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Activation, Aggregation, and Product Inhibition of Cobra Venom Phospholipase A_2 and Comparison with Other Phospholipases*

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The kinetics of phospholipid hydrolysis by cobra venom phospholipase A_2 were examined and compared to those of phospholipase A_2 from porcine pancreas, *Crotalus adamanteus* (rattlesnake) venom, and bee venom. Only the enzyme from *Naja naja naja* (cobra) venom was found to be activated significantly by phosphorylcholine-containing compounds when hydrolyzing phosphatidylethanolamine. The cobra venom enzyme was also the only one in which these activators induced protein aggregation. The parrallel specificity for activators and aggregators suggests that these two phenomena are linked. Product effects were also shown to vary between these four phospholipases. These effects manifest themselves in nonlinear time courses, in changes in steady state velocity, and in the differential effects of serum albumin on reaction rates. Different effects were even seen for the same enzyme when acting on different substrates. A model is presented to account for these observations; its main features are enzyme activation by an activator molecule, whose specificity depends on the enzyme, and an activator-induced aggregation of the enzyme.

There is currently considerable interest in elucidating the detailed mechanism of action of the extracellular phospholipase A_2 as a paradigm for the action of lipolytic enzymes and membrane-bound enzymes at the lipid-water interface (1). Phospholipase A_2 (EC 3.1.1.4) is ubiquitous in nature, and extracellular forms are found abundantly in the mammalian pancreas and in the venom of bees and snakes. It catalyzes the hydrolysis of the *sn*-2 fatty acid in phospholipids that may contain various polar head groups. Its catalytic activity is strongly dependent on the physical state of the phospholipid substrate and is usually several orders of magnitude higher toward aggregated phospholipids than toward monomeric ones. In order to better understand the relationship between enzymatic activity and the lipid-water interface, the kinetic behavior of the following enzymes was compared: the phospholipases from porcine pancreas (2, 3), *Crotalus adamanteus* (4), *Naja naja naja* (1, 5), and bee venom (3, 6). The first three enzymes show considerable homologies in primary structure while the bee venom enzyme shows no obvious homologies (1, 7). The four enzymes differ in several other important ways. The *C. adamanteus* enzyme is a dimer, the pancreatic and bee venom enzymes are monomers, and the *N. naja naja* enzyme can undergo a concentration and lipid-dependent oligomerization (see below). The two enzymes from snake venom are strongly acidic (pI 4.5–5.0), the pancreatic enzyme is slightly acidic (pI 6.3), and the bee venom enzyme is strongly basic (pI 10.5) (2, 4–6). Thus, these four enzymes span the spectrum of extracellular phospholipase A_2 characteristics, and a comparison of their kinetics should give insight into the kinetic consequences of the structural variations.

We will compare two kinetic phenomena: substrate specificity and product effects. The substrate specificity of the cobra venom enzyme shows an unusual activation phenomenon (8–12). While phosphatidylcholine (PC[1]) is a much better substrate than phosphatidylethanolamine (PE), the latter becomes the preferred substrate when the enzyme acts on mixtures of the two. Investigations in our laboratory (8–10, 12) have shown that the enzymatic hydrolysis of PE can be activated by a variety of compounds that contain a phosphorylcholine group and a hydrophobic residue. We will compare this behavior with that of the other enzymes listed above and will also investigate the effects of these activators on the aggregation state of the enzymes. Finally, we will examine the effects of various activators, detergents, and reaction products on the enzymatic activity. A detailed mathematical analysis is complicated by the fact that, for each compound, both bulk and surface concentrations have to be considered (9–11).

Although some kinetic properties of pancreatic and snake venom phospholipases have been compared on monolayers and bilayers (reviewed in Ref. 3), no investigation has yet been reported that compares these phospholipases under identical assay conditions. In fact, a major impediment to correlating the work from different laboratories has been this diversity of assay conditions. In the study reported here, the mixed micellar system was chosen because of the advantages it offers in providing a similar physical state and surface regardless of which phospholipids or effectors are present. The identical nature of the substrate structures presented to the enzymes should allow a meaningful comparison of these four enzymes.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized *N. naja naja* venom (Lot No. NNP9STLZ) was obtained from the Miami Serpentarium, and phospholipase A_2 was purified as described elsewhere (5, 13). Protein concentrations were determined either by the Lowry procedure, using the appropriate correction factor (5, 13), or by optical density. Other purified phospholipase A_2 was obtained from Millipore Corp. (*C. adamanteus*),

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DTAPS, *N,N*-dimethyl-*N*-tetradecyl-1-ammonio-propane-3-sulfonate.

Calbiochem-Behring (bee venom), and Boehringer Mannheim (porcine pancreas).

PC was purified from egg yolks by the method of Singleton *et al.* (14). PE, prepared by transesterification of egg PC, was obtained from Avanti Biochemicals. Gas chromotography (15) showed that, within experimental error, the PE and PC used here had identical fatty acid compositions in the *sn*-1 position as well as in the *sn*-2 position (data not shown). Dibutyryl-PC, lyso-PC, and lyso-PE were prepared as described elsewhere (16, 17). All phospholipids gave a single spot on thin-layer chromatography. Oleic acid and DTAPS were obtained from Calbiochem-Behring, bovine brain sphingomyelin from Avanti, defatted bovine serum albumin from Sigma, and Triton X-100 from Rohm and Haas. Dodecyl phosphorylcholine was the gift of Professor H. Stuart Hendrickson, St. Olaf College, Northfield, MN. Mixed micelles were prepared by the addition of detergent solutions to dry phospholipids which were then mixed by vigorous vortexing. The foam was allowed to subside before the sample was used.

pH-stat—Enzymatic hydrolysis was followed by pH-stat (5, 18). 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 Autoburette, and a recorder. Standard assays were conducted at pH 8.0, 40 °C, and contained 5 mM egg phospholipid, 20 mM Triton X-100, and 10 mM $CaCl_2$ in a total volume of 2.0 ml. For time courses, a typical plot from the pH-stat is shown directly. For rates, the average of at least duplicate experiments is reported. Usually, rates were reproducible within ±5%, although individual points were occasionally found to vary considerably more.

Chromatography—Chromatography on Sephadex G-75 (40–120 μm) was performed according to the manufacturer's instructions. A peristaltic pump was used to control the flow at an even rate (about 20 ml/h). The column (90 cm × 1 cm or 45 cm × 1.5 cm) was silanized by treatment with a solution of dichlorodimethylsilane in toluene to obtain a more even packing with better resolution and to minimize adherence of the enzyme to the glass (19). Rabbit muscle aldolase, hen ovalbumin, α-chymotrypsinogen, equine heart cytochrome *c*, and *p*-toluenesulfonate or ADP were used as molecular weight standards. Control experiments in a column equilibrated with *n*-dodecyl phosphorylcholine in the absence and presence of aldolase or *p*-toluenesulfonate showed that the elution volume of phospholipase A_2 was not changed by the inclusion of the molecular weight markers. Therefore, in the actual experiments, aldolase and *p*-toluenesulfonate were always included for added accuracy as void volume and total volume markers. In all experiments, the column was pre-equilibrated with 1.5–2 column volumes of buffer at the same flow rate, and phospholipase A_2 was always loaded in 600 μl at a concentration of 100 μg/ml. The elution of the enzyme was followed by UV absorption at 280 nm and was confirmed by activity assays with the pH-stat.

