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INTERACTION OF ENZYMES WITH MIXED MICELLES OF PHOSPHOLIPID AND DETERGENT: Analysis of the Phospholipase A_2—Dipalmitoyl Phosphatidylcholine—Triton X 100 System

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Physical studies on the formation and structure of mixed micelles of the nonionic surfactant Triton X-100 and phospholipids and enzymatic studies on the action of phospholipase A_2 toward these mixed micelles are presented. Results of nmr intensity, line width, and T_1 determinations, as well as gel chromatography and centrifugation experiments on the interaction of Triton X-100 with egg and dipalmitoyl phosphatidylcholine, are presented and discussed. The structure of mixed micelles is discussed in terms of a working schematic model which is consistent with the experimental results. Kinetic studies on phospholipase A_2 (Naja naja) action are then analyzed in terms of this model. The temperature dependence of phospholipase A_2 action toward dipalmitoyl phosphatidylcholine is considered in terms of the effect of thermotropic phase transitions on mixed micelle formation. The phospholipase A_2-dipalmitoyl phosphatidylcholine-Triton X-100 system is then considered as an artificial model system for studying the effect of lipid phase separations on biological activity.

For the last few years, our laboratory has been studying the action of phospholipase A_2 toward phosphatidylcholine as substrate in mixed micelles with the surfactant Triton X-100, employing largely kinetic techniques to characterize the enzyme (1, 2) and nuclear magnetic resonance (nmr) techniques to characterize the substrate (3–5). Our orientation has been that of attempting to develop a suitable in vitro system for studying the detailed mechanism of action of enzymes of lipid metabolism; these enzymes normally act on substrates that are part of complex structures rather than ones which are molecularly dispersed (6–8). In addition, these studies provide: a) information on the use of and possible effect of Triton and other surfactants (often referred to as "detergents") in the assay of enzymes that act on or require lipids (9), b) information about the process of protein solubilization, since mixed micelles are formed when surfactants are employed

in the solubilization of membrane-bound proteins (10), c) information about the action of enzymes that act *in* or *on* natural membranes which is of particular importance for the proper use of phospholipases in studying membrane asymmetry (11) and membrane fusion (12), and finally d) these studies provide an artificial, characterizable system for studying the effects of thermotropic phase transitions and lipid phase separations on biological activity – effects which have been suggested to occur in more complicated natural membranes (13). For the purposes of this article, these latter applications will be emphasized in considering our work on the phospholipase A_2-dipalmitoyl phosphatidylcholine-Triton X-100 system.

PHYSICAL CONSIDERATIONS

Interaction of Triton X-100 and Egg Phosphatidylcholine

Dilute unsonicated dispersions of egg phosphatidylcholine in water form smectic mesophases or multilamellar liposomes containing phospholipid bilayers in a multilamellar arrangement (14, 15); for simplicity we will refer to such preparations as "phospholipid bilayers." Triton X-100 is a commercial, polydisperse preparation (16, 17) of p, t-octylphenoxypolyethoxyethanols containing an average of about 9.5 oxyethylene units per molecule and an average molecular weight of about 628 (18); it may also contain some heterogeneity in its hydrophobic moiety (16). This nonionic surfactant has a CMC of about 0.2–0.3 mM (19, 20). At concentrations above its CMC Triton forms micelles, and at high concentrations the number of molecules existing as monomers is negligible compared to the number in micelles. Nmr intensity and linewidth measurements at 220 MHz have been employed to follow the changes which occur in various protons in the hydrophobic and hydrophilic portions of the phospholipid bilayers and Triton micelles when they are mixed (3). These studies have led to the suggestion that at molar ratios above about 2:1 Triton/phospholipid, all of the phospholipid is converted to mixed micelles as shown schematically in Fig. 1.

