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Alkaline Hydrolysis of Phospholipids in Model Membranes and the Dependence on Their State of Aggregation†

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ABSTRACT: The rate of alkaline hydrolysis of phospholipids in different model membranes was studied as a probe of the phospholipid conformation and packing and for a better understanding of the effect of phospholipid aggregation on hydrolysis by phospholipase A_2. The products of hydroxide attack on phosphatidylcholine were free fatty acids and glycerophosphorylcholine, with lysophosphatidylcholine as an intermediate. The kinetics of phospholipid hydrolysis could be analyzed as a pseudo-first-order reaction by having the hydroxide concentration in large excess. Egg phosphatidylcholine dispersed in Triton X-100 mixed micelles at a mole ratio of 8:1 detergent:phospholipid was hydrolyzed with a second-order rate constant of 14.7×10^{-3} s^{-1} M^{-1}. Egg phosphatidylcholine dispersed in single bilayer vesicles and multibilayers was hydrolyzed at rates 7–11- and 3-fold lower, respectively. The calculation of these rates had to take into account the extent of inaccessibility of the inner layers to hydroxide which was measured by the fluorescence of a pH-sensitive probe (pyranine) trapped inside the vesicles and multibilayers. The monomeric phospholipid dihexanoylphosphatidylcholine was hydrolyzed at a rate 7-fold higher than when this lipid was incorporated into Triton X-100 micelles. The activation energy for alkaline hydrolysis of egg phosphatidylcholine in Triton X-100 micelles was about 10 kcal/mol whereas it was about 19 kcal/mol in vesicles. Monomeric dihexanoylphosphatidylcholine was hydrolyzed with an activation energy of about 13 kcal/mol. When hydrolysis at the *sn*-1 and *sn*-2 positions of dipalmitoylphosphatidylcholine was followed independently, the rates were similar within experimental error at each position for both mixed micelles and vesicles over the temperature range of 15–60 °C. An Arrhenius plot of the hydrolysis of dipalmitoylphosphatidylcholine vesicles indicated that the phospholipid phase transition exerted a small but detectable effect on the rate of hydrolysis. As with phospholipase A_2, hydroxide-catalyzed hydrolysis rates depend critically on the aggregation state of the phospholipid.

Information about the conformation and packing of phospholipids at the lipid/water interface of model membranes has generally been obtained by physical techniques such as ^1H NMR (Roberts et al., 1978a), ^2H NMR (Seelig & Seelig, 1974), Raman spectroscopy (Gaber & Peticolas, 1977), and X-ray crystallography (Hitchcock et al., 1974). However, the relative rates of reactions on the phospholipid in various model membranes, such as hydrolysis of the fatty acyl bonds, may also be a useful probe of the properties of the phospholipid in the interfacial region. For example, the activity of the enzyme phospholipase A_2 (which specifically catalyzes hydrolysis of the *sn*-2 fatty acid ester bond) has been shown to be sensitive to the nature of the phospholipid/water interface (Verger, 1980) and thermotropic phase transitions of the phospholipid (Kensil & Dennis, 1979). Certain aggregation states, particularly phospholipid–detergent mixed micelles of the nonionic detergent Triton X-100, appear to be favored as substrates for the enzyme from cobra venom (Deems et al., 1975; Kensil & Dennis, 1979). Nonenzymatic reactions may also be sensitive to phospholipid conformation and packing. For example, Wells (1974) reported that alkaline hydrolysis of monomeric dibutyrylphosphatidylcholine is faster than that of dioctanoylphosphatidylcholine micelles, suggesting that the attack of hydroxide on phospholipid is affected by the aggregation state although an effect of fatty acyl chain length on the rate was not ruled out. In order to better understand phospholipase A_2 specificity (Adamich et al., 1979; Roberts et al., 1979) and phospholipid conformation and packing and to further probe the phospholipid/water interface, we have determined hydrolysis rates and activation energies for hydroxide ion attack on phospholipids in various aggregation states (mixed micelles, multibilayers, vesicles, monomers) and found them to depend critically on the lipid aggregation state.

Experimental Procedures

Lipids and Detergents. Egg phosphatidylcholine (PC)[1] was purified from egg yolks (Singleton et al., 1965). L-α-Dimyristoyl-, L-α-dipalmitoyl-, and L-α-dilauroyl-PC were purchased from Calbiochem. If more than a single spot was apparent after thin-layer chromatography in $CHCl_3$–CH_3OH–H_2O (65:25:4 v/v), the phospholipids were purified by column chromatography on Unisil silicic acid (100–200 mesh) obtained from Clarkson Chemicals. Egg PE (transesterified from egg PC) was purchased from Avanti Biochemicals. 1-Palmitoyl-2-[1-^{14}C]palmitoyl-PC (specific activity 59

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[1] Abbreviations used: PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; LPC, monoacyl-*sn*-glycero-3-phosphorylcholine; GPC, *sn*-glycero-3-phosphorylcholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine; LPE, monoacyl-*sn*-glycero-3-phosphorylethanolamine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; T_c, thermotropic phase transition.

