Phospholipid Activation of Cobra Venom Phospholipase A_2. 1. Lipid–Lipid or Lipid–Enzyme Interaction†

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ABSTRACT: In individual phospholipid–Triton X-100 mixed micelles, phospholipase A_2 hydrolyzes phosphatidylcholine at a tenfold higher rate than phosphatidylethanolamine, while in binary phospholipid mixtures phosphatidylcholine activates the enzyme toward phosphatidylethanolamine so that it becomes the preferred substrate. This specificity reversal has now been observed in anionic and zwitterionic as well as in nonionic detergent mixed micelle systems. Detailed studies on the physical characteristics of Triton X-100/phospholipid mixed micelles were conducted to ascertain their role in the activation process. The size of Triton mixed micelles containing phosphatidylcholine, phosphatidylethanolamine, or cholesterol, individually and in combination, was determined by agarose gel chromatography. Micelle size depends only on the total mole fraction of lipid, not on the phospholipid head group or whether the lipid is cholesterol, and the Stokes radius increases by about one-third in going from a mole fraction of lipid of 0.06 to 0.20. ^{31}P nuclear magnetic resonance spectroscopy was used to study lipid hydrolysis by phospholipase A_2 and to determine the importance of lipid–lipid interactions. Preferential hydrolysis of phosphatidylethanolamine is independent of the mole ratio of Triton/phospholipid and the presence of cholesterol. The $^{31}P\{^1H\}$ nuclear Overhauser effect enhancements for phosphatidylcholine and phosphatidylethanolamine alone and for the two in binary lipid mixed micelles have the same frequency dependence, maximum at methylene protons adjacent to the phosphate, suggesting that no strong intermolecular lipid head-group interactions occur. These data demonstrate that mixed micelle size, shape, surface charge, and lipid–lipid interactions are not responsible for specificity reversal. The observation that the soluble, monomeric phospholipid dibutyrylphosphatidylcholine, which is shown to not form mixed micelles with Triton, activates phospholipase A_2 toward phosphatidylethanolamine suggests a direct lipid–enzyme interaction as the cause of the activation process.

Using mixed micelles composed of Triton X-100 and binary mixtures of phosphatidylethanolamine and phosphatidylcholine, Adamich & Dennis (1978a) have shown that cobra venom phospholipase A_2 (EC 3.1.1.4) preferentially hydrolyzes phosphatidylethanolamine. They have also found phosphatidylethanolamine to be the preferred substrate of the enzyme when it acts on endogenous phospholipids in human erythrocyte ghosts and Triton–phospholipid mixed micelles prepared from the ghost membranes (Adamich & Dennis, 1978b). These findings contrast markedly with the specificity of phospholipase A_2 toward individual phospholipids in Triton mixed micelles,[1] where phosphatidylethanolamine is a much poorer substrate than phosphatidylcholine (Roberts et al., 1978a). The nature of this specificity reversal by phospholipase A_2 in lipid mixtures has direct relevance to the mechanism of action of this enzyme (Verger & de Haas, 1976) and to the use of phospholipases to probe the distribution of phospholipids across native and model membranes (Zwaal et al., 1975; Martin et al., 1975).

Possible explanations for the phosphatidylcholine-enhanced hydrolysis of phosphatidylethanolamine include (i) direct phospholipid–phospholipid interactions which change the conformation or environment of phosphatidylethanolamine so that it becomes an effective substrate, (ii) changes in the surface environment or micelle structure which make phosphatidylethanolamine the preferred substrate, or (iii) a direct phosphatidylcholine–enzyme activation.

There is a variety of evidence in the literature which suggests that phosphatidylethanolamine can form structures considerably different than phosphatidylcholine and that in certain systems interactions between the two lipids can occur (Cullis & de Kruijff, 1978; Jendrasiak & Mendible, 1976; Roberts & Dennis, 1977). Recently Yeagle et al. (1977) showed that, in phosphatidylethanolamine/phosphatidylcholine sonicated vesicles, measurable intermolecular phospholipid interactions do occur. Whether such lipid–lipid interactions or lipid–detergent surface changes occur in Triton-mixed micelles has not been examined.

We have now investigated the role of phospholipid–phospholipid and detergent–phospholipid interactions in the activation phenomenon. Physical characterization of the Triton X-100–phospholipid mixed micelles by ^{31}P NMR spectroscopy and agarose gel filtration suggests that no strong phosphatidylethanolamine–phosphatidylcholine interactions occur in this system and that the two lipids behave in a similar fashion in the Triton matrix. Observation of the same specificity reversal in other detergent systems (anionic and zwitterionic as well as nonionic), which would be expected to differ radically in surface microenvironment and structure, points to a lipid–enzyme interaction as the primary cause of the activation phenomenon. A direct demonstration of this effect is provided by experiments with the synthetic short-chain, soluble phospholipid dibutyrylphosphatidylcholine,[2] which will

† From the Department of Chemistry, University of California at San Diego, La Jolla, California 92093. *Received September 15, 1978; revised manuscript received March 1, 1979.* This research was supported by National Institutes of Health Grant GM-20501 and National Science Foundation Grant PCM 76-21552; the NMR spectrometer was supported by National Institutes of Health Grant RR-00708.

‡ Postdoctoral Fellow of the National Institutes of Health, Grant GM-05910.

§ Postdoctoral Fellow of the National Institutes of Health, Grant GM-06071.

[1] When one species (i.e., head group) of phospholipid is inserted into a detergent matrix, the mixed micelles are said to contain individual phospholipids. When two or more phospholipids differing in head group are present in the mixed micelle system, it will be referred to as a binary, ternary, etc., mixture.

[2] Abbreviations used: dibutyrylphosphatidylcholine, 1,2-dibutyryl-*sn*-glycerol-3-phosphorylcholine; Triton, Triton X-100; $C_{12}EO_8$, *n*-dodecyloctaethylene glycol; OPE-9, *p-tert*-octylphenoxynonaoxyethylene glycol; DOC, sodium deoxycholate; DTAPS, 3-(dimethyltetradecylamino)propane-1-sulfonate; NOE, nuclear Overhauser effect; cmc, critical micelle concentration.

