

Cobra venom phospholipase A₂: A review of its action toward lipid/water interfaces

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Summary

This review focuses on the mechanism of action of phospholipase A₂ from cobra venom (*Naja naja naja*) toward the lipid/water interface. Particular points of interest include dramatic changes in the enzyme activity if the physical state of its substrate is altered and the activation of the enzyme by phosphorylcholine containing lipids. The experimental findings include the following: Micellar substrates are hydrolyzed faster by the enzyme than various bilayer forms of substrate aggregation. The activity of the enzyme toward short chain phospholipids increases suddenly above their critical micelle concentrations. An abrupt change in susceptibility to the enzyme is observed at the thermotropic phase transition of phospholipid vesicles. The enzyme shows the kinetic phenomena of surface dilution and activation by certain lipids, which suggest a two-step mechanism of action. A model is discussed which accommodates the present data both for the action of this enzyme at various lipid/water interfaces as well as its interaction with synthetic monomeric ligands and substrates.

Introduction

Lipolytic enzymes act *in vivo* on triglycerides and phospholipids in structures such as mixed micelles with bile salts in the digestive tract, in lipoproteins and triglyceride droplets during the general metabolism of fats, and on phospholipids in membranes during the biosynthesis and degradation of membrane lipid. Most of what we know about enzyme mechanisms comes from the study of water-soluble enzymes which act on water-soluble substrates. Yet those enzymes which act in or on membranes and other lipid-water interfaces raise new and challenging questions (1). Phospholipase A₂ is one such enzyme. While it is water-soluble and carries out a classical hydrolytic reaction as shown in Fig. 1, it acts best on its substrate phospholipid when the substrate is in an aggregated structure such as a micelle or membrane. The activity of phospholipase A₂ is generally highest in the presence of

detergent which forms mixed micelles with its lipid substrate (2).

Phospholipase A₂ is very widespread in nature (for a recent review, see (3)). It occurs in tissues as diverse as pancreas and lung. It has been implicated as the control step in the generation of arachidonic acid for prostaglandin biosynthesis in human

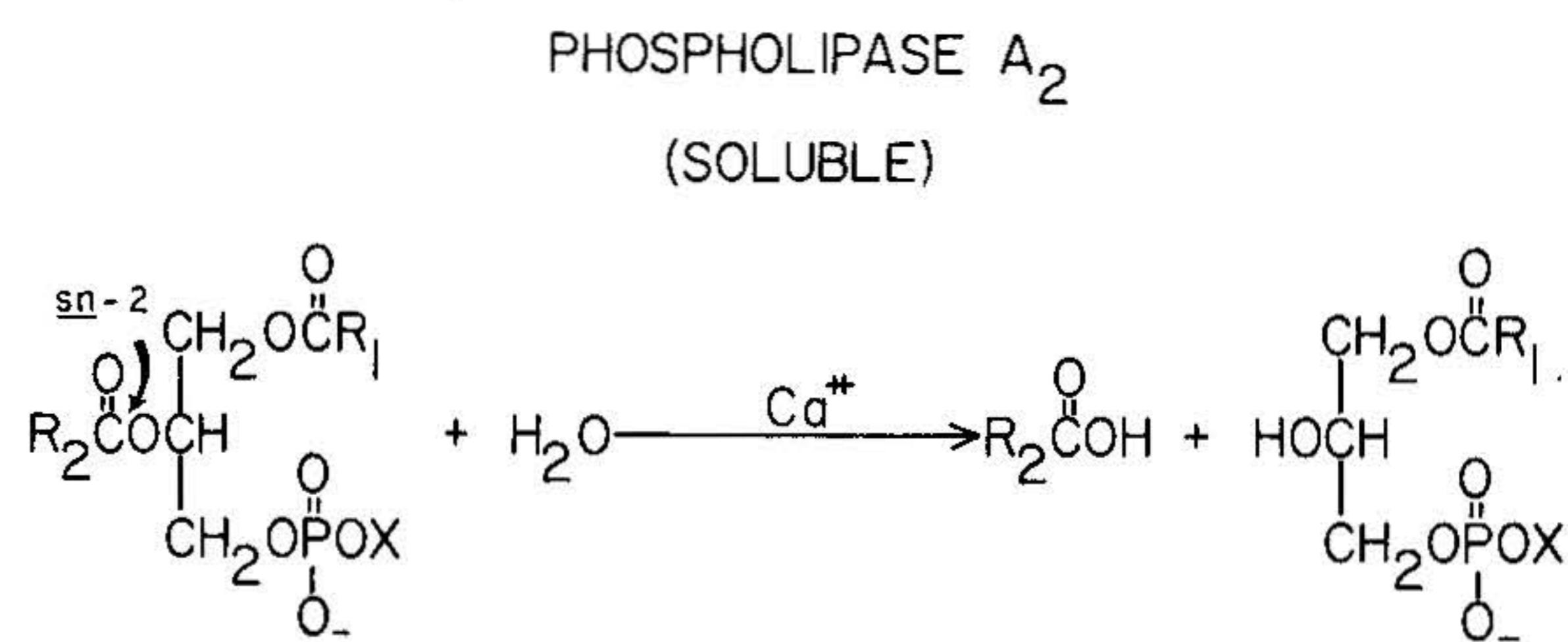


Fig. 1. Reaction of phospholipase A₂ (E.C. 3.1.1.4) at the *sn*-2 position of phospholipid to produce lyso-phospholipid and fatty acid as products. The enzyme has an absolute requirement for Ca⁺⁺.

tissues. It is also found in abundance in bee and snake venoms. Our laboratory has been studying the enzyme obtained from the venom of the Indian cobra of the *Naja naja naja* species (4, 5). This enzyme can be considered as a paradigm for lipid and membrane requiring enzymes because it interacts with phospholipids. However, unlike many membrane enzymes, phospholipase A₂ is particularly suited for study because it is a small, soluble protein of molecular weight 13 000, it has a turnover number of 50 000, and is highly resistant to denaturation presumably due in part to its 7 disulfide bonds (5, 6). It is the mode of action of this enzyme on mixed micelles and other membrane models that is the subject of this review.

An important observation made in the early 1970's by De Haas & coworkers (7) was that when synthetic water-soluble phospholipids containing short chain fatty acid groups were used as substrates for phospholipase A₂, the enzyme hydrolyzed the phospholipids appreciably only when they were at concentrations significantly above the critical micelle concentration (cmc) of the lipid. That is, the enzyme acted very poorly on monomeric phospholipids compared with micellar phospholipids. Over the years, several explanations have evolved to account for the requirement of phospholipase A₂ for substrate in aggregated forms and the importance of the interface including:

(i) An alteration of the physical state, such as conformation, hydration or orientation of the substrate phospholipid in going from monomers to micelles.

(ii) A conformational change and activation of the enzyme as a result of binding to phospholipid, whether it be interfacial or monomeric.

(iii) An activation of the enzyme resulting from its association with a lipid/water interface, such as penetration of the hydrophobic core of micelles.