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mCi/mmol) was obtained from Amersham. Phospholipase A_2 hydrolysis of this DPPC indicated that 99% of the label was in the *sn*-2 position. The nonionic detergent Triton X-100 was purchased from Rohm and Haas. Dihexanoyl-PC was synthesized from the GPC–cadmium chloride complex purchased from Calbiochem and hexanoic anhydride purchased from Aldrich (Patel et al., 1979).

Phospholipid Dispersions. Triton X-100–phospholipid mixed micelles were formed by dispersal of a film of lipid with the addition of a Triton X-100 solution to give a final mole ratio of 8:1 detergent:phospholipid. Typical phospholipid concentrations were 12 mM. Multibilayers were formed by suspending dry lipid into water or buffer. Small unilamellar vesicles were formed by sonication and centrifugation to remove multibilayers as previously described (Kensil & Dennis, 1979). Multibilayers and vesicles were always prepared above the phase transition of the phospholipid.

Alkaline Hydrolysis Assays. The standard alkaline hydrolysis reaction for long-chain phospholipids was initiated by addition of 0.5 mL of a base solution of 0.132 M NaOH and 0.05 M KCl to 0.5 mL of lipid disperision, resulting in a final phospholipid concentration of 6 mM at 40 °C unless otherwise indicated. The activity of 0.066 M hydroxide at an ionic strength of 0.091 is 0.050 M (Kielland, 1937), from which a pH value of 12.7 can be calculated. This agrees with the measured pH value of 12.7. The pH drop after 50% hydrolysis of 6 mM PC was less than 0.05 pH unit. The reaction was stopped by addition of 0.5 mL of $CHCl_3$–CH_3OH–CH_3CO-OH (2:4:1 v/v) followed by 0.3 mL of $CHCl_3$. PC, LPC, and fatty acids were extracted into the chloroform phase while GPC was partitioned quantitatively in the aqueous phase. The quantity of GPC produced was determined by a phosphate assay (Eaton & Dennis, 1976) on the aqueous phase. Fatty acids, LPC, and PC were separated on Brinkmann silica gel 60 thin-layer chromatography plates in the solvent system $CHCl_3$–CH_3OH–H_2O (65:25:4 v/v) or $CHCl_3$–CH_3OH–CH_3COOH–H_2O (25:15:4:2 v/v). The phospholipids were visualized in an iodine tank and were quantitated by removal of the silica gel which was then analyzed for phosphorus or radioactivity. When a sample contained PC, LPC, PE, and LPE, these lipids were separated by two-dimensional thin-layer chromatography (Turner & Rouser, 1970).

To distinguish *sn*-1 from *sn*-2 hydrolysis, DPPC, labeled with ^{14}C in the *sn*-2 fatty acid chain, was hydrolyzed as described above. *sn*-1 hydrolysis of this phospholipid produces ^{14}C-labeled LPC, while *sn*-2 hydrolysis does not. Thus, the amount of radioactive LPC present at the end of the reaction is an accurate measure of the *sn*-1 reaction. When this is compared to the total amount of LPC present, as determined by phosphate analysis, the ratio of the *sn*-1 to *sn*-2 reactions can be calculated.

DHPC in benzene was dried under N_2 and vacuum; to measure alkaline hydrolysis, 0.5 mL of 25 mM 3-(cyclohexylamino)propanesulfonic acid buffer, pH 10.8 (Calbiochem), was added to start the reaction. The lower hydroxide concentration was used due to the faster rate of hydrolysis of the short-chain PC compared to the long-chain PCs. The reaction was stopped by neutralization to pH 7 with 1 mL of 100 mM 3-(*N*-morpholino)propanesulfonic acid buffer (Calbiochem), followed by freezing in a dry ice–acetone bath and lyophilization. The reaction products were redissolved in H_2O–CH_3OH (1:4 v/v) and spotted on silica gel 60 plates. The solvent system consisted of $CHCl_3$–CH_3OH–CH_3CO-OH–H_2O (25:15:4:2 v/v). PC, LPC, and GPC were well separated and were quantitated by a phosphate analysis. The Triton X-100 micelle rate constants for duplicate sets of experiments varied by ±8% or less. The vesicle and DHPC rate constants varied by ±25% or less.

Measurement of Internal pH and Accessible Phospholipid. Trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate, a pH-sensitive fluorescent dye (also known as pyranine), was obtained from Eastman. Multibilayers and vesicles were formed in the presence of the dye in 10 mM Tris and 100 mM NaCl, pH 7.10. In the multibilayers, external dye was removed by centrifugation at 12 000*g* for 30 min followed by resuspension of the pellet in H_2O or in 10 mM Tris and 100 mM NaCl, pH 7.10. This process was repeated for a total of two centrifugation steps. The small unilamellar vesicles were separated from external dye and multibilayers by passage of the sonicated vesicle preparation over a (2.0 × 50 cm) CL-Sepharose 4B column (Pharmacia). The vesicles were used immediately to minimize leakage of dye from the vesicles. For determination of the pH inside the vesicles and multibilayers, the excitation spectrum for the fluorescence at 511 nm was scanned from 300 to 500 nm with a Perkin-Elmer MPF-44 spectrofluorometer using 5-nm bandwidths for both excitation and emission. The ratio of the peak intensities due to excitation at 408 (un-ionized dye) and 469 nm (ionized dye) was measured. This ratio allowed determination of the pH inside the vesicles from pH 7 to 10.

