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Role of Monomeric Activators in Cobra Venom Phospholipase A₂ Action[†]

Andreas Plückthun[†] and Edward A. Dennis*

ABSTRACT: Phospholipase A₂ from cobra venom (*Naja naja naja*), which acts poorly on phosphatidylethanolamine (PE) in mixed micelles, is activated toward PE by the monomeric phospholipid dibutyrylphosphatidylcholine (dibutyryl-PC) which is an even poorer substrate. Phosphorus-31 nuclear magnetic resonance spectroscopy was employed to show that only PE is hydrolyzed in mixtures of PE and dibutyryl-PC of various concentrations. The activation shows saturation behavior, and the fully activated enzyme hydrolyzes PE at a rate similar to its optimal substrate PC containing long chain fatty acid groups. Because dibutyryl-PC is not incorporated into the micelles, these results are consistent with a mechanism of direct activation of the enzyme by dibutyryl-PC rather than a change in the properties of the interface being responsible

for the activation of phospholipase A₂. Furthermore, if either PC or PE as substrate is dispersed in mixed micelles, increasing amounts of the detergent Triton X-100 decrease the hydrolysis rate. The same detergent effect occurs if PE hydrolysis is activated by sphingomyelin (SPH). However, if the enzyme is activated by the monomeric dibutyryl-PC, this detergent effect can be overcome at high enough dibutyryl-PC concentrations. The hydrolysis of the monomeric dibutyryl-PC can also be stimulated by SPH in mixed micelles. This reaction shows no effect of detergent. Several models are considered to explain these observations, and it is suggested that the enzyme has two types of functional sites: an activator site and a catalytic site.

There is considerable current interest in understanding the role of the lipid/water interface in the mechanism of action of lipolytic enzymes such as the lipases and phospholipases (Dennis et al., 1981; Verger, 1980). Important information

has been obtained from kinetic studies on monomeric phospholipids such as dibutyryl-PC,¹ which are very poor substrates for phospholipase A₂ (de Haas et al., 1971; Wells, 1974). Interestingly, this phospholipid appears to be able to activate

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¹ Abbreviations: PC, phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphorylcholine); lyso-PC, lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphorylcholine); PE, phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine); lyso-PE, lysophosphatidylethanolamine (1-acyl-*sn*-glycero-3-phosphorylethanolamine); SPH, sphingomyelin; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane.

the phospholipase A₂ from cobra venom toward PE hydrolysis (Roberts et al., 1979). This report focuses on the mechanism of this activation and its implications for interfacial catalysis by phospholipase A₂.

Phospholipase A₂ from cobra venom (EC 3.1.1.4) hydrolyzes PC at a 10–20-fold higher rate than PE in micelles containing Triton X-100² and a single³ type of phospholipid (Roberts et al., 1978b). In binary phospholipid mixtures, PC activates the enzyme toward PE so that the latter becomes the preferred substrate (Adamich et al., 1979; Roberts et al., 1979). In these binary mixtures, PE is hydrolyzed at approximately the same rate as PC in single-phospholipid mixed micelles, and PC is hydrolyzed more slowly than in single-phospholipid mixed micelles. In preliminary experiments (Roberts et al., 1979), an increased rate of phospholipase A₂ catalyzed hydrolysis of PE was also observed in the presence of dibutyl-PC. Using ³¹P NMR, we have now been able to follow the time course of phospholipase A₂ catalyzed hydrolysis of mixtures of PE and dibutyl-PC for each phospholipid simultaneously. We (Plückthun & Dennis, 1981) have also shown that the dibutyl-PC is monomeric in the presence of a large excess of Triton X-100 even in mixtures containing PE. This allows us to now characterize the activation process and its implication for the mechanism of action of phospholipase A₂.

Experimental Procedures

Materials. Lyophilized cobra venom, *Naja naja naja* (Pakistan) lot. no. NNP8STLZ, was obtained from the Miami Serpenterium. The phospholipase A₂ was purified as described elsewhere (Deems & Dennis, 1975, 1981). Protein was determined by the Lowry procedure using the appropriate correction factor (Deems & Dennis, 1981). PC was obtained from egg yolks by the method of Singleton et al. (1965). PE, prepared by transesterification of egg PC, and bovine brain sphingomyelin (SPH) were obtained from Avanti Biochemicals. Dibutyl-PC was obtained from Calbiochem and purified by silicic acid chromatography (Unisil, Clarkson Chemical Co.) or prepared by the method of Patel et al. (1979). Lyso-PE and lysobutyl-PC for NMR standards were prepared from the above phospholipids by the action of phospholipase A₂ as described elsewhere (Plückthun & Dennis, 1981).

1-Acyl-2-propionyl-*sn*-glycero-3-phosphorylcholine was prepared from lyso-PC, obtained from egg PC as described previously (Plückthun & Dennis, 1981), and propionic anhydride following the method of Khorana and co-workers (Gupta et al., 1977). After purification on silicic acid chromatography with a CHCl₃–CH₃OH gradient, the product gave a single spot on thin-layer chromatography (CHCl₃–CH₃OH–H₂O, 65:25:4 v/v/v). The product was characterized by ¹H NMR (10 mM in CDCl₃, chemical shifts relative to tetramethylsilane): ω-CH₃ of long fatty acyl chain, 0.89 ppm; ω-CH₃ of propionyl chain, 1.10 ppm; –CH₂– of long fatty acyl chain, 1.25 ppm; β-CH₂ of long fatty acyl chain, 1.60 ppm; α-CH₂ (*sn*-1), 2.28 ppm (t); α-CH₂ (*sn*-2), 2.33 ppm (q); –N(CH₃)₃, 3.36 ppm; –CH₂N–, 3.80 ppm; –CH₂CH₂N–, 4.30 ppm; –CH₂OCO–, 4.10 ppm; –CHOCO–, 5.21 ppm; –CH₂OP–, 3.95 ppm.

² Triton X-100 is a polydisperse preparation of *p*-(1,1,3,3-tetramethylbutyl)phenoxy poly(oxyethylene) glycols, containing an average of 9.5 oxyethylene units per molecule.

³ When one species (i.e., head group) of phospholipid is present in a detergent matrix, the mixed micelles are referred to as single-phospholipid mixed micelles. When two phospholipid species differing in head group are present in the mixed micelle system, it is referred to as a binary mixture.