(iv) A concentration effect, whereby the enzyme experiences a higher local concentration of substrate as a result of substrate aggregation, causing the enzyme to be closer to saturation. The effect could be brought about by: (a) An initial binding to interfacial phospholipid holding the enzyme close to the concentrated substrate which it then binds in its active site in a subsequent step. (b) An initial binding to the interface, followed by catalytically productive association with the concentrated substrate. This would be similar to

situation (a), but with the initial absorption to the interface being nonspecific as with an interfacial recognition site. (c) A higher probability of the enzyme reacting with further substrate merely as a result of there being regions in the solution (micelle, vesicles, etc.) of high substrate concentration and proximity, as opposed to the situation with monomeric substrate which is randomly dispersed throughout the solution. For a more detailed review of the experiments leading to some of these explanations, see Verger (8).

We have recent NMR evidence that there are differences in the conformation and three-dimensional structure between monomers and micelles (9) which support explanation (i), but we also have evidence that there is a phospholipid induced enzyme conformational change (10, 11) as in explanation (ii). De Haas & coworkers (12, 13) have conducted experiments with the enzyme from porcine pancreas which they have interpreted in terms of explanation (iii) involving an interfacial recognition site at the N-terminal end of the protein (14). Explanation (iv) is also consistent with some experimental observations. Clearly, any one or some combination of all of these explanations could account for the enhanced activity of phospholipase A₂ toward aggregated structures. We will first examine the aggregated structures formed by the phospholipid substrate.

Phospholipid substrate structure

We have recently used NMR to study the α -methylene protons adjacent to the carbonyl group on the fatty acid chains of phospholipid designated *sn*-1 and *sn*-2 as shown in Fig. 2. For phospholipids in micelles, the two groups are shifted 0.1 ppm from one another (15) and the *sn*-2 protons give rise to an AB quartet (9). The NMR studies show (9) that (i) the *sn*-1 and *sn*-2 α -methylene groups exhibit much greater chemical shift differences in micelles and mixed micelles than in monomers, (ii) the two *sn*-2 α -methylene protons give rise to the AB quartet system and are only observed to be non-equivalent in mixed micelles, and (iii) the two *sn*-1 α -methylene protons do not show the AB pattern in any system and their upfield chemical shift in mixed micelles suggests a more hydrophobic environment. We have recently found that all of this is the

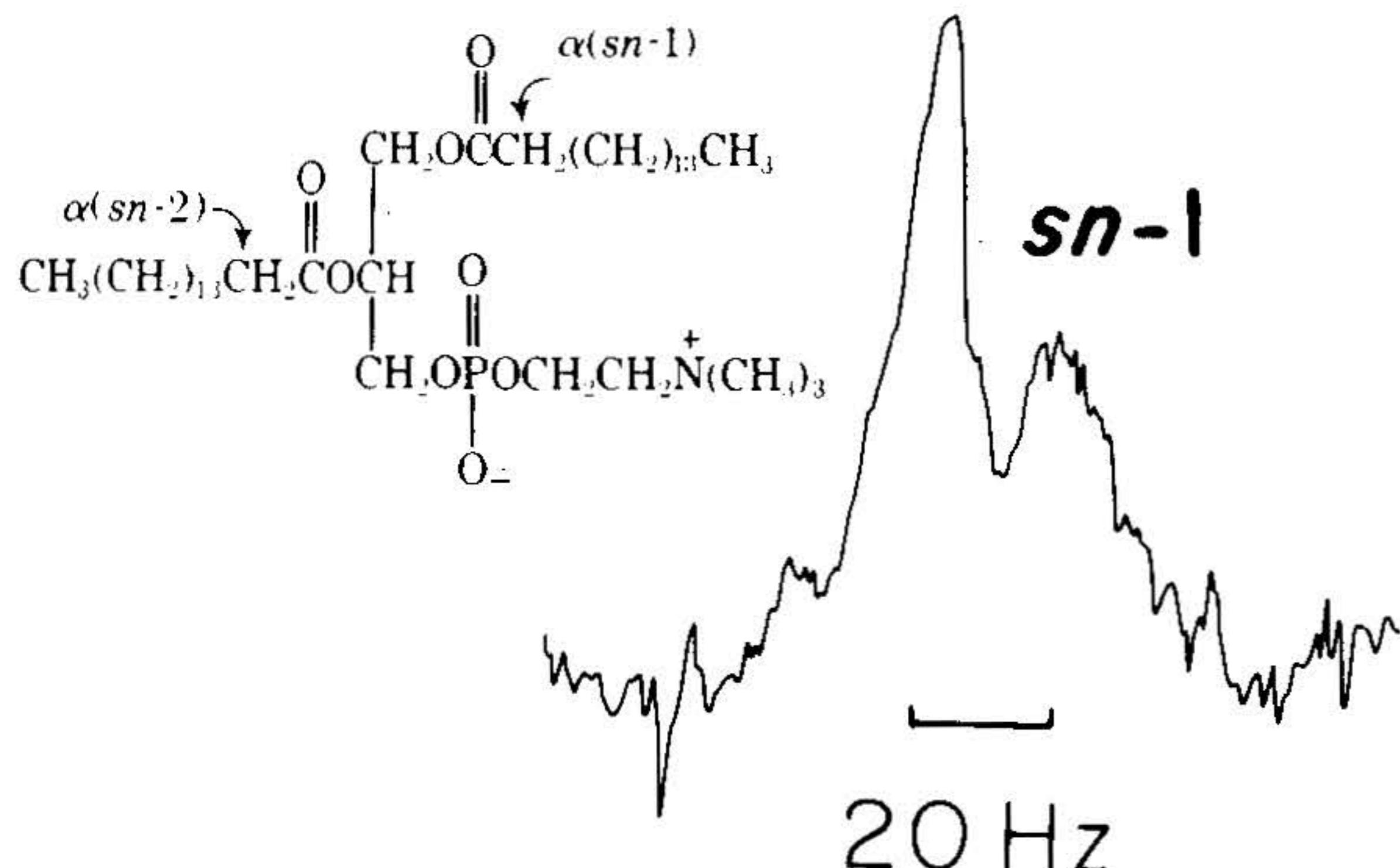
sn-2

Fig. 2. ^1H -NMR at 220 MHz of the α -methylene protons of phosphatidylcholine in mixed micelles with Triton X-100 at a molar ratio of Triton: phospholipid of 8:1. The protons on the *sn-1* and *sn-2* fatty acid chains are separated by about 0.1 ppm. From reference (15).

(16). Since the *sn-2* carbonyl appears to be at the interface and conformationally different from the *sn-1* carbonyl, this conformation could contribute to the *sn-2* specificity of the enzyme. However, we have recent evidence that the enzyme is also specific for the *sn-2* position in monomers although it acts poorly on these (A. Plückthun & E.A. Dennis, unpublished). In summary, Fig. 3 shows a model for a phospholipid in a lipid-water interface such as a nonionic surfactant micelle. This representation appears to be a very good working model for the conformation of phospholipids in aggregated systems be they natural membranes, crystals, multibilayers, vesicles, micelles or mixed micelles with detergents, but not necessarily for monomers (16, 17).