Phospholipase A_2 treatment of multibilayers was used to determine the amount of accessible phospholipid. The extent of hydrolysis was determined as described previously (Kensil & Dennis, 1979) by using enzyme prepared from cobra venom (*Naja naja naja* (Deems & Dennis, 1981)).

Results

Product Formation. Attack of hydroxide at the *sn*-1 and *sn*-2 positions of PC (Figure 1) in parallel reactions is expected to produce the 2-acyl- and 1-acyl-LPCs, respectively. These monoacylphosphatides are not resolved by the usual thin-layer chromatography systems. Therefore, the rate constant k_1 (where $k_1 = k_1' + k_1''$) is the sum of the rate constants for hydrolysis of PC into LPC (eq 1) by attack at either the *sn*-1

$$-d[PC]/dt = k_1[OH^-][PC] \quad (1)$$

or *sn*-2 position. Hydrolysis of the second fatty acid from either LPC should produce the same product, GPC. However, the rate constant for hydrolysis of the second fatty acid, k_2, probably reflects mainly hydrolysis of LPC acylated at the *sn*-1 position due to rapid acyl migration within LPC favoring the 1-acyl derivative (Martin, 1953; Wolfenden et al., 1964). In this paper, only the rate constant k_1 which represents the first step in hydrolysis is considered in detail.

Most previous studies of alkaline hydrolysis of phospholipids were carried out in methanol, and the rate of LPC production from PC (k_1) was not measured (Baer & Kates, 1950; Hübscher et al., 1960; Marriott, 1969). However, Marinetti (1962) demonstrated that LPC was an intermediate in the sodium methoxide catalyzed methanolysis of PC with subsequent production of GPC. The further hydrolysis of GPC is quite slow compared to acyl hydrolysis and has been reviewed elsewhere (Hanahan, 1960). In the present study, LPC was shown clearly to be an intermediate in the alkaline hydrolysis of phospholipids in aqueous solution. Figure 2 shows the disappearance of PC dispersed in Triton X-100 and the appearance of LPC and GPC as the alkaline hydrolysis reaction proceeded. As expected for series pseudo-first-order reactions, GPC is not produced until the LPC concentration becomes significant. No phosphatidic acid appeared, so, as expected, the phosphate–choline linkage was not hydrolyzed. The

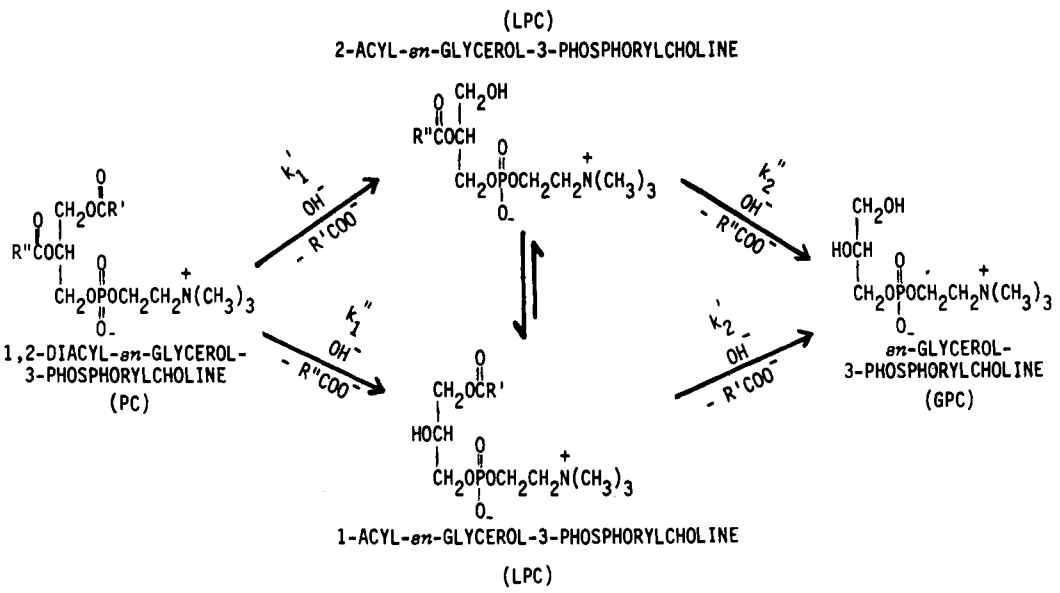

FIGURE 1: Alkaline hydrolysis of phosphatidylcholine.