0006-2960/79/0418-3301$01.00/0

not form mixed micelles with Triton, but will activate phosphatidylethanolamine hydrolysis. Further characterization of this lipid–enzyme interaction is reported in the accompanying manuscript (Adamich et al., 1979).

Experimental Procedure

Materials. Lyophilized cobra venom, *Naja naja naja* (Pakistan), lot no. NNP45-1Z, was obtained from the Miami Serpentarium. The phospholipase A_2 was purified as described elsewhere (Deems & Dennis, 1975; Roberts et al., 1977).

Egg phosphatidylcholine was prepared from fresh egg yolks by the method of Singleton et al. (1965). Egg phosphatidylethanolamine, *N*-methylphosphatidylethanolamine, and *N,N*-dimethylphosphatidylethanolamine, prepared by transesterification of egg phosphatidylcholine, were obtained from Avanti Biochemicals. Lysophospholipids were prepared by phospholipase A_2 treatment of diacylphospholipids in Triton-mixed micelles. Other lipids used included dihexanoylphosphatidylcholine (Avanti), dibutyrylphosphatidylcholine (Supelco), and cholesterol (Calbiochem). All phospholipids were routinely checked for purity by one-dimensional thin-layer chromatography on silica gel plates (Brinkmann) with $CHCl_3$:CH_3OH:H_2O (65:25:4 v/v/v) as the developing solvent. Large batches of phosphatidylethanolamine and phosphatidylcholine were further checked by two-dimensional thin-layer chromatography as described previously (Adamich & Dennis, 1978b). ^{31}P NMR was also used to screen phospholipid samples for phosphorus containing impurities. Two commercially available phospholipids (*N*-methylphosphatidylethanolamine and *N,N*-dimethylphosphatidylethanolamine) were found to contain 3–5% lysophospholipid. These impurities were removed by column chromatography on Unisil silicic acid (100–200 mesh) (Clarkson Chemicals). All phospholipid samples used gave a single spot on thin-layer chromatography.

Nonionic detergents employed in the formation of mixed micelles included polydisperse Triton X-100 (Triton) (Rohm and Haas) and the homogeneous, monodisperse compounds *n*-dodecyloctaethylene glycol ($C_{12}EO_8$) (Nikol Chemical) and *p-tert*-octylphenoxynonaoxyethylene glycol (OPE-9) synthesized by the procedure of Robson & Dennis (1978). Sodium deoxycholate (DOC) (Sigma) and 3-(dimethyltetradecylamino)propane-1-sulfonate (DTAPS), provided by Dr. Ammon Gonenne, University of California at San Diego, were also used to form mixed micelles. Mixed micelles were prepared by adding solutions of detergent to dry phospholipids; mixing was achieved by a few strokes with a Potter-Elvejhem homogenizer, or by vigorous shaking and stirring and then allowing the foam to subside before the sample was used. The final concentration of phospholipid in each mixed micelle sample was determined by phosphorus analysis of sample aliquots (Eaton & Dennis, 1976); solubilization was generally complete.

Blue dextran 2000 (Pharmacia), AMP (Sigma), yeast alcohol dehydrogenase (Worthington), *Escherichia coli* β-galactosidase (Boehringer), bovine serum albumin (Mann), and Bio-Gel A-5m (Rio-Rad) which is a 6% agarose gel, 100-200 mesh, were used as obtained.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were obtained at 40.5 MHz and 40 °C with a JEOL PFT-100 system equipped with a Nicolet 1085 computer and disk. The Hetero spin decoupler JNM-SD-HC unit was used with a Schomandl ND 100M frequency generator. Phospholipid samples usually contained 30% D_2O for an internal lock. Chemical shifts were measured relative to an external sample of 50 mM phosphate buffer, pH 7.5, and the chemical shifts are reported relative to the phosphatidylethanolamine sample; resolution is ±0.02 ppm. For enzymatic assays, mixed micelles consisted of 6 mM total phospholipid and 48 mM Triton X-100 in 50 mM Tris-HCl and 10 mM $CaCl_2$. Assays were conducted in duplicate at pH 8.0 and 40 °C. All phospholipase A_2 assays reported in this manuscript employed this method and these conditions unless noted otherwise. The concentration of phospholipase A_2 was such that the time necessary to acquire a spectrum was small compared with the entire enzymatic time course. Normally, 256 transients of 1.6 s, each generated by a 60–70° pulse, were accumulated for each reaction time point. Hydrolysis was quantified by integration of lysophospholipid and parent phospholipid peaks by using a planimeter. Results are the average of duplicate determinations.

For nuclear Overhauser effect (NOE) and spin–lattice (T_1) relaxation measurements, samples consisted of 10 mM phospholipid, 40 mM EDTA, 50 mM Tris-HCl, pH 8.0 (meter value), in D_2C NOE measurements were made by the gated decoupler method (Freeman et al., 1972). T_1 measurements were made by using a $180° - \tau - 90°$ pulse sequence with broad-band proton decoupling. The delay between pulse sequences was the greater of 4 T_1 (^1H) and 4 T_1 (^{31}P). For NOE frequency-dependence measurements, weak continuous-wave proton decoupling rather than strong broad-band decoupling was used. The power level of the proton decoupler was adjusted to be the minimum power that would decouple the phosphorus of trimethyl phosphate in D_2O. Irradiation of the trimethyl phosphate protons (3.75 ppm) was used to calibrate the frequency of the proton decoupler.

Agarose Chromatography. All agarose chromatography experiments were conducted on a single 2.5 × 50 cm water-jacketed column of 6% agarose (Robson & Dennis, 1978). The column was preequilibrated and then run with buffer consisting of 10 mM Tris-HCl, 100 mM NaCl, 0.02% sodium azide, 0.4 mM Triton X-100, pH 8.0. The flow rate was between 0.5 and 1.0 mL min^{-1}. Samples (1.0 mL) of 50 mM Triton plus appropriate concentrations of lipid as mixed micelles prepared in this buffer were applied to the column. Blue dextran 2000 and AMP were used in most samples as markers for the void volume and total volume, respectively. Phospholipid content of samples was determined by digestion in perchloric acid and a phosphate analysis according to Eaton & Dennis (1976); Triton X-100 content was determined by absorbance (Robson & Dennis, 1978). The recovery of both phospholipid and Triton was within ±5% of that applied to the column and did not vary from the first to the last column. Standardization of the agarose column was accomplished with bovine serum albumin, yeast alcohol dehydrogenase, and *E. coli* β-galactosidase.