Cobra venom phospholipase A₂ action on bilayer membranes

Even if the conformation discussed in the last section holds in all kinds of aggregated structures, there must be more subtle differences in the phospholipid packing in different sorts of membranes as judged by their susceptibility to phospholipase A₂ attack (18). As shown in Table 1, phospholipids packed in sonicated vesicles and in multibilayers are much poorer substrates than mixed micelles when compared by their V_{\max} 's. Furthermore, the physical state of the phospholipid in small unilamellar vesicles affects the activity of the enzyme (18) as shown in Fig. 4. If the activity were not affected by a physical state change caused by the thermotropic phase transition, a continuous line would be expected in the Arrhenius plot. However, a discontinuity in the Arrhenius plot was observed near the transition temperature because the apparent initial rates of activity are greater at temperatures below the thermotropic phase transi-

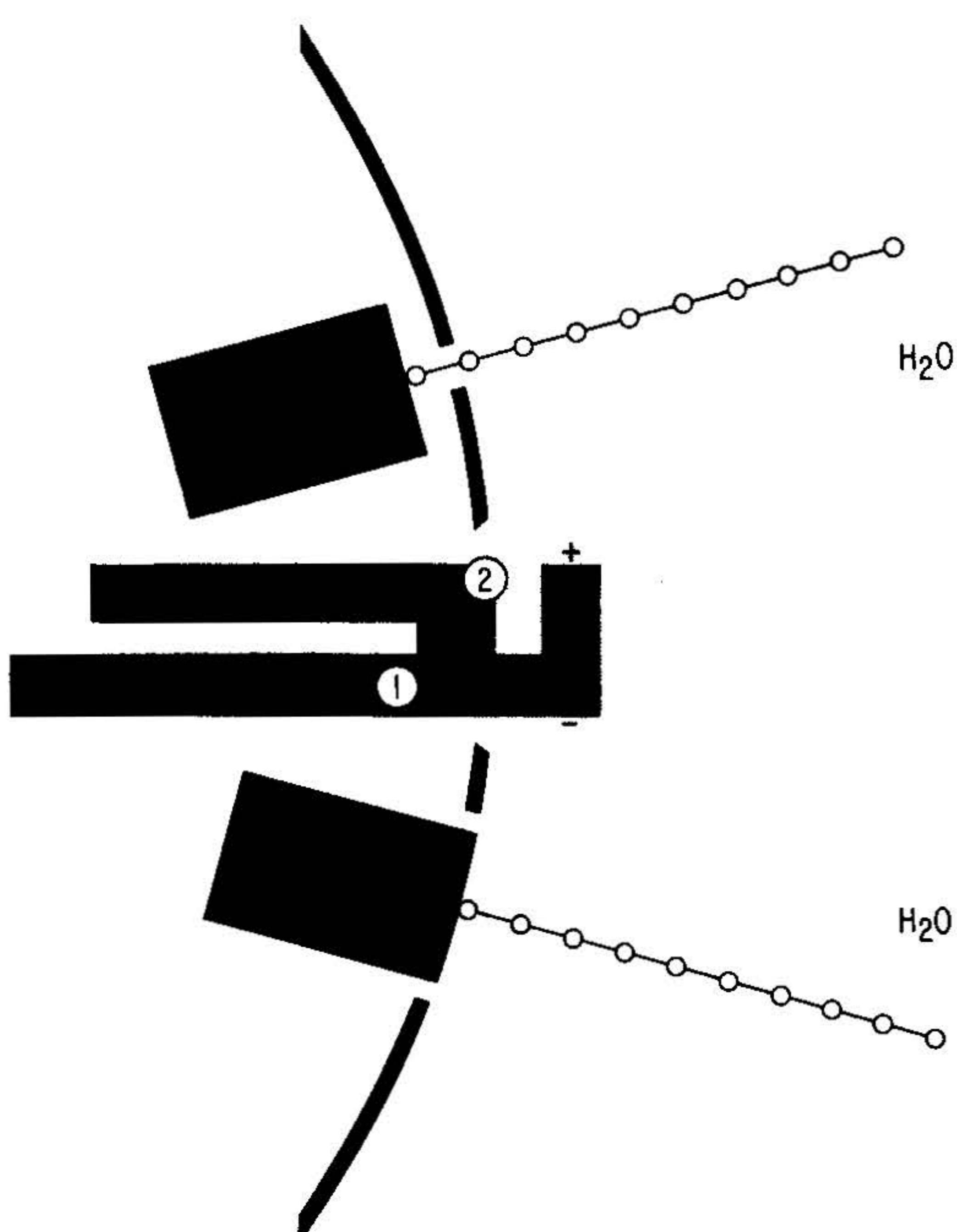


Fig. 3. 'Pipewrench' model for a phospholipid such as phosphatidylcholine in a lipid/water interface with Triton X-100. ① and ② refer to the α -methylene protons on the *sn-1* and *sn-2* chain, respectively. From reference (16).

case for sonicated vesicles as well (J. deBony & E.A. Dennis, unpublished). From these studies which show that the two α -methylene groups are magnetically non-equivalent, we have suggested the sort of picture shown in Fig. 3 for the conformation of the phospholipid in a mixed micelle interface

Table 1. Cobra venom phospholipase A₂ activity toward egg phosphatidylcholine

Aggregated state	V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_M (mM)
Vesicles	100	4.4
Multibilayers	125	7.7
Mixed micelles (Triton X-100)	4000	2.0