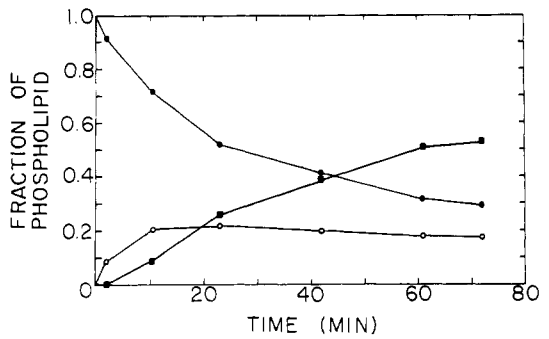

FIGURE 2: Reaction time course of alkaline hydrolysis of Triton X-100–dipalmitoylphosphatidylcholine mixed micelles (mol ratio 8:1). (●) Phosphatidylcholine; (○) lysophosphatidylcholine; (■) glycerophosphorylcholine.

Table I: Effect of Triton X-100 on Alkaline Hydrolysis of Egg Phosphatidylcholine Dispersed in Mixed Micelles

mol ratio Triton/phospholipid	$k_1 \times 10^3$ (s^{-1} M^{-1})	relative rates
4.5	12.3	1.0
7.8	15.0	1.2
12.0	15.9	1.3
15.0	16.2	1.3

water-soluble product of the reaction cochromatographed with a GPC standard on thin-layer chromatography in CH_3OH–H_2O (7:3 v/v).

When a negligible back-reaction is assumed, the rate of conversion of PC to LPC (k_1) can be calculated from the disappearance of PC (which should not be affected by the production of GPC from LPC). The reaction was found to be first order for both PC and hydroxide, as expected for alkaline hydrolysis of an ester. Typically, the hydroxide concentration was in excess so that pseudo-first-order rate constants could be determined from the disappearance of PC.

Effect of Triton X-100 and Divalent Cations. The effect of varying the Triton X-100 to PC ratio in the mixed micelles on alkaline hydrolysis was determined as shown in Table I. The egg PC concentration was held constant while the Triton X-100 concentration was varied. The rate of alkaline hydrolysis increased slightly as the relative proportion of detergent to phospholipid in the micelles increased. For other alkaline hydrolysis studies on the mixed micelles, the detergent:

Table II: Effect of Fatty Acid Composition on Alkaline Hydrolysis of Phosphatidylcholine in Mixed Micelles

phosphatidylcholine	$k_1 \times 10^3$ (s^{-1} M^{-1})	relative rates
egg	14.1	1.0
dipalmitoyl-	13.2	0.9
dimyristoyl-	15.0	1.1
dilauroyl-	16.0	1.2

Table III: Effect of Phospholipid Head Group on Hydrolysis of Mixed Micelles

phospholipid	$k_1 \times 10^3$ (s^{-1} M^{-1})	relative rates
egg phosphatidylcholine	14.0	1.0
egg phosphatidylethanolamine	4.6	0.3
egg phosphatidylcholine[a]	9.4	0.7
egg phosphatidylethanolamine[a]	5.5	0.4

[a] Activity toward the phospholipid specified which was in an equimolar mixture of egg PC and egg PE.

phospholipid mole ratio was set at 8:1.

Ca^{2+} has been reported to bind poorly to PC (McLaughlin et al., 1978; Seimiya & Ohki, 1973). Binding of Ca^{2+} to the phospholipid at the micelle interface would cause a modification of the surface charge, resulting in a change in rate. However, the presence of 10 mM $CaCl_2$ did not significantly affect the rate of alkaline hydrolysis of egg PC in Triton X-100 micelles.

Effect of Phospholipid Composition on Rate. The rate constants of alkaline hydrolysis of PC of various chain lengths are listed in Table II. For this experiment, the average error of duplicate determinations was $\pm 0.2 \times 10^{-3}$ s^{-1} M^{-1} and a t test showed that the differences between the data sets were significant with a confidence limit of about 80%. Thus, although the data suggest that as the chain length decreases, the rate of hydrolysis increases slightly, it is not clear that the small differences are meaningful.

The effect of the head group on the hydrolysis of the fatty acyl chains was investigated (Table III). The head groups compared were phosphorylcholine and phosphorylethanolamine. Egg PC was hydrolyzed at a rate 3 times higher than the rate at which PE was hydrolyzed. This should not be due to gross differences in the fatty acid chains as the PE was prepared from egg PC by transesterification.

Table IV: Relative Rates of Alkaline Hydrolysis of Phosphatidylcholine in Various Aggregation States

	$k_1 \times 10^3$ (s^{-1} M^{-1})					
		mixed micelles		vesicles		
phosphatidylcholine	monomers	obsd	calcd[a]	100% accessibility	68% accessibility	multibilayers
dihexanoyl-	370	77	48			
egg		14.7		1.4	2.2	4.1
dipalmitoyl-		13.8		0.7	1.1	

[a] See text for assumptions used in the calculation of k_1 from the observed rate constant.