Light scattering studies suggest that the average molecular weight of Triton micelles is about 67,000–153,000 (21–23). We have now shown that on agarose gel chromatography, Triton micelles and mixed micelles at a molar ratio of about 10:1 Triton/phospholipid elute at about the same volume, as shown in Fig. 2, suggesting that they comprise a similar size range. At lower molar ratios, where the size and polydispersity of the mixed micelles are larger and below a molar ratio of about 2:1 Triton/phospholipid, two fractions are separated: phospholipid bilayers which contain Triton at a molar ratio of about 1:1 Triton/phospholipid, and Triton micelles which contain phospholipid at a molar ratio of about 2:1 Triton/phospholipid and which will be referred to as mixed micelles. Bilayers containing Triton and mixed micelles can also be separated by centrifugation techniques and the Triton and phospholipid content in each fraction quantitated directly. The chromatographic and centrifuge experiments will be considered in greater detail elsewhere by us (E. A. Dennis, manuscript in preparation), but the results are summarized schematically in Fig. 3. Thus, phospholipid dispersions are able to incorporate a certain amount of Triton (presumably as the monomer) into their structure, but above a certain concentration, additional Triton forms mixed micelles. Thus, over a certain limited range

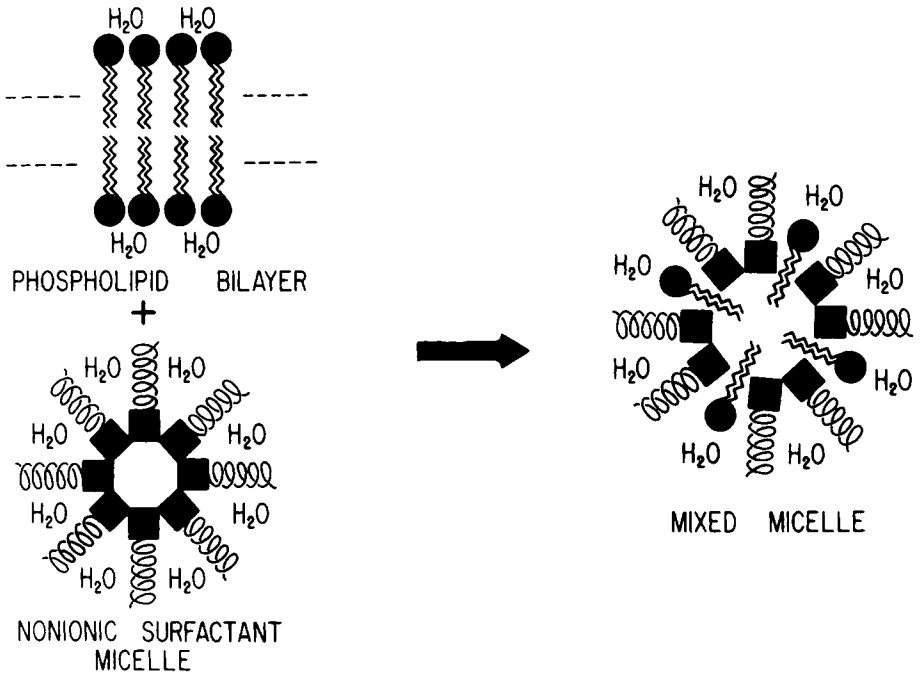

Fig. 1. Schematic diagram of the conversion of egg phosphatidylcholine bilayers and Triton X-100 micelles into mixed micelles.

of Triton concentrations, both bilayers and mixed micelles coexist, but above a molar ratio of about 2:1 Triton/phospholipid, additional Triton serves to dilute the phospholipid in the mixed micelles. It should be noted that similar changes probably occur in the structure of membrane phospholipids when surfactants are employed in the solubilization of membrane-bound proteins (10).

Effect of Thermotropic Phase Transitions on Mixed Micelle Formation

Phospholipids containing saturated fatty acids undergo thermotropic phase transitions from a gel phase (also referred to as an ordered or solid phase) to a lamellar phase (also referred to as a disordered, fluid, or liquid-crystalline phase) at characteristic temperatures (24–27). For dipalmitoyl phosphatidylcholine, this temperature is about 41°C and there is a pretransition at about 34°C (25); these temperatures may be lowered in the presence of other materials such as Triton X-100. Nmr intensity and linewidth studies at 37°C suggest that above a molar ratio of about 2:1 Triton/phospholipid, all of the phospholipid and Triton is in mixed micelles (5). At higher temperatures, studies are complicated by the fact that Triton X-100 has a cloud point (28, 29) (separation of a Triton-rich and Triton-poor phase at higher temperatures) and the cloud point is lowered in the presence of phospholipid (4). At temperatures well below the phase transition, mixed micelle formation is greatly inhibited so that a large excess of Triton is required to convert the gel phase phospholipid into mixed micelles; an approximate phase diagram for the dipalm-