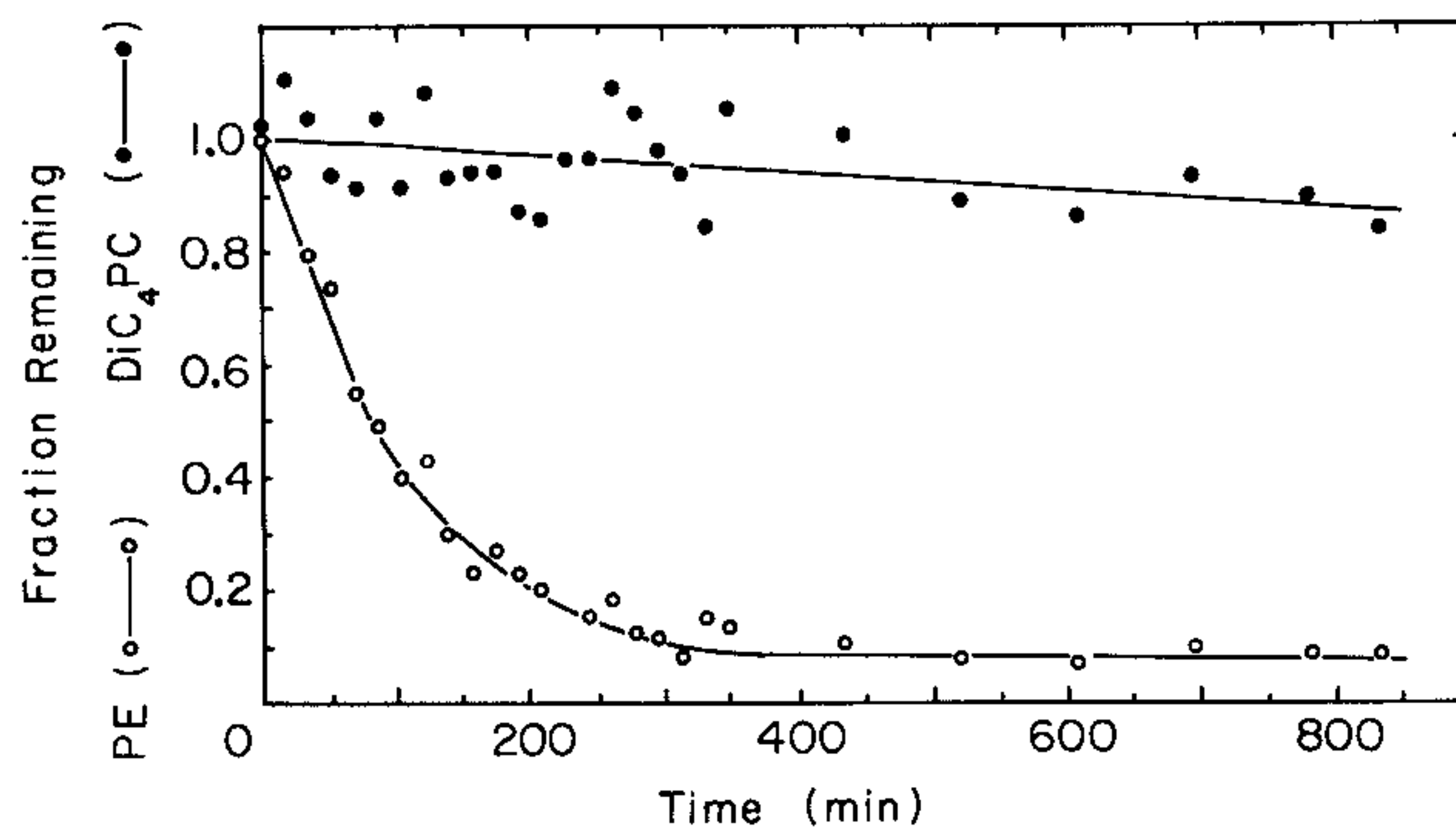


FIGURE 1: Time course for a typical hydrolysis of a binary mixture of 6.5 mM PE and 6.1 mM PC (DiC₄PC) in 96 mM Triton X-100 as followed by ³¹P NMR spectroscopy. The fraction of remaining substrate is calculated for PE from the integrated intensities of the PE signal and the lyso-PE signal as described under Experimental Procedures. For dibutyl-PC, no lyso product was observed, and therefore the intensity of the dibutyl-PC signal relative to that at zero time is plotted.

Dodecylphosphorylcholine was the generous gift of Dr. H. S. Hendrickson, Saint Olaf College, Northfield, MN. Triton X-100 was obtained from Rohm and Haas. Mixed micelles were prepared by the addition of solutions of detergent to dry phospholipids; mixing was achieved by vortexing and then allowing the foam to subside before the sample was used.

³¹P NMR. ³¹P NMR spectra were obtained at 40.3 MHz and 40 ± 0.5 °C with a JEOL PFT-100 system equipped with a modified Nicolet 1080 computer and disk. The JNM-SD-HC hetero spin decoupler unit was used with a Schomandl ND-100M frequency generator. The phospholipid sample contained 30% D₂O for an internal lock. The assay contained phospholipid and Triton X-100 as indicated, 50 mM Tris-HCl, 10 mM CaCl₂, and 0.11 μg of phospholipase A₂. The measured pH was 8.0. The concentration of phospholipase A₂ employed was such that the time to acquire a spectrum was small compared to the entire enzymatic time course. The spin-lattice relaxation times are 2.5 s for PE and 12.4 s for dibutyl-PC under the experimental conditions employed (Plückthun & Dennis, 1981). For optimization of the signal-to-noise ratios within a given time period, a delay time equal to the longer relaxation time was used. Integration via an electronic planimeter (Tallos Systems) was used to quantitate hydrolysis. When sufficient lyso product was produced, the fractional intensity (integral of lyso product divided by the sum of the integrals of lyso product and unhydrolyzed phospholipid) was used as a measure of the extent of reaction. If no lyso product could be detected, the integral of the unhydrolyzed phospholipid was monitored. The latter values were compared with results obtained by computing the fractional intensity as unhydrolyzed phospholipid intensity divided by the sum of the integrals of all phospholipid peaks in order to test for machine instabilities. Both methods gave identical results.

pH Stat. Enzymatic hydrolysis was followed by the pH-stat technique (Dennis, 1973a; Deems & Dennis, 1981) where indicated. Standard assay conditions were 5 mM PC or 5 mM PE, dibutyl-PC, SPH, or dodecylphosphorylcholine where indicated, 20 mM Triton X-100, 10 mM CaCl₂, pH 8.0, and 40 °C. Except where indicated, 0.11 μg of phospholipase A₂ was used. All experiments were conducted in at least duplicate, and the average values are reported.

Results

Activation by Dibutyl-PC. In Figure 1, the time course for the enzymatic hydrolysis of an equimolar mixture of PE