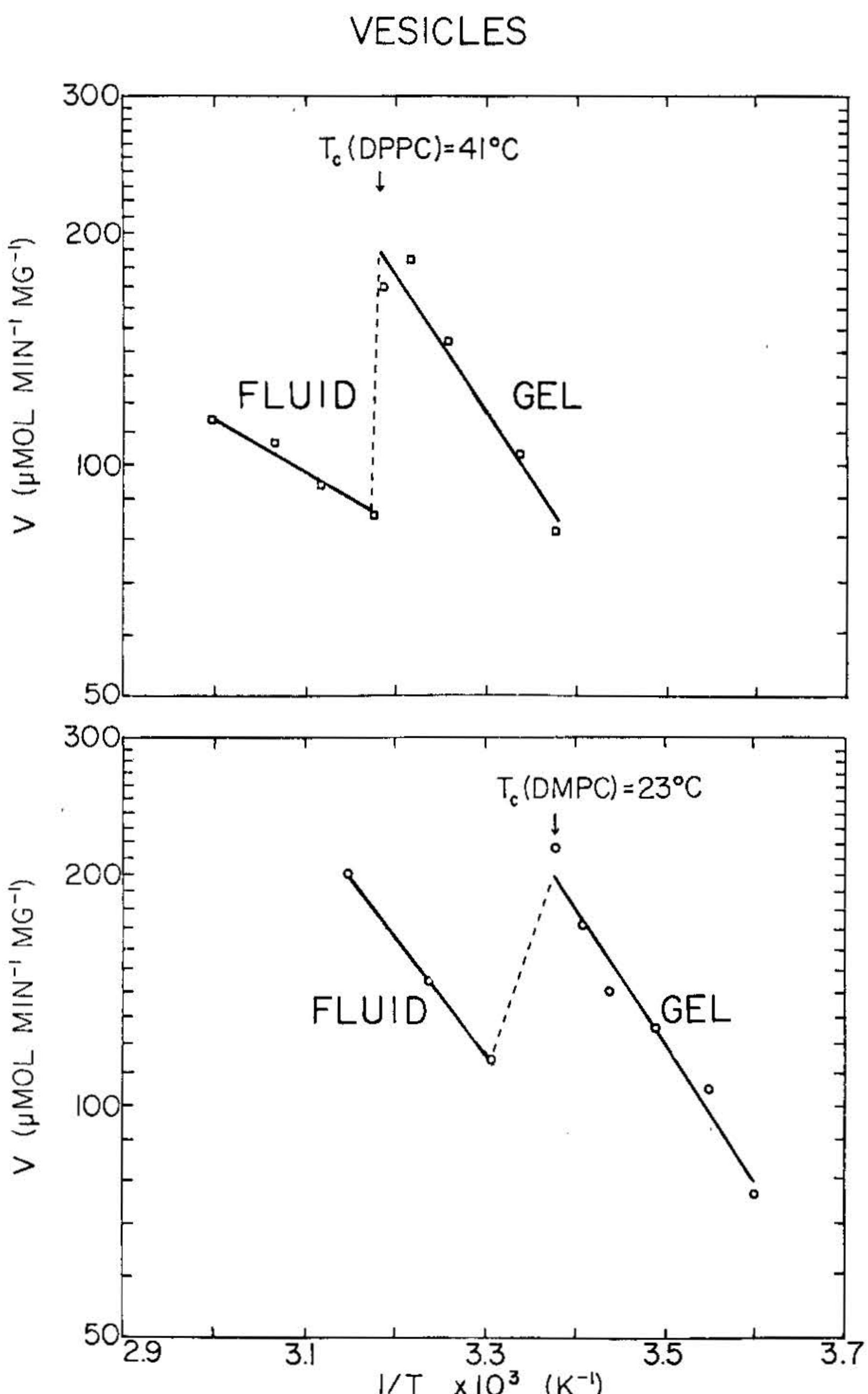


Fig. 4. Arrhenius plots of the initial rates of cobra venom phospholipase A₂ action toward dipalmitoyl phosphatidylcholine (top) and dimyristoyl phosphatidylcholine (bottom) in sonicated unilamellar vesicles. The phase transition, T_c , for each phospholipid is indicated. From reference (18).

tion where the lipid is in the gel state than above the transition where the lipid is in the fluid or liquid crystalline state. There is an increase in the phospholipase activity at long reaction times above the transition temperature, but not below it. This may be due to a change in the physical state of the phospholipid due to the presence of products above the transition temperature, but not below it. Alternatively, the initial slow rate above the transition temperature followed by increased activity may reflect a slow approach to the steady-state above the transition temperature while the steady-state rate is reached quickly below the transition temperature. At present, it has not been

Table 2. Hydrolysis of erythrocytes by cobra venom phospholipase A₂ (2 μG, 10 min, 37 °C). More complete data is given in reference 19.

	Phospholipid (mol fraction)		Phospholipid hydrolyzed
	PE	PC	Total
No enzyme	0.00	0.00	0
Intact erythrocytes	<0.01	<0.01	<1%
Ghost membranes	0.28	0.10	38%
Erythrocytes/Triton	0.28	0.05	33%

determined which of these possibilities is responsible for this effect.

Another sort of dependence on physical state is observed with natural membranes (19) as shown in Table 2. Here, one can see that the enzyme works poorly on intact red blood cells, but quite well on ghost membranes and Triton-disrupted erythrocytes. We attribute these differences to subtle changes in the membrane structure which occur upon ghosting or to a higher susceptibility to attack of the inside of the bilayer compared to the outside of the bilayer. We are currently trying to elucidate these changes more precisely. In the ghosts and in the presence of Triton, the enzyme acts much better toward phosphatidylethanolamine than phosphatidylcholine. This led to some new ideas about the specificity of the enzyme which will be discussed in the next section.

Mechanism of cobra venom phospholipase A₂ action

Activation of phosphatidylethanolamine hydrolysis

In mixed micelles, the enzyme works well on phosphatidylcholine, but poorly on phosphatidylethanolamine unless phosphatidylcholine or a phosphorylcholine containing lipid is also present (11), as shown in Fig. 5. In an equimolar mixture of phosphatidylcholine and phosphatidylethanolamine, phosphatidylethanolamine is a much better substrate than phosphatidylcholine. Phosphatidylcholine activates the enzyme toward phosphatidylethanolamine as substrate and reverses the normal specificity of the enzyme.