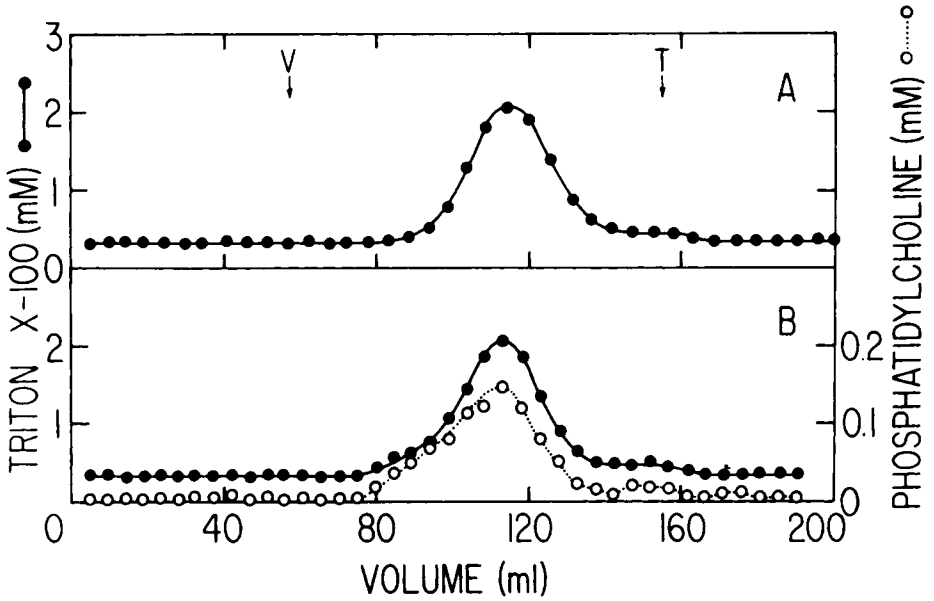

Fig. 2. Elution profile of A) Triton micelles (45 mM) and B) mixed micelles with egg phosphatidylcholine at a molar ratio of about 10:1 Triton/phospholipid on 6% agarose which was pre-equilibrated with and the elution carried out with buffer containing 0.35 mM Triton X-100. ^{3}H-Triton was detected by its radioactivity and the phospholipid by a phosphorus determination. The void volume (V) and the total volume (T) are indicated.

itoyl phosphatidylcholine-Triton X-100-water system is presented elsewhere (5). Gel phase phospholipid and mixed micelles of dipalmitoyl phosphatidylcholine and Triton X-100 can be separated by centrifugation techniques as summarized and compared with egg phosphatidylcholine in Table I. It should be noted that the mixed micelles formed with saturated phospholipids below their thermotropic phase transition may not be stable with time. (E. A. Dennis, manuscript in preparation.)

Structure of Mixed Micelles

At temperatures above the range of the thermotropic phase transition of the phospholipid and below the range of the cloud point of Triton, mixed micelles are formed at molar ratios above about 2:1 Triton/phospholipid. Under these conditions, the protons in both the Triton and phospholipid give rise to high-resolution nmr spectra with full intensities (within experimental error) and narrow linewidths, as illustrated in Fig. 4, whereas in phospholipid bilayer preparations, the phospholipid peaks are quite broad and lack full intensities (30). The intensities and linewidths due to Triton in the mixed micelles are similar to those observed in pure Triton micelles (3, 5). Furthermore, the spin-lattice relaxation times (T_1) of various Triton protons in the mixed micelles and pure Triton micelles are similar and the T_1 values for various protons in the phospholipid in mixed micelles are similar to those reported (31, 32) for sonicated vesicles which con-