Sephadex G-100 Chromatography. The partitioning of dibutyrylphosphatidylcholine into Triton X-100 micelles was measured by the equilibrium gel filtration technique of Hummel & Dreyer (1962). A Sephadex G-100 column (1.5 × 50 cm) was equilibrated with 0.52 mM dibutyrylphosphatidylcholine in 50 mM Tris-HCl, pH 8.0. A 1.0-mL sample of the column buffer containing 200 mM Triton X-100 was loaded onto the column. The concentration of phospholipid in the eluate was determined by phosphorus analysis (Eaton & Dennis, 1976), while the Triton X-100 was measured by its absorbance at 276.5 nm.

pH-Stat Assay. The effect of cholesterol on phospholipase A_2 activity was determined by the pH-stat procedure described previously (Dennis, 1973; Roberts et al., 1978a). Assays were conducted under the same conditions as the ^{31}P NMR assay except that the buffer was omitted.

Table I: ^{31}P NMR Chemical Shifts of Phospholipids (6 mM) in Mixed Micelles with Triton X-100 (48 mM)

phospholipid[a]	δ_P (ppm)[b]
phosphatidylcholine	+0.63
dihexanoylphosphatidylcholine (micelle)[c]	+0.52
dihexanoylphosphatidylcholine (monomer)[d]	+0.34
dibutyrylphosphatidylcholine (monomer)[e]	+0.35
lysophosphatidylcholine	+0.17
N,N-dimethylphosphatidylethanolamine	+0.33
lyso-*N,N*-dimethylphosphatidylethanolamine	−0.15
N-methylphosphatidylethanolamine	+0.20
lyso-*N*-methylphosphatidylethanolamine	−0.26
phosphatidylethanolamine	0.00
lysophosphatidylethanolamine	−0.44

[a] All phospholipids are derived from egg phosphatidylcholine by transesterification (and phospholipase A_2 treatment for the lyso derivatives), except for the two synthetic short-chain phosphatidylcholines. [b] Chemical shifts are reported relative to phosphatidylethanolamine. Upfield shifts are positive. [c] At 8:1 Triton:dihexanoylphosphatidylcholine, nearly all of the short-chain phospholipid is taken up into the Triton micelle (Roberts et al., 1978b). [d] Dihexanoylphosphatidylcholine at 6 mM in water (no surfactant) exists as a monomer. [e] The chemical shift of 6 mM dibutyrylphosphatidylcholine did not change upon the addition of Triton.

Thin-Layer Chromatographic Assay. This assay was employed where indicated by using thin-layer chromatography to separate the reaction products and phosphorus determination for quantitation as described elsewhere (Adamich & Dennis 1978a,b). Assays were conducted under the same general conditions as the ^{31}P NMR assay.

Results

^{31}P NMR Assay of Phospholipase A_2 Action. The action of phospholipase A_2 toward phospholipid mixtures can be followed conveniently by ^{31}P NMR spectroscopy. This technique has previously been used to follow phospholipase A_2 action on lipoproteins (Henderson et al., 1975; Brasure et al., 1978). The primary advantage of this assay is that no physical separation of products and reactants is necessary, so that it is especially useful for mixtures of phospholipids. The reaction progress can be sampled periodically and a time course can be obtained simultaneously for each lipid.

The ^{31}P NMR chemical shifts of various phospholipids and their lysophospholipids are presented in Table I. At 40.5 MHz, phosphatidylethanolamine and phosphatidylcholine are separated by 26 Hz (0.63 ppm), while the line widths in the mixed micelle systems are 3–4 Hz. These are not as narrow as those reported by London & Feigenson (1979) for detergent-dispersed phospholipids (1 Hz) in which 50 mM EDTA was present. However, phospholipase A_2 requires Ca^{2+} for activity; thus, large amounts of EDTA cannot be used in our case. Lysophospholipids are always 0.44–0.48 ppm downfield from the parent phospholipids, regardless of head group. Methylation of the phosphatidylethanolamine nitrogen atom increases the shielding of the phosphorus atom (shifting the phospholipid signal upfield). The ^{31}P chemical shift is also sensitive to the aggregation of the phospholipid. The chemical shift for dibutyryl- and dihexanoylphosphatidylcholine, as monomers, is 0.18 ppm downfield of micellar dihexanoylphosphatidylcholine and 0.28 ppm downfield of egg phosphatidylcholine in Triton X-100. Hence, ^{31}P NMR can be used to follow micellization of short-chain phospholipids.

In order for ^{31}P NMR to reflect phospholipase A_2 activity accurately, it is necessary to know the T_1 and NOE for each lipid component. One must be sure that no phospholipid signal is being saturated, and that, if large NOE differences occur, the data are corrected for them. Otherwise gated decoupling must be used at the expense of signal to noise. In Triton X-100 with 2 mM EDTA, the T_1 is 2.5 s for phosphatidylcholine and 2.2 s for phosphatidylethanolamine. T_1(phosphatidylethanolamine) is always slightly shorter than T_1(phosphatidylcholine), even in mixtures. The nuclear Overhauser effect enhancements for phosphatidylethanolamine, phosphatidylcholine, and their lyso compounds range between 0.6 and 0.7. This is a reasonable range for NOE measurements, reflecting instrumental variations and possible differences in sample preparation (phosphates are very sensitive to metal ions, and only a minimum amount of EDTA is present as in the hydrolysis samples). Nonetheless, the reproducibility was ±6% for similarly prepared samples run within the same experiment, indicating that phosphatidylcholine and phosphatidylethanolamine have the same NOEs within experimental error. Thus, broad-band proton decoupling was routinely used in the ^{31}P NMR assays.