Rates were also determined on PC and PE as a mixture. This is of interest because phospholipase A_2 preferentially hydrolyzes PC when the two lipids are separate (Roberts et al., 1978b) but prefers PE when the two lipids are mixed (Adamich et al., 1979). It has been suggested that this effect is due to an interaction of PC with the enzyme, but a lipid–lipid effect on the conformation of the phospholipids has not been ruled out entirely (Roberts et al., 1979). In a mixture, the rate of PC hydrolysis decreases somewhat, although it is still higher than that of PE. No significant increase in alkaline hydrolysis of PE in the mixture was observed (contrary to the previous results with the enzyme hydrolysis).

Permeation of Base into Vesicles and Multibilayers. By use of the fluorescent probe pyranine, it had been shown for negatively charged vesicles consisting of DPPC, cholesterol, and sodium dicetyl phosphate that a pH gradient is maintained when vesicles which are prepared at pH 7 are transferred into a buffer of pH 10 (Kano & Fendler, 1978). If a significant pH gradient is maintained during alkaline hydrolysis of vesicles and multibilayers, only the external monolayer of phospholipid is available for hydrolysis. Therefore, hydroxide permeation was measured for vesicles and multibilayers under alkaline hydrolysis conditions.

Egg PC multibilayers with entrapped pyranine were mixed with hydroxide resulting in a bulk pH of 12.7. The average internal pH as measured by pyranine typically increased from pH 7.0 to 7.7 within the first minute. The pH remained unchanged over the next 24 h (Figure 3). The initial increase in pH can be accounted for by residual pyranine which remained bound to the outer monolayer of phospholipid after the washing steps as determined by quenching with the paramagnetic ion Co^{2+}. For vesicles, the pH rose much more rapidly. Within 5–10 min, the internal pH increased to 9.5, which is the highest pH measurable by this method. It was therefore impossible to determine by this method the time necessary for complete equilibration of the internal pH to 12.7. The more rapid base influx into the vesicles compared to the multibilayers may be a reflection of the curvature of the vesicle and/or the increased number of permeability barriers in a multibilayer compared to the single bilayer in a vesicle. The slower influx of hydroxide into the multibilayers compared to vesicles agrees with the data of Kano & Fendler (1978). The shielding of dye from the external pH suggests that the exposure to an alkaline pH does not immediately disrupt the vesicles and multibilayers and must leave the structures relatively intact. This was further corroborated by exposing egg PC vesicles to pH 12.7 for a 10-min period followed by acidification to pH 8. The elution profile of these vesicles on a CL-Sepharose 4B gel filtration column was similar to that of vesicles which had not been exposed to a high pH.

Phospholipid Aggregation State. If the accessibility of the carbonyl group to water and hydroxide differs when the phospholipid is dispersed in various aggregation forms, the rate constants measured for alkaline hydrolysis would be expected

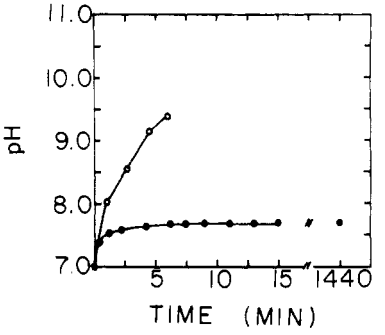

FIGURE 3: pH of interior of egg phosphatidylcholine vesicles (○) and multibilayers (●) as a function of time after addition of base. Interior pH was determined by pyranine fluorescence. Bulk pH = 12.6.

to differ. To test this, long-chain PC was dispersed in multibilayers, small unilamellar vesicles, and Triton X-100–PC mixed micelles. These rates (Table IV) are highest for the mixed micelles. For determination of the rate constant, the fraction of phospholipid which is accessible to hydroxide must be included in the calculation. Thus, the concentration of accessible rather than bulk phospholipid is used as the initial phospholipid concentration. For phospholipid dispersed in Triton X-100 micelles, the true rate constant equals the observed rate constant due to the presumed accessibility of 100% of the substrate to the hydroxide catalyst. However, in a small unilamellar vesicle, only 68% of the phospholipid is on the external monolayer (Yeagle et al., 1976). If the vesicles are impermeable to hydroxide, the rate constant of alkaline hydrolysis of egg PC in vesicles is 2.2×10^{-3} s^{-1} M^{-1} (due to the initial concentration of PC being 68% of the bulk concentration). If 100% of the PC is subjected to pH 12.7, the rate constant is 1.4×10^{-3} s^{-1} M^{-1}. The rate constant for the vesicles is between these two values since there is a slow permeation of hydroxide into the vesicles during the incubation period of the assay. The rate constant for the vesicles is thus 7–11-fold lower than the micellar rate constant.

