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### Authors

Roberts, MF  
Otnaess, AB  
Kensil, CA  
et al.

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# The Specificity of Phospholipase A<sub>2</sub> and Phospholipase C in a Mixed Micellar System\*

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MARY FEDARKO ROBERTS,‡ ANNE-BRIT OTNAESS,§ CHARLOTTE A. KENSIL, AND EDWARD A. DENNIS¶

From the Department of Chemistry and Department of Pediatrics, University of California at San Diego, La Jolla, California 92093

The activities of cobra venom phospholipase A<sub>2</sub> (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) toward different phospholipids in mixed micelles with Triton X-100 are reported. The nonionic surfactant acts as an inert matrix which solubilizes the phospholipids in similar structures; thus, the observed activities can be compared directly. Knowledge of phospholipid specificity is important in interpreting the action of these enzymes on natural membranes. Phospholipase A<sub>2</sub> prefers phosphatidylcholine as its substrate; it hydrolyzes phosphatidylethanolamine poorly. The enzyme requires Ca<sup>2+</sup> for activity and its true specificity toward phosphatidylserine is difficult to interpret because this negatively charged phospholipid forms complexes with Ca<sup>2+</sup>. Sphingomyelin is not hydrolyzed at all. Phospholipase C, which is a Zn<sup>2+</sup> metalloenzyme, hydrolyzes both phosphatidylcholine and phosphatidylethanolamine at almost equivalent rates; phosphatidylserine is a 2- to 3-fold poorer substrate than these other lipids. Sphingomyelin does not serve as a substrate.

Both enzymes show an effect of fatty acid chain length: as the chain length is reduced, enzyme activity increases. This trend is observed with both phosphatidylcholine and phosphatidylethanolamine and probably reflects optimization of the interfacial properties of the phospholipid. NMR studies on phosphatidylcholine in mixed micelles have shown that the  $\alpha$ -methylene groups of the *sn*-1 and *sn*-2 fatty acids are chemically shifted from one another and that the ethanolamine moiety alters this chemical shift pattern of the fatty acid  $\alpha$ -methylene groups somewhat from that observed in phosphatidylcholine. This difference between the two phospholipids may correlate with the specificity results reported here for phospholipase A<sub>2</sub> catalysis, but does not for phospholipase C catalysis.

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‡ Postdoctoral Fellow of the National Institutes of Health GM-07,169 and GM-05,190.

§ Recipient of United States Public Health Service International Research Fellowship F65-2373 and support from the Norwegian Council for Science and Humanities. Present address, Pediatric Department, Ullevål Hospital, Oslo, Norway.

¶ To whom correspondence should be addressed at Department of Chemistry (M-001), University of California at San Diego, La Jolla, Calif. 92093.

Both phospholipase A<sub>2</sub> (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) are widely used as tools in membrane studies. Interpretation of such studies must be viewed in terms of the relative specificity of these enzymes on individual phospholipids. Although the phospholipase A<sub>2</sub> literature is extensive, only a few studies have attempted to determine the substrate preferences of this enzyme. These studies compared enzyme activity toward phospholipids prepared in varying ways (1), solubilized in ethereal solutions (2), in deoxycholate (3-5), or toward synthetic phosphatidylcholines containing short chain fatty acids as aqueous dispersions (6, 7). The structure of the aggregated phospholipid substrate in each of these systems is either unknown or would differ greatly depending on the net charge of the head group or the fatty acid chain length. Often phospholipid specificities were compared below their thermotropic phase transition temperatures (8) and we (9) have previously shown that this can have an enormous effect on rate. In early studies, enzyme preparations contaminated with other proteins were frequently used and often assays were not conducted under initial rate conditions. Such problems make it difficult to interpret the observed preferences of phospholipase A<sub>2</sub> in the earlier studies. Similarly, with purified *B. cereus* phospholipase C, substrate preferences have only been determined for sonicated phospholipids containing deoxycholate (10) and for synthetic phosphatidylcholines containing various short chain fatty acids (11).

The Triton X-100/phospholipid mixed micelle system has the advantage for specificity studies of offering a well defined inert surfactant matrix (12), whose gross structure should not be altered when small amounts of various phospholipids are inserted into it. Furthermore, small environmental or conformational differences of the various phospholipids can be compared directly in these mixed micelles (13). Using the mixed micellar system, which also has advantages for kinetic analysis (14), the activity of phospholipases toward different phospholipids can be directly compared and evaluated.