RESULTS

Time Dependence of Enzymatic Activity—The reaction time courses of the four enzymes toward PC and PE were compared and are shown in Figs. 1 and 2. The bee venom enzyme was the only one that had linear time courses for both PC and PE hydrolysis (Figs. 1*A* and 2*A*). The pancreatic enzyme's activity gradually increased until a maximum rate was achieved, and then declined again. This apparent lag in maximum activity was found for both substrates (Figs. 1*B* and 2*B*). Both of the snake venom enzymes showed marked decreases in activity with time; however, this inhibition occurred only with the PC substrate (Fig. 1, *C* and *D*). When acting on PE, the *C. adamanteus* time course was linear (Fig. 2*D*) while the *N. naja naja* time course (Fig. 2*C*) exhibited a lag period resembling that of the pancreatic enzyme. To obtain further information on the nature of these results, the effect of products on the lag period and steady state rate was investigated.

Product Inhibition—For the *N. naja naja* phospholipase A_2 we found that the addition of fatty acid, a reaction product, lowers the initial rate (Fig. 3*A*). The magnitude of this inhibition was comparable to that found in the time course (Fig. 1*C*) for similar amounts of fatty acid. The error in these experiments was quite large and was presumably due to the

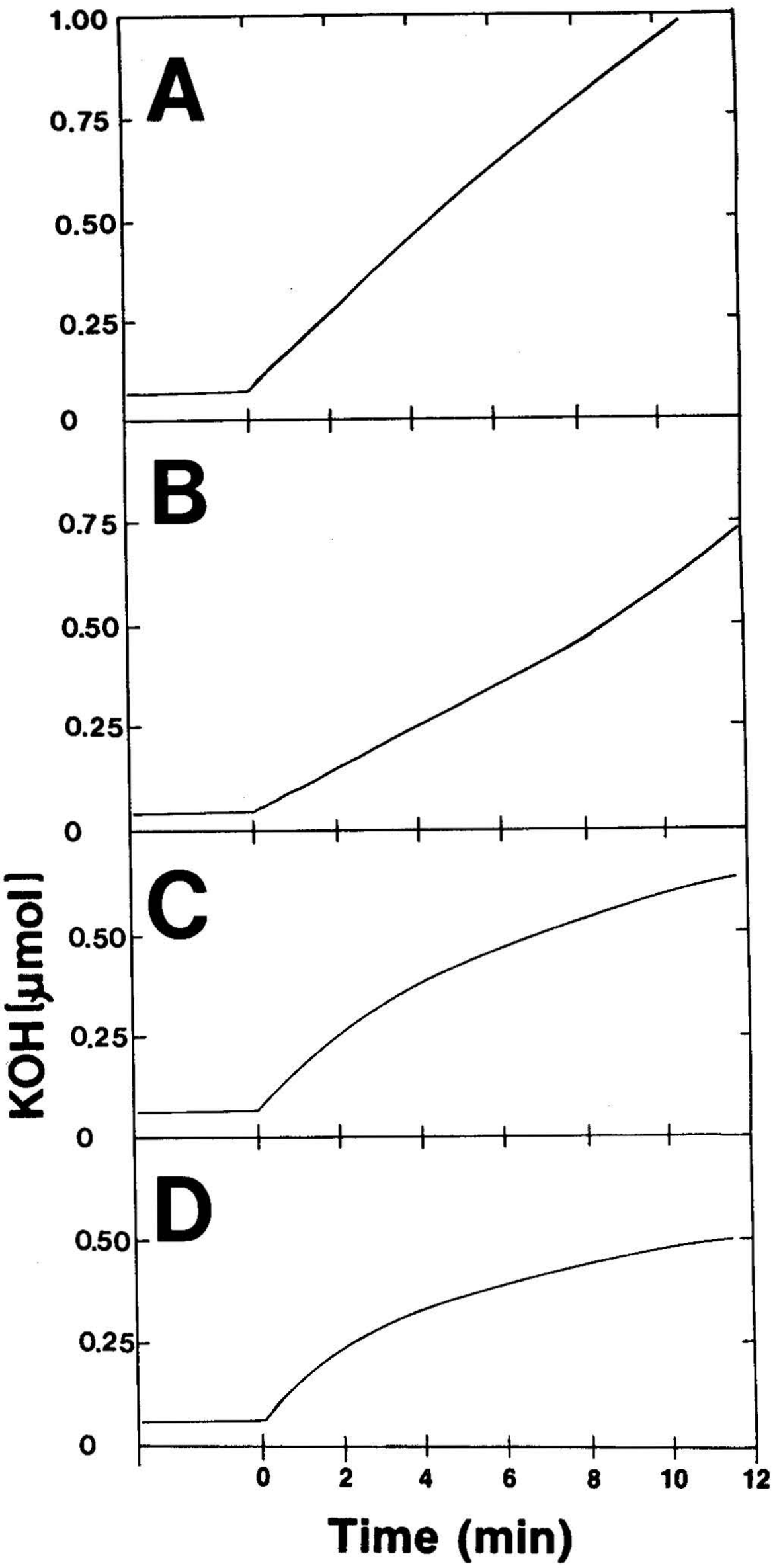

FIG. 1. **Time course of hydrolysis of aliquots from the same stock solution of mixed micelles consisting of PC (5 mM) and Triton X-100 (20 mM).** Enzyme from the following sources was employed: phospholipase A_2 from (*A*) bee venom (0.4 μg), (*B*) porcine pancreas (40 μg), (*C*) *N. naja naja* (0.1 μg), and (*D*) *C. adamanteus* (0.25 μg).

presence of fatty acid concentrations close to the solubility of their calcium soaps. The progress curve of reaction mixtures containing added fatty acid sometimes still appear curved. It is not clear why this occurs since the amount of enzymatically produced fatty acid should be insignificant compared to the amount added. It is possible that a high local concentration of fatty acid builds up and that the diffusion of the fatty acid is hindered by the interaction with Ca^{2+}.

Since the cobra venom enzyme has an isoelectric point of about 5 and is negatively charged at pH 8.0, repulsion by a negatively charged surface might conceivably cause this inhibition. However, small amounts of negatively charged so-