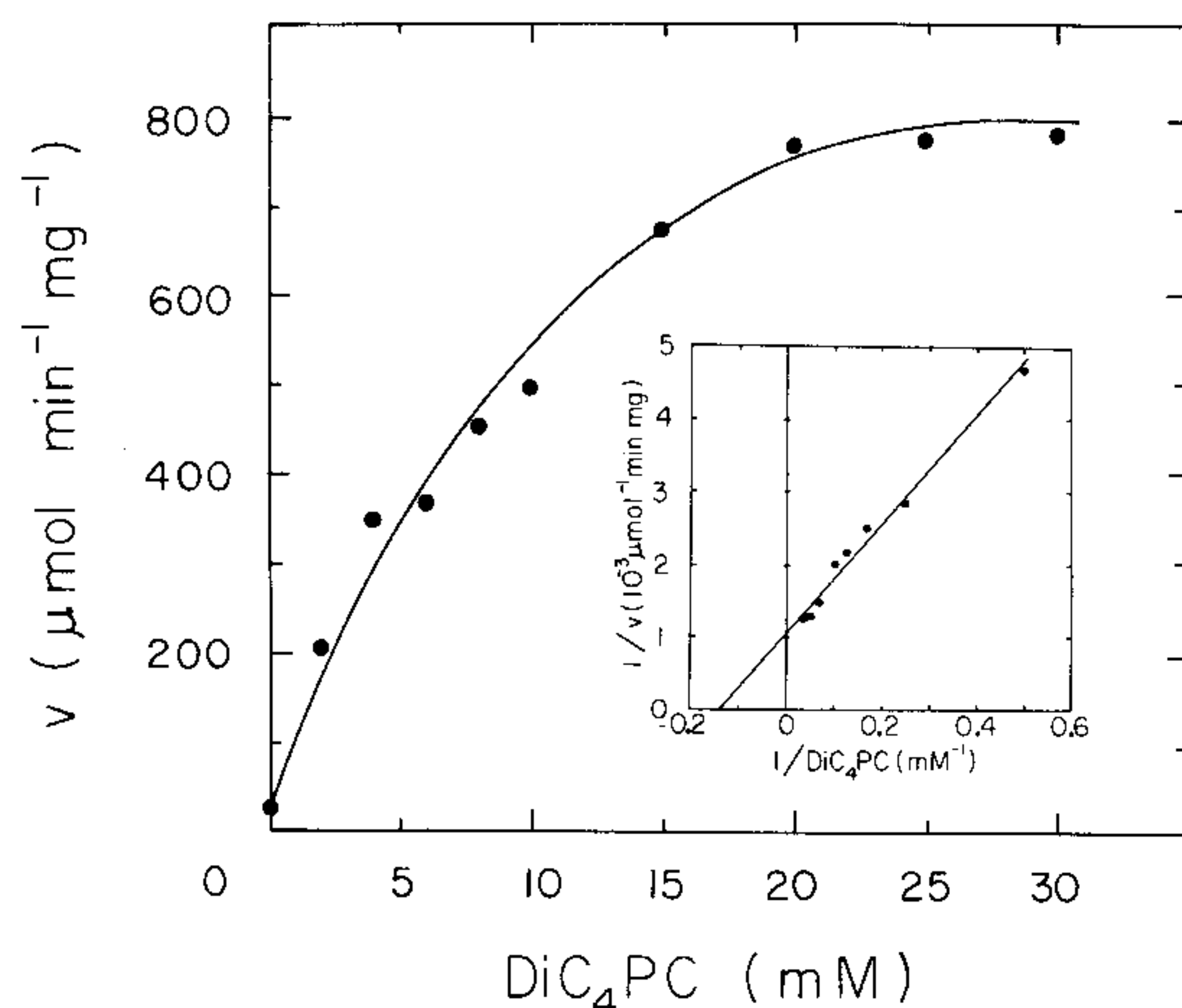


FIGURE 2: Specific activity of phospholipase A_2 catalyzed hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of the concentration of added dibutyl-PC (DiC_4PC) is shown. The pH-stat assay was employed. The insert is the double-reciprocal plot of the results.

and dibutyl-PC is shown as determined by ^{31}P NMR. While the PE is rapidly hydrolyzed, the rate of hydrolysis of dibutyl-PC is very low. From the line shown in Figure 1, it can be seen that less than 10% was hydrolyzed in 800 min. This can be accounted for mainly by nonenzymatic base hydrolysis using the value of base-catalyzed dibutyl-PC hydrolysis of about $1.5 M^{-1} s^{-1}$ calculated from the data of Wells (1974). Thus, it appears that only a negligible amount of enzymatic hydrolysis of this compound occurs during activated PE hydrolysis. In a similar experiment, the enzymatic hydrolysis of an equimolar mixture of dibutyl-PC and egg PC was followed by ^{31}P NMR, and as expected, only the egg PC was hydrolyzed (data not shown). On the other hand, 1-acyl-2-propionyl-PC (derived from egg PC, the *sn*-1 position contains predominantly palmitic acid) is hydrolyzed at about 35% of the rate of egg PC, whereas dibutyl-PC is hydrolyzed at less than 1% of this rate. This confirms that the low rate against monomeric phospholipids is not due primarily to a specificity of the enzyme for the cleavage of a long chain fatty acid. The very high rate of enzymatic hydrolysis of this mixed-acyl phospholipid is presumably mainly due to its quantitative incorporation into the Triton micelles.

Figure 2 shows the rate of enzymatic hydrolysis of PE as a function of the concentration of dibutyl-PC. Apparently, sufficiently large amounts of dibutyl-PC can bring the enzyme to a fully activated state where the rate reaches a plateau region and the enzyme exhibits saturation behavior, with an apparent K_d of 7 mM. The maximum activity is about equal to the activity the enzyme has with egg PC alone as a substrate. Control experiments with identical mixtures assayed by ^{31}P NMR or in the absence of PE by pH stat show that no significant amount of dibutyl-PC is hydrolyzed on this time scale even at the highest concentrations employed in Figure 2.

Effect of Triton X-100. The effect of the addition of Triton X-100 on the rate of hydrolysis of PE both in the absence and in the presence of several activators is shown in Figure 3. The enzymatic hydrolysis of PE and of PE in the presence of 10 mol % sphingomyelin shows a similar decrease as more Triton X-100 is added. Similar behavior had previously been found for dipalmitoyl-PC as a substrate in single-phospholipid mixed micelles (Dennis, 1973a). However, when the enzymatic hydrolysis of PE was activated by the monomeric activator dibutyl-PC (30 mM), the dependence on Triton concentration was less, and it was nearly completely eliminated when