For egg PC multibilayers, the proportion of phospholipid on the external monolayer ranged from 9% to 14% as measured for our preparations by determining the total hydrolysis of the multibilayers by an excess of phospholipase A_2 [which has been shown to hydrolyze only the outer monolayer of vesicles (Kupferberg & Kezdy, 1978)]. During alkaline hydrolysis of a given multibilayer preparation, the fraction of phospholipid on the outer monolayer was determined by phospholipase A_2 treatment of a sample from the same preparation; the fraction of phospholipid hydrolyzed by the hydroxide never exceeded that determined by phospholipase A_2. Due to the impermeability of the multibilayers to hydroxide, the fraction of external phospholipid determined by phospholipase A_2 treatment was used to calculate the concentration of phospholipid which was accessible to the hydroxide. The rate constant of alkaline hydrolysis of egg PC in multibilayers is 4.1×10^{-3} s^{-1} M^{-1}, which is over 3-fold lower than in the Triton X-100 micelles.

Table V: Positional Specificity of Alkaline Hydrolysis of Dipalmitoylphosphatidylcholine

aggregation state	temp (°C)	*sn*-1 hydrolysis (%)	*sn*-2 hydrolysis (%)	k_1'/k_1''
Triton X-100 micelles[a]	40	52	48	1.1
vesicles	19	50	50	1.0
	40	49	51	1.0
	50	49	51	1.0

[a] Triton X-100:phosphatidylcholine mol ratio 4:1.

Table VI: Activation Energies for Alkaline Hydrolysis of Phosphatidylcholine in Various Aggregation States

phosphatidylcholine	aggregation state	E_{act} (kcal/mol)
dipalmitoyl-	Triton X-100 micelles	11.5
	vesicles	17
egg	Triton X-100 micelles	10.2
	vesicles	19.4
dihexanoyl-	monomers	12.6

This difference in rates between the various aggregation states must be due to properties of the interfacial region.

DHPC can exist as a monomer below the critical micelle concentration of 10–15 mM (Tausk et al., 1974; Plückthun & Dennis, 1981) and in a Triton X-100 micelle. Alkaline hydrolysis rates were measured toward this phospholipid as monomer and in the presence of Triton X-100. Unlike the long-chain phospholipids, the DHPC does not partition completely into the micelle at the concentrations of detergent used in this study. Therefore, the Triton X-100:phospholipid mole ratio was increased to 36:1 to increase the amount of phospholipid which had partitioned into the micelle. At a Triton X-100 concentration of 218 mM and a phospholipid concentration of 6 mM, the equilibrium concentration of DHPC in the micelle was 5.5 mM (91 mol %) (Plückthun & Dennis, 1981). The remaining 9% in aqueous solution would be hydrolyzed at the rate expected for monomer hydrolysis. This background monomer hydrolysis was subtracted from the total hydrolysis. The remainder of hydrolysis was assumed to be hydrolysis of DHPC which had partitioned into the Trition X-100 micelle and was used to calculate a micellar rate constant. Rates are faster toward the monomer DHPC than toward the micellar substrate. For long-chain PC, the critical micelle concentration is approximately 10^{-8} M to 10^{-10} M (Smith & Tanford, 1972; Martin & MacDonald, 1976). In the alkaline hydrolysis assays, the total phospholipid concentration is 6 mM, so hydrolysis of monomers cannot account for a significant contribution to the observed rate.

sn-1 vs. sn-2 Alkaline Hydrolysis. NMR studies have suggested that in Triton X-100–PC mixed micelles, the two fatty acid chains are nonequivalent with respect to their position relative to the interface (Roberts & Dennis, 1977; Roberts et al., 1978a). The α-methylene group of the two fatty acid chains are in different environments, with the *sn*-2 α-methylene apparently being in a more hydrophilic environment compared to the *sn*-1 α-methylene. Although the chemical reactivity of the two carbonyls with hydroxide may be intrinsically different, relative rates of alkaline hydrolysis at the *sn*-1 and *sn*-2 positions may also reflect the relative accessibilities of the carbonyls to water (and the hydroxide ion) in the micelle and vesicles. Thus, we compared these rates in Triton X-100–DPPC micelles and DPPC vesicles.

By using DPPC preferentially labeled with ^{14}C in the *sn*-2 fatty acid chain, the fractions of hydrolysis at the *sn*-1 and *sn*-2 positions were determined as shown in Table V. Base-catalyzed acyl migration in monoglycerides is reported to have a rate constant 6500 times higher than hydrolysis of monoglycerides at a given pH (Wolfenden et al., 1964). This would cause the LPC formed in the first reaction to be equivalently labeled with the remaining ^{14}C in the *sn*-1 and *sn*-2 positions before further hydrolysis to GPC. Thus, a specific hydrolysis of 1-acyl- or 2-acyl-LPC will not affect the results obtained for the first step of the reaction. For the Triton X-100 micelles, rates of *sn*-1 and *sn*-2 hydrolysis at 40 °C were similar within experimental error. For DPPC vesicles (which undergo a thermotropic phase transition near 40 °C), several temperatures were examined to determine if the physical state of the phospholipid bilayer (i.e., gel, liquid crystalline, or a mixture) might be reflected in the relative proportions of *sn*-1 and *sn*-2 hydrolysis. However, the rates were again similar within experimental error at each temperature, and there is no significant difference between *sn*-1 and *sn*-2 hydrolysis in either mixed micelles or DPPC vesicles. It has not been determined whether this result is due to similar intrinsic chemical reactivity of the two carbonyl groups, interfacial factors common to both vesicles and mixed micelles, or a compensation for differing chemical reactivities by interfacial factors.