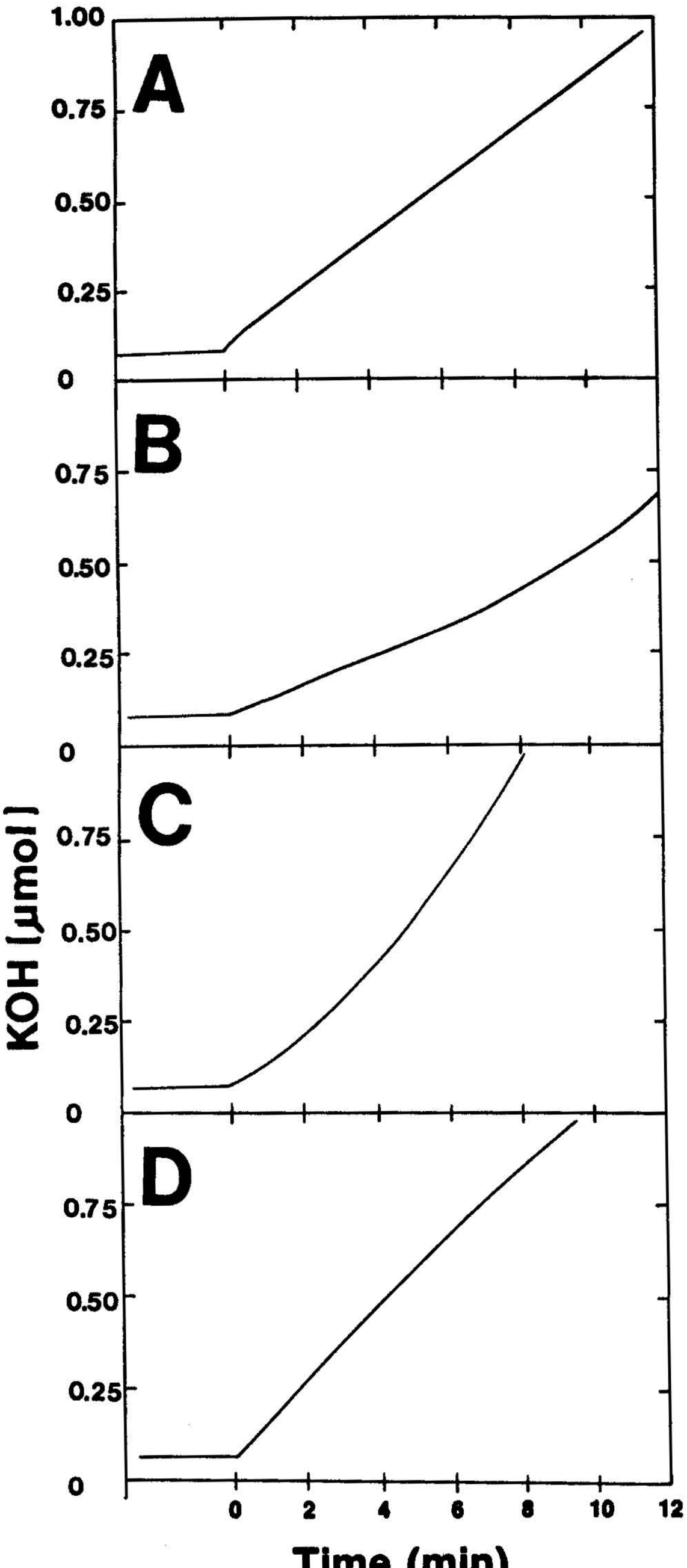

FIG. 2. **Time course of hydrolysis of aliquots from the same stock solution of mixed micelles consisting of PE (5 mM) and Triton X-100 (20 mM).** Enzyme from the following sources was employed: phospholipase A_2 from (*A*) bee venom (0.2 μg), (*B*) porcine pancreas (40 μg), (*C*) *N. naja naja* (1.5 μg), and (*D*) *C. adamanteus* (2.2 μg).

dium dodecyl sulfate had no measurable effect on the rate (Fig. 3*B*), nor did the positively charged cetyltrimethylammonium bromide. Thus, the inhibition must be caused by a more specific interaction of the reaction product with the enzyme. In contrast to fatty acid, neither lyso-PC nor lyso-PE decreased the hydrolysis rate at similar concentrations (Fig. 3*C*). The combined addition of both lyso-PC and fatty

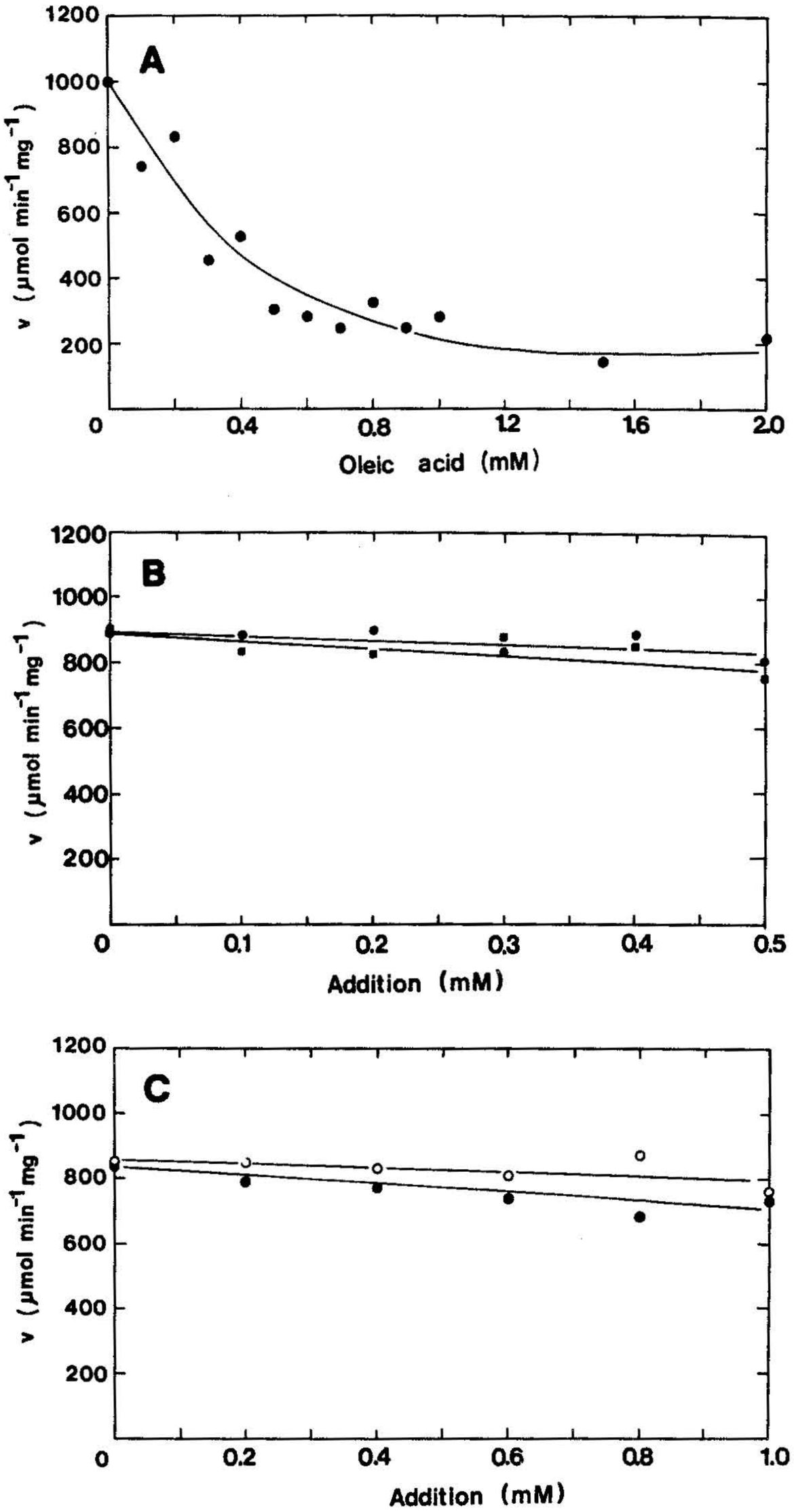

FIG. 3. **Specific activity of cobra venom phospholipase A_2 for the hydrolysis of PC (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of (*A*) oleic acid, (*B*) cetyltrimethylammonium bromide (■) and sodium dodecyl sulfate (●) concentration, and (*C*) lyso-PC (○) and lyso-PE (●).**

acid did not give rise to a synergistic effect at low concentrations (data not shown). Similar observations were made for the enzyme from *C. adamanteus* (data not shown).

The presence of albumin significantly reduced the curvature in the time course of PC hydrolysis for both the *N. naja naja* and *C. adamanteus* phospholipase A_2 (Fig. 4). Albumin is known to interact with both fatty acids and lysophospholipids (20), but since the rate decrease with time is caused by the fatty acid produced, the critical step must be the removal of fatty acids. Very little is known about the mechanism by which albumin extracts the fatty acids from the micelle. The effects of albumin can be partially reversed by the addition of excess fatty acid, but not by that of lyso-PC (data not shown).