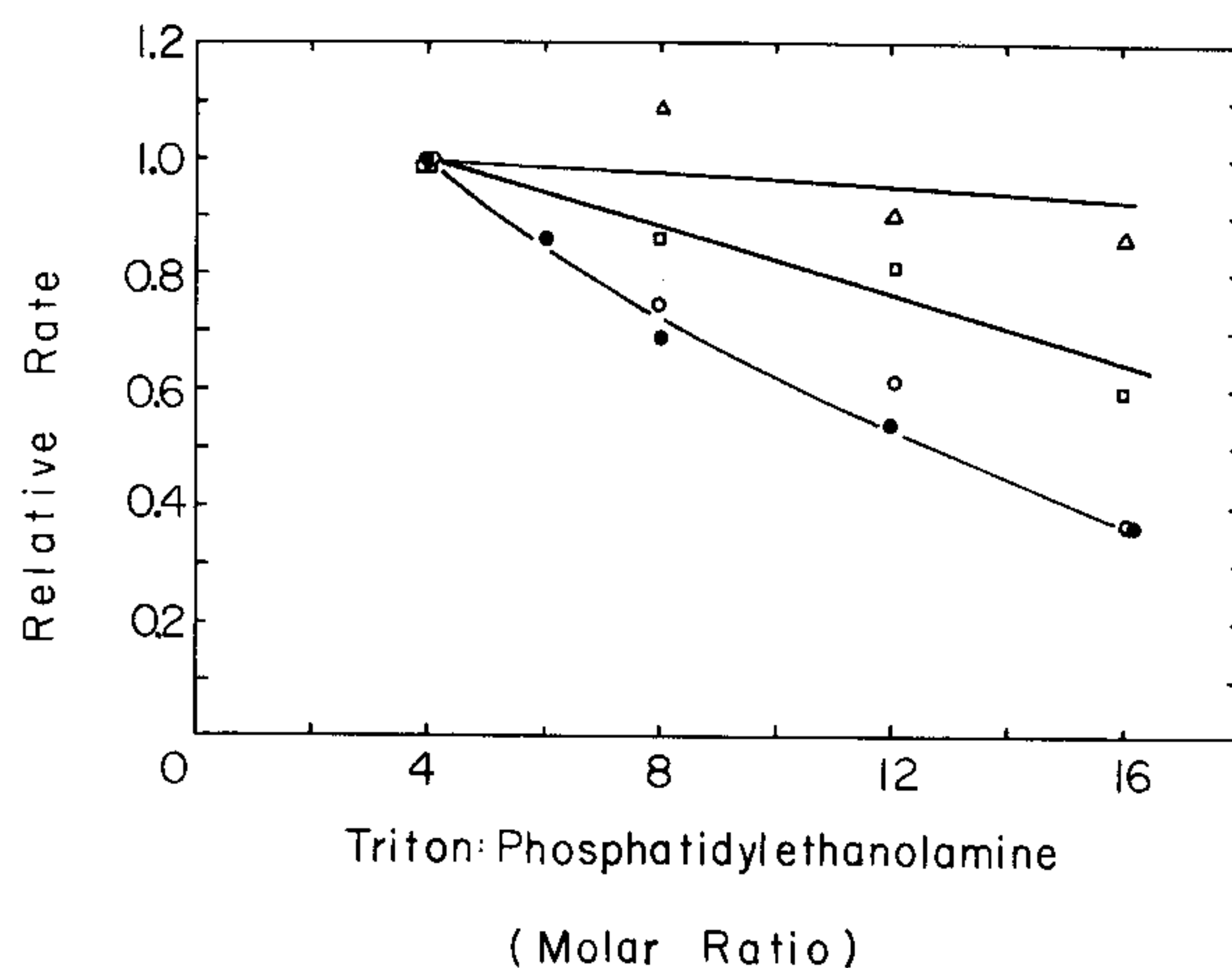


FIGURE 3: Rate of phospholipase A_2 catalyzed hydrolysis of PE as a function of the detergent-PE molar ratio. The pH-stat assay was employed with 5 mM PE as substrate and Triton X-100 at the mole ratio indicated. The Triton X-100 effect on the hydrolysis of PE (●), PE activated by 0.5 mM SPH (○), PE activated by 30 mM dibutyl-PC (□), and PE activated by 60 mM dibutyl-PC (Δ) is shown. The hydrolysis rate at a Triton to PE ratio of 4:1 was set at 1.0 in each case. For the nonactivated reaction, 1.3 μg of phospholipase A_2 was employed in each assay, whereas for the SPH and dibutyl-PC activated reactions, 0.26 and 0.13 μg , respectively, of phospholipase A_2 were employed.

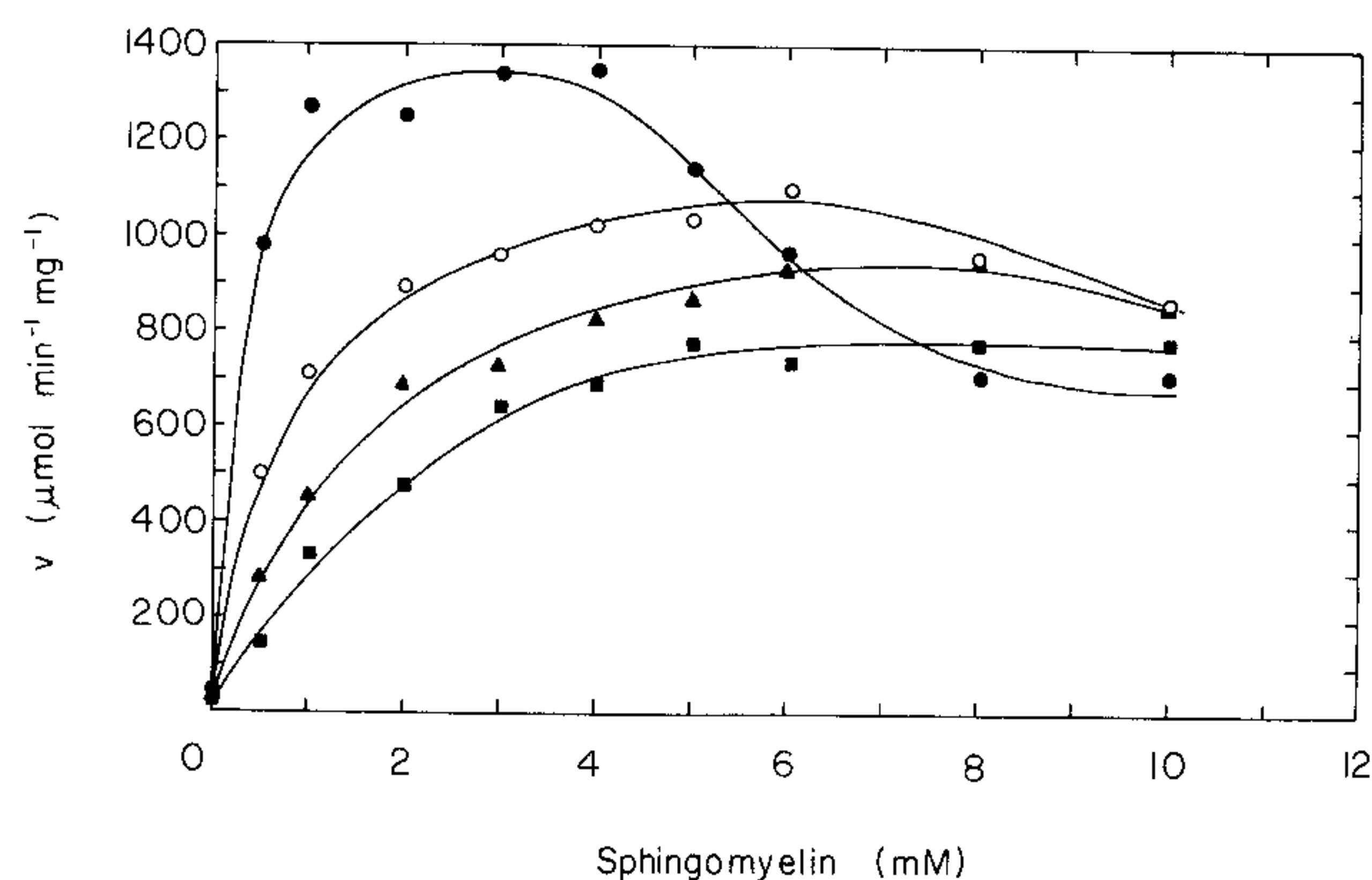


FIGURE 4: Specific activity of phospholipase A_2 catalyzed hydrolysis of PE (5 mM) as a function of SPH concentration in the presence of Triton X-100: (●) 20 mM; (○) 40 mM; (▲) 60 mM; (■) 80 mM. The pH-stat assay was employed.

60 mM dibutyl-PC was employed. Additionally, there was only a very small increase in rate when the substrate was increased from 2 mM PE to 10 mM PE, at a constant PE-Triton ratio of 8:1, both in the presence and in the absence of dibutyl-PC, suggesting that the phospholipase is close to saturation with substrate in all experiments in Figure 3.