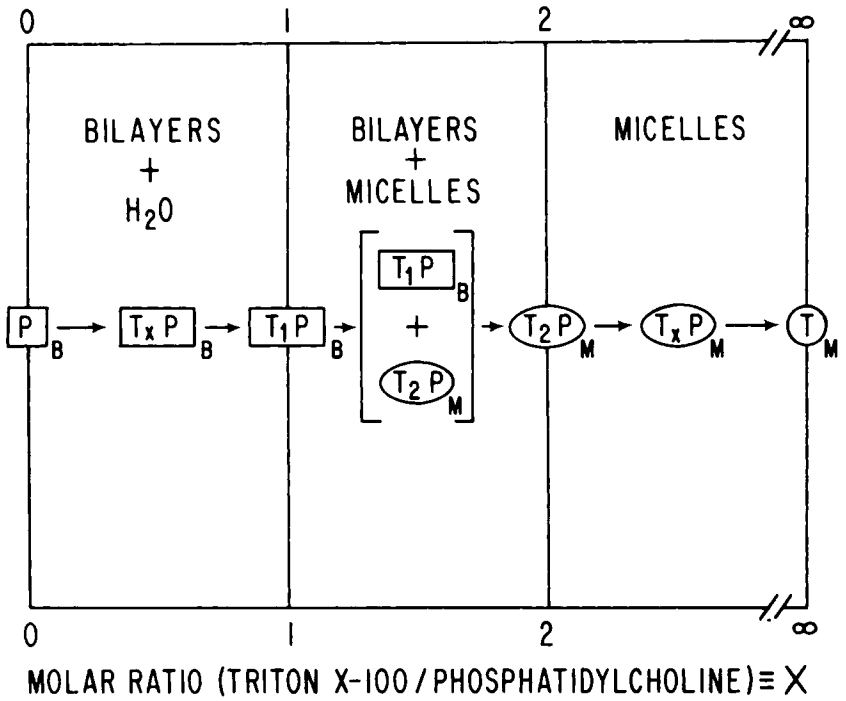

Fig. 3. Schematic diagram of the approximate changes which occur when increasing molar ratios of Triton X-100 (T) to egg phosphatidylcholine (P) are present in an excess of water. For simplicity, the stoichiometry of the phospholipid bilayers (B) in the presence of an excess of Triton is assumed to be 1:1 and the stoichiometry of the Triton micelles (M) in the presence of an excess of phospholipid is assumed to be 2:1.

TABLE I. Centrifugation of Phospholipid–Triton Mixtures*

	Molar Ratio (Triton/Phospholipid)		
Phospholipid	Original	Supernatant	Pellet
Egg PC	1.6	2.4	0.63
Dipalmitoyl PC	5.1	13	0.14

*Centrifugation of 2.0 ml samples of 43 mM Triton X-100 and phospholipid at the molar ratios indicated in a Sorvall centrifuge model RC2-B at 35,000 × g for 1 hr at room temperature. Under these conditions, the phospholipid was about equally divided between the supernatant and pellet. With egg phosphatidylcholine, the supernatant fraction chromatographed as mixed micelles and the pellet fraction (taken up in buffer) chromatographed as bilayers. For dipalmitoyl phosphatidylcholine, the molar ratio of Triton/phospholipid in the gel phase bilayers may be less than that indicated due to contamination of the pellet with a small amount of supernatant.

