

Short-Chain Phosphatidylethanolamines: Physical Properties and Susceptibility of the Monomers to Phospholipase A₂ Action[†]

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ABSTRACT: The homologous series of optically active short-chain phosphatidylethanolamines (PE) from dibutyryl-PE to dioctanoyl-PE was synthesized. In addition, two monomeric short-chain phospholipid analogues that are not degraded by phospholipase A₂ (1,2-bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphocholine and the corresponding ethanolamine derivative) were synthesized. In contrast to the short-chain phosphatidylcholines (PC), short-chain PE's have defined solubilities in water. No break below the solubility limit was found in surface tension plots, suggesting that these compounds exist as monomers in aqueous solution. Only when a significant fraction of the molecules is negatively charged can they form micelles by themselves. Cobra venom phospholipase A₂ hydrolyzes monomeric short-chain PE's at about the same rate as short-chain PC's but hydrolyzes long-chain PC's much more rapidly than long-chain PE's. The hydrolysis of short-chain PE's is found to be activated by phosphocholine-containing compounds only in the presence of an interface; in its absence phosphocholine-containing compounds can act as competitive inhibitors. Possible explanations for this phenomenon are considered.

Synthetic phospholipids containing short fatty acid chains have unique properties that make them very useful as model membrane systems and as substrates for phospholipases. The physical chemical properties of short-chain phosphatidylcholines (PC)¹ have been previously investigated by Tausk et al. (1974a-c). These compounds are water soluble and form micelles above their cmc. Studies using short-chain phosphatidylcholines as substrates (Pietersen et al., 1974; Wells, 1972, 1974) or activators (Plückthun & Dennis, 1982b) have provided valuable information on the mechanism of action of phospholipase A₂ (Dennis, 1983), since their aggregation state can easily be varied. In particular, the poor hydrolysis of phosphatidylethanolamine (PE) by cobra venom phospholipase A₂ was found to be activated 10–20-fold by phosphocholine-

containing compounds including dibutyryl-PC. Short-chain PC that is monomerically dispersed below its cmc is generally a poor substrate for phospholipases but does allow the use of the standard kinetic treatments for homogeneous enzymatic reactions. It is for this reason that we synthesized and studied the short-chain PE as substrate for phospholipase A₂, in particular to obtain kinetic information on the activation phenomenon (Plückthun & Dennis, 1982b). In addition, two nonhydrolyzable short-chain urethane analogues (with choline and ethanolamine head groups) were synthesized, and their effect on the enzymatic reaction was examined.

The physical properties of long-chain PE and PC are known to differ from one another in many respects, i.e., phase dia-

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¹ Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; cmc, critical micelle concentration; Triton, Triton X-100; HPLC, high-pressure liquid chromatography; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

grams, hydration, and head group conformations (Hauser, 1976; Hauser et al. 1981), and it was of interest to determine whether these differences can also be observed for the short-chain compounds. Therefore, the surface tensions, solubilities, and pK_a values were also examined.

EXPERIMENTAL PROCEDURES

Materials. Propionic, butanoic, and hexanoic anhydrides were obtained from Aldrich, pentanoic and heptanoic anhydrides were from Tridom/Fluka, and octanoic anhydride was from Nu-check-Prep. *p*-Pyrrolidinopyridine and *p*-(*N,N*-dimethylamino)pyridine, butyl isocyanate, and trityl bromide were purchased from Aldrich. Triton X-100 was obtained from Rohm and Haas. Dibutyryl-PC and sphingomyelin were obtained from Calbiochem, and egg PE (transesterified) was from Avanti Biochemicals. Silicic acid chromatography was performed on Biosil A (Bio-Rad). Rexyn ion-exchange resins were obtained from Fisher Scientific Co. DEAE-cellulose (DE-23) was from Whatman. Other chemicals were of analytical grade. Thin-layer chromatography was carried out on silica gel 60 plates (Merck); the solvent was $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65:25:4 v/v/v). Compounds were detected with either I_2 vapor, the appropriate molybdate spray (Dittmer & Lester, 1964; Cubero Robles & Roels, 1971; Hanes & Isherwood, 1949), or ninhydrin.

Purification of Natural PE. (A) *From Egg Yolk.* In an adaptation of the method of Singleton et al. (1965), the yolks from 6 dozen fresh eggs were stirred thoroughly in 1 L of acetone and were then left to stand for 1 h. The resulting precipitate was washed with three portions of 400 mL of acetone previously cooled to 10–15 °C. The precipitate was then suspended in 2 L of CHCl_3 and allowed to stand for 1 h. The supernatant was decanted and the precipitate extracted again with 1 L of CHCl_3 . The extracts were combined, and the solvent was removed in vacuo. The crude phospholipids were dissolved in 600 mL of petroleum ether and vacuum filtered if necessary. The volume was reduced to approximately 400 mL on a rotary evaporator and the solution slowly poured into acetone at about 15 °C with rapid stirring. The mixture was allowed to stand until the supernatant cleared (4 h to overnight). The supernatant was decanted and discarded and a second petroleum ether extraction carried out in the same manner. Typical yield was about 90–100 g of crude phospholipids. Purification of both PC and PE from the mixture was achieved by either HPLC or column chromatography. For HPLC, a Waters Prep LC/500 liquid chromatography system was used with two radially compressed PrepPAK 500 silica gel cartridges in series in a two-step purification as described by Patel & Sparrow (1978). For column chromatography, 1500 g of silicic acid was used for 90 g of crude lipids in a 100 × 10 cm column with a $\text{CHCl}_3-\text{CH}_3\text{OH}$ step gradient (Sweeley, 1969). PE was eluted in the 20% CH_3OH fraction. From either purification method, about 18–20 g of PE and 35–50 g of PC were isolated (each with >95% purity).