For determination of the dependence of the rate decrease on SPH concentration (both bulk and surface), a more extensive study was carried out. In Figure 4, activation profiles with SPH are shown for four different detergent concentrations. It appears that the amount of the rate decrease with increasing Triton becomes smaller with higher activator concentrations (going vertically between the lines). It must be noted, however, that at these low ratios of detergent to total phospholipid, the system is below the lamellar to micelle transition ratio (e.g., at 10 mM sphingomyelin, the ratios in Figure 4 are 1.33:1, 2.66:1, 4:1, and 5.33:1), so that the first two are expected to give anomalous results and lower rates (Dennis, 1973a,b; Robson & Dennis, 1979) because of incomplete solubilization of the phospholipid. Therefore, additional experiments were conducted with much higher detergent concentrations to overcome this problem.

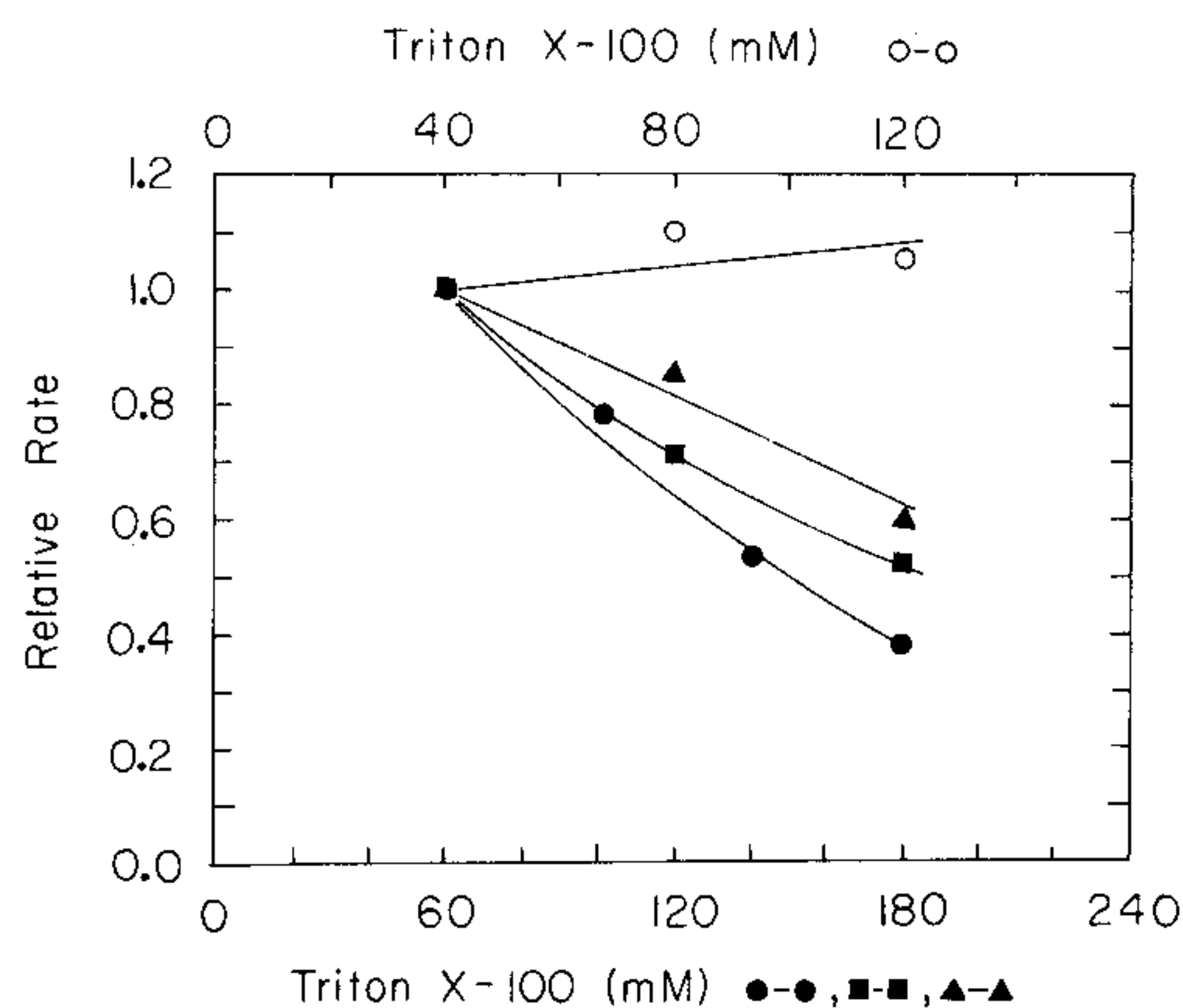


FIGURE 5: Effect of surface concentration of substrate PE and activator SPH on the hydrolysis rate of PE as a function of Triton X-100 concentration under four conditions: (i) (●) The bulk concentration of both PE (5 mM) and SPH (10 mM) are kept constant, and the amount of detergent is increased as indicated; hence, both activator and substrate are diluted in the surface. (ii) (■) The bulk concentration of PE is kept constant (10 mM), whereas the bulk concentration of SPH is increased with increasing Triton X-100 to keep the ratio of Triton-SPH constant at 12:1; hence, to a first approximation, only the substrate is diluted in the surface. (iii) (▲) The bulk concentration of SPH is kept constant (10 mM), whereas the bulk concentration of PE is increased with increasing Triton to keep the ratio of Triton-PE constant at 12:1; hence, to a first approximation only the activator is diluted in the surface. (iv) (○) The ratio of PE-SPH-Triton is kept constant at 1:1:8, but all bulk concentrations are increased; hence, the surface concentrations of both the substrate and the activator remain constant. Note that in all four experiments, the initial detergent-total lipid ratio was set at 4:1 and the concentration of detergent was varied over a 3-fold range. The pH-stat assay was employed.

If the bulk concentrations of both PE (substrate) and SPH (activator) are kept constant and the concentration of Triton is increased, a rate decrease identical with that in Figure 3 is observed as shown in Figure 5 (experiment i). It should be noted that the detergent effect appears similar, whether the dilution is carried out from 20 to 80 mM Triton (Figure 3) or from 60 to 180 mM (Figure 5). Although a change in micelle structure might occur over the range of Triton concentrations in each study, it would not be expected to be large (Robson & Dennis, 1978; Roberts et al., 1979), and therefore a dilution effect of the phospholipids in the surface is the simplest consistent explanation for the observed phenomena.

For examination of the kinetics when only one of the two phospholipids is surface diluted, the detergent and one phospholipid were added at a fixed ratio while keeping the bulk concentration of the other phospholipid constant as shown in Figure 5 (experiments ii and iii). Although these types of curves can be obtained in principle from the data in Figure 4 by going diagonally between the drawn lines, it must be noted that the dramatic dependence of the rate on bulk concentration of activator at low activator concentrations and the low detergent-phospholipid ratio at high activator concentrations prevent the data in Figure 4 from being used for this purpose. Therefore these investigations were carried out at both high activator concentrations (so that a plateau region of rate vs. bulk concentration is reached) and at high detergent concentrations. Since at even higher detergent concentrations, problems with the viscosity of the sample would arise, the range of useful concentrations of all components for kinetic experiments is limited.