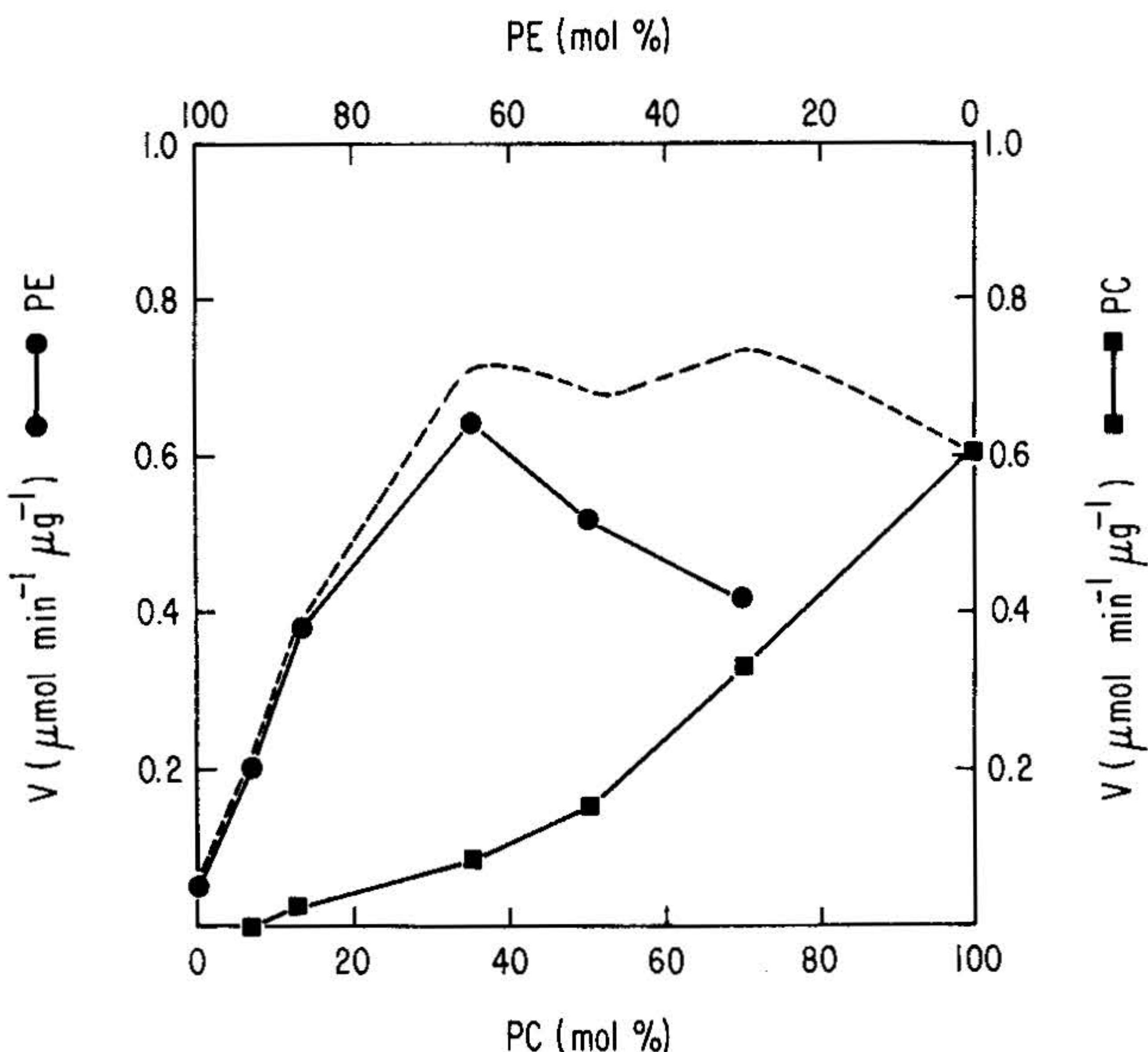


Fig. 5. Activity of phospholipase A₂ toward mixtures of phosphatidylcholine and phosphatidylethanolamine in Triton X-100 micelles. The mole ratio of Triton to total phospholipid was maintained at 8:1. The dashed line is the sum of the activity toward both phospholipids. From reference (11).

Activation toward phosphatidylethanolamine can be achieved by a variety of lipids including phosphatidylcholine, sphingomyelin, and lyso-phosphatidylcholine (11) as shown in Table 3. Most interestingly, the enzyme can be activated toward phosphatidylethanolamine by dibutyryl phosphatidylcholine. This is a monomeric phospholipid that is not incorporated into the micelles containing

Table 3. Effect of ligands on phosphatidylethanolamine hydrolysis by phospholipase A₂. Experimental conditions are given elsewhere (11).

Ligand	Rate (μmol min⁻¹ mg⁻¹)
None	30
Phosphorylcholine	30
Glycerol phosphorylcholine	35
Dibutyryl phosphatidylcholine	126
Egg phosphatidylcholine	244
β-Dipalmitoyl phosphatidylcholine	250
D-Dipalmitoyl phosphatidylcholine	271
Lyso phosphatidylcholine	246
Sphingomyelin	642
CTAB	34
Oleic acid	53
Phosphatidylserine	25
Phosphatidylglycerol	32

the substrate phospholipid. These studies led us to suggest that the enzyme contains two functional sites: an *activator* site with minimum specificity for a phosphorylcholine group attached to a hydrophobic chain and a *catalytic* site with less specificity for the polar group. The activator molecule presumably binds directly to the enzyme causing a conformational change.

There are alternative explanations (10) for the phosphatidylcholine activation of phosphatidylethanolamine hydrolysis such as: (i) direct lipid-lipid interactions in which phosphatidylcholine alters in some manner the conformation of phosphatidylethanolamine, or (ii) a subtle change in the micelle structure or surface charge induced by phosphatidylcholine occurs even though it is zwitterionic itself. Our hypothesis of a direct activator-enzyme interaction rests on proof that dibutyryl phosphatidylcholine is not incorporated into the micelle, yet activates phosphatidylethanolamine hydrolysis.

To prove that dibutyryl phosphatidylcholine is not in the micelle, our strategy was to study dihexanoyl phosphatidylcholine which is incorporated into Triton micelles, and show that dibutyryl phosphatidylcholine is not incorporated under the same conditions. The following three techniques were used which all show differences between monomers and micelles (20): (i) ³¹P-NMR chemical shift differences between monomers and micelles, (ii) ¹H-NMR chemical shift differences between the *sn*-1 and *sn*-2 α-methylene groups as well as the AB quartet characteristic of the *sn*-2 α-methylene protons in micelles, and (iii) direct binding studies using gel chromatography. For dihexanoyl phosphatidylcholine, the partitioning of it into micelles over this concentration range can be followed by these techniques, while dibutyryl phosphatidylcholine is not significantly incorporated into the micelles, even when phosphatidylethanolamine is included. At 20 mM Triton, which was used in activation studies, less than 2% dibutyryl phosphatidylcholine is incorporated as shown by ³¹P-NMR. Within error limits of 5%, similar results were found by the other techniques (20). Therefore, lipid-lipid interactions or micelle alterations are very unlikely to be the cause of the activation process.

Phospholipase A₂ hydrolysis of phosphatidylethanolamine as a function of added dibutyryl