(B) *From Soybeans.* The starting material was soybean phospholipid (Sigma soy bean lecithin, commercial grade). It was extracted with acetone to remove most neutral fats, and the insoluble residue was treated with 90% ethanol to obtain a PE-rich precipitate (Aneja et al., 1969c). To obtain PE, preparative HPLC was used on a benzene solution of 70 g of this precipitate as described above, by employing the same two-step procedure. It was not found to be possible to separate PE from phosphatidylinositol (PI) present in the soybean lipid mixture. Therefore, the enriched PE from HPLC was dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1 v/v) and slowly poured onto

100 g of DEAE-cellulose (DE-23, Whatman) in a Büchner funnel and washed with 500 mL of the same solvent. The DEAE-cellulose had been washed, defined, and equilibrated with organic solvent as described by Rouser et al. (1969). Under these conditions, anionic phospholipids bind to the resin, but zwitterionic phospholipids are eluted. The yield was about 20 g of PE.

N-Tritylphosphatidylethanolamine. The amino group of the purified PE was protected with trityl bromide (Aneja et al., 1969b). *N*-Trityl-PE was purified by chromatography on silicic acid impregnated with triethylamine (Aneja et al., 1969b). It gave a single spot on TLC ($R_f \approx 0.8$). The yield of purified material was about 90%.

N-Trityl-sn-glycero-3-phosphoethanolamine. The procedure reported here is an adaptation of the methods that Chadha (1970) or Brockerhoff & Yerkowski (1965) used for the preparation of glycerophosphocholine. The *N*-trityl-PE (18 mmol) from above was taken to dryness under reduced pressure. It was redissolved in anhydrous ether (250 mL) and filtered free of insoluble material. Tetrabutylammonium hydroxide (25% in CH_3OH , 25 mL) was then added. The mixture was shaken for 2 min and allowed to stand for 8 h, with occasional shaking. When TLC indicated the completion of the reaction, the supernatant was decanted off, and the residue was repeatedly washed with 100-mL portions of anhydrous ether to remove fatty acids, always allowing the precipitate to settle fully. *N*-Trityl-GPE was obtained as a yellowish oil. A TLC analysis showed one spot ($R_f \approx 0.7$). The *N*-trityl-GPE was not further purified but was used in the reacylation reaction directly.

N-Trityl-1,2-diacyl-sn-glycero-3-phosphoethanolamine. These procedures are adaptations of the acylation reactions of Gupta et al. (1977) or Patel et al. (1979). The oily *N*-trityl-GPE was dried and reacylated by one of the following two methods.

Method A (Preferred). *N*-Trityl-GPE was dissolved in 1:1 dry $\text{CHCl}_3/\text{benzene}$ and dried in vacuo 3 times. The dry *N*-trityl-GPE (10 mmol) was dissolved in about 100 mL of dry ethanol-free CHCl_3 . Then the appropriate fatty acid anhydride (40 mmol) and *p*-pyrrolidinopyridine (20 mmol) or *p*-(*N,N*-dimethylamino)pyridine (20 mmol) were added. The reaction flask was flushed with N_2 or Ar, sealed, and protected from light. The reaction mixture was stirred for 24–48 h until TLC indicated completion of the reaction. The product was then taken to dryness.

Method B. The *N*-trityl-GPE (10 mmol) was dissolved in 25 mL of boiling H_2O , and 15 mL of a CdCl_2 solution (40% in H_2O , w/v) was added. This solution was allowed to stand overnight at 0–5 °C. A yellowish precipitate formed, which was vacuum filtered. This CdCl_2 adduct was dried over P_2O_5 in vacuo overnight and then over P_2O_5 in a vacuum oven at 40 °C for 2 h. The compound was added to dry Me_2SO (20 mL) at 40 °C, and a solution of fatty acid anhydride (40 mmol) and *p*-pyrrolidinopyridine (20 mmol) in 20 mL of dry benzene was added. The mixture was stirred for 24–48 h until TLC showed the completion of the reaction. Most of the solvent was evaporated in vacuo, and most of the water was removed by azeotropic evaporation with benzene. This material was used directly for detritylation with acetic acid (method B below).

1,2-Diacyl-sn-glycero-3-phosphoethanolamine. Detritylation was accomplished by either one of the following methods.

Method A. The *N*-trityl-PE was taken to complete dryness by freeze-drying from benzene. (This was possible only if the reacylation was carried out in CHCl_3 .) The flask was cooled

in an ice bath, and anhydrous trifluoroacetic acid (about 1 mL/mmol of *N*-trityl-PE) was added, with care taken to dissolve the lipid completely. After 30 min the trifluoroacetic acid was removed under high vacuum, still cooling the flask in ice/water. Under these conditions, complete removal of the trityl group was observed with only a negligible amount of ester hydrolysis. The lipid was then chromatographed as described below.

Method B. *N*-Trityl-PE was taken to dryness, or if the solvent could not be removed completely, its volume was reduced as much as possible. Then, 90% acetic acid was added (20 mL/mmol of *N*-trityl-PE), and the solution was boiled under reflux for 5 min. Under these conditions, the trityl group was removed nearly completely, whereas only a negligible amount of hydrolysis of the fatty acids was observed. At room temperature, even after 48 h, there was only incomplete loss of the trityl group and also traces of ester hydrolysis. Most of the acetic acid was then removed in vacuo. The lipid was then chromatographed as described below.