The data in Figure 5 show that if only the substrate PE is surface diluted, but the ratio of Triton to SPH is kept constant, the rate still decreases with increasing Triton (experiment ii). The same effect is observed if only the activator is surface

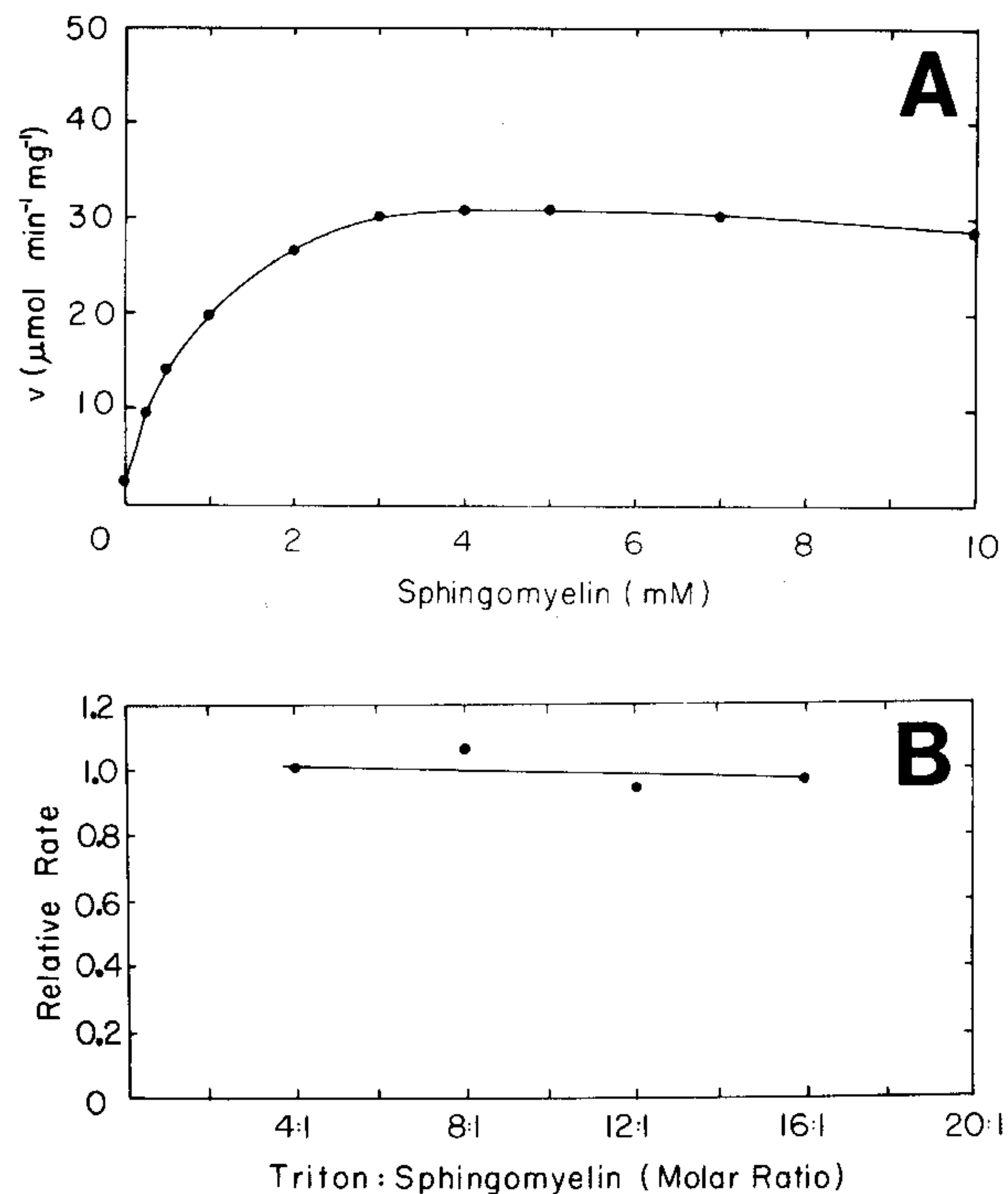


FIGURE 6: (A) Rate of phospholipase A₂ catalyzed hydrolysis of dibutyl-PC (5 mM) in the presence of Triton X-100 (20 mM) as a function of SPH concentration. The pH-stat assay was employed. (B) Relative rate of phospholipase A₂ catalyzed hydrolysis of dibutyl-PC (5 mM) in the presence of SPH (5 mM) as a function of the Triton X-100-SPH molar ratio. The pH-stat assay was employed.

diluted, but the ratio of Triton to PE is kept constant (experiment iii). As expected, if the surface concentration of both PE and SPH is kept constant, no decrease in the rate is observed with increasing Triton (experiment iv).

Activation of Dibutyl-PC Hydrolysis by Sphingomyelin. It was found that the very slow hydrolysis of dibutyl-PC can be significantly activated by the presence of SPH/Triton mixed micelles as shown in Figure 6A. ³¹P NMR shows that in these mixtures only dibutyl-PC is hydrolyzed, yet the amount of dibutyl-PC that is incorporated in the mixed micelles must be very small. The saturation behavior of the activation is a further indication that it is most probably due to a direct effect of SPH on the enzyme. In this system, with 5 mM dibutyl-PC and 5 mM SPH, no change in rate was observed with increasing Triton concentrations (Figure 6B). This experiment suggests that there is no effect of detergent on the reaction when the substrate is monomeric and the activator is micellar. It is unlikely that the addition of SPH increases the very small amount of dibutyl-PC in the micelle to a significant level and that it really is the micelle-bound dibutyl-PC that is the substrate since the ³¹P NMR chemical shift of this compound under these conditions was that of the monomer within experimental error (Plückthun & Dennis, 1981). However, if such an increase in solubilization did occur, the small amount of dibutyl-PC should be proportional to the Triton concentration, and therefore, to a crude approximation, its surface concentration would stay constant. The surface concentration of SPH would, however, be diluted, and consequently a surface dilution effect analogous to Figure 5 (experiment iii) would be expected. This is not observed in Figure 6B. It can be seen from the data in Figures 3-6 that in order for the detergent to show surface dilution kinetics, both activator and substrate phospholipid must be micellar. If either the activator is monomeric (Figure 3) or the substrate is monomeric (Figure 6B), there is no surface dilution effect.