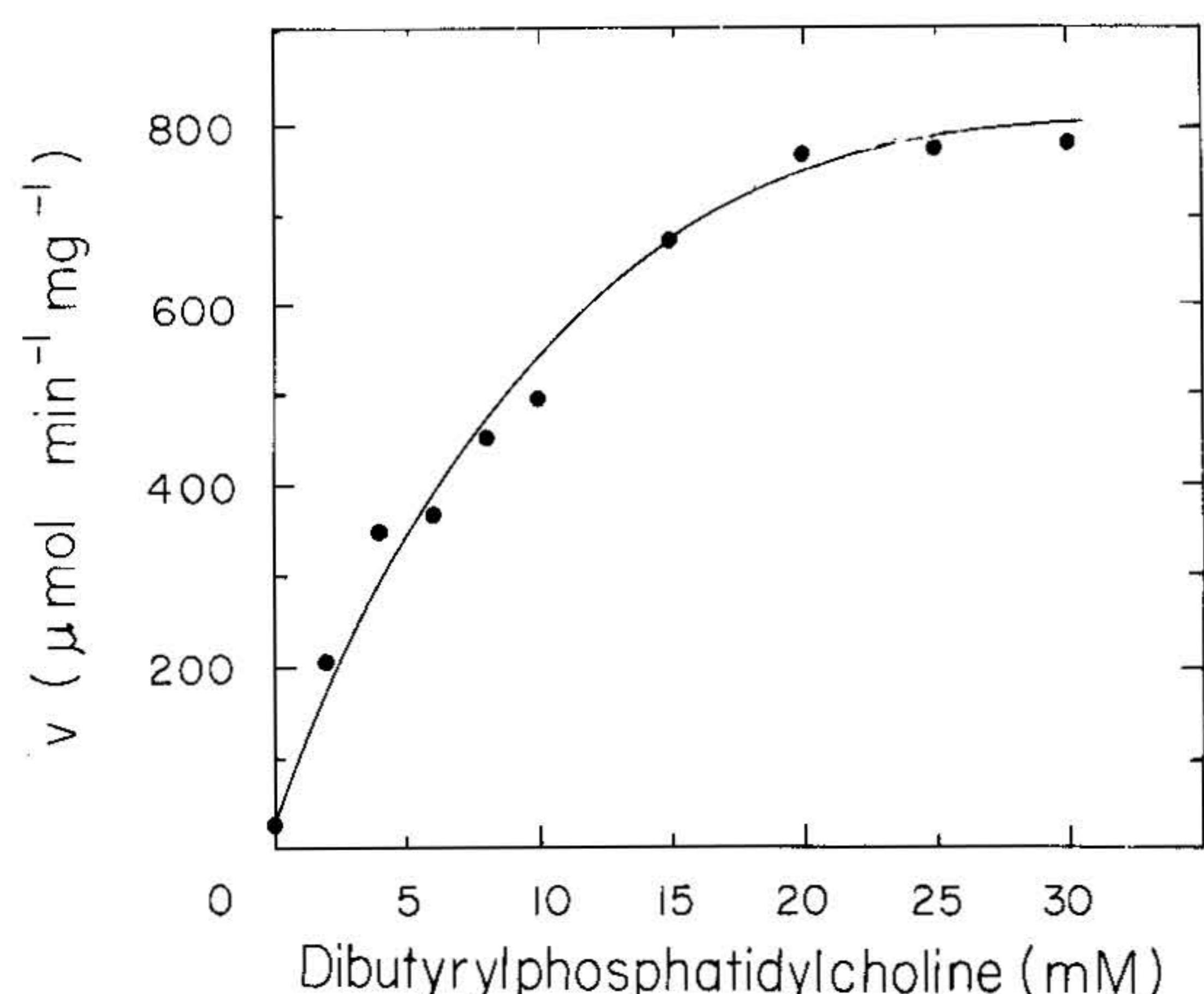


Fig. 6. Activity of phospholipase A₂ toward phosphatidylethanolamine in mixed micelles with Triton X-100 as a function of added dibutyryl phosphatidylcholine.

phosphatidylcholine in the presence of 20 mM Triton is shown in Fig. 6. The dibutyryl phosphatidylcholine is not hydrolyzed, and the activation shows saturation behavior (A. Plückthun & E. A. Dennis, unpublished). These rates were followed by ³¹P-NMR which differentiates phosphatidylethanolamine, phosphatidylcholine, and the lyso products. Because dibutyryl phosphatidylcholine activates, but is not incorporated into the micelle itself, activation by dibutyryl phosphatidylcholine is only consistent with a direct enzyme interaction and two functional sites: an

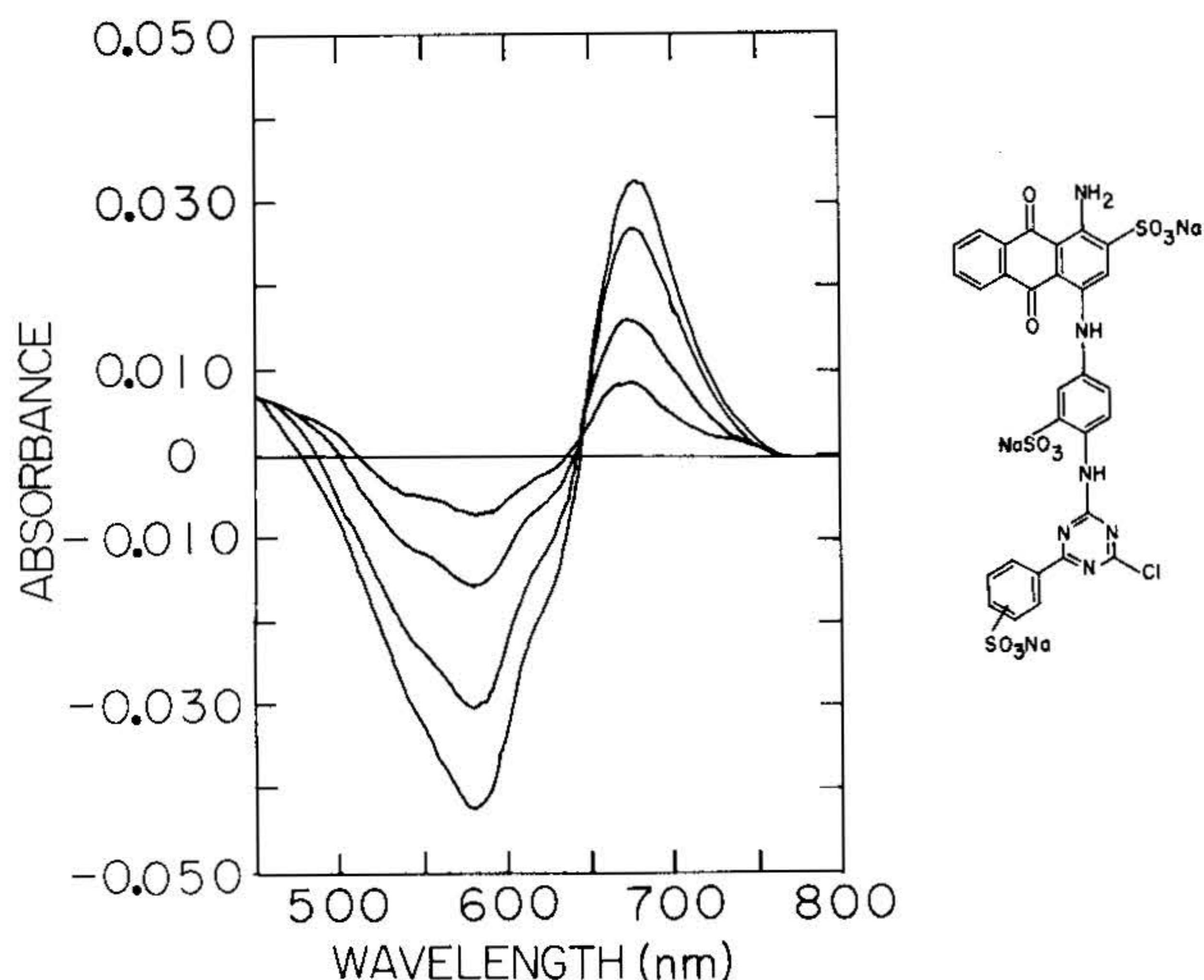


Fig. 7. UV difference spectra of Cibacron Blue F3GA whose chemical formula is shown, in the presence of increasing amounts of Cibacron Blue F3GA: 2.26, 5.94, 12.26 and 28.1 μM. From reference (21).

activator site and a catalytic site.