Chromatographic Purification of Diacyl-PE. The synthetic diacyl-PE was taken up in CHCl₃/CH₃OH/H₂O (4:10:1 v/v/v) and passed through a mixed-bed ion-exchange column (Rexyn I-300, 50 ml of resin/mmol of PE) by washing with 2 column volumes of the same solvent. (The bubbles that are generated on the column by exposure to this solvent, even when degassed, do not interfere with the desired purification.) The solvent was removed under reduced pressure. This material was chromatographed on a silicic acid column (100 g of absorbent/g of lipid) with a CHCl₃/CH₃OH step gradient. The products generally eluted at the following CHCl₃/CH₃OH ratios (v/v): dioctanoyl-PE, 6:4; diheptanoyl-PE, 1:1; dihexanoyl-PE, 4:6; dipentanoyl-PE, 4:6; dibutyryl-PE, 3:7. The products so obtained had a slightly yellowish color and, on an overloaded TLC, occasionally showed a trace impurity running slightly above the respective short-chain PE (both ninhydrin and molybdate positive). When this was the case, the product was further purified by either reverse-phase or normal-phase HPLC. Reverse-phase HPLC was carried out on a Waters Prep LC/500 system [equipped with a PrepPAK C₁₈ cartridge; solvent was CH₃OH/H₂O (4:1 v/v)], and the product was only slightly retarded and free of the trace impurity. For smaller amounts, normal-phase HPLC was carried out on a semipreparative Waters μ -Porasil column, with the solvent system hexane/2-propanol/water (6:8:0.75 v/v/v). The pure, colorless product was freeze-dried from benzene and stored dissolved in CHCl₃ at -20 °C. Yields were 35–55%.

Analysis of 1,2-Diacyl-sn-glycero-3-phosphoethanolamines. All products were Cd²⁺ free as determined by atomic absorption (detection limit: about 1 mol Cd²⁺/4000 mol of PE). They were free from long chain containing PE, as determined by GC (estimated detection limit: 0.05 mol %). Neither ¹H NMR nor ³¹P NMR showed any indication of phosphoryl migration (Plückthun & Dennis, 1982a); the detection limit was about 1%. All products gave single spots on TLC. ¹H NMR of dibutyryl-PE [CDCl₃/CD₃OD (1:1 v/v)] showed the following: 0.9 (2 t, ω -CH₃), 1.64 [m, (CO)CH₂CH₂], 2.30 and 2.32 [2 t, O(CO)CH₂], 3.15 (m, CH₂N), [4.02 (m), 4.05 (m), 4.2–4.4 (m)], [CH₂OP, CH₂CH₂N, CH₂OCO], and 5.22 ppm (m, CHOCO). The ¹H NMR spectra of the other short-chain PE's were identical except for the appropriate fatty acid signals. Specific rotations [all c 1, CHCl₃/CH₃OH (1:1 v/v)] were as follows: for dibutyryl-PE, $[\alpha]^{22}_D +13.0$; for dipentanoyl-PE, $[\alpha]^{22}_D +11.4$; for dihexanoyl-PE, $[\alpha]^{22}_D +10.8$; for diheptanoyl-PE, $[\alpha]^{22}_D +10.0$; for dioctanoyl-PE, $[\alpha]^{22}_D +9.7$. Dioctanoyl PE was not studied further.

1,2-Bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphocholine. This compound was prepared from *sn*-glycero-3-phosphocholine (CdCl₂ adduct) and butyl isocyanate by the method utilized by Gupta & Bali (1981) for longer chain isocyanates and purified by ion-exchange chromatography and silicic acid chromatography as described above.

1,2-Bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphoethanolamine. 1,2-Bis[(butylcarbamyl)oxy]-*sn*-glycero-[(*N*-tritylamino)ethyl phosphate] was prepared from *N*-trityl-*sn*-glycero-3-phosphoethanolamine and butyl isocyanate by the method utilized by Gupta & Bali (1981) for the preparation of long-chain urethane derivatives of PC. After completion of the reaction, the solvent was removed and the raw product taken up in 90% acetic acid (20 mL/mmol) and boiled under reflux for 5 min. The acid was then removed under high vacuum without heating. The material was dissolved in CHCl₃/CH₃OH/H₂O (4:10:1, v/v/v) and then passed through a mixed-bed ion-exchange column (Rexyn I-300, 50-mL bed volume/mmol of PE). The eluant was taken to dryness by repeated evaporation with benzene and purified by silicic acid chromatography with a CHCl₃/CH₃OH step gradient. The product showed a single spot on TLC. ¹H NMR (D₂O) showed the following: 0.90 (t, ω -CH₃), 1.32 (m, CH₂CH₃), 1.45 (m, CH₂CH₂CH₃), 3.10 [m, O(CO)NDCH₂], 3.25 (m, CH₂ND₃), [4.02 (m), 4.06 (m), 4.1–4.3 (m)], [CH₂CH₂ND₃, CH₂OP, CH₂OCO], and 5.07 ppm (m, CHOCO).

Surface Tension and cmc Measurements. Surface tension measurements were carried out by determining the maximum bubble pressure with an apparatus developed by Dr. K. I. Mysels (Mysels, 1981; Roda et al., 1983). The maximum pressure at which a small bubble is stable at the end of a capillary tube submerged in a liquid is proportional to the surface tension, except for a hydrostatic correction. Once the bubble becomes unstable, its volume and surface increase suddenly by several orders of magnitude as the bubble detaches. The sample (0.50 mL) was contained in a 7.5-mm diameter test tube cut to about 2-cm length. This test tube was placed in a small plastic holder on a laboratory jack. A capillary tube, made from a siliconized micropipet broken to about 6-cm length and fixed in an inclined position, could be lowered into the solution with a micrometer screw. The capillary has a hydrophilic face and hydrophobic lumen, thus anchoring the meniscus at the edge. The capillary tube was connected to a supply of charcoal-filtered air at regulated pressure whose flow was controlled by a precision value. The capillary was also connected to a pressure transducer (based on a metal diaphragm with electronic detection, amplification, and suppression). The resulting signal was observed on a storage oscilloscope. The apparatus was protected from vibrations by placement on heavy lead blocks with rubber feet for damping and from pressure fluctuation by a plastic cover enclosing the whole measuring device.

