoyl-PC (5 mM) in SLUVs prepared with different long-chain phospholipids (20 mM): (●) dipalmitoyl-PC; (□) dimyristoyl-PC; (○) egg PC; (▲) sphingomyelin, (■) dihexadecyl-PC. The solid line represents the fit to data for gel phase and liquid-crystalline phase (>5 °C above T_m) SLUVs of saturated lecithins and egg PC. The dashed line indicates the fit for sphingomyelin SLUVs.

In contrast to phospholipase A_2, phospholipase C shows no marked increase or decrease for diheptanoyl-PC hydrolysis in SLUVs made with sphingomyelin at about 35 °C (Figure 7A). Essentially all the phospholipase C data as a function of temperature can be fit with similar E_a's in the gel and liquid-crystalline states (Figure 8). Hydrolysis of diheptanoyl-PC in gel phase dipalmitoyl-PC has an E_a of 4.5 kcal/mol, similar to the E_a of 4.7 kcal/mol for the short-chain lecithin in egg PC SLUVs. Phospholipase C hydrolysis of diheptanoyl-PC in sphingomyelin has a slightly higher E_a (6.2 kcal/mol). All values are considerably less than what is obtained for diheptanoyl-PC micelles. For the two saturated long-chain lecithins dipalmitoyl-PC and dimyristoyl-PC, there is a peak in activity around T_m which decreases in the fluid matrix to values expected, given the temperature dependence of diheptanoyl-PC in gel phase matrices.

Discussion

SLUVs are composed of two different lipids which alone manifest drastically different kinetic behavior with water-soluble phospholipases. Generally, short-chain lecithin micelles are excellent substrates for both phospholipase A_2 and phospholipase C (El-Sayed & Roberts, 1985), while single-component lecithin bilayers are poor substrates (Densil & Dennis, 1979; DeBose & Roberts, 1983). On the other hand, SLUVs are good substrates in which the short-chain lecithin is preferentially hydrolyzed. Kinetic parameters, K_m and V_{max}, for diheptanoyl-PC hydrolysis in SLUVs are similar to values observed with short-chain lecithin micelles except that the long-chain lipid matrix phase state has some influence on enzymatic rates. Diheptanoyl-PC can exist in three distinct states in SLUVs: (i) surrounded by gel-like long-chain phospholipids; (ii) interspersed with solid and fluid patches of long-chain lipid around its phase transition temperature; and (iii) embedded in a liquid-crystalline long-chain lipid milieu. Understanding diheptanoyl-PC hydrolysis in SLUVs

Table III: Enzymatic Hydrolysis of Diheptanoyl-PC (5 mM) in SLUVs as a Function of Long-Chain Phospholipid (20 mM)

long-chain phospholipid	T (°C)	physical state[a]	vesicle diameter (Å)	sp act. (μmol min^{-1} mg^{-1})	
				PLA_2	PLC
dilauroyl-PC	25	lc		2250	
dimyristoyl-PC	15	gel	340[b]	550	1400
	25	T_m	270; lge[c]	1100	3200
	30	lc		300	1900
dipalmitoyl-PC	25	gel	170;[c] 220[b]	550	1590
	40	T_m	170[c]	1600	5500
	45	lc	lge[d]	1200	3000
dihexadecyl-PC	25	gel		300	1500
	40	T_m		900	
	45	lc		600	
distearoyl-PC	25	gel	160[c]	720	2780
egg PC	25	lc	830[c]	80	1500
sphingomyelin	25	gel	150[c]	1100	1300
	35	T_m	–[e]	250	1720
	45	lc	lge[d]	1200	2600

[a] Determined by the relation of the assay temperature to the T_m, gel-to-liquid-crystalline phase transition of the long-chain phospholipid in SLUVs; lc, liquid-crystalline phase ($T_{assay} > T_m$). [b] Determined by quasi-elastic light scattering. [c] Average diameter determined from electron micrographs of SLUV mixtures (Gabriel & Roberts, 1986). [d] Sample dominated by large aggregates, as monitored by ^{31}P NMR spectra (Gabriel & Roberts, 1986). [e] No large change in particle size from 25 to 35 °C as monitored by ^{31}P NMR (Gabriel & Roberts, 1986).

in these three states provides important clues for the mechanism of interfacial activation of phospholipases.