## EXPERIMENTAL PROCEDURES

**Materials**—Lyophilized cobra venom, *Naja naja naja* (Pakistan), Lot. No. NNP45-1Z, was obtained from the Miami Serpentarium. The phospholipase A<sub>2</sub> was purified according to the procedure of Deems and Dennis (15) as modified by Roberts *et al.* (16). Phospholipase C was purified from the growth media of *Bacillus cereus* (ATCC 10987 AB-1) using affinity chromatography as described by Little *et al.* (17).

Dipalmitoyl phosphatidylcholine,<sup>1</sup> dimyristoyl phosphatidylcho-

<sup>1</sup> Dipalmitoyl phosphatidylcholine refers to 1,2-dipalmitoyl-*sn*-

line, dilauryl phosphatidylethanolamine, bacterial phosphatidylethanolamine, and bovine brain sphingomyelin were obtained from Calbiochem. Egg phosphatidylethanolamine, prepared by transesterification of egg phosphatidylcholine, was obtained from Avanti Biochemicals. Egg phosphatidylcholine was prepared from fresh egg yolks by the method of Singleton *et al.* (18). Phosphatidylserine was obtained from fresh bovine brains by a Folch (19) extraction and subsequent purification and characterization as described in detail by Warner and Dennis (20). Dioctanoyl phosphatidylcholine, prepared by acylation in the presence of sodium methylsulfinylmethide (21), was kindly supplied by Dr. Thomas G. Warner, University of California, San Diego. Dihexanoyl phosphatidylcholine was the gift of Dr. Michael Wells, University of Arizona, Tucson. Dilauryl phosphatidylcholine and dimyristoyl phosphatidylethanolamine were the gift of Dr. Craig Jackson, Washington University, St. Louis. All phospholipids employed gave a single spot upon thin layer chromatography on precoated layers of Silica Gel G on glass plates (Brinkmann) using a chloroform:methanol:water (65:25:4, v/v/v) solvent system and visualization with iodine vapors. Triton X-100 was obtained from Rohm and Haas and all other chemicals were reagent grade. Glass distilled water was used routinely.

**Assay**—Phospholipase A<sub>2</sub> was assayed by the pH-stat procedure described in detail by Dennis (22). Phospholipase C was assayed by the same procedure (22); the application of this technique to phospholipase C is discussed elsewhere (23). Assays for both enzymes were conducted at 40°, pH 8.0, with mixed micelles composed of 48 mM Triton X-100 (except where noted) and 6 mM phospholipid and sufficient enzyme to hydrolyze at most 0.2  $\mu\text{mol min}^{-1}$  phospholipid. Assays were conducted in a 2-ml volume and the amount of product produced in 2 min was used to calculate the rates so that at most a few per cent of phospholipid was hydrolyzed (initial rate conditions). For phospholipase A<sub>2</sub>, 10 mM CaCl<sub>2</sub> was included. Phospholipase C is a Zn<sup>2+</sup> metalloenzyme (24), and the enzyme is stored in 1 mM Zn<sup>2+</sup> for added stability (25); the final concentration of Zn<sup>2+</sup> in the assay mixture was 0.25  $\mu\text{M}$ . Additional Zn<sup>2+</sup> was not added to the assay mixture, except where indicated. In some experiments 10 mM CaCl<sub>2</sub> was also included during phospholipase C studies as indicated. Controls without added enzyme were subtracted from the observed results. Hydrolysis rates are the average of two or more experiments; the average error between duplicate experiments was approximately  $\pm 5\%$ . Measurements conducted at different times were normalized to dipalmitoyl phosphatidylcholine.

## RESULTS

**Cobra Venom Phospholipase A<sub>2</sub> Specificity toward Long Chain Phospholipids**—The results of incubating pure phospholipase A<sub>2</sub> with various phospholipids inserted into the Triton X-100 matrix at a surfactant:phospholipid mole ratio of 8:1 are summarized in Table I. It should be noted that all phospholipids, except dimyristoyl phosphatidylethanolamine, were above their thermotropic phase transition temperature under the standard assay conditions. This ensured that the phospholipids were in mixed micelles and that phase changes which could occur below that temperature and affect rates (9) would not have to be considered. Dimyristoyl phosphatidylethanolamine, whose phase transition is 47.5° (27), was assayed at 50° and compared to dilauryl phosphatidylethanolamine and dipalmitoyl phosphatidylcholine mixed micelles at that temperature. The zwitterionic phosphatidylcholines were the most effective substrates, whereas all phosphatidylethanolamines were poor substrates. Determining phosphatidylethanolamine hydrolysis by phospholipase A<sub>2</sub> is complicated by a pH-dependent nonenzymatic breakdown of the phospholipid. For phosphatidylethanolamine isolated directly from egg yolk, this background reaction is of the same order of magnitude as the enzymatic reaction at pH = 8 and standard assay conditions, so that phosphatidylethanolamine from other sources

glycerophosphorylcholine, where the fatty acids are in the *sn*-1 and *sn*-2 positions; analogous nomenclature is employed for the other phospholipids.