After the sample was placed into the shortened test tube, the capillary tube was lowered with the micrometer screw to the point where the solution would suddenly form a meniscus with the capillary tip. From this point, the capillary was lowered exactly 5.0 mm into the solution with the micrometer screw. The plastic cover was placed over the instrument and the flow of air was regulated such that a steady stream of bubbles was obtained (about 1 bubble per 0.5–5 s). When the rate and pressure had stabilized (after about 5 min), the rate and the maximum pressure were recorded from the oscilloscope. This is the pressure at the moment the bubble leaves the capillary, before the pressure drops again. The surface tension measurement depends on the age of the surface and

thus on the rate of bubble formation due to the time it takes to establish the equilibrium surface tension. Therefore, measurements at several rates were carried out and interpolated to the same rate (usually to 1.0 bubble per second) for all experimental points. In principle, for a two-component system an infinitely slow bubble rate would most accurately give the true thermodynamic surface tension value, but in practice, much faster rates are advantageous. Small traces of very surface active impurities have a much larger influence on the surface tension value at very slow bubble rates than at faster ones. Very fast rates, on the other hand, lead to foaming and therefore substantial instabilities in the oscilloscope readings and, in addition, cause measurements to be further from the equilibrium values. Because of these considerations, the optimum rates were found to be about 1 bubble per 1–5 s. All samples were prepared with glass-distilled water. cmc values were obtained from break points in the slope of plots of surface tension vs. the logarithm of concentration of the surfactant. Therefore, the small and constant hydrostatic correction need not be applied.

Solubilities. An appropriate amount of lipid in organic solvent was placed in a 13 × 100 mm test tube and dried under nitrogen and finally under high vacuum. Tris buffer, 50 mM, pH 8.0 (1.0 mL), was added, and the solution was vigorously vortexed. It was finally heated to 50–60 °C and vortexed again to dislodge material adhering to the sides of the test tube. After cooling to room temperature, the solution was centrifuged at 1000g for 30 min at room temperature. An aliquot of the clear supernatant was then carefully removed without disturbing the pellet for phosphate analysis (Eaton & Dennis, 1976). These experiments were carried out at least in triplicate.

Phospholipase A₂ Assay. Phospholipase A₂ from cobra venom (*Naja naja naja*) was purified as described previously (Deems & Dennis, 1981; Darke et al., 1980). The enzymatic hydrolysis was followed by pH stat (Dennis, 1973; Deems & Dennis, 1981). The automatic titration apparatus consisted of a Radiometer PHM 62 pH meter equipped with a combination glass electrode, a Radiometer TTT 60 titrator, a Radiometer ABU 13 autoburet, and a recorder. Standard assays were conducted in a volume of 2 mL, at pH 8.0, 40 °C, and contained 5 mM phospholipid and 10 mM CaCl₂, unless otherwise noted, and other compounds as indicated. The average of at least duplicate experiments is reported. Titration curves were also obtained with the pH stat.

RESULTS

Solubility and pK_a. The homologous series of phosphatidylethanolamines from dibutyryl- to dioctanoyl-PE was synthesized to compare their properties to the corresponding short-chain phosphatidylcholines and to select the compounds most suitable for studies with phospholipase A₂. As expected, all compounds were found to be soluble in CHCl₃, CH₃OH, and CH₃OH/H₂O (1:1, v/v), but the short-chain PE's differ drastically from the short-chain PC's in their behavior in water. All short-chain PE's were found to have defined solubilities ranging from 1.1 mM for diheptanoyl-PE to >100 mM for dibutyryl-PE (Table I). The solubility of dihexanoyl-PE agreed with that previously reported (Baer & Gröf, 1960; Maurukas et al., 1963). Above those concentrations, a precipitate formed. The structure of the lipid-rich phase was not further determined. Neither sonication, heating/cooling cycles, nor prolonged stirring (up to 48 h) changed the concentration of phospholipid in the aqueous supernatant, indicating that thermodynamic equilibrium was reached. The pK_a of monomeric dihexanoyl-PE (5 mM in H₂O) was found to be 9.6

Table I: Solubility and Break Point in Surface Tension for Short-Chain PE's and Phospholipid Urethane Analogs

phospholipid	solubility (mM)	break point (mM)
dibutyryl-PE	>100	>100
dipentanoyl-PE	50	45
dihexanoyl-PE	10	10
diheptanoyl-PE	1.1	0.8
1,2-bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphocholine	>100	>100
1,2-bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphoethanolamine	>100	>100