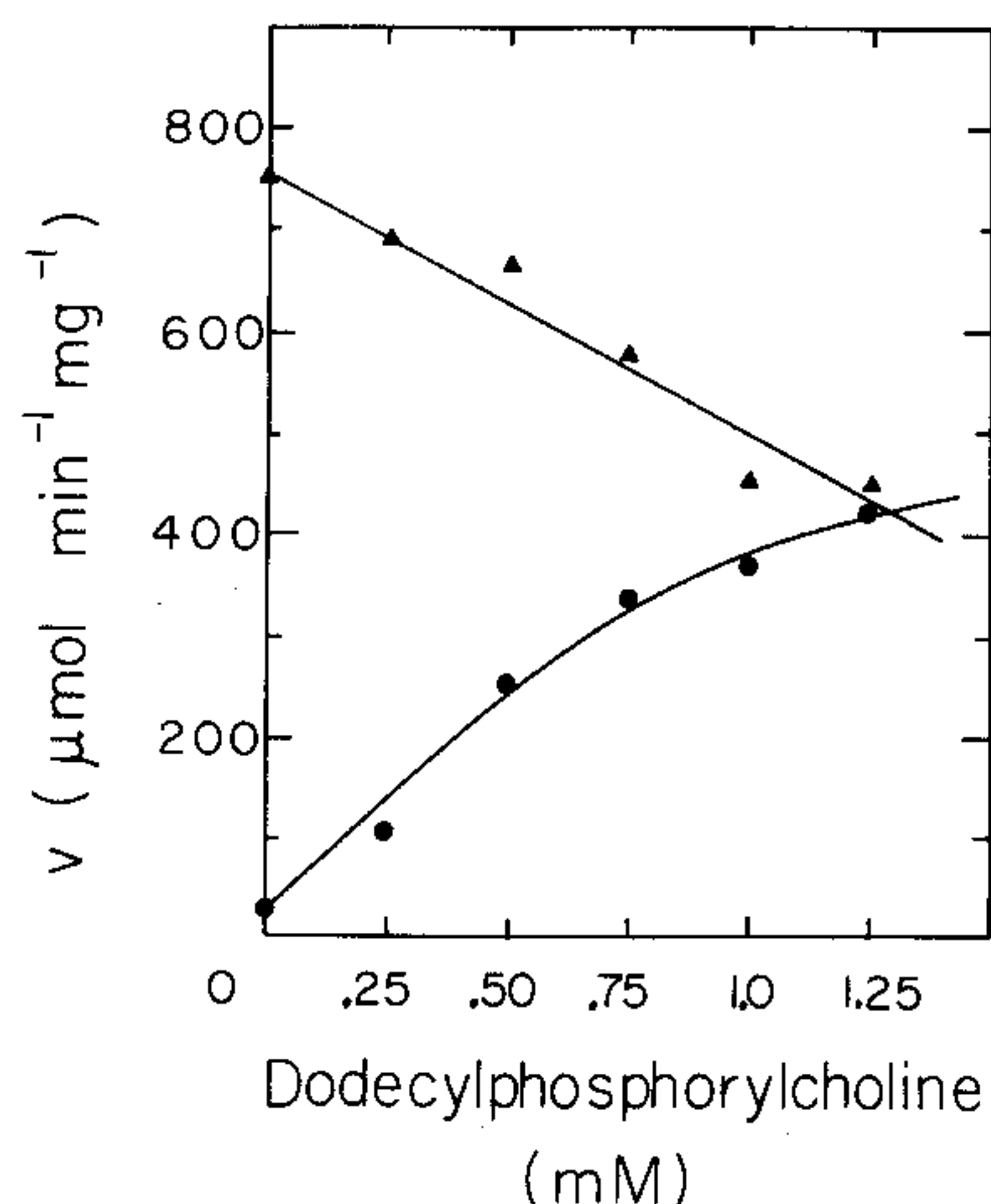


FIGURE 7: Rate of phospholipase A_2 hydrolysis as determined by pH stat is shown for mixed micelles composed of 20 mM Triton X-100 and either 5 mM PC (▲) or 5 mM PE (●) as a function of dodecylphosphorylcholine concentration.

Dodecylphosphorylcholine Effects. Various *n*-alkylphosphorylcholines have been used to protect the active site of phospholipase A_2 against chemical modification (van Dam-Mieras et al., 1975), as substrate analogues to measure binding to the enzyme (van Dam-Mieras et al., 1975; Verheij et al., 1980), as a detergent (Slotboom et al., 1976), or to determine the physical properties of micelle-enzyme complexes (Soares de Araujo et al., 1979). We tested the effect of dodecylphosphorylcholine on the rate of enzymatic hydrolysis of PC and PE. PC hydrolysis shows the expected decrease in rate with increasing amounts of dodecylphosphorylcholine (probably because of competitive inhibition). On the other hand, the hydrolysis of PE is activated by this compound (Figure 7). This observation shows that the minimum structural requirement for a molecule to act as an activator is the phosphorylcholine moiety and at least one hydrophobic chain, either a fatty acid or even an *n*-alkyl residue. Glycerophosphorylcholine alone has no effect on the rate. All attempts to activate the enzyme by mixtures of glycerophosphorylcholine and butyric acid, oleic acid, or oleoyl alcohol, with concentrations of glycerophosphorylcholine between 0.6 and 30 mM, butyric acid between 0.6 and 30 mM, oleic acid between 0.5 and 5 mM, or oleoyl alcohol between 0.6 and 2 mM, did not lead to a rate increase and in some cases led to a small rate decrease equivalent to that caused by the fatty acid or alcohol alone.

Discussion

Dibutyl-PC as Monomeric Activator or Substrate. Dibutyl-PC has now been shown by three independent methods to not be solubilized by Triton X-100 micelles (Plückthun & Dennis, 1981): (i) In a gel chromatography column that was equilibrated with dibutyl-PC, and through which Triton X-100 micelles were passed, no binding of the phospholipid to the detergent micelles could be detected (Roberts et al., 1979), whereas with dihexanoyl-PC binding did occur. (ii) The ^1H NMR chemical shift difference between the α -methylene protons of the *sn*-1 and *sn*-2 fatty acid chains of dibutyl-PC is 6–7 Hz and does not increase significantly upon the addition of Triton X-100, whereas for dihexanoyl-PC the chemical shift difference increases from 7–8 Hz to 17–18 Hz upon the addition of the same amount of Triton. This increase in chemical shift difference is presumably caused by a greater difference in environments of the *sn*-1 and *sn*-2 α -methylene groups in mixed micelles than in monomeric form (Roberts et al., 1978a; Burns & Roberts, 1980). (iii) ^{31}P

NMR studies showed a negligible change in the chemical shift of dibutyl-PC upon the addition of Triton X-100, even when 5 mM PE was also present, whereas the chemical shift of dihexanoyl-PC moves upfield by about 0.19 ppm in going from a monomeric state to the complete solubilization of all dihexanoyl-PC by Triton X-100. We therefore conclude that dibutyl-PC is present as a monomer in solution under all experimental conditions employed in the work presented here.

Phospholipase A_2 catalyzed PE hydrolysis can be activated both by compounds that are quantitatively incorporated into Triton X-100 micelles (SPH and dodecylphosphorylcholine) and by monomeric compounds that are incorporated into the micelles to only a negligible extent (dibutyl-PC). None of these activators is significantly hydrolyzed. Whereas the presence of the micelle-bound SPH did not alter the decrease in hydrolysis rate of PE with increasing concentrations of Triton X-100, the presence of monomeric dibutyl-PC as either activator or substrate eliminates this detergent dependence. The rate of long chain substrate hydrolysis at full activation is about the same in all cases.