TABLE I  
Phospholipase A<sub>2</sub> hydrolysis of various phospholipids in Triton/  
phospholipid mixed micelles

Phospholipid	Rate $\mu\text{mol min}^{-1}$ $\text{mg}^{-1}$	Relative rate
Egg phosphatidylcholine	440	0.9
Dipalmitoyl phosphatidylcholine	490	1.0
Dimyristoyl phosphatidylcholine	820	1.7
Dilauryl phosphatidylcholine	950	1.9
Dioctanoyl phosphatidylcholine	6500	13
Dihexanoyl phosphatidylcholine	410	0.84
Egg phosphatidylethanolamine	50	0.10
Bacterial phosphatidylethanolamine	72	0.15
Dimyristoyl phosphatidylethanolamine	— <sup>a</sup>	0.15
Dilauryl phosphatidylethanolamine	110	0.23
Bovine brain phosphatidylserine	— <sup>b</sup>	0.05–0.15
Bovine brain sphingomyelin	0 <sup>c</sup>	0.00

<sup>a</sup> Because of the high thermotropic phase transition temperature of dimyristoyl phosphatidylethanolamine, assays were conducted at 50° with this lipid and compared to dipalmitoyl phosphatidylcholine at the same temperature.

<sup>b</sup> 0.5 mM CaCl<sub>2</sub> was employed. The relative rate is reported compared to dipalmitoyl phosphatidylcholine in the presence of 0.5 mM CaCl<sub>2</sub> (380  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) instead of 10 mM CaCl<sub>2</sub>. The range is given which was found for several determinations using both the pH-stat assay and an assay based on phosphorus quantitation (26) of the hydrolysis products which were extracted (16) and then separated by thin layer chromatography using the conditions described under "Experimental Procedures" for determining phospholipid purity.

<sup>c</sup> Hydrolysis was checked by thin layer chromatography after 1-h incubation using the conditions indicated under "Experimental Procedures" for determining phospholipid purity.

or prepared by transesterification of egg phosphatidylcholine was employed in the studies reported here. With the bacterial source, transesterified egg, or dilauryl phosphatidylethanolamine, the background reaction is considerably slower and initial rates of enzyme hydrolysis can be determined accurately. The bacterial and transesterified egg phosphatidylethanolamines have enzymatic hydrolysis rates 0.10 to 0.15 that of phosphatidylcholine. As for phosphatidylcholine hydrolysis (16), phospholipase A<sub>2</sub> was found to require Ca<sup>2+</sup> to hydrolyze phosphatidylethanolamine ( $K_{Ca^{2+}}$  is 0.1 to 0.2 mM).

Fatty acid chain length, as well as head group, influences the ability of phospholipase A<sub>2</sub> to hydrolyze phospholipids. The rate of degradation of phosphatidylcholines increases by a factor of 1.9 as the fatty acid chain lengths decreased from 16 to 12 carbons. Comparison of the hydrolysis rates for dimyristoyl (130  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) and dilauryl phosphatidylethanolamine (180  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ) at 50° also shows an increase in activity as the chain length is decreased. The 8-carbon phospholipid, dioctanoyl phosphatidylcholine, is an exceptionally good substrate for the enzyme. Shortening the chain length to 6 carbons decreases the activity to the level of long chain phosphatidylcholines.

The negatively charged phosphatidylserine is harder to analyze because of the enzyme requirement for Ca<sup>2+</sup>. In the presence of 10 mM Ca<sup>2+</sup>, the phosphatidylserine precipitated from the mixed micelle; the phase change is suggestive of the multilamellar structures detected by Papahadjopoulos *et al.* (28) under similar conditions. When the Ca<sup>2+</sup> concentration was decreased to 0.5 mM or less, the lipid usually appeared to remain solubilized. Under these conditions, the measured

enzymatic activity was 0.05 to 0.15 that of dipalmitoyl phosphatidylcholine in the presence of 0.5 mM Ca<sup>2+</sup>. The interpretation of this relative activity is hampered by the fact that both enzyme and phospholipid are competing for Ca<sup>2+</sup>. The dissociation constant for Ca<sup>2+</sup> to enzyme is 0.15 mM (16). The dissociation constant for Ca<sup>2+</sup> to phosphatidylserine in mixed micelles has not been reported, although a 2:1 phospholipid:Ca<sup>2+</sup> stoichiometry has been suggested for phosphatidylserine in vesicles (28) and the tight binding has been shown in monolayers (29). If the Ca<sup>2+</sup>-phospholipid interaction is comparable to or greater than that of the enzyme, the majority of the Ca<sup>2+</sup> will be bound by the phospholipid and not the enzyme. Hence, little hydrolysis would be expected. Since Ca<sup>2+</sup> was not saturating the enzyme in the experiments reported here, slight variations in conditions could account for the range of activities observed with this substrate.