We conclude that significant product inhibition occurs only by fatty acid and not by lysophospholipid. Half-inhibition by the former under these assay conditions occurs at about 0.5

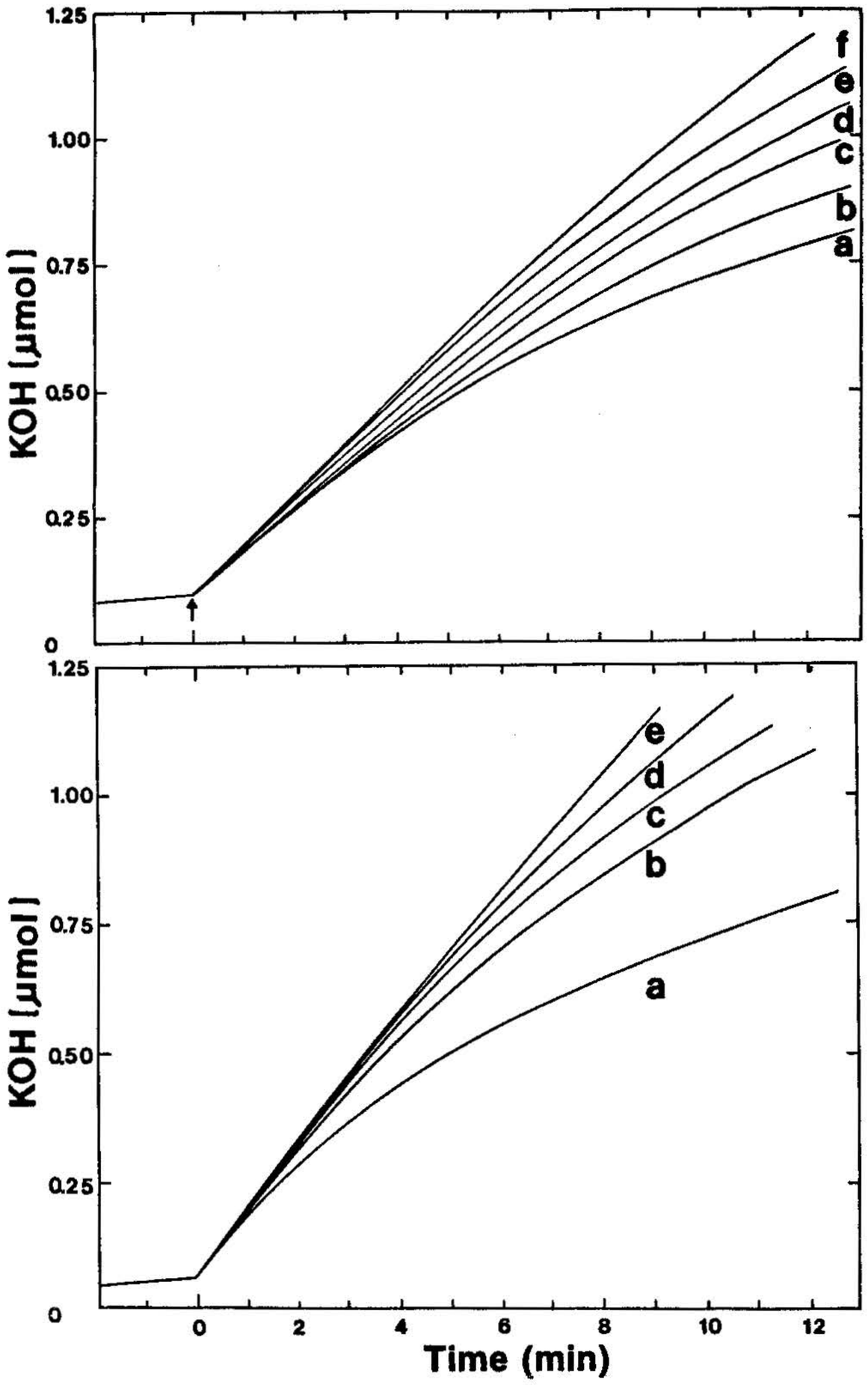

FIG. 4. Time course of the hydrolysis of PC (5 mM) in mixed micelles with Triton X-100 (20 mM) catalyzed by (*top panel*) cobra venom phospholipase A_2 in the presence of various concentrations of bovine serum albumin: *a*, no albumin; *b*, 0.2 mg/ml; *c*, 0.4 mg/ml; *d*, 0.6 mg/ml; *e*, 0.8 mg/ml; *f*, 2.0 mg/ml. In the *bottom panel* are shown similar data for the enzyme from *C. adamanteus* in the presence of the following concentrations of bovine serum albumin: *a*, no albumin; *b*, 0.25 mg/ml; *c*, 0.5 mg/ml; *d*, 0.75 mg/ml; *e*, 2.0 mg/ml.

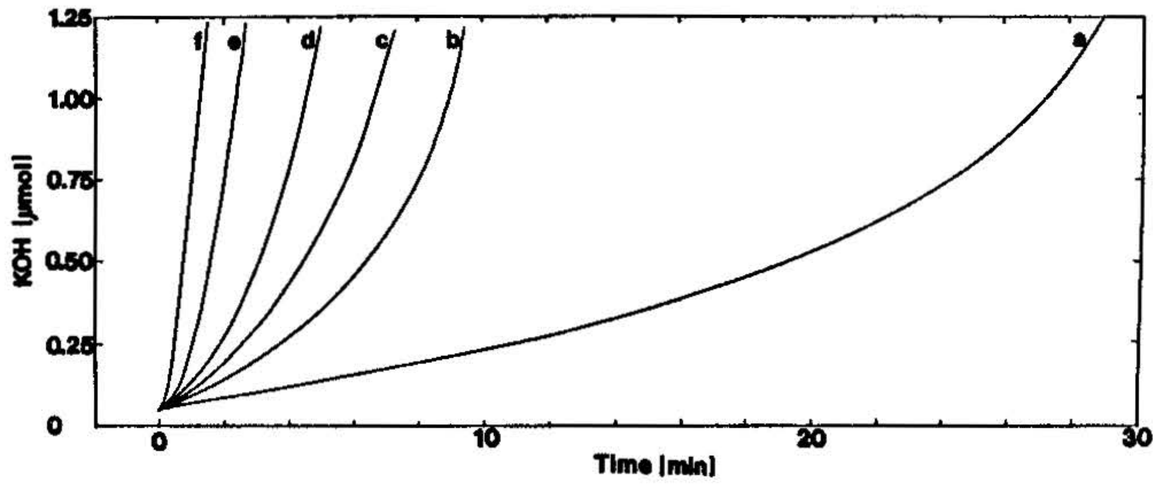

FIG. 5. **Time course of the hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) by pancreatic phospholipase A_2 as a function of the oleic acid concentration.** *a*, no fatty acid; *b*, 0.2 mM; *c*, 0.4 mM; *d*, 0.6 mM; *e*, 0.8 mM; *f*, 1.0 mM.