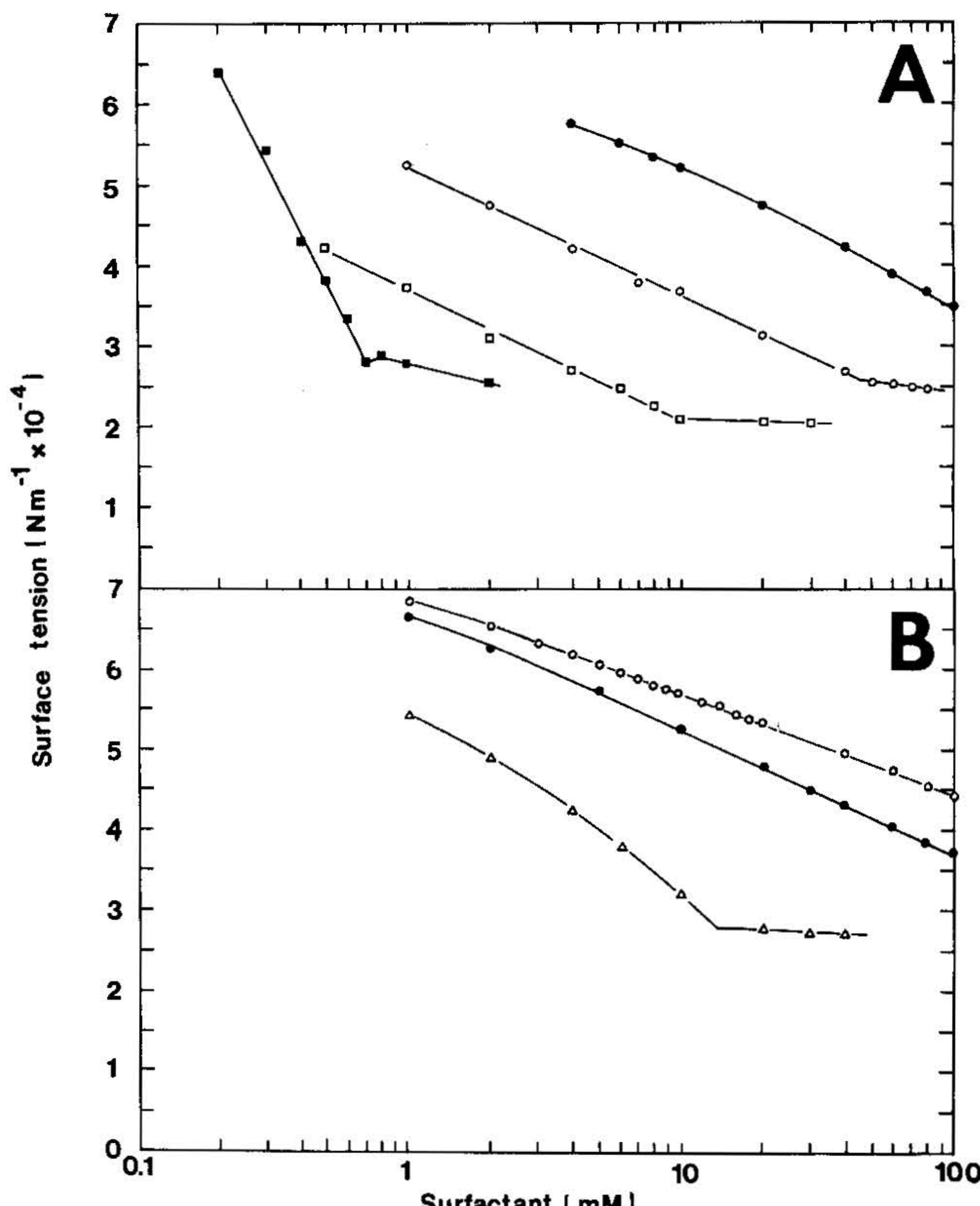


FIGURE 1: Surface tension for several short-chain phosphatidylethanolamines and phospholipid analogues as a function of concentration in distilled H₂O as determined by the maximum bubble pressure method. (A) Dibutyryl-PE (●), dipentanoyl-PE (○), dihexanoyl-PE (□), and diheptanoyl-PE (■); (B) 1,2-bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphoethanolamine (○), 1,2-bis[(butylcarbamyl)oxy]-sn-glycero-3-phosphocholine (●), and dihexanoyl-PE at pH 11.0 (in 0.1 M CAPS buffer) (Δ).

± 0.2 and therefore not significantly different from aggregated PE in mixed micelles (5 mM egg PE, transesterified, 40 mM Triton X-100, pK_a = 9.8 ± 0.2). Therefore, PE must be dispersed by Triton sufficiently to make an electrostatic surface contribution to the pK_a negligible. In PE bilayers at low ionic strength, much higher pK_a values are found (Seddon et al., 1983; Cevc et al., 1981).

Surface Tension Measurement. Surface tension measurements were carried out to determine whether or not breaks in the surface tension plots occur below the solubility limit, which would indicate a micellization preceding the precipitation. The plots of surface tension vs. the logarithm of concentration are shown in Figure 1. It can be seen that the breaks in surface tension (listed in Table I) approximately coincide with the measured solubility. The slope of those plots below the break point is what would be expected for surface active monomers (Tausk et al., 1974a–c). This does not rule out aggregates of very small size, but makes the presence of

micelles in this concentration range unlikely.

The solubilities of the short-chain PE's were strongly dependent on pH. Since the precipitate of dihexanoyl-PE could be dissolved at a pH greater than about 10 and reversibly precipitated below this value, one can conclude that the short-chain PE must be negatively charged to become water soluble, whereas it has limited solubility under conditions where it is zwitterionic. The precipitation of dihexanoyl-PE could not be reversed by the addition of EDTA, and atomic absorption measurements (see Experimental Procedures) showed that Cd^{2+} , which was used in the synthesis, was not present. It would appear, therefore, that divalent cations are not responsible for the precipitation. However, at low pH, lower concentrations of Ca^{2+} in the enzyme assay mixtures resulted in greater solubility of the dihexanoyl-PE. For this reason, only 1 mM Ca^{2+} was used in the kinetic assays of dihexanoyl-PE discussed below.