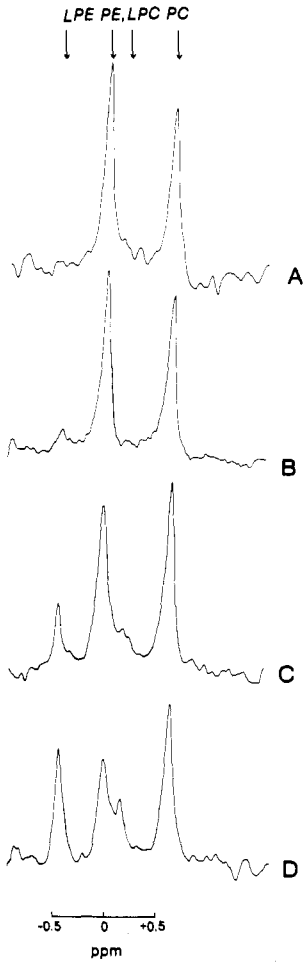

FIGURE 1: Phospholipase A_2 (0.05 μg) was added to a 1.4-mL solution containing egg phosphatidylcholine (2.6 mM)–phosphatidylethanolamine (3.0 mM) in 48 mM Triton X-100, 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0, 40 °C. ^{31}P NMR spectra are shown at the following times after the initiation of reaction: (A) 0; (B) 7 min; (C) 27 min; (D) 87 min. Peaks corresponding to lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), and phosphatidylcholine (PC) are indicated.

In preliminary experiments, we showed, using a thin-layer chromatography assay, that phosphatidylcholine activates phospholipase A_2 hydrolysis of phosphatidylethanolamine (Adamich & Dennis, 1978a). This activation is confirmed with the ^{31}P NMR assay. In Figure 1 are typical spectra obtained when cobra venom phospholipase A_2 is incubated with mixed micelles of Triton/phosphatidylethanolamine/phosphatidylcholine. Although the chemical shifts for lyso-

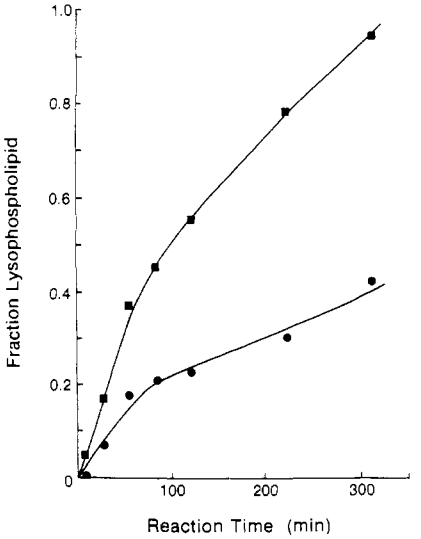

FIGURE 2: Time course of the phospholipase A_2 reaction shown in Figure 1. Egg phosphatidylcholine (●); egg phosphatidylethanolamine (■).

phosphatidylcholine and phosphatidylethanolamine are similar, they can usually be resolved. For 5–20% hydrolysis, the reaction with both lipids appears to be linear with time. Hydrolysis levels of less than 5% could not be measured accurately, but the average error for duplicate determinations at longer times was ±10%. The hydrolysis reaction for both lipids can be followed to near completion as indicated in Figure 2. We have used this ^{31}P NMR assay to study the activation of phosphatidylethanolamine hydrolysis by phosphatidylcholine under various conditions and to characterize the mixed micelles.

Physical Characterization of Triton–Phospholipid Mixed Micelles. (i) Agarose Gel Chromatography. We have previously shown by column chromatography that at very low mole fractions of lipid, phosphatidylcholine with various fatty acid compositions forms mixed micelles that elute at a similar size to micelles of pure surfactant (Dennis, 1974). As the mole fraction of lipid increases, the "mixed micelles" become larger and a second population of structures occurs, termed "quasi-micelles".

We have performed column chromatography studies on Triton X-100/phosphatidylethanolamine and/or phosphatidylcholine mixed micelles. The sizes of the micelles relative to standards have been determined by using the center of the peak corresponding to the mixed micelles. At low mole fractions, most of the Triton and phospholipid elutes as the mixed micelle fraction, with the quasi-micelle peak being only a shoulder on the high molecular weight side of the peak. As the mole fraction of phospholipid increased above 0.2, the quasi-micelle peak becomes a significant fraction of the total detergent and phospholipid applied. The mole fraction of lipid in the mixed micelle fraction, however, remains very close to the mole fraction of lipid applied to the column. With OPE-9 mixed micelles (Robson & Dennis, 1978), quasi-micelles are not observed under these conditions, suggesting that they are due to the polydispersity of the Triton and that quantitation of the mixed micelle peak with Triton is valid as discussed elsewhere (Robson & Dennis, 1979). Figure 3 is a plot of K_{av} as a function of the mole fraction of total lipid for mixed micelles of Triton/egg phosphatidylcholine, Triton/phosphatidylethanolamine, Triton/phosphatidylcholine/phosphatidylethanolamine, Triton/cholesterol, and Triton/phosphatidylcholine/cholesterol. There is a regular decrease of K_{av} as the mole fraction of lipid is increased, regardless of the

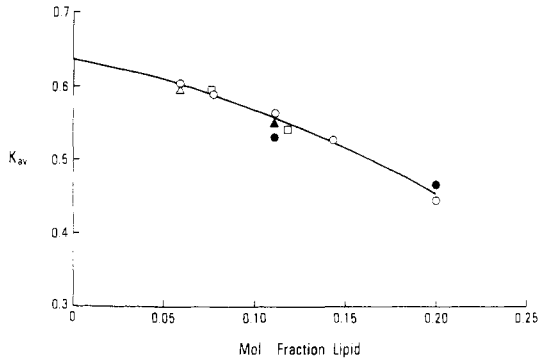

FIGURE 3: A plot of the K_{av} of the most predominant eluted peak from the agarose columns as a function of the mole fraction of lipid applied, lipid/(lipid + Triton X-100), at 28 °C keeping the Triton X-100 concentration at 50 mM. Data are shown for Triton X-100/phosphatidylcholine (○), Triton X-100/phosphatidylethanolamine (□), Triton X-100/phosphatidylcholine/phosphatidylethanolamine with both lipids in equimolar ratios (▲), Triton X-100/phosphatidylcholine/cholesterol with both lipids in equimolar ratios (●), and Triton X-100/cholesterol (△).