*B. cereus* Phospholipase C Specificity—When mixed micelles of Triton and phospholipid (mole ratio 8:1) were treated with phospholipase C, transesterified egg phosphatidylethanolamine and egg phosphatidylcholine were almost equivalent substrates for the enzyme (Table II). Decreasing the length of the fatty acid in phosphatidylcholines resulted in increased reaction rates. Thus, the rate ratios observed in going from dipalmitoyl to dilauryl phosphatidylcholine increase for both phospholipase C and phospholipase A<sub>2</sub>. For phosphatidylethanolamines, where it was necessary to determine the reaction rates at 50° due to the phase transition of dimyristoyl phosphatidylethanolamine, the rate for dimyristoyl phosphatidylethanolamine (1900 μmol min<sup>-1</sup> mg<sup>-1</sup>) was less than that for dilauryl phosphatidylethanolamine (4500 μmol min<sup>-1</sup> mg<sup>-1</sup>). Thus, the same chain length dependence holds for the phosphatidylethanolamines. Phosphatidylserine was hydrolyzed more slowly than phosphatidylcholine and phosphatidylethanolamine, while sphingomyelin was not a substrate.

In several experiments, 10 mM Ca<sup>2+</sup> was included during the incubation (Table II) so that assay conditions would be identical with those employed with phospholipase A<sub>2</sub>. In no case was there any effect on activity of the phospholipase C. Phospholipase C is a Zn<sup>2+</sup> metalloenzyme which has 2 mol of

Zn<sup>2+</sup>/mol of enzyme tightly bound (24). Free Zn<sup>2+</sup> concentration was 0.25 μM during assay. Additional Zn<sup>2+</sup> did not lead to enhanced activity with sonicated phospholipids containing deoxycholate (10) and we found no stimulation by the addition of small amounts of Zn<sup>2+</sup> when dipalmitoyl phosphatidylcholine was used as substrate in mixed micelles with Triton X-100. In fact, with high levels of Zn<sup>2+</sup> (0.05 to 0.5 mM), an apparent inhibition of activity was observed. Similar effects had also been encountered in earlier studies on phospholipase C action toward phosphatidylcholine determined by pH-stat techniques at pH = 8.0 (23) where Zn(OH)<sub>2</sub> formation or Zn<sup>2+</sup> effects on the electrode were considered as possible causes for the apparent inhibition (23). With phosphatidylserine, it is possible that this anionic phospholipid would compete for the enzyme-bound Zn<sup>2+</sup> in an analogous fashion to its competition for Ca<sup>2+</sup> in assays of phospholipase A<sub>2</sub>. However, the addition of 0.01 mM Zn<sup>2+</sup> with phosphatidylserine did not lead to an enhancement of activity as indicated in Table II. Unfortunately, when we attempted to study the effect of higher concentrations of Zn<sup>2+</sup> (0.05 to 0.5 mM) on enzyme activity toward phosphatidylserine, spurious activities were often observed, and no clear pattern of activation or inhibition emerged.

*Phospholipase A<sub>2</sub> Activity toward Short Chain Phosphatidylcholines*—Two synthetic short chain phosphatidylcholines were examined as substrates for phospholipase A<sub>2</sub>. Unlike the long chain phosphatidylcholines investigated, these compounds can form micelles when dispersed in water without surfactants (30, 31). The apparent molecular weight of dioctanoyl phosphatidylcholine micelles has been determined by light scattering and is very large and concentration dependent, spanning the range 5 × 10<sup>6</sup> to 20 × 10<sup>6</sup> (32). This phospholipid tends to form cloudy (two phase) solutions in water. Phospholipase A<sub>2</sub> hydrolyzed these dispersions approximately 13 times faster than dipalmitoyl phosphatidylcholine in Triton X-100 mixed micelles at a Triton/phospholipid mole ratio of 8:1 (approximately 7 times faster than for Triton/dipalmitoyl phosphatidylcholine mole ratio of 2:1). When dioctanoyl phosphatidylcholine was inserted into Triton micelles, the solution immediately clarified. NMR experiments suggested that all of the phospholipid was then in mixed micelles.<sup>2</sup> The activity of the enzyme toward these mixed micelles was also large; at a Triton/phospholipid mole ratio of 2:1, the activity was slightly higher than toward an aqueous solution of the phospholipid as shown in Table III. However, as the Triton/phospholipid ratio was increased (at a given phospholipid concentration), the activity decreased. This results from the "surface dilution phenomenon" described previously (9, 14).