The surface tension of dihexanoyl-PE was measured at pH 11 (Figure 1B). The solution remained clear even at a concentration of 50 mM, but a break in surface tension was observed at about 14 mM. This suggests the formation of micelles above this concentration from negatively charged short-chain PE. The exact value of the cmc of the dihexanoyl-PE anion cannot be easily determined, since some hydrolysis does occur under these conditions. Surface tension readings changed, however, only negligibly over 3 h.

The enzymatically nonhydrolyzable urethane analogues of dihexanoyl-PC and dihexanoyl-PE (1,2-bis[(butylcarbamyl)-oxy]-*sn*-glycero-3-phosphocholine and 1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphoethanolamine) were found to give no break in surface tension plots below 100 mM (Figure 1B) and are therefore most probably present as monomers in aqueous solution.

Action of Phospholipase A₂ on Dibutyryl-PE. The hydrolysis of dibutyryl-PC by cobra (*Naja naja naja*) venom phospholipase A₂ was compared with that of dibutyryl-PE over a concentration range of 1–20 mM substrate. Double-reciprocal plots yielded approximate kinetic constants of $K_M = 40$ mM and $V_{max} = 14 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for dibutyryl-PC and $K_M = 14 \text{ mM}$ and $V_{max} = 24 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for dibutyryl-PE. Dibutyryl-PC is by no means a better substrate than dibutyryl-PE, in dramatic contrast to the enzymatic hydrolysis of the long-chain phospholipids (Plückthun & Dennis, 1982b). In fact, dibutyryl-PE shows an approximately 2-fold higher V_{max} and an approximately 2-fold lower K_M . The influence of the urethane analogue of dihexanoyl-PC (1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphocholine) and the urethane analogue of dihexanoyl-PE (1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphoethanolamine) on the hydrolysis rate of dibutyryl-PE was also determined. This experiment was carried out with 5 mM dibutyryl-PE and concentrations of the urethane analogues ranging between 1 and 10 mM. Both compounds inhibit the hydrolysis somewhat, but most interestingly, the phosphocholine-containing compound does not activate the enzyme toward PE monomers. This inhibition is again in contrast to the ability of phosphocholine-containing lipids to activate the hydrolysis of micellar PE. The mode of inhibition of dibutyryl-PE by the urethane analogue of dihexanoyl-PC was investigated, and the results in Figure 2 show that the data are consistent with a simple competitive inhibition.

To delineate the effects of the presence of a surface on the behavior of phosphocholine-containing compounds toward the hydrolysis of PE's, further experiments were carried out with dihexanoyl-PE (Table II). In the presence of a molar excess