Table II: Stokes Radii (R_s) for Triton X-100 Micelles and Mixed Micelles with Phospholipids and Cholesterol[a]

lipid	T (°C)	mole fraction lipid	R_s (Å)
none	28	0.00	43–44
phosphatidylcholine	28	0.06	47
cholesterol	28	0.06	48
phosphatidylethanolamine	28	0.08	48
phosphatidylcholine	28	0.08	48
phosphatidylcholine	28	0.11	52
phosphatidylcholine/phosphatidylethanolamine	28	0.11	54
phosphatidylcholine/cholesterol	28	0.11	56
phosphatidylethanolamine	28	0.12	54
phosphatidylcholine	28	0.14	56
phosphatidylcholine/cholesterol	28	0.20	64
phosphatidylcholine	28	0.20	67
none	40	0.00	56
phosphatidylcholine	40	0.07	67
phosphatidylcholine/cholesterol	40	0.11	69
phosphatidylcholine	40	0.13	74
phosphatidylethanolamine	40	0.14	79

[a] When more than one lipid species is present in the micelles, they are in equimolar concentrations.

nature of the polar head group and whether cholesterol is present. Table II shows the calculated Stokes radii for pure Triton micelles and various mixed phospholipid/cholesterol micelles at 28 and 40 °C, calculated by using the equation $(K_{av})^{1/3} = a - bR_s$ (Porath, 1963). Here a and b are measured constants for the column and R_s is the Stokes radius. Mixed micelles at similar mole fractions of lipid and detergent and temperature have similar elution profiles and Stokes radii, independent of the identity or number of the lipid components.

Evidence that the phosphatidylethanolamine to phosphatidylcholine ratio in Triton/binary lipid mixed micelles is constant across the elution profile was obtained by using radioactive phosphatidylcholine. Both radioactivity determinations and phosphorus analyses were performed. Total phospholipid phosphorus and phosphatidylcholine radioactivity cochromatographed, indicating that the ratio of the two lipids is unchanged upon their incorporation into mixed micelles.

(ii) NOE Frequency Dependence of Phospholipid Phosphorus Nuclei. The $^{31}P\{^1H\}$ NOE has previously been explored for phospholipids, and the results suggest that intermolecular interactions occur in sonicated vesicles of phosphatidylcholine and in mixed vesicles of phosphatidylcholine–phosphatidyl-

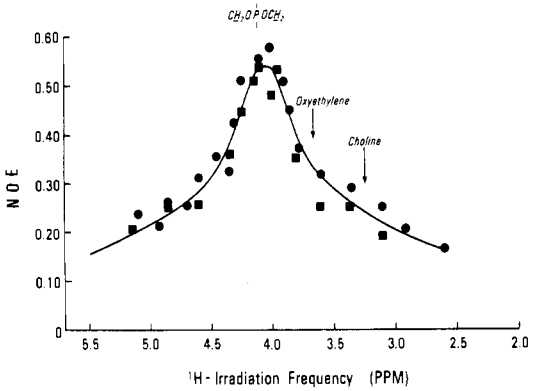

FIGURE 4: Frequency dependence of $^{31}P\{^1H\}$ NOE of 10 mM egg phosphatidylcholine (●) and 10 mM egg phosphatidylethanolamine (■) in 40 mM Triton X-100 plotted as a function of the continuous wave proton decoupler frequency. The chemical shifts for choline methyl groups, methylene groups adjacent to the phosphate of the phospholipid, and Triton oxyethylene groups are indicated in ppm from tetramethylsilane.

Table III: Effect of Surface Dilution on the Activation of Phosphatidylethanolamine Hydrolysis by Phosphatidylcholine Determined by ^{31}P NMR Spectroscopy[a]

Triton: phospholipid[b] (mole ratio)	phospholipid	rate (μmol min^{-1} mg^{-1}) PE	rate (μmol min^{-1} mg^{-1}) PC	rel rate[c] PE	rel rate[c] PC
4	PC		850		1.00
	PE	70		0.08	
	PE:PC (1:9)	220	680	0.26	0.80
	PE:PC (9:1)	460	ND	0.54	ND
	PE:PC (1:1)	730	260	0.86	0.31
8	PC		500		1.00
	PE	50		0.10	
	PE:PC (1:9)	ND	400	ND	0.80
	PE:PC (9:1)	250	ND	0.50	ND
	PE:PC (1:1)	470	240	0.94	0.48
15	PC		310		1.00
	PE	35		0.11	
	PE:PC (1:9)	75	260	0.24	0.84
	PE:PC (9:1)	150	ND	0.48	ND
	PE:PC (1:1)	260	170	0.85	0.55

[a] Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine. [b] Total phospholipid concentration was kept constant at 6 mM; Triton X-100 varied from 24 mM to 90 mM. [c] The relative rate is determined with respect to pure PC at the appropriate *T/P* ratio.

ethanolamine (Yeagle et al., 1977). Phospholipids in mixed sonicated vesicles must be sufficiently close that choline methyl groups dominate the NOE of the phosphatidylethanolamine phosphorus. We find that the NOE frequency profile for phospholipids in Triton mixed micelles is considerably sharper than in sonicated vesicles. The peak has been shifted to about 4 ppm from Me_4Si for phosphatidylcholine and for phosphatidylethanolamine, both individually in Triton (Figure 4) and as a binary mixture (data superimposable on Figure 4). Thus, the maximum contribution to the NOE is from methylene protons adjacent to the phosphate moiety.

Activation of Phosphatidylethanolamine Hydrolysis by Phosphatidylcholine under Various Conditions. Variations in the surface concentration of phospholipid could also be involved in the activation phenomenon. Table III shows the results of ^{31}P NMR monitored Triton dilution experiments where the concentration of total phospholipid was maintained at 6 mM and the concentration of Triton varied from 24 to 90 mM. In all cases, specificity reversal is detected and the relative rate increases are observed to be the same.

Table IV: Effect of Cholesterol on the Activity of Phospholipase A_2 toward Triton-Phospholipid Mixed Micelles

mixed micelle lipids[a]	total rate (μmol mg^{-1})[b]	ratio (+cholesterol/−cholesterol)
PC	533	
PC:CHOL (1:1)	287	0.54
PE	45	
PE:CHOL (1:1)	19	0.42
PE:PC (1:1)	954	
PE:PC:CHOL (1:1:1)	655	0.69
(1:1:2)	402	0.40

[a] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHOL, cholesterol. [b] The activity was determined by pH-stat on mixed micelles of 8:1 Triton/total phospholipid with 6 mM total phospholipid.