Activation Energies for Alkaline Hydrolysis. Arrhenius plots for the temperature dependence of alkaline hydrolysis in vesicles, micelles, and monomers are shown in Figure 4. From the Arrhenius plots, activation energies of alkaline hydrolysis could be determined and are summarized in Table VI. For DPPC vesicles (Figure 4A), there is a small but measurable break in the Arrhenius plot in the area of the phase transition. The lines in this figure are linear least-squares lines and the fit is better when the vesicle data are considered in two parts, one from 40 to 60 °C and the other between 15 and 33 °C. The 36 °C point may be a transition between these two lines. While the line calculated with all of the data points is not an unreasonable one, it does not fit the data as well as the two separate lines. The activation energies of the lines both above and below the phase transition are about 17 kcal/mol within experimental error, whereas the least-squares fit for all data points assuming no phase transition effect gives an activation energy of about 21 kcal/mol. The overall activation energy was lower for alkaline hydrolysis of DPPC in Triton X-100 micelles than for small unilamellar vesicles. Similar results were determined for egg PC (Figure 4B) in vesicles and micelles. The activation energy for hydrolysis of monomer DHPC (Figure 4C) is slightly higher than that of the long-chain PCs in the Triton X-100 mixed micelles. However, the activation energy was not determined for DHPC in Triton X-100 micelles as the proportion of monomers in solution (partition coefficient) may vary with temperature and complicate the calculation.

The observed increase in the activation energy of vesicles over micelles of about 10 kcal/mol would be expected to decrease the hydrolysis rate approximately 10^7 at 40 °C if the entropy of activation were the same in both vesicles and micelles. However, the Arrhenius plots do show a change in the entropy and the observed rates differ by only a factor of 10 at 40 °C.

Discussion

Phospholipid/Water Interface. It is apparent that the attack of hydroxide on phospholipid is very sensitive to the conditions of the reaction and the substrate aggregation state. The dispersal of the phospholipid (as monomer, small unila-

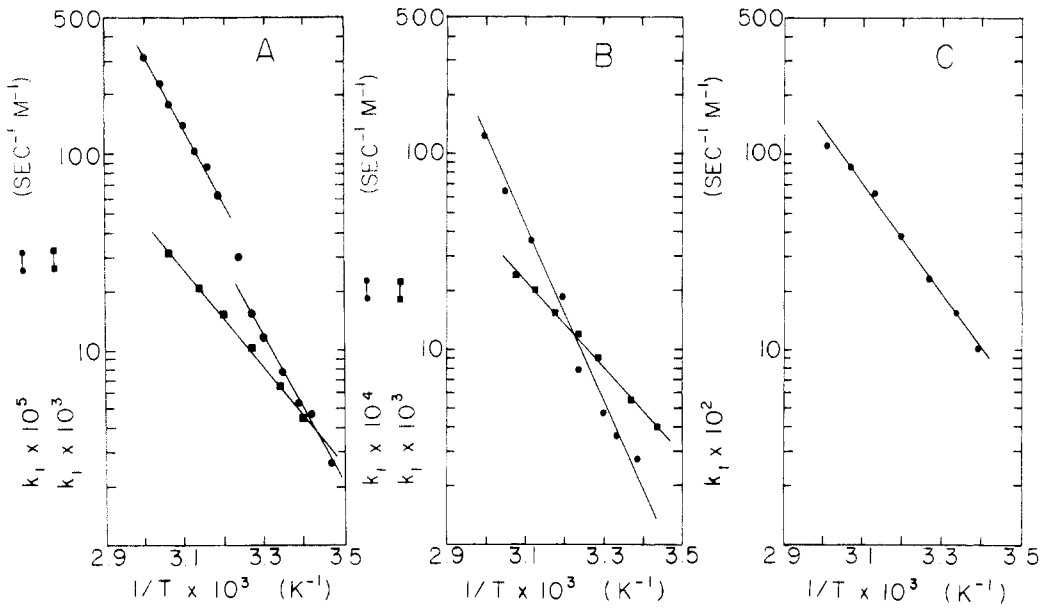

FIGURE 4: Typical Arrhenius plots of the temperature dependence of the rates of alkaline hydrolysis of phosphatidylcholine dispersed in various aggregation states. (A) (●) Dipalmitoylphosphatidylcholine vesicles; (■) Triton X-100–dipalmitoylphosphatidylcholine mixed micelles (mol ratio 8:1). (B) (●) Egg phosphatidylcholine vesicles; (■) Triton X-100–egg phosphatidylcholine mixed micelles (mol ratio 8:1). (C) (●) Monomer dihexanoylphosphatidylcholine. The rate constants for the vesicles were calculated by assuming 100% accessibility to hydroxide.

mellar vesicle, multibilayer, or Triton X-100 micelle) affects reaction rates greatly. This effect is due to properties of the phospholipid/water interface.