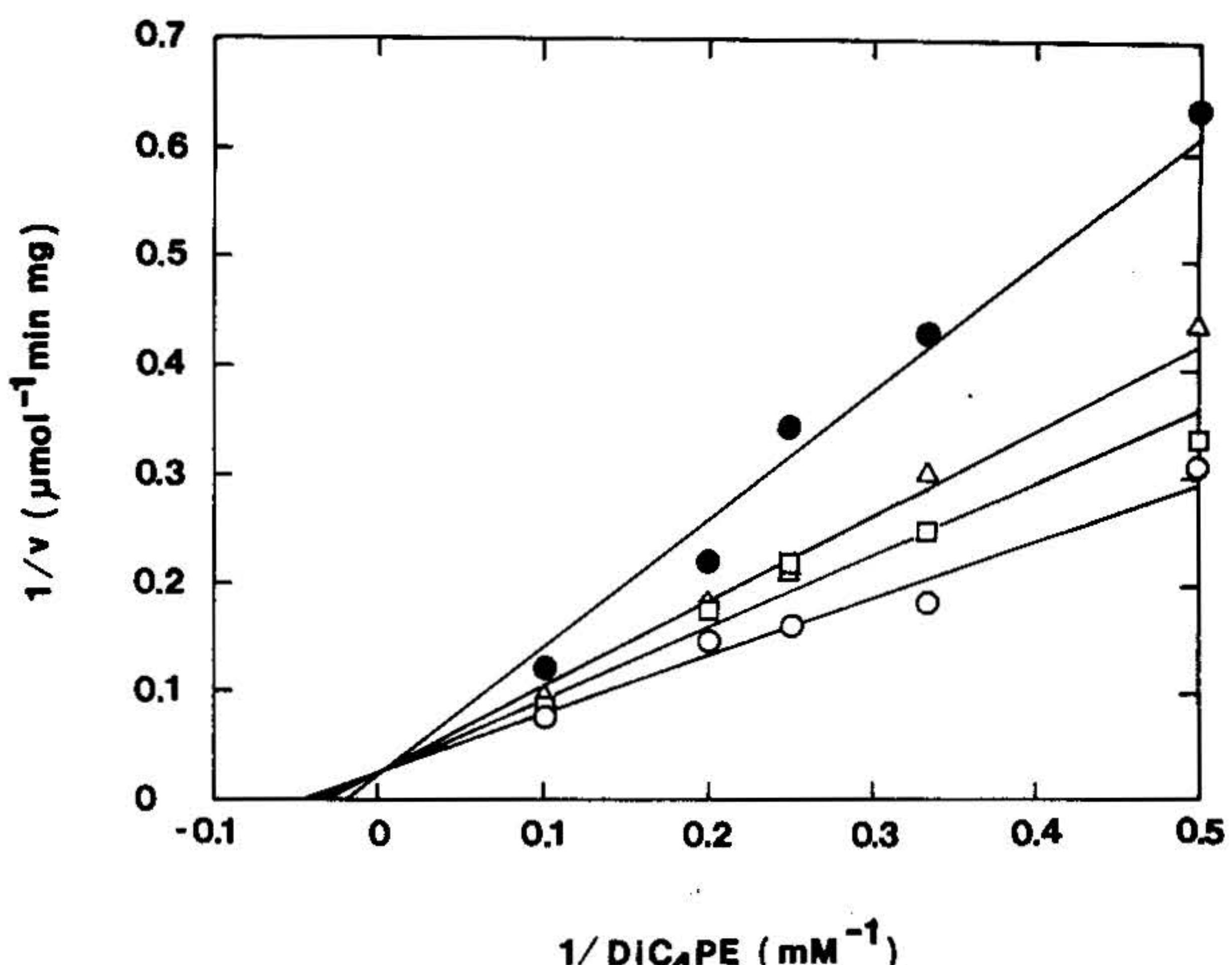


FIGURE 2: Inhibition of phospholipase A₂ hydrolysis of dibutyryl-PE by 1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphocholine. A Lineweaver-Burk plot is shown with the following inhibitor concentrations: 2 (○), 4 (□), 6 (Δ), and 8 mM (●).

Table II: Rate of Dihexanoyl-PE^a Hydrolysis by Cobra Venom Phospholipase A₂ in the Presence of Various Phosphocholine-Containing Compounds

addition	concn (mM)	Triton X-100 (8 mM)	sp act. ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
none		—	20 ± 3
none		+	40 ± 4
1,2-bis[(butylcarbamyl)-oxy]- <i>sn</i> -glycero-3-phosphocholine	5.5	—	25 ± 0
1,2-bis[(butylcarbamyl)-oxy]- <i>sn</i> -glycero-3-phosphocholine	5.5	+	283 ± 3
sphingomyelin	1	+	866 ± 12

^a Each assay included 1 mM dihexanoyl-PC and 1 mM Ca^{2+} . Average of duplicates or triplicates reported.

of detergent, dihexanoyl-PE is presumably comicellized, and this makes it possible to study the effect of the presence of a surface. In sharp contrast to the effect of Triton on dihexanoyl-PC (Roberts et al., 1978), the addition of detergent to dihexanoyl-PE does not itself greatly increase the rate of hydrolysis. Similarly, in the absence of micelles, the urethane analogue of PC does not appreciably change the rate of dihexanoyl-PE hydrolysis. But in the presence of mixed micelles of PE and Triton, the soluble urethane analogue causes a more than 11-fold activation when compared to the system without Triton, or 7-fold when compared to that with Triton and without activator. Additionally, sphingomyelin, present in mixed micelles with the substrate and Triton, activates the enzyme to an even greater extent. From these data, one must conclude that an interface is necessary for the activation of PE hydrolysis to occur.

DISCUSSION

Synthesis of Short-Chain Phosphatidylethanolamines. In adapting a procedure for the synthesis of short-chain phosphatidylethanolamines, the following points had to be considered: (i) If the amino group has to be protected, the deprotection must be mild enough to ensure only a small amount of hydrolysis of the ester bonds, which are considerably more sensitive to hydrolysis in short-chain lipids than in long-chain ones (Kensil & Dennis, 1981). (ii) The intermediates with short acyl chains do not crystallize nor can they be extracted in organic solvents, making purification difficult. (iii) Intermediates, in which acyl migration between vicinal hydroxyl groups can lead to isomers, must be avoided, since acyl mi-

gration is more rapid in short-chain lipids than in long-chain ones (A. Plückthun and E. A. Dennis, unpublished results). (iv) The procedure must allow the convenient and economical synthesis of large amounts of very pure optically active short-chain PE's to be useful for the preparation of substrates for phospholipase studies.

Phosphatidylethanolamines have been synthesized from 1,2-diglycerides (Hirt & Berchtold, 1957; Baer & Gröf, 1960; Eibl, 1978, 1980; Eibl & Niksch, 1978), 1,2-diacyl-*sn*-glycero-3-halohydrins (Billimoria & Lewis, 1968; Daemen, 1967; Chadha, 1968; Maurukas et al., 1963), diacyl-phosphatidic acids (Aneja et al., 1969b, 1970; Barzilay & Lapidot, 1969, 1971), the corresponding PC's by using phospholipase D (Eibl & Kovatchev, 1981), and natural PE (Aneja et al., 1969a,c; Chakrabarti & Khorana, 1975). A route starting from natural PE with the trityl protective group was found to fulfill all the above criteria and was used here for a convenient synthesis of optically active short-chain PE's.

Short-Chain PE vs. Short-Chain PC. The short-chain PC's, in contrast to the PE's discussed here, form micelles above the cmc (Tausk et al., 1974a-c) and remain water soluble. This cmc decreases monotonically with chain length. The uniformity of the micelles, the problem of premicellar aggregation, and the dependence of cmc and micelle size on chain length are still only poorly understood (Johnson et al., 1981; Allgyer & Wells, 1979b).

The differences in behavior between the short-chain PE's and short-chain PC's are paralleled by the differences between the long-chain phospholipids (Hauser, 1976; Hauser et al., 1981). The phase properties of both lipid classes are quite distinct (Shipley, 1973). Although the phase diagrams are not yet complete, it is clear that PE (especially unsaturated PE) can form hexagonal-phase (H_{II}) structures over wide concentration and temperature ranges (Cullis & de Kruijff, 1979; Boggs et al., 1981). For PC, a hexagonal phase is limited to a narrow high-concentration, high-temperature region and does not occur in excess water. Sonicated vesicles are not formed from PE at neutral pH (Szoka & Papahadjopoulos, 1980; Stollery & Vail, 1977). Their formation is possible when at least a small percentage of the PE is negatively charged (at basic pH) or another phospholipid is included in the vesicle. The hydration of PE and PC is also different: PE contains less bound water than PC (Hauser, 1975, 1976; Hauser et al., 1981; Finer, 1973; Finer & Drake, 1974), but the lamellae of PE appear to show a significant increase in swelling at pH greater than 10 (Papahadjopoulos & Miller, 1967), when the phospholipid is negatively charged. PE also has significantly higher gel-liquid crystal phase transition temperatures than PC with the same fatty acid composition (Ladbrooke & Chapman, 1969; Szoka & Papahadjopoulos, 1980), and this temperature drops when PE is negatively charged (Stümpel et al., 1980). In conclusion, it appears that the cohesive forces in crystalline PE are stronger than those in crystalline PC.