Table V: Phosphatidylcholine Activation of Phosphatidylethanolamine in Other Detergent Micelle Systems[a]

detergent	rate (μmol min^{-1} mg^{-1}) PE	rate (μmol min^{-1} mg^{-1}) PC	rate (μmol min^{-1} mg^{-1}) PE (+10% PC)	rel rate[d] PE	rel rate[d] PE (+10% PC)
$C_{12}EO_8$	70	840	475	0.08	0.57
DTAPS	152	309	600	0.19	0.74
DOC[b]	82	344	240	0.24	0.70
OPE-9[c]	58	682	964	0.08	1.4

[a] Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine. The ^{31}P NMR assay was used with 6 mM PE or PC and the detergent indicated at a detergent:phospholipid ratio of 4:1 except where noted. [b] The detergent:phospholipid ratio was 3:1 and only 0.5 mM Ca^{2+} was employed. [c] The thin-layer chromatography assay was employed by using the same experimental conditions as with the ^{31}P NMR assay. [d] Rates relative to rate with PC alone.

Cholesterol is ubiquitous in mammalian membranes and has been shown to interact specifically with phospholipids in sonicated vesicles (Van Dijck et al., 1976). This led us to determine its possible effect on phospholipase A_2 action. The effect of cholesterol in ternary lipid mixtures in Triton-mixed micelles containing equimolar concentrations of phosphatidylethanolamine, phosphatidylcholine, and cholesterol as well as in a 50/50 mol % mixture of cholesterol with either individual or binary mixtures of phosphatidylcholine and phosphatidylethanolamine is shown in Table IV. The results show that, while considerable hydrolysis of phospholipid occurs in the presence of cholesterol, its presence does appear to affect the absolute rate of hydrolysis of the two phospholipids. However, in binary phospholipid mixtures with 33 and 50 mol % cholesterol, the ^{31}P NMR assay shows that the rate for phosphatidylethanolamine hydrolysis is still twice that for phosphatidylcholine. Thus, activation still occurs in the presence of cholesterol.

Triton X-100 is a polydisperse, nonionic detergent, and a possible explanation for the activation phenomenon is that a small change in the microenvironment occurs in the phosphatidylethanolamine–Triton surface structure when phosphatidylcholine is added. The activity of phospholipase A_2 toward mixtures of phospholipids in other detergent systems is summarized in Table V. Enzyme activation toward phospholipids in the homogeneous nonionic surfactants $C_{12}EO_8$ and OPE-9 is the same as toward phospholipids in Triton-mixed micelles. Activation is observed in the zwitterionic DTAPS mixed micelles, although phosphatidylethanolamine

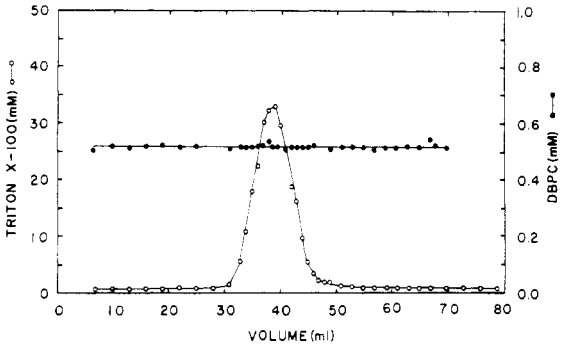

FIGURE 5: Elution profile of Triton X-100 (○) on a Sephadex G-100 column which was preequilibrated with and then run with buffer containing dibutyrylphosphatidylcholine (●).

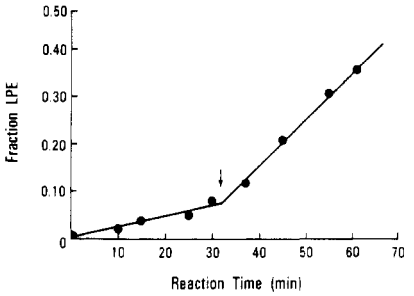

FIGURE 6: Soluble phosphatidylcholine activation of phospholipase A_2 hydrolysis of 6 mM phosphatidylethanolamine in 48 mM Triton X-100. Dibutyrylphosphatidylcholine (1.6 mM) was added to the Triton/phosphatidylethanolamine mixed micelles after a 32-min reaction (indicated by arrow). The fraction of lysophosphatidylethanolamine (LPE) produced is shown.

alone is hydrolyzed more rapidly in this system than in the nonionic mixed micelles. It is also observed in deoxycholate/phospholipid micelles, although it could only be studied at low Ca^{2+} concentrations (0.5 mM) because at 1 mM or greater a gel phase was formed.

Activation of Phosphatidylethanolamine Hydrolysis by Monomeric Phosphatidylcholine. The synthetic short-chain compound dibutyrylphosphatidylcholine does not intercalate into Triton micelles, as shown by equilibrium gel filtration (Hummel & Dreyer, 1962) in Figure 5. This is also shown by a comparison of the ^{31}P NMR chemical shift of dibutyrylphosphatidylcholine in D_2O (where it exists as a monomer) and in the presence of Triton-mixed micelles. The ^{31}P chemical shift (Table I) is not altered for this four-carbon fatty acid phospholipid, whereas for the six-carbon synthetic phosphatidylcholine a significant upfield shift of 0.18 ppm is observed upon micellization by Triton. Thus, dibutyrylphosphatidylcholine is not solubilized by Triton X-100.

The ability of the monomer phosphatidylcholine to activate phosphatidylethanolamine hydrolysis is shown in Figure 6. The addition of 28 mol % dibutyrylphosphatidylcholine increases phosphatidylethanolamine hydrolysis fourfold. Addition of 10 mol % dibutyrylphosphatidylcholine increases phosphatidylethanolamine hydrolysis twofold. By comparison, 28 mol % egg phosphatidylcholine causes a 10–11-fold increase in phosphatidylethanolamine hydrolysis; while 10 mol % causes a 4–5-fold rate increase. These rate increases are the average of two separate experiments for each phosphatidylcholine concentration.