There are several properties of the interfacial region which may affect hydroxide-catalyzed hydrolysis of ester bonds. The dielectric constant is lower at micellar interfaces than in the bulk aqueous solution (Mukerjee & Ray, 1966). A decrease in dielectric constant decreases the rate of alkaline hydrolysis of esters [reviewed in Kirby (1972)]. Thus, the lower rate constant observed toward PC in Triton X-100 micelles compared to monomeric PC may be due in part to this effect. There is little information on the polarity at a bilayer surface relative to a micelle surface. However, a decreased polarity at the bilayer surface could account for the lower rate on the PC in vesicles and multibilayers compared to that in Triton X-100 micelles.

A second factor to consider is the interfacial concentration of hydroxide. A charged interface, according to Gouy–Chapman theory, results in a surface potential and an increased concentration of counterions at the surface. For a positively charged interface, interfacial hydroxide ion concentration should be increased relative to the bulk concentration. The opposite effect occurs for a negatively charged interface. An example of this electrostatic effect is the decrease in hydroxide hydrolysis rate of monoalkyl sulfonates when they micellize to form a negatively charged surface (Kurz, 1962). This accounts for the lower rate of alkaline hydrolysis of PE (which is negatively charged at the high pH of this study) compared to the zwitterionic PC and for the somewhat decreased hydrolysis of PC when dispersed in a Triton X-100 micelle with PE. However, other contributing effects, such as packing differences between the two phospholipids and differences in accessibility to solvent, cannot be ruled out.

Both a PC interface and an interface of PC and nonionic Triton X-100 have an overall neutral charge. This overall neutrality suggests that the surface potential cannot be used to account for the difference in hydrolysis rates between Triton X-100 micelles, vesicles, multibilayers, and monomers. However, a microscopic surface potential effect due to the negatively charged phosphate group (the charge closest to the fatty acid ester bond) on the same molecule or on an adjacent molecule cannot be ruled out. In this case, the close packing of the head groups in a bilayer would repel hydroxide ions more than in a detergent micelle where the charges are spaced farther apart. The slight increase in rate as PC is diluted with detergent supports a microscopic charge effect as a possible cause of the decrease in rate when PC is dispersed in a bilayer structure.

Water penetration of the micelles may contribute to the rate enhancement as hydroxide must penetrate near the carbonyl group to effect hydrolysis of the fatty acid chain. There is uncertainty about the extent of water penetration in both ionic and nonionic micelles. Some investigators have postulated that in an ionic micelle, the methylenes of the alkyl group are in a hydrocarbon environment (Stigter, 1974) while others have postulated that at least some of the methylenes are exposed to the aqueous environment (Menger et al., 1978). For phospholipids, ^{13}C NMR studies have suggested that the carbonyls of monomer DHPC are exposed to the solvent water for a fraction of time 1.7 longer than when it is micellized and 2.5 longer than DPPC carbonyls in bilayers (Schmidt et al., 1977). The carbonyl group in bilayers should thus be less accessible to hydroxide than the carbonyls of monomer phospholipid. Water penetration could account for at least part of the difference in alkaline hydrolysis rates of phospholipid in various aggregation states, although this explanation would only account for a 2.5-fold higher rate constant for monomer PC over bilayer PC.

The packing of the phospholipids must also be considered. It appears that the phospholipids are more loosely packed in the Triton X-100 micelle than in vesicles and multibilayers (Ribeiro & Dennis, 1975; Dennis et al., 1979), and this may play some role in the hydrolysis, although it appears that the "pipewrench" structure found for an individual phospholipid by X-ray crystallography (Hitchcock et al., 1974) occurs in all of these structures. A tetrahedral intermediate might fit less well in a closely packed bilayer than in a Triton X-100 micelle. It is also possible that the aggregated structures provide steric hindrance to the approach of the hydroxide to the ester bond.

Relevance to Phospholipase A_2 Action. In both hydroxide-catalyzed and phospholipase-catalyzed hydrolysis of

phospholipids, rates are lower when the substrate is dispersed as a bilayer vesicle rather than in a Triton X-100 mixed micelle. Thus, both mechanisms are sensitive to some property of the interface. However, the underlying mechanisms may not be identical. For example, the activation energies for phospholipase A_2 catalysis of both vesicles and micelles are similar, in contrast to the result observed with alkaline hydrolysis. The increased rate of hydrolysis of gel phase PC compared to liquid-crystalline phase PC by phospholipase A_2 (Kensil & Dennis, 1979) was not observed in this study for the hydroxide-catalyzed hydrolysis, suggesting that the nonenzymatic reaction is affected differently than the enzymatic reaction by changes in the properties of the phospholipid/water interface caused by the phase transition. Further work is required to determine the interfacial factors affecting alkaline hydrolysis and whether or not they are the same as those affecting enzyme hydrolysis.

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