The activity of phospholipase A<sub>2</sub> was also examined toward dihexanoyl phosphatidylcholine. At a concentration of 6 mM, this phospholipid should exist as a monomer (30). The enzyme showed little activity toward this material, the rate being 0.09 that of dipalmitoyl phosphatidylcholine in mixed micelles at a mole ratio of 8:1 (0.04 that of dipalmitoyl phosphatidylcholine in mixed micelles at a mole ratio of 2:1). When Triton is added to the solution of dihexanoyl phosphatidylcholine monomers at a mole ratio of 2:1 or greater, the phospholipid is taken up by the detergent micelle.<sup>2</sup> This form of the phospholipid was a much better substrate for phospholipase A<sub>2</sub>. For a Triton/phospholipid mole ratio of 4:1, the relative rate increased 17-fold. At a diluted mole ratio of 8:1, the increased

TABLE II

*Phospholipase C hydrolysis of various phospholipids in Triton/  
phospholipid mixed micelles*

Phospholipid	Ca <sup>2+</sup>	Rate	Relative rate
		μmol min <sup>-1</sup> mg <sup>-1</sup>	
Egg phosphatidylcholine	—	1500	0.94
	+	1500	0.94
Dipalmitoyl phosphatidylcholine	—	1600	1.0
	+	1600	1.0
Dimyristoyl phosphatidylcholine	—	2000	1.3
Dilauryl phosphatidylcholine	—	4600	2.9
Egg phosphatidylethanolamine	—	1300	0.81
Dimyristoyl phosphatidylethanolamine	—	— <sup>a</sup>	0.67
Dilauryl phosphatidylethanolamine	—	2500	1.6
Bovine brain phosphatidylserine	—	590	0.37
	— <sup>b</sup>	590	0.37
Bovine brain sphingomyelin	—	0	0.0

<sup>a</sup> Because of the high thermotropic phase transition temperature of dimyristoyl phosphatidylethanolamine, assays were conducted at 50° with this lipid and compared to dipalmitoyl phosphatidylcholine at the same temperature.

<sup>b</sup> Zn<sup>2+</sup> (0.01 mM) was included in the assay mixture.

<sup>2</sup> M. F. Roberts and E. A. Dennis, manuscript in preparation.

TABLE III

Comparison of phospholipase A<sub>2</sub> activity toward phosphatidylcholine as monomers, micelles, and mixed micelles with Triton X-100

The phospholipid concentration was 6 mM and the Triton X-100 concentration was 48 mM (mole ratio 8:1), 24 mM (mole ratio 4:1), or 12 mM (mole ratio 2:1) or it was omitted (mole ratio 0).

Phospholipid	Triton: phospholipid	Rate $\mu\text{mol min}^{-1}$ $\text{mg}^{-1}$
	mole ratio	
Dihexanoyl phosphatidylcholine	0	45
	4:1	780
	8:1	410
Dioctanoyl phosphatidylcholine	0	8200
	2:1	8500
	4:1	8300
	8:1	6500
	8:1	6500
Dipalmitoyl phosphatidylcholine	2:1	1200
	4:1	1100
	8:1	490

activity is comparable to that of the long chain dipalmitoyl phosphatidylcholine in Triton micelles.

## DISCUSSION

*Advantage of Mixed Micelles for Specificity Studies*—In order to determine phospholipid preferences of enzymes which work on aggregated substrates, one needs a well defined matrix which is independent of phospholipid structure. The nonionic surfactant Triton X-100 serves this purpose. At 8:1 Triton/phospholipid, although enzyme activity is much lower than toward 2:1 mixed micelles because of surface dilution (9, 14), there is little perturbation of the basic Triton micelle structure (12). We have now found for a monodisperse Triton analogue that all phospholipids, regardless of head group, form similarly sized micelles at this surfactant/phospholipid ratio.<sup>3</sup> Also, the large excess of Triton molecules should serve to decrease any patching or clustering of phospholipid molecules in the micelle surface which might occur. Triton X-100 is also useful for comparing enzyme activity on synthetic short chain phospholipids. When Triton, with its very low critical micelle concentration (0.3 mM), is combined with zwitterionic short chain phosphatidylcholines, the critical micelle concentration of the phospholipid is reduced sharply so that assays can be conducted at phospholipid concentrations in which all of the phospholipid is micellar. This makes it easy to compare enzyme activity toward phospholipids of varying chain length without the complication of different micellar structures, sizes, or residual phospholipid monomers.