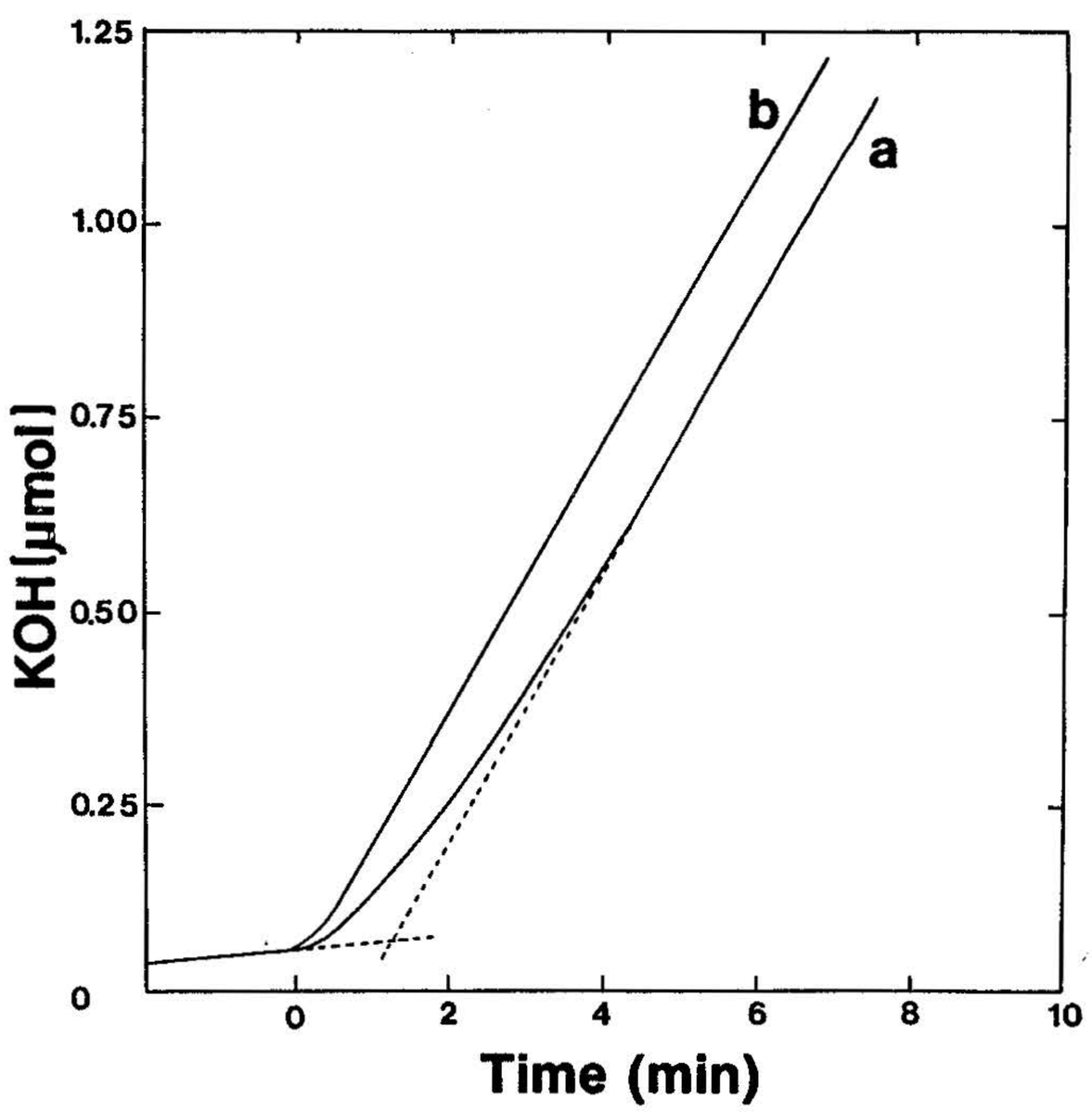

FIG. 6. **Time course for the hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (40 mM) by cobra venom phospholipase A_2 in the absence (*a*) and presence (*b*) of 1 mM oleic acid.**

mM fatty acid, but a proper quantitative determination of the K_I would be quite complex since both bulk and surface concentrations of substrate, activator, and product inhibitor must be considered (10–12), and the potential accuracy of the measurements does not warrant such an effort at this time.

Lag Period—A long lag period was found for pancreatic phospholipase A_2 activity toward both PC and PE (Figs. 1*B* and 2*B*). This lag period could be reduced by the addition of fatty acid (Fig. 5) or other negatively charged detergents, *i.e.* sodium dodecyl sulfate (data not shown). This was true for either substrate. While the lag period was reduced, the maximum rates remained unchanged (data not shown). This affinity for negatively charged surfaces is not surprising since the pancreatic enzyme acts *in vivo* on negatively charged micelles of bile acids and phospholipids. Similar amounts of lyso-PC had no measureable effect on the rate of PC hydrolysis by the pancreatic phospholipase A_2 (data not shown). These experiments indicate that the fatty acid product acts as an activator for porcine pancreatic phospholipase A_2.

The *N. naja naja* enzyme also showed a short lag period in its time course for PE hydrolysis (Fig. 2*C*). This was quite different from the strong inhibition exhibited during PC hydrolysis where no lag period was detected. This lag period could be overcome by either fatty acid (Fig. 6) or sodium dodecyl sulfate (not shown), again with only a small effect on the maximum rate portion of the time course. An identical fatty acid effect was observed when the homogeneous dilauryl-PE served as a substrate instead of transesterified egg PE, thus eliminating inhomogeneity of the substrate as an explanation (data not shown).

Since the *N. naja naja* venom enzyme is the only one to show a pronounced activation phenomenon with PE and since the activation might conceivably be reflected in pre-steady state kinetics, the nature of this lag phase was investigated more thoroughly. First, it was established that the lag phase is not artifactual. The enzymes from bee and *C. adamanteus* venom do not show such an effect on the identical substrate mixture, thus eliminating a buffer effect on the pH-stat (21) as a conceivable cause. This was verified by the fact that the lag phase could also be observed by using a thin layer chromatography assay (data not shown). Phosphorylcholine-containing activators, including lyso-PC, also appear to eliminate the lag period but, in contrast to fatty acid, also have a very dramatic effect on the maximum rate portion of a time course. Lyso-PE, on the other hand, has no effect on either the lag

phase or the maximum rate. Neither the *C. adamanteus* nor the bee venom enzymes showed a lag period or an inhibition when acting on PE. In agreement with this, no fatty acid effects were observed on their initial rates (data not shown).

In the three cases where there is a lag phase and where an apparent accumulation of fatty acid in the surface increases the initial rate (pancreatic enzyme acting on PC, pancreatic enzyme acting on PE, cobra venom enzyme acting on PE), the inclusion of albumin (2 mg/ml) did not appear to measurably increase the time of the lag phase or decrease the initial rate. Since the same concentration of albumin almost completely eliminated the inhibition of PC hydrolysis by fatty acid, the binding capacity of the amount of albumin employed would seem to be sufficient, although the exact binding stoichiometries and binding constants for fatty acids to albumin are still a matter of debate (22). A very small effect on the lag phases might have escaped detection.

Specificity—These enzymes differ in another, even more dramatic way: substrate specificity. Tables I and II demonstrate the large variability in specifity of these enzymes toward PC and PE. In light of the nonlinear time courses discussed above, the following conventions were used to calculate the specific activities reported in these tables. When inhibition was present, the rates were calculated from the base consumption after 1.5 min (<1% hydrolysis). If a lag period existed, the "initial rate" was determined from the base consumption after 1.0 min (<1% hydrolysis), but the reaction was allowed to continue until a "maximum rate" was observed (this rate eventually declined again); both rates are reported. As is discussed above, these curvatures are mainly due to fatty acid effects; thus, very small amounts of hydrolysis products in the substrate could substantially alter the measured rates. Because of this complication, the reported specific activities should be viewed as only approximate.