Experiments which directly differentiate lipid binding to the two putative sites would be desirable. Phospholipase A₂ binds to the dye Cibacron Blue (21) as illustrated in the series of difference spectra shown in Fig. 7. Several lines of evidence indicate that this relatively large dye covers (or is in) the active site of the enzyme (21). Furthermore, the substrate dihexanoyl phosphatidylcholine, when at monomer concentrations, can displace the dye in the absence of metal ions, but using the method of Kitz and Wilson (23), it does not compete with the active site reagent *p*-bromophenacyl bromide (22) (P.L. Darke, unpublished observations). Thus, the dye binding is sensitive to a phospholipid binding site separate from the site of *p*-bromophenacyl bromide reaction (an active site histidine). This indicates either binding to an activation site, or association of the substrate with a region of the protein near the active site in a non-specific or poorly oriented fashion.

Surface dilution kinetics

The activity of phospholipase decreases as the molar ratio of detergent to phospholipid is increased (2, 24). In addition to phosphatidylcholine, this also occurs for phosphatidylethanolamine alone as well as when the enzyme is activated by sphingomyelin (A. Plückthun & E. A. Dennis, unpublished). An explanation for this observation is that the enzyme first binds to the interface in some manner and then binds a phospholipid in its active site to carry out hydrolysis. In the presence of long chain phospholipid, the activation step would also at the same time attach the enzyme to the interface. Then its binding to the substrate phospholipid would depend on the phospholipid concentration in the two-dimensional interface. Thus as more Triton is added, surface dilution reduces the activity. However, when sufficient dibutyryl phosphatidylcholine is present to fully activate the enzyme, but it is not in the micelles, surface dilution does not occur (A. Plückthun & E. A. Dennis, unpublished).

Surface dilution is illustrated in Fig. 8. These are schematic drawings based upon theoretical calculations (25, 26) for the structure of the mixed micelles. An important feature of the mixed micelles is that the surfactant forms an inert matrix

Monomer/dimer equilibrium of phospholipase A₂

Cobra venom phospholipase A₂ is monomeric at low concentrations, but forms dimers at high concentrations (4, 5). Cross-linking experiments with dimethyl suberimidate suggest that substrate phospholipid induces aggregation at monomeric concentrations (29). In the presence of divalent metal ion alone or Triton and phospholipid alone, there is little dimer formation. But in the presence of mixed micelles composed of Triton, phospholipid and Ba⁺⁺, there is a large increase in the dimer to monomer ratio indicating an increase in the level of aggregation. This suggests that the enzyme may actually function as a dimer when bound to phospholipid. Recent experiments from other laboratories on phospholipases from other snake venoms support this idea (M. Wells, personal communication). There is also evidence that the active form of the enzyme from *Crotalus adamanteus* venom is a dimer (30, 31).

Dual phospholipid model

In summary, the following phenomena bear on the mechanism we would like to suggest to explain our experimental results: (i) phospholipid induced aggregation of the enzyme suggesting the possibility of functional dimers, (ii) surface dilution kinetics suggesting a two-step mechanism, and (iii) phosphatidylcholine activation of phosphatidylethanolamine hydrolysis suggesting two sites, one for activation and one for catalysis. In Fig. 9 is shown the "dual phospholipid" model which we proposed several years ago (29) as a working model for the action of this enzyme. This model is consistent with the recent experiments showing separate activator and catalytic sites involving the binding to two phospholipid molecules. This model could explain why phosphatidylcholine activates the enzyme toward phosphatidylethanolamine as a substrate if phosphatidylethanolamine cannot cause the prerequisite conformational change or does so only slowly, but can serve well as a substrate.

Although the model accommodates our data at this point, it includes several steps which are hypothetical, and thus provides a framework for further experimentation. The phenomena of

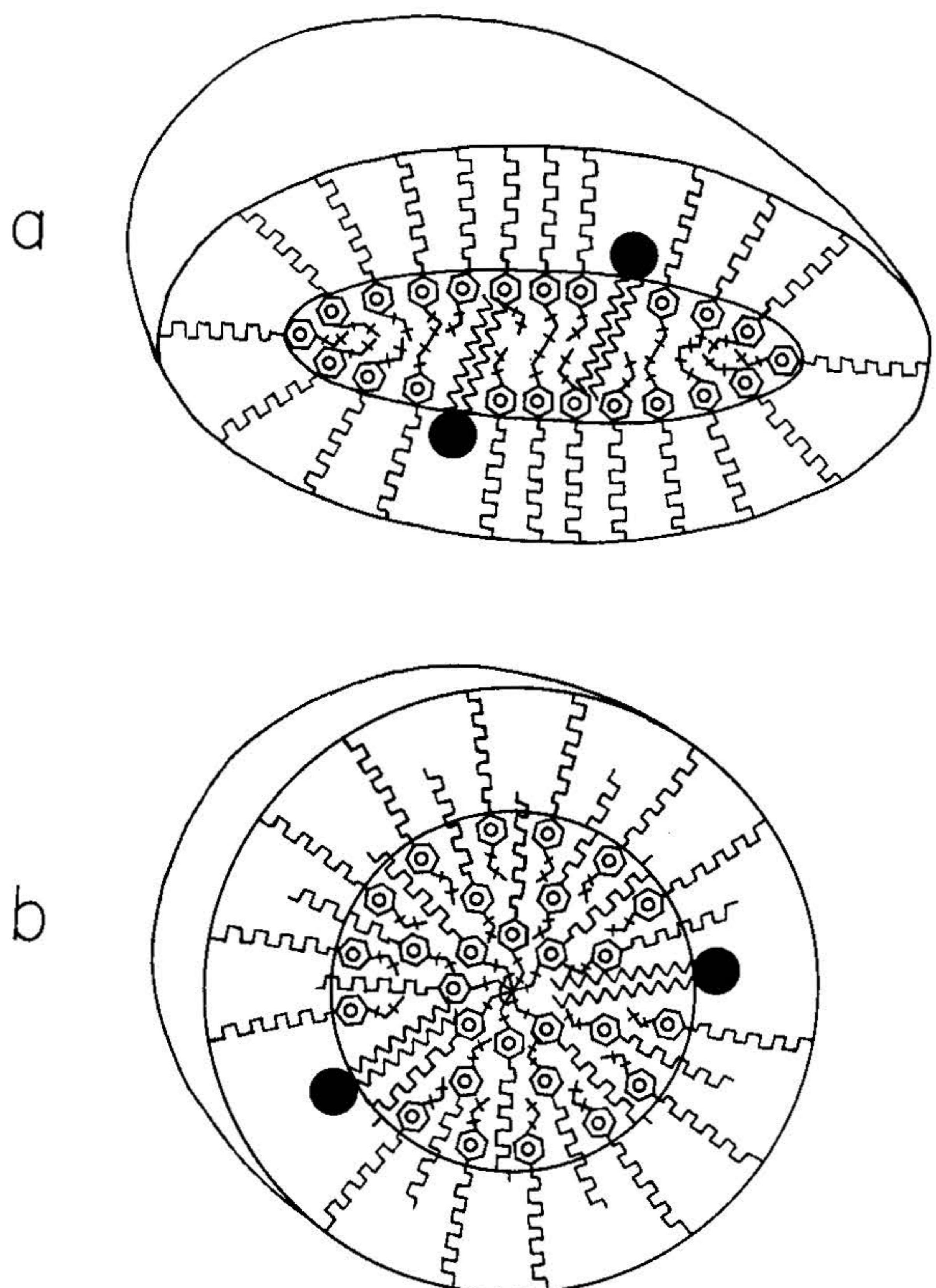


Fig. 8. Models for the structure of Triton X-100 micelles based on theoretical calculations at a high molar ratio of Triton to phospholipid. The structure is either a classical oblate ellipsoid (a) or a non-classical spherical micelle (b) which contains a non-distinct hydrophobic-hydrophilic interface region in which some of the oxyethylene chains are embedded in the hydrophobic region. From reference (26).