*Phospholipase A<sub>2</sub> Specificity*—Cobra venom phospholipase A<sub>2</sub> has been used to investigate the sidedness of phospholipid distribution in red blood cells and other natural membrane systems. Using a large excess of enzyme, it was shown that in red blood cells, phosphatidylcholine was hydrolyzed before phosphatidylethanolamine or phosphatidylserine (33, 34). If these results are real indications of membrane asymmetry, one must be sure that the relative activity of phospholipase A<sub>2</sub> toward the different phospholipids in these membranes is not very different. The results reported here show different apparent specificities for phospholipase A<sub>2</sub> toward the different phospholipids when each is assayed separately in the Triton model system. In order to generalize to membranes, it will be necessary to examine specificities in mixtures of

phospholipids and in preparations containing other components as these too can conceivably affect the structure of the substrate and enzyme activity.<sup>4</sup>

Earlier work on snake venom specificity showed certain trends: phosphatidylcholine and phosphatidylethanolamine were good substrates, while phosphatidylserine was hardly hydrolyzed. Dawson (35) used an ethereal solution at 30° with 0.45 mM Ca<sup>2+</sup>, but found hydrolysis of phosphatidylethanolamine occurred without added Ca<sup>2+</sup> and also without ether. Ibrahim *et al.* (3) found similar results using deoxycholate micelles and no added Ca<sup>2+</sup>. These results differ dramatically from those reported in this study. It is likely that the discrepancy results from the nonenzymatic hydrolysis of phosphatidylethanolamine which was apparently not followed in the latter studies and which we have shown is quite rapid at pH = 8 in the Triton mixed micelle system. Cobra venom phospholipase A<sub>2</sub> (*Naja naja naja*) was found here to require Ca<sup>2+</sup> for phosphatidylethanolamine hydrolysis in mixed micelles. In a more recent study, Salach *et al.* (8) assayed in the presence of Triton X-100 and found phosphatidylethanolamine to be a poor substrate, but their experiments were done at 25°, a temperature well below the thermotropic phase transition temperature (36, 37) of several of the saturated phospholipids they studied. In fact, one of the major conclusions of that study was that saturation of the fatty acid in the *sn*-2 position results in a large decrease in the hydrolysis rate without an increase in *K<sub>m</sub>*. At that temperature, dipalmitoyl phosphatidylcholine/Triton X-100 mixtures actually phase separate (12, 38) and this apparently decreased the hydrolysis rate (9).

In all studies phosphatidylserine was found to be a poor substrate. The usual explanation given is that a net negatively charged substrate adversely affects the enzyme which is specific for zwitterionic substrates. An alternate reason is that phosphatidylserine, unlike the choline- or ethanolamine-containing phospholipids, binds Ca<sup>2+</sup> tightly, and under the conditions of most phospholipase A<sub>2</sub> assays, the large excess of Ca<sup>2+</sup> forms insoluble complexes with phosphatidylserine. This insoluble or precipitated material would be inert to phospholipase A<sub>2</sub> action. It is only as one lowers the Ca<sup>2+</sup> concentration so that the phosphatidylserine stays in solution that the inherent enzyme activity can be measured. We have found that the rate is 0.05 to 0.15 that for dipalmitoyl phosphatidylcholine at 0.5 mM Ca<sup>2+</sup>. This is a lower limit for the relative activity: phospholipase A<sub>2</sub> has an absolute requirement for Ca<sup>2+</sup> and will have to compete with phosphatidylserine for Ca<sup>2+</sup> ions. If the phospholipid-Ca<sup>2+</sup> affinity were known, one could calculate the true activity of phospholipase A<sub>2</sub> toward phosphatidylserine in Triton/phospholipid mixed micelles or other structures.

The results with synthetic short chain phospholipids indicate that the cobra venom phospholipase A<sub>2</sub> is not very active toward monomers, as is the case for phospholipases from most other sources (6, 7). The enzyme greatly prefers micellar substrates, either Triton/phospholipid mixed micelles or pure phospholipid micelles (as for dioctanoyl phosphatidylcholine). Furthermore, at a mole ratio of 2:1, the Triton mixed micelles show comparable hydrolysis rates to pure dioctanoyl phosphatidylcholine micelles.

*Phospholipase C Specificity*—Purified phospholipase C from *Bacillus cereus* is known to hydrolyze phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in natural

<sup>3</sup> R. J. Robson and E. A. Dennis, manuscript in preparation.