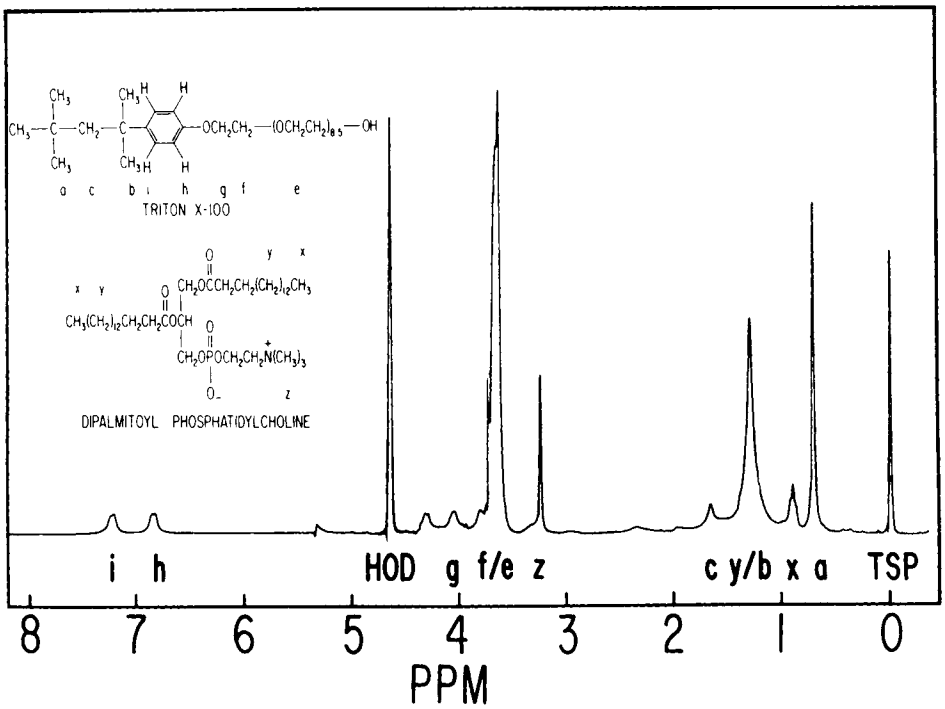

Fig. 4. 220 MHz ^{1}H-nmr spectrum recorded at 37°C of a mixture of 200 mM Triton X-100 and 100 mM dipalmitoyl phosphatidylcholine in D_2O and containing TSP (3-trimethylsilyl tetradeutero sodium propionate). See Refs. 3–5 for assignment of peaks.

sist of a single bilayer of phospholipid (A. A. Ribeiro and E. A. Dennis, unpublished experiments). This is illustrated for the nine-proton singlet due to the t-butyl group (peak a) of Triton and for the terminal methyl (peak x) and choline methyl (peak z) of the phospholipid in Table II. The relaxation mechanisms for phospholipids in sonicated vesicles have been discussed in the recent literature (31–37). It is probable that the relaxation processes which are indicated by T_1 values are similar for the phospholipid in mixed micelles and sonicated vesicles, but the determination of the precise state of motion of the phospholipid in mixed micelles requires further studies, including T_2 determinations; such studies are in progress.

The nmr criteria suggest that the Triton molecules are in similar environments in pure Triton micelles and in mixed micelles and that the phospholipid molecules are in a very different environment in mixed micelles than they are in phospholipid bilayer preparations. Additionally, column chromatography shows that mixed micelles at a molar ratio of about 10:1 Triton/phospholipid are similar in size to pure Triton micelles. Thus, it is reasonable to assume that the structure of the mixed micelles is fairly similar to that of pure Triton micelles. Tanford (38) has recently suggested on the basis of geometrical considerations that amphiphiles containing two hydrocarbon chains (such as phospholipids) pack best in bilayer structures, whereas amphiphiles containing one hydrocarbon chain (such as sodium dodecyl sulfate) pack best in micellar structures. We would like to

TABLE II. Spin-Lattice Relaxation Times (T_1)*

Sample	T_1 Values (sec)		
	t-Butyl	Terminal Methyl	Choline Methyl
Triton	0.26	–	–
Triton + egg PC	0.25	0.64	0.41
Triton + dipal PC	0.27	0.56	0.49
Egg PC vesicles (31)	–	0.54	0.39
Dipal PC vesicles (31)	–	0.42	0.38

*T_1 values obtained at 100 MHz and 40°C on 100 mM Triton X-100 in D_2O and containing phospholipid at a molar ratio of 3:1 Triton/phospholipid where indicated.

suggest on the basis of our results that micelle-forming amphiphiles (such as Triton X-100) can hold a certain limited amount of bilayer-forming amphiphile within their structure. Thus, at a molar ratio of 10:1 Triton/phospholipid, Triton micelles contain for every 10 molecules of Triton an extra molecule of phospholipid intercalated into their structure. At smaller molar ratios than 10:1, the mixed micelles are larger in size, but presumably they still have a micellar rather than a lamellar structure.