Discussion

Phosphatidylethanolamine and Phosphatidylcholine in Triton X-100 Mixed Micelles. There are several possible explanations for the preference of cobra venom phospholipase A_2 for phosphatidylcholine over phosphatidylethanolamine in individual phospholipid mixed micelles and the striking specificity reversal observed in binary lipid mixed micelles. For example, the two phospholipids might form different micellar structures. ^1H NMR studies of the phospholipid α-methylene groups of phosphatidylcholine and phosphatidylethanolamine in Triton X-100 suggest that the two lipids differ somewhat in conformational or environmental details (Roberts & Dennis, 1977). The overall size and molecular packing might differ dramatically for the two mixed micelles, whereby the Triton matrix causes a phosphatidylethanolamine conformation that makes it a poor substrate or phosphatidylethanolamine might associate in nonrandom arrays that are not effectively hydrolyzed. Phosphatidylcholine could then activate phosphatidylethanolamine hydrolysis by altering the micelle size, or by forming a mixed lipid complex which either directly alters phosphatidylethanolamine conformation or disrupts phosphatidylethanolamine patches.

The results of agarose gel chromatography rule out major micelle size changes as the cause of the activation since the elution profiles and micelle sizes are similar for both phosphatidylethanolamine and phosphatidylcholine as the individual phospholipid in Triton and for binary mixtures of the two. Micelle size depends only on the mole fraction of total phospholipid. This behavior emphasizes the inert nature of the nonionic detergent matrix. Surface dilution of the phospholipid affects micelle size (Robson & Dennis, 1978) and the specific activity of the enzyme (Deems et al., 1975); yet, the activation of phosphatidylethanolamine hydrolysis by phosphatidylcholine is always observed. In dilute Triton/phosphatidylethanolamine (90 mM:6 mM) micelles, as little as 10 mol % phosphatidylcholine causes a 4.3-fold increase in the hydrolysis rate of phosphatidylethanolamine.

A nonrandom distribution of phospholipids in Triton-mixed micelles due to strong "bilayer-like" lipid–lipid interactions is inconsistent with the phosphorus NOE frequency dependence experiments. The methylene protons adjacent to the phosphate group are the major source of phosphorus NOE for phosphatidylethanolamine and phosphatidylcholine individually and in binary mixtures in Triton. This contrasts markedly with similar studies of sonicated vesicles (Yeagle et al., 1977) where the choline methyl group was found to be the primary source of NOE enhancement in pure phosphatidylcholine vesicles and for the phosphatidylethanolamine phosphorus in mixed vesicles with phosphatidylcholine, supporting the idea of intermolecular complexes. Interestingly, the results for phospholipids in Triton-mixed micelles also suggest that strong interactions between detergent oxyethylene groups and the phospholipid head group do not occur.

While there is no evidence for phospholipid–phospholipid interactions in Triton-mixed micelles, there is some evidence that sterol–phospholipid complexes may exist. Preliminary experiments[3] suggest that Triton/cholesterol-mixed micelles at a low molar ratio can be prepared by our standard procedure only in the presence of phospholipid. Although the incorporation of cholesterol does not alter mixed micelle size, it does decrease phospholipase A_2 activity 2–2.5-fold when present equimolar with total phospholipid. For phosphatidylcholine–cholesterol bilayers, both Brockerhoff (1974) and Huang (1976) have proposed that the cholesterol hydroxyl group hydrogen-bonds to a fatty acyl carbonyl group of the phospholipid. Phospholipase A_2 catalyzes the hydrolysis of phospholipids at the *sn*-2 fatty acyl chain. If phospholipid–cholesterol complexes exist in Triton mixed micelles, they

[3] R. J. Robson and E. A. Dennis, unpublished results.

might be expected to affect enzyme activity by altering the accessibility of this ester group. However, even with cholesterol present, activation is still observed.

Micelle Surface Microenvironment and Possible Role in Specificity Reversal. Phosphatidylethanolamine contains a titratable amino group whose pK_a might be altered in the Triton interface. At pH 8, where activation has been studied, the phosphatidylethanolamine/Triton micelle could be negatively charged or have a negative ζ potential, and this could adversely affect phospholipase A_2 activity. The addition of phosphatidylcholine could then "dilute the high negative surface charge density". Such an effect was proposed by Bangham & Dawson (1962) to explain why phosphatidylcholine enhanced somewhat the susceptibility of phosphatidylethanolamine as a substrate for *Cl. perfringes* phospholipase C. However, the pK_a of phosphatidylethanolamine has recently been determined in Triton X-100 mixed micelles by ^{31}P NMR techniques (London & Feigenson, 1979) and it is about 9.5. Furthermore, activation occurs over the entire bulk pH range of 5 to 9 (Adamich et al., 1979) and Dawson (1963) showed that there was no correlation of the ζ potential with cobra venom phospholipase A_2 hydrolysis of phosphatidylcholine.

If the surface charge at the phosphatidylethanolamine/Triton-mixed micelle is inhibiting phospholipase A_2, then one might expect different relative rates for phosphatidylethanolamine and phosphatidylcholine hydrolysis as individual phospholipids and as binary mixtures in micelle systems of ionic detergents, particularly anionic detergents. The activation of phosphatidylethanolamine hydrolysis of phosphatidylcholine is seen in micelle systems of different surface charges including zwitterionic DTAPS and anionic DOC, although it is most pronounced in the nonionic systems. Therefore, surface microenvironment changes are not the primary cause of the activation phenomenon.

It is interesting to speculate on the reason why nonionic detergents show the largest difference in activity of phospholipase A_2 toward phosphatidylethanolamine and phosphatidylcholine. There may be *subtle* conformational or environmental differences between phosphatidylcholine and phosphatidylethanolamine in nonionic micelles that are not present in the ionic ones, so that to some degree micelle structure may influence enzyme reactivity toward a particular phospholipid. Previous ^1H NMR data suggest that there is a subtle difference in these two phospholipids in Triton mixed micelles (Roberts & Dennis, 1977). ^{31}P NMR data also indicate a slightly more rigid structure for the phosphorylethanolamine head group in Triton. In the ionic detergent micelles both phosphatidylethanolamine and phosphatidylcholine appear to be the same in the interfacial region as judged by the α-methylene proton resonance observed by ^1H NMR.[4]

A preference for a modified phosphatidylethanolamine over phosphatidylcholine has been noted in sonicated vesicles where the phospholipid is packed in bilayers (Sundler et al., 1977). Phosphatidylethanolamine is also the preferred substrate in human erythrocyte ghosts where proteins and other lipids are present (Adamich & Dennis, 1978b). Thus, the nature of the interface appears to be secondary and this lends credence to the possibility of a specific phosphatidylcholine–enzyme interaction as the cause of the activation phenomenon.