For phospholipase C catalyzed hydrolysis of the short-chain lecithin in gel-state SLUVs, the long-chain phospholipid can be treated as a competitive inhibitor of the good short-chain substrate with an apparent $K_m \simeq 0.03$ mM and $K_i \simeq 1$ mM for these species. There is an enzymatic preference for diheptanoyl-PC in gel phase SLUVs made with longer acyl chains: SLUVs made with distearoyl-PC are better substrates than ones with dipalmitoyl-PC, which are better than dimyristoyl-PC (Table III). Therefore, chain mismatch between the two components in SLUVs appears to be important. In Triton X-100 mixed micelles, the shorter the substrate chain length, the higher the observed phospholipase specific activities (Roberts et al., 1978), for phospholipid fatty acyl chains of six or more carbons (El-Sayed et al., 1985). In SLUVs, the long-chain lipid is not a good substrate; hence, making its chains longer should weaken its interactions with short-chain lecithins and further weaken its inhibition of phospholipase C. SLUV size seems to have no effect on phospholipase C activity.

Phospholipase A_2 activity toward diheptanoyl-PC in gel phase SLUVs cannot be treated as simply as that of phospholipase C, because the long-chain species is in fact a substrate. There is a much wider variation in V_{max}. Some of this may reflect vesicle size. Phospholipase A_2 appears to show a preference for smaller SLUVs (diheptanoyl-PC/sphingomyelin-PC SLUVs have an average diameter of ~150 Å while diheptanoyl-PC/dipalmitoyl-PC or diheptanoyl-PC/distearoyl-PC SLUVs are on average larger).

Both phospholipases show a pronounced activation toward diheptanoyl-PC hydrolysis around the gel-to-liquid-crystalline transition of the long-chain lipid matrix. If one compares enzyme specific activity 5–10 °C below and above T_m, one sees an activity increase with phospholipase C that is consistent with an activation energy of 4.5–6 kcal/mol for that enzyme—i.e., no difference in rates that can be ascribed to diheptanoyl-PC in a fluid vs gel matrix. Phospholipase A_2 shows no large (or consistent) effect in observed rates in fluid long-chain matrices, although long-chain lecithins are hydrolyzed at higher rates in liquid-crystalline SLUVs. These rates are still well below diheptanoyl-PC hydrolysis rates. This behavior contrasts with that of dipalmitoyl-PC-sonicated vesicles where gel phase molecules are better substrates than those in a fluid bilayer above T_m (Kensil & Dennis, 1979). The most dramatic change both enzymes show toward liquid-crystalline SLUVs is not an effect in rates but in the amount of diheptanoyl-PC hydrolyzed. All diheptanoyl-PC in a gel phase matrix can be hydrolyzed by phospholipase C. Only a fraction of this is accessible to the enzyme when the nonsubstrate matrix becomes liquid crystalline.

There are three possible explanations for this behavior. (i) A morphological change in vesicles occurs as the long-chain phospholipid melts. For example, the head-group area may expand such that the short-chain lecithin is now driven toward the inner monolayer of liquid-crystalline SLUVs. (ii) Two pools of diheptanoyl-PC occur: a "loose" or uncoupled diheptanoyl-PC which is noninteractive with neighboring long-chain species (and which is the preferred substrate by both enzymes) and a "restrained" diheptanoyl-PC which interacts more strongly with long-chain species (and is hence not a good substrate). In gel phase SLUVs, the loose diheptanoyl-PC pool is dominant while the restrained pool becomes significant in liquid-crystalline SLUVs. (iii) Product inhibitioon becomes stronger for the short-chain diglyceride or heptanoate/lysoheptanoyl-PC molecules in fluid SLUV particles. Physical studies have shown that there is no dramatic change in dipalmitoyl-PC/diheptanoyl-PC vesicle sizes before, at, and right after the T_m (although vesicle aggregation does occur 5–10 °C above T_m) (Gabriel & Roberts, 1986). Sidedness NMR studies of diheptanoyl-PC in SLUVs are difficult since the *N*-methyl line widths increase around T_m (Gabriel & Roberts, 1986, 1987) and short-chain lecithin choline methyls are only moderately affected by lanthanides (Gabriel & Roberts, 1987). At least in gel phase SLUVs, the short-chain choline methyls have a different chemical shift from long-chain species and appear able to adopt a trans rather than gauche orientation of the choline to maximize the distance of the positively charged $N(CH_3)_3$ from the Pr^{3+}, which interacts strongly with the negatively charged phosphate. Therefore, we cannot rule out a change in the asymmetric distribution of diheptanoyl-PC across the bilayer in liquid-crystalline SLUVs, although if this occured it would do so initially without altering vesicle sizes. Phospholipase C can hydrolyze all diheptanoyl-PC in gel phase SLUVs, implying that vesicle reorganization does occur with hydrolysis. In fact, significant vesicle changes occur with ~20% short-chain lecithin hydrolyzed. If diheptanoyl-PC on the inner monolayer is made accessible to the enzyme in gel SLUVs, it seems unlikely that a shift in its initial distribution will prevent phospholipase C from acting on all of the short-chain lecithin. The idea that product inhibition is accentuated in fluid SLUVs is a less plausible explanation for the drop in extent of diheptanoyl-PC hydrolyzed, because diglycerides and triglycerides should be more soluble in liquid-crystalline vs gel phase bilayers. If it becomes more difficult to partition hydrophobic products from phospholipases back into the bilayer, one would expect a drop in the observed specific activity but not a change in the extent of hydrolysis. The possibility of two discrete types of environment for diheptanoyl-PC molecules is the most reasonable explanation. From the amount of diheptanoyl-PC left unhydrolyzed in egg PC or liquid-crystalline dipalmitoyl-PC SLUVs (1:4, short-chain/long-chain), we can estimate that the restrained complex consists of five to six long-chain molecules and one short-chain