<sup>4</sup> M. Adamich and E. A. Dennis, manuscript in preparation.

membranes (39-42). Studies on red cell ghosts indicate that phosphatidylserine is hydrolyzed more slowly than the other lipids (39). As with phospholipase A<sub>2</sub>, this could be due to a greater availability of phosphatidylcholine and phosphatidylethanolamine in the membrane or to a preference of phospholipase C for these two lipids.

With sonicated phospholipid preparations containing deoxycholate, Otnaess *et al.* (10) found that egg phosphatidylethanolamine and brain phosphatidylserine were hydrolyzed more slowly than dipalmitoyl phosphatidylcholine. With the Triton-phospholipid mixed micelle system, the activity of phospholipase C toward phosphatidylethanolamine was only slightly less than toward phosphatidylcholine and its activity toward phosphatidylserine was only 2 to 3 times less than toward phosphatidylcholine. Unfortunately, the fatty acid composition of the phosphatidylserine is not exactly the same as any of the phosphatidylcholines, so a precise evaluation of the specificity toward this phospholipid must be performed on synthetic phosphatidylserines which have low thermotropic phase transition temperatures; such phospholipids have not been available. The possibility of Zn<sup>2+</sup> chelation by phosphatidylserine could also complicate the interpretation of phosphatidylserine hydrolysis by phospholipase C in analogy to Ca<sup>2+</sup> chelation with phospholipase A<sub>2</sub>, although this is less likely with phospholipase C since the Zn<sup>2+</sup> is quite tightly bound to this enzyme (24).

*Comparison of Phospholipase A<sub>2</sub> and C Specificities in Mixed Micelle System*—These two pure phospholipases show different head group specificities toward individual phospholipids in the well characterized Triton X-100 mixed micelle system. Phospholipase A<sub>2</sub> prefers phosphatidylcholine; phospholipase C will hydrolyze phosphatidylcholine and phosphatidylethanolamine at similar rates. Ca<sup>2+</sup> does not affect phospholipase C activity so that the assay mixture containing mixed micelles and 10 mM Ca<sup>2+</sup> was identical for evaluating the different specificities toward phosphatidylcholine and phosphatidylethanolamine of the two enzymes. Recently, we (13) showed that <sup>1</sup>H NMR detects differences in the α-methylene groups of the *sn*-1 and *sn*-2 fatty acid chains in phosphatidylcholine and phosphatidylserine. Phosphatidylethanolamine shows a broadened pattern, indicating a local conformation or environment different from the other two phospholipids. This is of particular interest because phospholipase A<sub>2</sub> catalyzes the hydrolysis of the fatty acid at the carbonyl carbon of the *sn*-2 chain. In the case of the two zwitterionic phospholipids, the conformational differences near the site of enzymatic attack may be important for phospholipase A<sub>2</sub> catalysis. Since this region is quite removed from the site of phospholipase C catalysis, it may not be important for that enzyme.

Both enzymes display less activity toward phosphatidylserine than toward phosphatidylcholine, but evaluation of this apparent specificity is complicated as discussed above for each enzyme. The similar effects of chain length on activity for both enzymes suggest that there is an optimum chain length for both phospholipases in this assay system. While this could fortuitously represent similar binding specificities of both enzymes, it is more likely that the interfacial properties of the phospholipid are optimized and this is equally important for both enzymes. If prior association of the enzyme with phospholipid in the mixed micelles is essential before Michaelis complex formation, as we (43) have recently suggested for phospholipase A<sub>2</sub> based on chemical modification (44) and other studies (14), then apparent fatty acid chain

length and polar head group preferences would not be simply interpretable in terms of active site binding specificities. Furthermore, mixtures of phospholipids and natural membranes could exhibit different specificities if the phospholipids affected each step differently. Thus, further work is required to delineate the precise cause of the apparent specificities reported here.