Specificity Reversal: A Phosphatidylcholine–Enzyme Activation. The synthetic short-chain phospholipid dibutyrylphosphatidylcholine has a very high cmc, around 80 mM (Wells, 1974). We have shown that it does not form mixed micelles with Triton X-100 at the concentrations employed. The observation that the soluble, monomeric phosphatidylcholine causes activation suggests a direct lipid–enzyme interaction. It is interesting to consider the role of phosphatidylcholine conformation in the enzyme activation phenomenon. We have shown that the α-methylene groups of the two fatty acid chains on the phospholipid are not equivalent in monomer phosphatidylcholine, but that this difference is accentuated when the phospholipid is organized at an interface (Roberts et al., 1978b). It was suggested that this difference between monomer and micelle might be important in the increased activity of the enzyme toward micellar phospholipid. The results reported here show that the change in phosphatidylcholine conformation is *not necessary* for that molecule to activate phospholipase toward phosphatidylethanolamine. Thus, the activating molecule could bind at a noncatalytic site on the enzyme where its detailed conformation would not necessarily be important.

The phenomenon of specificity reversal observed here is one in which an enzyme is specifically activated by one substrate toward another "potential" substrate. It is unique because most enzymes which are activated by phospholipids are membrane associated and the phospholipid is a cofactor and not a substrate (Coleman, 1973). The phosphatidylcholine activation of soluble phospholipase A_2 toward phosphatidylethanolamine is somewhat reminiscent of the cooperative kinetics one observes with multisubunit enzymes. It is an unusual form of enzyme regulation and may be important for the action of this enzyme on natural membranes.

[4] M. F. Roberts and E. A. Dennis, unpublished results.

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Phospholipid Activation of Cobra Venom Phospholipase A_2. 2. Characterization of the Phospholipid–Enzyme Interaction†

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ABSTRACT: Activation of cobra venom phospholipase A_2 toward phosphatidylethanolamine by phosphatidylcholine in mixed micelles has been suggested to be caused by a direct phosphatidylcholine–enzyme interaction. Comparable activation of phospholipase A_2 toward phosphatidylethanolamine also occurs with nonsubstrates sphingomyelin and lysophosphatidylcholine. Phospholipids with anionic head groups and water-soluble phosphorylcholine or glycerol phosphorylcholine alone are nonactivators. Hydrolysis of phosphatidylethanolamine is 0.06 that of phosphatidylcholine, and the rate increases abruptly (0.38) when two methyl groups are present on the nitrogen. The hydrolysis of all phospholipids examined is enhanced dramatically in the presence of activator lipid. The activation profile for phosphatidylethanolamine hydrolysis in the presence of sphingomyelin parallels cosubstrate phosphatidylcholine activation. Phospholipid binding studies reveal a similar apparent K_D (1 mM) for all phospholipids examined. Enzyme inactivation by *p*-bromophenacyl bromide, a reagent which inactivates the enzyme through modification of a histidine in the active site, is significantly protected by lipid substrates and fatty acids, while nonsubstrate activator lipids do not protect. The pK_a controlling phosphatidylethanolamine hydrolysis was found to shift from 6.5 to 5.8 in response to phosphatidylcholine activation of the enzyme, suggesting that the activator lipid causes a conformational change in the enzyme. These results can be explained by the existence of two binding sites: (i) an activator site which requires a lipid molecule containing the phosphorylcholine moiety and a fatty acyl chain and (ii) a head-group nonspecific catalytic site.

In human erythrocyte ghosts and in phosphatidylethanolamine/phosphatidylcholine/Triton X-100 mixed micelles, phosphatidylethanolamine is the preferred substrate of cobra venom phospholipase A_2 (*Naja naja naja*) (EC 3.1.1.4) (Adamich & Dennis, 1978a,b). This specificity for phosphatidylethanolamine is in direct contrast to that found if the individual[1] phospholipids in Triton X-100 mixed micelles are treated with phospholipase A_2 (Roberts et al., 1978b). Such behavior has been examined in a variety of other mixed micellar systems as well (Roberts et al., 1979). The activation of phosphatidylethanolamine hydrolysis with cosubstrate phosphatidylcholine occurs under a variety of micelle sizes, surface charges, and concentrations of phospholipid in the surface. Furthermore, monomeric dibutyrylphosphatidylcholine[2] activates phosphatidylethanolamine hydrolysis. These findings suggest that the activation phenomenon is the result of a direct interaction of the phosphatidylcholine molecule with phospholipase A_2 as discussed in the accompanying manuscript (Roberts et al., 1979).

The nature of the lipid–enzyme activation has now been examined in greater detail. Structural features required for the activator molecule are the phosphorylcholine moiety and at least one fatty acyl chain. Phospholipids with head groups other than choline are comparatively poor substrates for phospholipase A_2, but they are hydrolyzed more efficiently when activator lipid is added. The kinetics of the phosphatidylethanolamine activation by both cosubstrate phospha-

† From the Department of Chemistry, University of California at San Diego, La Jolla, California 92093. *Received September 15, 1978; revised manuscript received March 1, 1979.* This research was supported by National Institutes of Health Grant GM-20501 and National Science Foundation Grant PCM 76-21552; the NMR spectrometer was supported by National Institutes of Health Grant RR-00708.

‡ Postdoctoral Fellow of the National Institutes of Health, Grant GM-06071.
§ Postdoctoral Fellow of the National Institutes of Health, Grant GM-05910.

[1] When one species (i.e., head group) of a phospholipid is inserted into a detergent matrix, the mixed micelles are said to contain individual phospholipids. When two or more phospholipids differing in head group are present in the mixed micelle system, it will be referred to as a binary, ternary, etc. mixture.

[2] Abbreviations used: diacylphosphatidylcholine, 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine; Triton, Triton X-100; CTAB, cetyltrimethylammonium bromide; Mes, 2-(*N*-morpholino)ethanesulfonate.

0006-2960/79/0418-3308$01.00/0