lecithin. These higher ratios of short-chain lecithin to long-chain molecules may naturally induce stronger short-chain lecithin/long-chain lipid interactions, rendering the diheptanoyl-PC unsuitable for phospholipase hydrolysis. In gel phase SLUVs, such a complex does not occur to any appreciable extent. Perhaps in the gel-state matrix the diheptanoyl-PC molecules cluster or patch as n-mers (where $n < 10$) and are to some extent phase-separated from the long-chain species. As SLUVs melt, diheptanoyl-PC mixes more randomly with the long-chain species, and interactions between the two components become stronger. Therefore, less diheptanoyl-PC is available with a K_m and V_{max} indicative of micellelike "uncoupled" behavior; hence, rates are not affected while the extent of hydrolysis is dramatically decreased. The result is that any matrix with substantial lipid–lipid interactions will be a poor substrate for water-soluble phospholipases. Micelles (either pure short-chain lecithins or detergent-mixed micelles) are good substrates because intermolecular interactions are relatively weak and the enzyme when bound to one molecule has access to several other phospholipids if more than one molecule is necessary for efficient hydrolysis, and because the micelle matrix is efficient at solubilizing products and hence removing them from the active site. Bilayers of long-chain phospholipids are poor substrates because of strong lipid–lipid intermolecular interactions. While phospholipases can bind to the surface, in some cases with an apparent K_m comparable to values in mixed micelles (Kensil & Dennis, 1979; Dennis, 1973b; Tinker et al., 1978; Upreti & Jain, 1978; Burns et al., 1982), this interaction cannot lead to catalysis unless the enzyme can "isolate" or "uncouple" the substrate molecule from its neighbors.