The X-ray crystallographic analysis of PE showed evidence for intermolecular interactions (electrostatic and/or hydrogen bonding) (Hitchcock et al., 1974) by the close distance between N of one molecule and O of the phosphate of the neighboring molecule. This cohesion makes PE difficult to hydrate (Phillips et al., 1972). A compact lattice with strong PO-H-N contacts is not possible for PC. Rather, water molecules partially shield electrostatic interactions in the head group lattice. In addition, steric hindrance forces (at least in the crystal) the choline group of PC to be almost parallel to the axis of the fatty acid chains (Pearson & Pascher, 1979), whereas the ethanolamine group of PE can fold almost perpendicularly to the chains in

the crystal (Hitchcock et al., 1974, 1975). 1H NMR studies by Hauser et al. (1980) suggested that the conformation of the polar head group of PC is very similar in monomers, in micelles, in organic solvents, and, presumably, in bilayers and is consistent with the crystal structure within the accuracy of the analysis. The question must now be addressed for PE as well. It is interesting that differences between PE and PC are also observed here for short-chain phospholipids. The failure of short-chain PE's to form micelles is probably another expression of the strong cohesion and small space requirement of the head groups with respect to the hydrocarbon chains.

Kinetic Studies with Phospholipase A₂. Cobra venom phospholipase A₂ has been shown to be activated toward the poor substrate, long-chain PE in mixed micelles with Triton, by the even poorer monomeric substrate dibutyryl-PC. Conversely, the hydrolysis of monomeric dibutyryl-PC can be activated by sphingomyelin in mixed micelles with Triton (Plückthun & Dennis, 1982b). In both of these cases, an interface was present. Since short-chain PE's are monomerically dispersed below their solubility limits, they can be used to examine activator effects in the absence of any interface. The solubility of dibutyryl-PE is greater than 100 mM, and this compound is, therefore, especially suited for these studies, but dihexanoyl-PE can also be conveniently studied below 10 mM. The synthetic compounds 1,2-bis[(butylcarbamyl)-oxy]-*sn*-glycero-3-phosphocholine and 1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphoethanolamine (non-hydrolyzable urethane analogues of dihexanoyl-PC and dihexanoyl-PE) are also monomerically dispersed at concentrations below 100 mM.

Hydrolysis of monomeric dibutyryl-PE and dihexanoyl-PE was not activated by the monomeric short-chain PC analogue. Furthermore, the results in Table II show that the effect of 1,2-bis[(butylcarbamyl)oxy]-*sn*-glycero-3-phosphocholine on the enzymatic hydrolysis of dihexanoyl-PE can be directly varied by omission or inclusion of detergent, which supplies an interface and incorporates either substrate or activator or both. These results show that the activation of cobra venom phospholipase toward PE requires an interface. Thus, it is clear that the simple allosteric treatment of the enzyme activation by monomeric phospholipids (two sites) is by itself insufficient to explain the activation phenomenon. The interface must also be included as a necessary factor in activation of PE hydrolysis by PC-containing lipids. These results demonstrate that, in the "dual phospholipid" model for phospholipase A₂ action (Roberts et al., 1977; Dennis, 1983), at least one of the phospholipids, either the activator or the substrate, must be interfacial.

The peculiar activation/inhibition phenomenon observed here must be attributed to surface phenomena. One possibility is a difference in binding between conformationally unrestrained monomers and densely packed, well-aligned micellar phospholipids to the enzyme. The enzyme might not be able to bind more than one monomer, so that different monomeric substrates simply compete for the binding. This loose binding could then result in the experimentally observed very high K_m and very low V_{max} for the monomers. On the other hand, the enzyme might bind more than one micellar phospholipid molecule, as long as one of them is PC. Activation, rather than inhibition, could then occur due to forced correct substrate positioning in the active site or a conformational change of the enzyme. Other possibilities to examine are difference in aggregation (dimerization or oligomerization) of the cobra venom enzyme in the presence of interfacial substrate or activator (Roberts et al., 1977) or a possible difference in the

rate-determining step (the chemical step or product removal) in the interfacial vs. homogeneous reaction. Experiments to delineate the rate-limiting step and the variation in enzyme aggregation with activation are currently in progress.

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Registry No. Dibutyryl-PE, 69078-12-2; dipentanoyl-PE, 96760-42-8; dihexanoyl-PE, 96893-06-0; diheptanoyl-PE, 96760-43-9; dioctanoyl-PE, 96760-44-0; 1,2-(Bu-NH-CO)₂-GPC, 96760-45-1; 1,2-(Bu-NH-CO)₂-GPE, 96760-46-2; N-trityl-GPE, 26432-20-2; [PrC(=O)]₂O, 106-31-0; [CH₃(CH₂)₄C(=O)]₂O, 2051-49-2; [BuC(=O)]₂O, 2082-59-9; [CH₃(CH₂)₅C(=O)]₂O, 626-27-7; [CH₃(CH₂)₆C(=O)]₂O, 623-66-5; BuNCS, 111-36-4; phospholipase A₂, 9001-84-7.

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