In considering the enzymatic studies, it would be useful to be able to picture the surface of the mixed micelles more precisely. The following considerations allow the formulation of a working model for the surface of the mixed micelles which is consistent with current knowledge, is useful in explaining kinetic data with phospholipase A_2, and is subject to further testing and modification, although it is speculative in nature: Triton X-100 and phosphatidylcholine molecules have similar average molecular weights. The surface areas of the molecules in mixed micelles are not known, but interfacial tension measurements suggest that Triton molecules have an average surface area of about 85 $Å^2$ in isooctane–water interfaces (19). The surface areas of phosphatidylcholine molecules in monolayers are pressure dependent, but the surface area per molecule corresponding to the surface pressure at which phospholipase A_2 acts maximally on monolayers of various phosphatidylcholines is about 80–90 $Å^2$ (6, 39, 40). Furthermore, the surface area per molecule of dioctanoyl phosphatidylcholine "micelles," toward which phospholipase A_2 acts optimally, has been estimated to be about 90 $Å^2$ by extrapolation of the surface areas of diheptanoyl and dihexanoyl phosphatidylcholine micelles and dinonanoyl phosphatidylcholine bilayers (6). Thus, Triton and phosphatidylcholine molecules appear to have similar average surface areas in separate interfaces and to a first approximation may have similar surface areas per molecule in mixed micelles. If the above considerations are correct and we use circles of the same size to represent each molecule in the surface of the mixed micelle, then the most reasonable representation of the average distribution of molecules in the micelle surface would be that in which every circle is surrounded by six other circles in a two-dimensional, closely packed manner as shown in Fig. 5.

While the surface areas of the Triton and phospholipid molecules may be nearly similar, the hydrophobic portions are vastly different: phosphatidylcholine contains two long hydrocarbon chains while Triton contains a short bulky group. Our results suggest that the minimum Triton necessary to form mixed micelles rather than bilayers is about 2:1 Triton/phospholipid. This presumably results from geometrical and thermo-

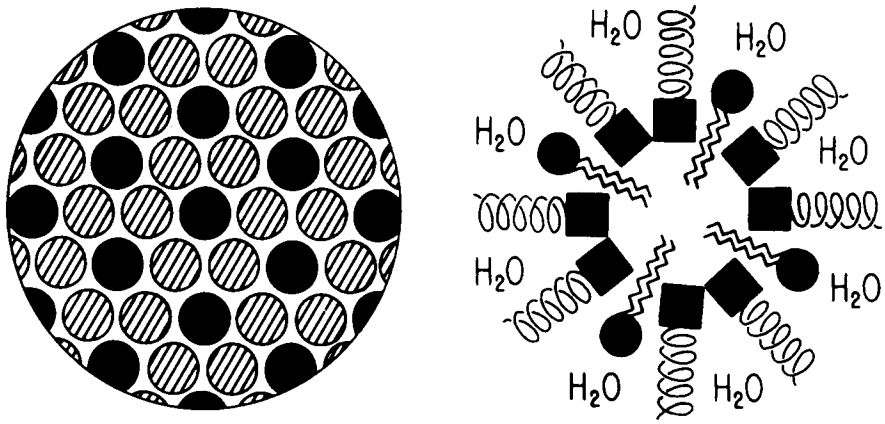

Fig. 5. Left: Schematic representation of a possible arrangement for the surface of mixed micelles. Black circles represent the hydrophilic end of the phospholipid molecules and cross-hatched circles represent the hydrophilic end of the Triton molecules. The stoichiometry is 2:1 Triton/phospholipid. Right: Stoichiometry is represented schematically in cross section using the symbols employed in Fig. 1.

dynamic packing considerations and additional phosphatidylcholine would cause less curvature in the surface and favor bilayer formation (38). At 2:1 Triton/phospholipid, the average distribution of phospholipid and Triton in the micelle surface is shown in Fig. 5. It is interesting to note that with this model and a molar ratio of 2:1 Triton/phospholipid, on the average every phospholipid molecule in the surface is separated from every other and surrounded by six Triton molecules, whereas each Triton molecule is surrounded by three phospholipid molecules and three Triton molecules.

ENZYMATIC CONSIDERATIONS

Phospholipase A_2 and Surface Dilution Kinetics

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acid at the C-2 position of diacyl phosphatidylcholines (1, 2-diacyl-sn-glycero-3-phosphorylcholine) and other phospholipids. We have been studying the enzyme purified from Naja naja cobra venom (41). It is a small, soluble, highly stable enzyme of molecular weight about 11,000 (42; R. A. Deems and E. A. Dennis, unpublished experiments); a phospholipase of similar molecular weight has been purified from sea snake venom (43). Similar enzymes (44) obtained from pancreas (6), molecular weight 14,000, and Crotalus adamanteus venom (45), a dimer consisting of two 15,000 molecular weight subunits, have been more extensively studied kinetically. It should be noted that in all of these sources, multiple forms of the enzyme have been found, and the origin of these forms is not clear at this time (45–48).