The bee venom phospholipase A_2 showed no preference for either PC or PE. Neither the initial nor maximum rates of the pancreatic enzyme demonstrated a preference for PC or PE. The pancreatic rates were also an order of magnitude lower than those of the venom enzymes in this assay system. Both of the snake enzymes clearly preferred PC. The *C. adamanteus* was about 4 times more active on PC while the *N. naja naja* preferred PC by a factor of 36.

TABLE I

Approximate specific activities of phospholipase A_2 toward PC and PE in mixed micelles

Enzyme source	Specific activity	
	PC	PE
	μmol min^{-1} mg^{-1}	
Bee venom	500	600
Porcine pancreas, initial rate	0.4	0.4
Maximal rate	~30	~40
N. naja naja, initial rate	900	25
Maximal rate		60
C. adamanteus	300	70

TABLE II

Effects of sphingomyelin and dibutyryl-PC on the relative rate of hydrolysis of PE by different enzymes

Enzyme source	Activator		
	None	Sphingomyelin	Dibutyryl PC
		1 mM	*5 mM*
Bee venom	1.0 ± 0.11[a]	0.85 ± 0.15	0.85 ± 0.05
Porcine pancreas	1.0 ± 0.2	1.15 ± 0.15	0.91 ± 0.15
N. naja naja	1.0 ± 0.11	21.6 ± 3.0	13.6 ± 3.0
C. adamanteus	1.0 ± 0.10	0.93 ± 0.07	0.98 ± 0.07

[a] Mean ± standard deviation (n = 3) in initial rates.

The activation of PE hydrolysis by phosphorylcholine-containing compounds was compared for all four enzymes. Only the cobra venom enzyme showed any significant activation by the addition of either the micelle-bound sphingomyelin or the monomeric activator dibutyryl-PC (Table II). Dibutyryl-PC is not hydrolyzed under these conditions (10). The specificity of activation is demonstrated by a comparison of two rather similar zwitterionic detergent-like molecules which differ in the relationship of the charges in the polar group. Dodecyl phosphorylcholine (critical micellar concentration, 1 mM (23)) is $CH_3(CH_2)_{11}O(PO_2^-)OCH_2CH_2^+N(CH_3)_3$ and DTAPS (critical micellar concentration, 0.33 mM (24)) is $CH_3(CH_2)_{13}^+N(CH_3)_2CH_2CH_2CH_2SO_3^-$. Only the phosphorylcholine-containing compound has a measureable effect on the activity of the cobra venom enzyme toward PE in mixed micelles (Fig. 7). This specificity also applied to the products of the reaction (Fig. 8). Lyso-PC activates PE hydrolysis, but lyso-PE and fatty acid do not. The addition of fatty acid to either lysophospholipid did not significantly alter that lysophospholipid's effect.

Aggregation—The results of gel filtration experiments on phospholipase A_2 from porcine pancreas, *C. adamanteus* venom, bee venom, and *N. naja naja* venom are shown in Table III. All phospholipase A_2 that have been sequenced fall into a range of molecular weights between 13,000 and 15,000 (1). It can be seen, however, that only the pancreatic enzyme elutes as a monomer with the expected molecular weight. The *C. adamanteus* enzyme elutes, as expected, as a dimer of 26,000

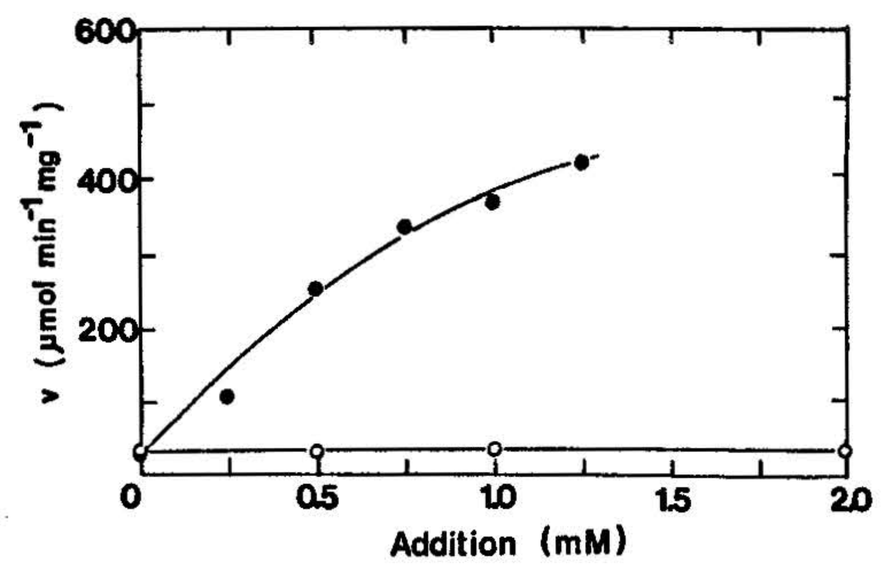

FIG. 7. **Specific activity of cobra venom phospholipase A_2 for the hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of dodecyl phosphorylcholine (●) or DTAPS (○).**

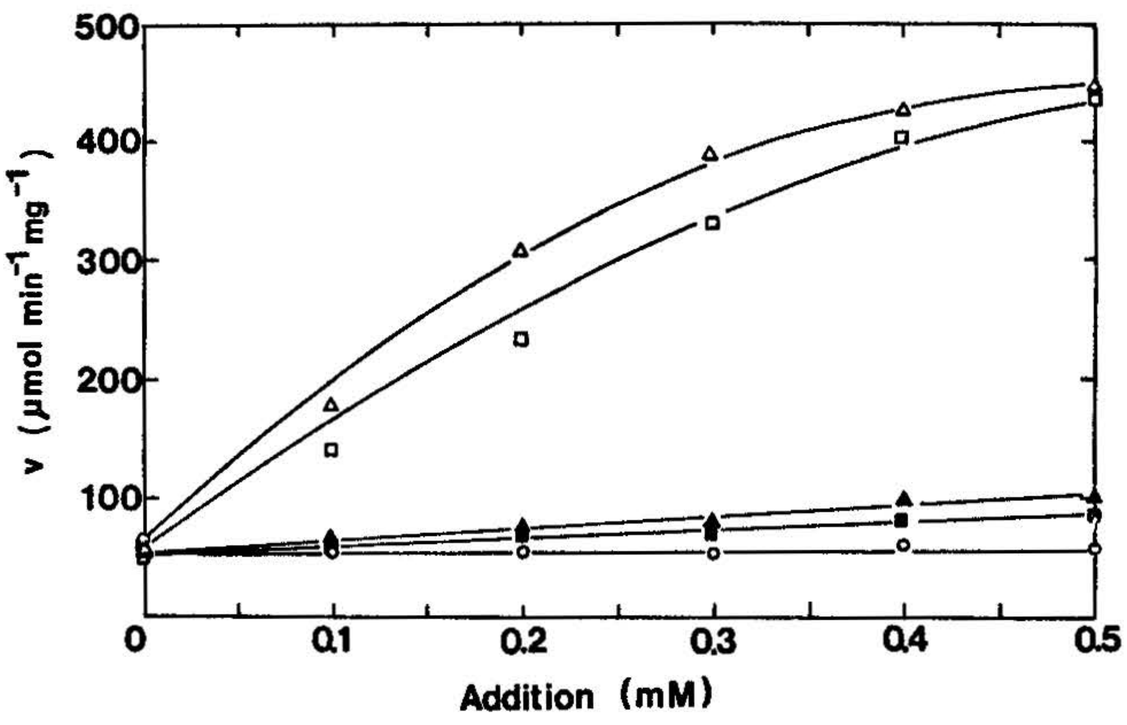

FIG. 8. **Specific activity of cobra venom phospholipase A_2 for the hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of the concentration of lyso-PE (○), oleic acid (■), lyso-PE and oleic acid (▲), each at the concentration indicated, lyso-PC (□), or lyso-PC and oleic acid (△).** In this experiment, initial rates were determined by measuring proton release after a fixed time period (2 min). Therefore, elimination of the lag phase would contribute to a small apparent rate increase in the data.