We now have a model for observed effects in gel or fluid vesicles and need to integrate the changes which occur around T_m. In general, there is a 2–3-fold increase in the observed specific activity for diheptanoyl-PC hydrolysis around the T_m of the long-chain matrix (Table III). This behavior differs from what is observed with cobra venom phospholipase A_2 hydrolysis of pure dipalmitoyl-PC or dimyristoyl-PC-sonicated vesicles around their transition temperature, where a discontinuity is seen such that the gel phase matrix is a better substrate than the liquid-crystalline phase (Kensil & Dennis, 1979). With pancreatic phospholipase A_2, anomalously high activity is detected around the T_m of saturated lecithins (Op den Kamp et al., 1974, 1975). As the long-chain lipids melt from an all-trans gel to a fluid, kinked phase, there is a large increase in the lateral pressure of the long-chain lecithin head groups. Perhaps the long-chain phospholipid molecules partially "squeeze out" the short-chain lecithins from their normal orientation in the SLUV bilayer. In such a state, the short-chain lecithins are more accessible to phospholipase binding, hydrolysis, and subsequent product release and absorption. SLUVs at the T_m are not lytic to erythrocytes; therefore, the diheptanoyl-PC is not forced into a micelle or monomer state but is still associated with the bilayer. Around the T_m there will also be an increase in defects where patches of gel and fluid long-chain lipid are juxtaposed. Jain has proposed that defects are critical to the binding and activity of pancreatic phospholipase A_2 (Jain et al., 1982). Bilayers become more permeable to small molecules around the T_m (Papahadjopoulos et al., 1973). However, the simple explanation of patches of gel and liquid-crystalline domains in the bilayer around T_m is not adequate. The anomalous increase in enzymatic hydrolysis of diheptanoyl-PC occurs over an 8–10 °C (or greater) range while the melting of the long-chain lecithin in SLUVs occurs over 3–4 °C (Gabriel & Roberts, 1986). Furthermore, the short-chain lecithin is already "uncoupled" or "isolated" from its long-chain neighbors (Gabriel & Roberts, 1987) in the gel state. Nagle and Scott (1978) point out that large fluctuations in the phospholipid area per molecule occur near the T_m, but over a broader (~10 °C) temperature range than the actual chain melting. One would also expect larger fluctuations for lipids moving vertically perpendicular to the membrane. Such fluctuations could enhance phospholipase binding to diheptanoyl-PC and/or product release. For phospholipase A_2 (*Naja naja naja*), binding of the enzyme to vesicles of the substrate analogue dihexadecyl-PC is constant over the temperature range 20–50 °C (DeBose, 1984). Therefore, any "enhanced", binding around T_m must represent a separate "productive" binding step. Molecular diffusion of both short-chain lecithin and long-chain lipid in the plane of the bilayer will also increase as the long-chain matrix melts. If multiple phospholipid molecules must be bound to a phospholipase for optimal catalysis, such an increase in lateral diffusion could increase phospholipase activity toward diheptanoyl-PC around T_m.

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A Study of the Schiff Base Mode in Bovine Rhodopsin and Bathorhodopsin†

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ABSTRACT: We have obtained the resonance Raman spectra of bovine rhodopsin, bathorhodopsin, and isorhodopsin for a series of isotopically labeled retinal chromophores. The specific substitutions are at retinal's protonated Schiff base moiety and include —HC=NH^+—, —HC=ND^+—, —$H^{13}C$=NH^+—, and —$H^{13}C$=ND^+—. Apart from the doubly labeled retinal, we find that the protonated Schiff base frequency is the same, within experimental error, for both rhodopsin and bathorhodopsin for all the substitutions measured here and elsewhere. We develop a force field that accurately fits the observed ethylenic (C=C) and protonated Schiff base stretching frequencies of rhodopsin and labeled derivatives. Using MINDO/3 quantum mechanical procedures, we investigate the response of this force field, and the ethylenic and Schiff base stretching frequencies, to the placement of charges close to retinal's Schiff base moiety. Specifically, we find that the Schiff base frequency should be measurably affected by a 3.0–4.5-Å movement of a negatively charged counterion from the positively charged protonated Schiff base moiety. That there is no experimentally discernible difference in the Schiff base frequency between rhodopsin and bathorhodopsin suggests that models for the efficient conversion of light to chemical energy in the rhodopsin to bathorhodopsin photoconversion based solely on salt bridge separation of the protonated Schiff base and its counterion are probably incorrect. We discuss various alternative models and the role of electrostatics in the rhodopsin to bathorhodopsin primary process.

When rhodopsin, the visual pigment protein, absorbs a photon, a species called bathorhodopsin is formed photochemically. Some two-thirds of the photon's energy is converted to chemical energy in the rhodopsin to bathorhodopsin photoreaction (Cooper, 1979; Cooper et al., 1986; Schick et al., 1987). At least two events are believed to occur in the rhodopsin to bathorhodopsin transformation [reviewed in Birge (1981) and Ottolenghi (1980)]. There is a large body of evidence that rhodopsin's chromophore photoisomerizes from an 11-cis to a distorted all-trans configuration. Probably following this, one or more protons of the apoprotein appear to translocate to a new position.

A few years ago, a model was proposed (Honig et al., 1979) to understand how so much of the available light energy could be converted to chemical energy in the primary process. The essential feature of this model was the movement of the positively charged Schiff base through space against an electrostatic field. Specifically proposed was the separation of the positively charged protonated Schiff base moiety from a nearby and presumably hydrogen-bonded negatively charged counterion. This charge separation of a salt bridge was presumed to be the consequence of retinal's photoisomerization. Taking Coulomb's law and assuming reasonable values for the partial charges on the Schiff base and counterion and the separation distance that could occur between the protonated Schiff base and its counterion in an 11-cis to all-trans isomerization, it was shown that a (later observed; Cooper, 1979)

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