## REFERENCES

1. Van Deenen, L. L. M., and de Haas, G. H. (1963) *Biochim. Biophys. Acta* 70, 538-553
2. Braganca, B. M., Sambray, Y. M., and Ghadially, R. C. (1969) *Toxicol.* 7, 151-157
3. Ibrahim, S. A., Sanders, H., and Thompson, R. H. S. (1964) *Biochem. J.* 93, 588-594
4. Uthe, J. F., and Magee, W. L. (1970) *Can. J. Biochem.* 49, 776-784
5. de Haas, G. H., Postema, N. M., Nieuwenhuizen, W., and Van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* 159, 103-117
6. de Haas, G. H., Bensen, P. P. M., Pieterse, W. A., and Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* 239, 252-266
7. Wells, M. A. (1974) *Biochemistry* 13, 2248-2257
8. Salach, J. I., Seng, R., Tisdale, H., and Singer, T. P. (1971) *J. Biol. Chem.* 246, 340-347
9. Dennis, E. A. (1973) *Arch. Biochem. Biophys.* 158, 485-493
10. Otnaess, A.-B., Little, C., Sletten, K., Wallin, R., Johnsen, S., Flengsrud, R., and Prydz, H. (1977) *Eur. J. Biochem.* 79, 459-468
11. Little, C. (1977) *Acta Chem. Scand.* B31, 267-272
12. Dennis, E. A. (1974) *Arch. Biochem. Biophys.* 165, 764-773
13. Roberts, M. F., and Dennis, E. A. (1977) *J. Am. Chem. Soc.* 99, 6142-6143
14. Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) *J. Biol. Chem.* 250, 9013-9020
15. Deems, R. A., and Dennis, E. A. (1975) *J. Biol. Chem.* 250, 9008-9012
16. Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *J. Biol. Chem.* 252, 6011-6017
17. Little, C., Aurebekk, B., and Otnaess, A.-B. (1975) *FEBS Lett.* 52, 175-179
18. Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965) *J. Amer. Oil Chem. Soc.* 42, 53-56
19. Folch, J. (1942) *J. Biol. Chem.* 146, 35-44
20. Warner, T. G., and Dennis, E. A. (1973) *J. Lipid Res.* 14, 595-598
21. Warner, T. G., and Benson, A. A. (1977) *J. Lipid Res.* 18, 548-552
22. Dennis, E. A. (1973) *J. Lipid Res.* 14, 152-159
23. Eaton, B. R. (1975) *Ph.D. dissertation*, University of California at San Diego; (1976) *Diss. Abstr. B Sci. Eng.* 36, 3351
24. Little, C., and Otnaess, A.-B. (1975) *Biochim. Biophys. Acta* 391, 326-333
25. Otnaess, A.-B., Prydz, H., Bjørklid, E., and Berre, A. (1972) *Eur. J. Biochem.* 27, 238-243
26. Eaton, B. R., and Dennis, E. A. (1976) *Arch. Biochem. Biophys.* 176, 604-609
27. Van Dijck, P. W. M., de Kruijff, B., Van Deenen, L. L. M., de Gier, J., and Demel, R. A. (1976) *Biochim. Biophys. Acta* 455, 576-587
28. Papahadjopoulos, D., Vail, W. J., Jacobson, K., and Poste, G. (1975) *Biochim. Biophys. Acta* 394, 483-491
29. Seimiya, T., and Ohki, S. (1973) *Biochim. Biophys. Acta* 298, 546-561
30. Tausk, R. J. M., Karmiggelt, J., Oudshoorn, C., and Overbeek, J. (1974) *Biophys. Chem.* 1, 175-183
31. Tausk, R. J. M., van Esch, J., Karmiggelt, J., Voordouw, G., and Overbeek, J. (1974) *Biophys. Chem.* 1, 184-203
32. Tausk, R. J. M., Oudshoorn, C., and Overbeek, J. (1974) *Biophys. Chem.* 2, 53-63
33. Zwaal, R. F. A., Roelofsen, B., Comfurius, P., and Van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta* 406, 83-96
34. Martin, J. K., Luthra, M. G., Wells, M. A., Watts, R. P., and Hanahan, D. J. (1975) *Biochemistry* 14, 5400-5408
35. Dawson, R. M. C. (1963) *Biochem. J.* 88, 414-423
36. Melchior, D. L., and Morowitz, H. J. (1972) *Biochemistry* 11, 4558-4562
37. Hinz, H.-J., and Sturtevant, J. M. (1972) *J. Biol. Chem.* 247,

- 6071-6075
38. Ribeiro, A. A., and Dennis, E. A. (1974) *Biochim. Biophys. Acta* 332, 26-35
39. Roelofsen, B., Zwaal, R. F. A., Comfurius, P., Woodward, C. B., and Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* 241, 925-929
40. Mavis, R. D., Bell, R. M., and Vagelos, P. R. (1972) *J. Biol. Chem.* 247, 2835-2841
41. Otnaess, A.-B., Krokan, H., Bjørklid, E., and Prydz, H. (1976) *Biochim. Biophys. Acta* 454, 193-206
42. Otnaess, A.-B., and Holm, T. (1976) *J. Clin. Invest.* 57, 1419-1425
43. Roberts, M. F., Deems, R. A., and Dennis, E. A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 1950-1954
44. Roberts, M. F., Deems, R. A., Mincey, T. C., and Dennis, E. A. (1977) *J. Biol. Chem.* 252, 2405-2411