TABLE III

Gel chromatography of phospholipase A_2 from different sources on sephadex G-75

Enzyme source	Addition[a]	Apparent M_r
Bee venom		2,000[b]
	Dodecyl phosphorylcholine	2,000
Porcine pancreas		12,000
	Dodecyl phosphorylcholine	12,500
N. naja naja		10,000
	Dodecyl phosphorylcholine	44,000
	DTAPS	10,000
	Dibutyryl-PC	10,500
C. adamanteus		26,000
	Dodecyl phosphorylcholine	26,000

[a] The buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM $BaCl_2$, and either 50 μM dodecyl phosphorylcholine, 50 μM DTAPS, or 5 mM dibutyryl-PC where indicated.

[b] From the known sequence of the bee venom enzyme, a M_r of about 14,000 is expected; see text for details.

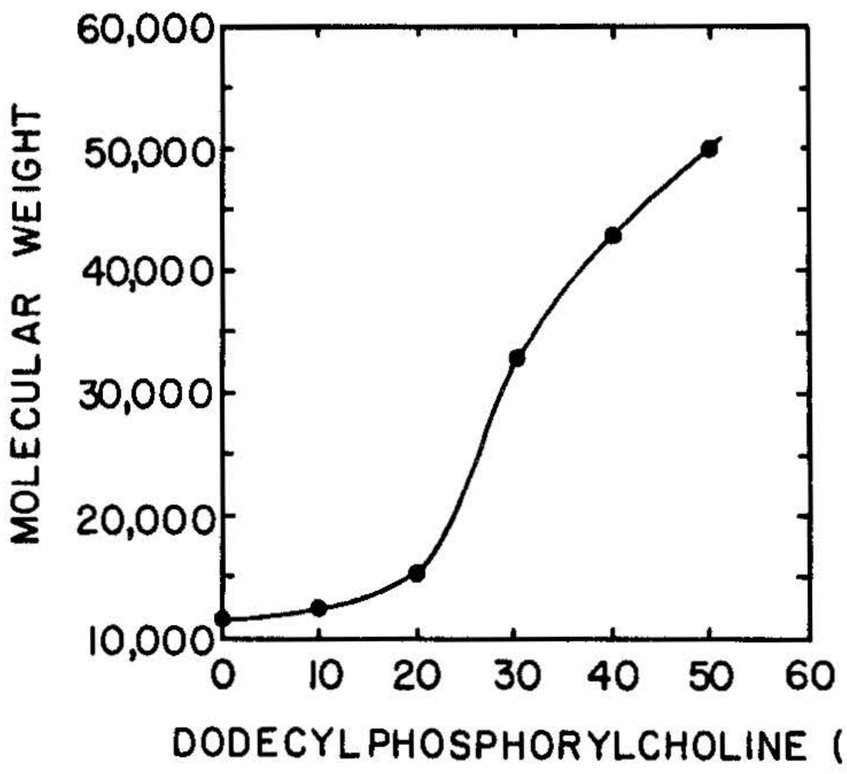

FIG. 9. **Apparent molecular weight of cobra venom phospholipase A_2 as a function of dodecyl phosphorylcholine concentration, as determined by gel chromatography on Sephadex G-75.** The buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM $BaCl_2$, and dodecyl phosphorylcholine as indicated.

daltons. The enzyme from *N. naja naja* is slightly retarded, suggesting adsorptive interaction with Sephadex. In contrast to the other enzymes, the enzyme from bee venom contains sugar residues; it shows strong adsorptive binding and is extremely retarded. The fact that the elution peak contains full enzymatic activity precludes that enzyme fragments are being eluted.

The substrate analog *n*-dodecyl phosphorylcholine, which was investigated as activator and inhibitor (10), was included in the column elution buffer at a concentration far below its critical micellar concentration of 1 mM (23). Of all the enzymes tested, only the phospholipase from *N. naja naja* showed a marked increase in molecular weight. The adsorptive interaction of the bee venom enzyme was not decreased, and the elution volumes of the enzymes from porcine pancreas and *C. adamanteus* remained those of a monomer and a dimer, respectively.

When the effect of *n*-dodecyl phosphorylcholine concentration on the apparent molecular weight of the phospholipase from *N. naja naja* was investigated, a gradual increase with increasing concentration was observed (Fig. 9). At even higher concentrations (125–500 μM), the enzyme eluted at the void volume of the column. It must be pointed out that there is considerable uncertainty in assigning aggregation numbers to molecular weights (even if the influence of the detergent molecules themselves is neglected), since they may assume an unusually extended structure and elute early. However, it appears extremely unlikely that a dimer could elute in the void volume; therefore, the existence of aggregates larger than dimers is reasonable.

DISCUSSION

Activation—The results reported here confirm that the *N. naja naja* enzyme catalyzed hydrolysis of PE is activated 20–30-fold by phosphorylcholine-containing lipids and that fatty acid, lyso-PE, and DTAPS do not activate. In addition, we found that the activator *n*-dodecyl phosphorylcholine also caused this enzyme to aggregate, thus linking aggregation and activation. This is consistent with a model containing two types of functional sites: an activator site and a catalytic site (9–11); the details of the model will be considered later.

Similar to the *N. naja naja* enzyme, the *C. adamanteus* enzyme shows a preference for PC, although the preference is only about 4-fold compared to the 30-fold preference of the *N. naja naja* enzyme. Since the *C. adamanteus* enzyme is also a dimer that presumably can bind two PC molecules, one might expect it to show activation, but no activation was observed under these experimental conditions. The fact that the *C. adamanteus* enzyme hydrolyzes PC only slightly faster than PE and is always a dimer could mean that it is always activated and that the rates observed are in fact the activated ones. Further experiments are required to clarify this question.

Neither the bee venom nor the pancreatic enzyme showed a preference for PC or PE as substrate nor did their relative rates change when a phosphorylcholine-containing activator was present. In addition, *N. naja naja* activators did not cause either enzyme to aggregate. We conclude that these two enzymes are not activated by activators of the *N. naja naja* enzyme. However, the pancreatic enzyme is activated by negatively charged surfactants. This activation seems to effect both PC and PE hydrolysis to the same extent. It is interesting to note that these negatively charged detergents and substrate analogs have now been found to aggregate the pancreatic enzyme (25–27).

It is possible that the only difference between the *N. naja naja* and the pancreatic enzymes lies not in the presence or absence of activation, but rather in the specificity of the activation. Since alternative substrates or products are involved, an unusual kinetic complexity results. For the *N. naja naja* enzyme, the activator is a phosphorylcholine-containing lipid, and PE hydrolysis is activated. For the pancreatic enzyme, the activator is fatty acid or bile salts, and both PC and PE hydrolysis are activated. Even the observation that the *N. naja naja* hydrolysis is only activated toward PE may be misleading. The fact that the pancreatic enzyme is activated by compounds which are not substrates allows both the activated and nonactivated rates for both PC and PE to be compared. Such is not the case for the *N. naja naja* enzyme. Since PC is both an activator and a substrate, it is not possible to measure the nonactivated rate toward PC, *i.e.* the activity toward PC, the substrate, in the absence of PC, the activator. Therefore, it is not possible to determine whether PC hydrolysis is activated or not. If PC does in fact activate itself, the activation of the enzyme from *N. naja naja* would be the same as the enzyme from the pancreas. This would leave the chemical composition of the activator as the only remaining difference between these two enzymes.