A consistent explanation for these effects is given by the model of "surface dilution" (Dennis, 1973b; Deems et al., 1975; Roberts et al., 1977). According to this model, for full activity, the enzyme must interact with an activator molecule prior to catalysis. This activator molecule can either be surface bound or monomeric. If the activator molecule is surface bound, then the activated enzyme is sequestered to the interface; the binding of the substrate molecule to the enzyme should then depend on the concentration of substrate in the two-dimensional interface, and a surface dilution effect could be responsible for the observed decrease in rate with increasing detergent concentration. If the activator molecule is monomeric, then the activated enzyme is not sequestered to the interface; thus the activity should not depend on the concentration of substrate in the interface, and surface dilution should not be observed. At 30 mM dibutyl-PC, this compound acts as an activator, and the surface effect is diminished; at 60 mM dibutyl-PC, the enzyme is completely activated and surface dilution eliminated. Even under these conditions, the dibutyl-PC shows only negligible hydrolysis. In this case, the surface dependence is overcome, because every enzyme molecule binding to substrate in the mixed micelles is already activated. The lack of complete elimination of surface dilution at 30 mM dibutyl-PC (which is about 4 times the apparent K_m) may be due to the presence of some PE in the activator site. With PC alone, this single type of phospholipid might serve as both an activator and a substrate for the enzyme, and full activity is expressed.

The concept of surface dilution is also consistent with the results on SPH/dibutyl-PC mixtures. When the enzyme is activated by SPH, the reaction should only depend on the bulk concentration of lipid and enzyme since the activation on the surface occurs by simple collision, and since the substrate is in solution, then a diffusion within the surface is not necessary. Therefore this case is the mirror image of the reaction with PE and dibutyl-PC.

The fact that surface dilution occurs only when both activator and substrate are surface bound is consistent with a model in which at least two phospholipid molecules are needed for catalysis. It also appears that both PC and PE can bind to the activation site since they both show surface dilution. The self-activation of the latter must, however, be poor or slow.

A particularly interesting case is obtained if only one of the two required phospholipids (either the activator or the substrate) is surface diluted (Figure 5, experiments ii and iii). In

both cases, the rate decrease is somewhat less than if both phospholipids are surface diluted (Figure 5, experiment i). Several possible contributory factors have to be considered: (a) If the bulk concentration of one phospholipid is increased, the enzyme gets closer to saturation, resulting in a slight rate increase (demonstrated in Figure 5, experiment iv). (b) If the enzyme binds both phospholipids in a random order, part of the time it will first bind the phospholipid whose bulk concentration is kept constant and therefore whose surface concentration is diluted. Thus, the phospholipid it binds in the second step would not be surface diluted, and no decrease in rate would be expected. The overall decrease in rate observed with added detergent would then reflect only that fraction of enzyme which first binds to the lipid that is not surface diluted (bulk concentration increased). Thus, the apparent decrease in rate with added detergent would be less than that in Figure 5 (experiment i). (c) Since the effective surface areas of both phospholipids and Triton are unknown, the effective surface concentrations of each component may be somewhat different in each experiment. This too would lead to differences in experiments i-iii.

The concept of surface dilution has been applied elsewhere (Wells, 1978) and has also been questioned (Sundler et al., 1978; Slotboom et al., 1976). While the kinetics of surface dilution may be indistinguishable from those of competitive inhibition (Dennis, 1973b), binding of the enzyme to Triton micelles devoid of phospholipid could not be detected (Roberts et al., 1977). Furthermore, if Triton were a competitive inhibitor of the enzyme, it is not clear how high concentrations of another phospholipid (dibutyl-PC), which is itself not a substrate under these conditions, could reverse this competitive inhibition, without itself inhibiting the reaction.

If Triton competes for the "activator site", on the other hand, it would be difficult to explain why the enzyme, which is activated to about the same steady-state rate toward PE by SPH or dibutyl-PC, is inhibited by Triton only in the case of SPH activation. Such a mechanism would only be consistent with the data reported here if SPH and dibutyl-PC do not bind to the same activator site or if sphingomyelin acts only by modifying the surface rather than interacting with the enzyme. However, having each activator act in a different manner seems unlikely. It should also be noted that the effect of Triton on phospholipase C hydrolysis of PC (Eaton, 1975) or of still another phospholipase C that is specific for phosphatidylinositol (Sundler et al., 1978) falls practically on the same curve as the effect on phospholipase A₂ (Dennis, 1973a) with PC, PE, or PE in the presence of SPH. On the other hand, not all phospholipase A₂'s from other sources showed the same behavior (A. Plückthun and E. A. Dennis, unpublished experiments), and we cannot exclude a very weak affinity of the enzyme for Triton.

Another explanation for the Triton effect would be that increasing amounts of Triton X-100 change the "quality of the interface" (Slotboom et al., 1976) and a two-step mechanism would not necessarily be required. The monomeric activator could then facilitate "penetration" of the interface by the enzyme (either by accelerating a slow surface binding step or by shifting a fast equilibrium), and no surface effect would be apparent. The interaction of the enzyme with the completely micelle-bound activator, on the other hand, would itself depend on the quality of the interface and therefore show a Triton effect. Since at the high dibutyl-PC concentrations employed in some experiments, the percentage of micelle-bound dibutyl-PC remains negligible but the total amount of dibutyl-PC bound to the micelle might not, effects by

micelle-bound dibutyl-PC to change the quality of the interface might also fortuitously reverse the effect of Triton X-100. These possibilities are very difficult to rule out unequivocally at present.

Direct Enzyme Activation. While an alteration in micelle structure with changing penetrability for the enzyme or a direct lipid-lipid interaction appears at first to be a simple explanation for the activation of the hydrolysis of PE, evidence against these possibilities arises from the following experimental results: (i) At a molar ratio of PE-dibutyl-PC of 1:1, the apparent hydrolysis rate is increased by about a factor of 10 compared to the hydrolysis of PE alone. The upper limit of dibutyl-PC that could be bound to detergent micelles under these conditions is about 5% (Plückthun & Dennis, 1981) and probably only 2%. At the same concentration ratio (1:1), egg PC activates the enzymatic hydrolysis of 5 mM PE in mixed micelles by a factor of about 10. Egg PC has, however, been shown to be incorporated practically quantitatively into Triton X-100 micelles or PE/Triton X-100 micelles (Roberts et al., 1979). Postulating a lipid-enzyme interaction in which the lipid activator is required to be part of the interface to cause the activation phenomenon would require dibutyl-PC to be a 20-fold better activator than egg PC. Although this does not rule out such a mechanism, it makes it appear very unlikely. (ii) Dodecylphosphorylcholine activates the enzymatic PE hydrolysis by a factor of about 4 at a concentration of only 5 mol % relative to PE and 1 mol % relative to Triton X-100, so that an effect of the activator on the substrate or the micelle structure is unlikely. Thus, all experimental results are consistent with a direct enzyme-activator interaction.

A model has been proposed for the mechanism of action of cobra venom phospholipase A₂ at lipid/water interfaces in which the enzyme undergoes a conformational change prior to catalysis, and this could involve a dimerization and/or an activator lipid binding step (Dennis et al., 1981). From the data presented here, PC would be a better substrate than PE for phospholipase A₂ according to this model not because it binds more tightly to the enzyme (the apparent dissociation constants of all phospholipids are about 1 mM) (Adamich et al., 1979) but rather because it is itself a good activator. The binding of more than one phospholipid molecule by the enzyme also appears very attractive in view of the fact that aggregated phospholipids are so much better substrates than monomeric ones.

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