in which the phospholipid is imbedded and this allows one to vary the concentration of phospholipid in a lipid-water interface by varying the surfactant to phospholipid ratio (27). The activity of the phospholipase depends directly on this concentration and this should occur if the enzyme binds to the interface in some manner (28) before binding its substrate phospholipid. This binding can be non-specific as de Haas & coworkers (12, 13) suggest with an interfacial recognition site which binds to the hydrophobic core, or it can constitute the binding to phosphatidylcholine in an activation step thereby linking the enzyme to the interface. Whatever the specific mode of attachment, this suggests a two-step mechanism which leads to 'surface dilution kinetics' (24, 28).

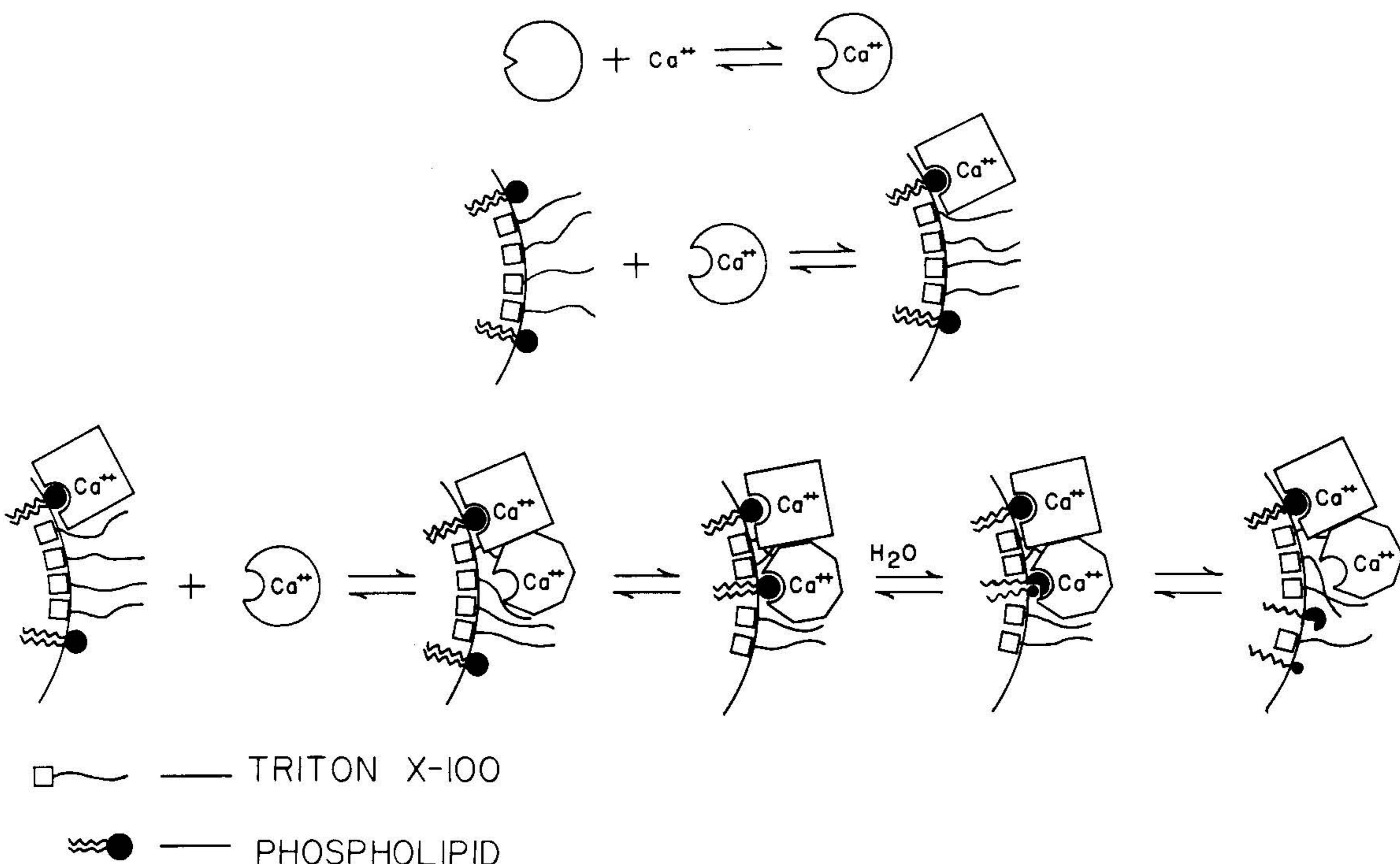


Fig. 9. The steps in the 'dual phospholipid' model in reference (29) for phospholipase A₂ action are shown: (i) Enzyme first binds Ca^{++} in an ordered reaction as shown in the top. (ii) This allows the enzyme to bind to phospholipid in the mixed micelles as shown in the second line. The phospholipid must be phosphatidylcholine or a related analogue to cause the enzyme to undergo the desired conformational change indicated by a circle to a square. With normal phospholipids, this binding automatically attaches the enzyme to the micelle, although with dibutyryl phosphatidylcholine, it would not. (iii) The activated enzyme may then form a dimer as shown on the third line. One subunit of this dimer is then responsible for the activation (square) while the other (octagon) catalyzes the hydrolysis of an accessible phospholipid. This can be phosphatidylcholine or most other phospholipids. Lateral diffusion of phospholipid in the mixed micelle may be involved before the catalytic subunit binds phospholipid. Alternatively, two micelle-bound enzyme molecules may dimerize directly. Once catalysis occurs, the products may diffuse away from the enzyme and either be retained in the mixed micelle or released into the solution.

enzyme activation toward phosphatidylethanolamine and surface dilution could both be explained by two sites on a single monomeric enzyme, in which case the octagon and the square in Fig. 9 would represent separate functional domains on a monomeric enzyme. We are currently trying to clarify whether or not phospholipid and activators induce dimerization of the protein at the low (100 ng/ml) protein concentrations used in kinetic experiments. However, even if activators induce dimerization, this does not rule out the monomer as an active unit. The stoichiometry of phospholipid and Ca^{++} association with the cobra venom enzyme also must be established. The extent to which the rate of catalysis is affected by conformational changes of the protein and changes in the physical state of its substrates must still be further elaborated.

Acknowledgements

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