The analysis of these effects poses a question which continually plagues the interpretation of effector studies on lipolytic enzymes. Does the activator produce its effect by interacting directly with the enzyme or by modifying the interface or the substrate, and thus the activity? Previous experiments have

shown that the activation of the *N. naja naja* enzyme is via a direct interaction between the effector and enzyme (10). First, monomeric activators have been found that are not part of the substrate interface and thus could not directly alter it. Second, several compounds activate at concentrations that are unlikely to alter the interface significantly (10). Third, the specificity resides in the phosphorylcholine moiety, and not in the hydrophobic portion of the activator. The hydrophobic part would be expected to have the larger effect on the surface structure (9).

Product Inhibition and Time Courses—We conclude that the significant differences between the shape of the time courses of the different enzymes are due mainly to the influence of the fatty acid products on the rate. The response of the pancreatic enzyme to fatty acid follows directly from its activation by fatty acid discussed above. The lag period is not a pre-steady state phenomenon. It simply represents the time required for the hydrolytic production of this activator and is not necessarily indicative of a slow penetration step, which other investigators have postulated (reviewed in Ref. 3).

More intriguing is the behavior of the *N. naja naja* enzyme which is both inhibited and activated by the same compound. Fatty acid inhibits the reaction on PC; yet, when PE is the substrate, fatty acid does not inhibit hydrolysis, but activates it. How does a single compound both activate and inhibit the same enzyme? A possible explanation lies in the activation of PE hydrolysis by phosphorylcholine-containing lipids. If PC requires activation and fatty acid, acting as a poor activator, competes with PC for the activator site, increasing the fatty acid concentration would decrease the concentration of activated enzyme and thus produce the observed inhibition. When PE is hydrolyzed in the absence of PC, the enzyme is not activated to begin with. The binding of fatty acid to the activator site would not inhibit the rate, but would increase it slightly. Again, the lag time represents the time required for the hydrolytic production of sufficient fatty acid activators to saturate the enzyme. This hypothesis can also explain why the pancreatic enzyme was not inhibited since the fatty acid is a powerful activator in this case.

The delineation of the activation mechanism is complicated by the fact that it is not known whether molecules other than the optimum activator bind to the activator site of the respective enzyme. If, in the cobra venom enzyme, PE did not bind to the activator site (or did so very weakly), the intrinsic binding constant of the fatty acid to the activator site would be observed. In the presence of a stronger activator, only an apparent binding constant for the fatty acid would be observed, depending on the amount and affinity of the other activator as in any competition experiment.

This complexity is illustrated in Fig. 8. If one adds equal concentrations of the poor activator fatty acid and the strong activator lyso-PC at concentrations below their approximate binding constants under these conditions (9–11), a slight further activation by fatty acid is observed over the effect of lyso-PC alone. If the enzyme is far from saturation with respect to either activator, the sum of activated species will be higher when both fatty acid and lyso-PC are present than in the presence of either activator alone. At higher concentrations of the better activator lyso-PC (or PC), inhibition of the enzyme saturated in the better activator by the poorer activator results. This is consistent with the results shown in Fig. 3*A*.

The enzyme from *C. adamanteus*, however, does not easily fit the same hypothesis. This enzyme also shows inhibition by fatty acid if the substrate is PC, but not if the substrate is PE. Phosphorylcholine-containing compounds do not dramatically activate this enzyme toward PE hydrolysis. If we assume the enzyme is permanently activated (*i.e.* does not require the binding of an activator), then the inhibition by fatty acid cannot be due to the competition for the activator site. If, on the other hand, the different rates toward PC and PE were due to differential activation by these compounds, one may explain the fatty acid effects in analogy to the cobra venom enzyme, but then, an effect of phosphorylcholine-containing compounds on PE hydrolysis would be expected. We cannot rule out a relatively small effect by other phosphorylcholine-containing compounds such as PC itself (8), but under the conditions reported here, no activation was detected.

An alternative explanation for these opposing effects of fatty acid on the snake venom phospholipase A_2 activity must also be considered. If the enzyme could bind PE and fatty acid simultaneously without interfering with the catalytic mechanism, but could not do so for PC and fatty acid because of the three additional methyl groups on PC, one might observe true product inhibition if PC is the substrate, but not if PE is the substrate. While this hypothesis explains the behavior of the *N. naja naja* and *C. adamanteus* enzymes, it does not explain why the pancreatic enzyme does not show inhibition. Of course, its active site could have slightly different binding characteristics. Another possibility for the pancreatic enzyme is that the activation by the fatty acid overshadows any product inhibition that is present.

Albumin reverses the inhibition of PC hydrolysis by fatty acid. We could not, however, demonstrate an effect of albumin on the hydrolysis of PE. Were the fatty acid a weak activator of PE hydrolysis, its removal should result in inhibition, *i.e.* a prolonged lag phase until the albumin is saturated. Such an inhibition, however, would not be observed if the affinity of the enzyme for fatty acid in the presence of PE is larger than in the presence of PC and large enough to compete successfully with albumin for fatty acid. If the intrinsic binding constant of fatty acid to the activator site is observed with PE but an apparent, weaker, binding constant is observed with a large excess of PC, which should compete for the activator site, then the differential affinity of fatty acid for the enzyme depending on substrate can be explained, and therefore the observed kinetic effect. It is, however, not known whether the equilibrium or kinetics of fatty acid extraction by albumin are different in PC or PE mixed micelles.

We note that although the initial rates can be obtained quite unequivocally in the presence of albumin, its inclusion brings no increase in precision between assays of identical composition (data not shown) and offers, therefore, no advantage that would justify its regular inclusion in routine assays. In fact, the addition of another protein into the assay would just increase the complexity of an already complex system.

Conclusion—The results reported here suggest that while these four phospholipase A_2 can carry out the same catalytic reaction on identical substrates, major differences in their kinetics do exist. We found that the dramatic activation of PE hydrolysis by phosphorylcholine-containing lipids was unique to the *N. naja naja* venom enzyme among those tested, that the pancreatic enzyme's hydrolysis of both PC and PE was activated by fatty acid but not phosphorylcholine-containing lipids, and that the PC, but not PE, hydrolysis catalyzed by both snake venom enzymes was strongly inhibited by fatty acid.

Can these diverse kinetics be explained by a single mechanistic model valid for all four enzymes, or are these enzymes in fact quite different and follow distinctly different mechanisms? While a final answer to this question is still not within

easy reach, there are some correlations that can be made. We have found that the apparent differences in the specificities of the *N. naja naja* and pancreatic enzymes orginate from similar activation phenomena. Both enzymes can be aggregated by their respective activators and the *C. adamanteus* enzyme is a dimer as well. It may be that all of these enzymes are active as dimers or higher oligomers (as we originally suggested by the "dual phospholipid" model (28)) and that they can be activated, albeit by different compounds. In addition, the snake venom enzymes' strong inhibition by fatty acid indicates a similarity in their mechanisms. The structural similarity between the phospholipase A_2 from *Crotalus atrox* and bovine pancreas is striking (1). These facts make a common model of interfacial catalysis involving both activation and enzyme aggregation both plausible and very attractive.

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