Phospholipase A_2 will act best toward phosphatidylcholine as substrate when it is in monolayer form (39, 49), when it is a synthetic compound made from short chain fatty acids and forms micelles (6), or when it is in mixed micelles with surfactants such as the bile salts (50) or Triton X-100 (1) rather than when it is molecularly dispersed

(6, 45). The utilization of substrate in the form of mixed micelles has advantages over other systems for the detailed study of this enzyme in that both natural and synthetic phospholipids can be employed as substrate and traditional bulk kinetic methods can be employed in the assay. The nonionic surfactant Triton X-100 has advantages over ionic surfactants in that it has a low CMC, does not introduce charged species into the system which can complicate the Ca^{2+} requirement of the enzyme, and generally it does not inactivate proteins. In kinetic studies with the Naja naja enzyme, we have found that this enzyme works poorly on phospholipid dispersions (bilayers) and optimally when Triton X-100 is present at a molar ratio of about 2:1 Triton/phospholipid, which corresponds to the conversion of phospholipid bilayers to mixed micelles (1). Surprisingly, however, at high surfactant concentrations a dramatic apparent inhibition of activity occurs; we have suggested that this is not a classical inhibition, but rather a consequence of the dilution of phospholipid in the surface of the mixed micelle (1, 2) as discussed below.

When the activity of phospholipase A_2 is determined at fixed molar ratios of Triton/phospholipid above 2:1 and the substrate is assumed to be the sum of the concentrations of phospholipid and Triton, the apparent K_m is about the same at each molar ratio, but the V_{max} at each molar ratio is directly proportional to the mole fraction of phospholipid in the phospholipid/Triton mixture (2). These results can most easily be explained in terms of the model for mixed micelles presented above. The enzyme presumably binds to the surface of the mixed micelle, but it cannot be distinguished at this time whether it recognizes and binds to the outer hydrophilic surface of the micelle or whether part of the enzyme penetrates the surface and binds to the hydrophobic core. The surface areas of the Triton and phospholipid molecules are probably similar to a first approximation and the similarity of the apparent K_m at various molar ratios of Triton/phospholipid is probably due to the fact that binding to the mixed micelle depends on the total surface area available. However, once the enzyme is bound to the mixed micelle (saturating conditions at a given molar ratio of Triton/phospholipid), the rate is reduced by the proportion of Triton present. This presumably occurs because the enzyme activity then depends on the proportion of time its active site is in contact with phospholipid and the phospholipid is diluted in the surface of the mixed micelles by Triton.

The significance of surface area in the action of lipolytic enzymes was first demonstrated by Benzonana and Desnuelle (51) in a different system. They showed that the activity of triglyceride lipase toward substrate emulsions depends on particle size and they calculated that the activity depended on the total surface area. However, because triglyceride–surfactant emulsions form structures in which all of the molecules are not in the surface, quantitation of the type done here would be difficult in that system. Finally, it should be noted that an apparent inhibition of activity at high surfactant concentrations is a frequently observed phenomenon in the assay of lipid enzymes. These studies suggest the possibility that dilution of the lipid in the surface of mixed micelles may be responsible for this apparent inhibition with other enzymes as well.

Effect of Thermotropic Phase Transitions on Enzymatic Activity

Several reports in the literature suggest that phospholipase A_2 acts toward saturated egg phosphatidylcholine or dipalmitoyl phosphatidylcholine at a much slower rate than

toward natural egg phosphatidylcholine – as much as 1/30 the rate for some forms of the enzyme (43, 52). On the other hand, we found that phospholipase A_2 acted at a similar rate toward egg phosphatidylcholine and dipalmitoyl phosphatidylcholine suggesting similar specificities for the two substrates (1). Our studies were conducted at a molar ratio of 2:1 Triton/phospholipid and 40°C, the temperature maximum of the enzyme; the other kinetic studies were conducted at 25°C and with a comparable amount of Triton X-100. We suggested that this apparent anomaly was due to an effect of the thermotropic phase transition of the saturated substrate. From the physical studies discussed earlier, it is now clear that at 40°C, mixed micelles containing a molar ratio of 2:1 Triton/phospholipid are present and, at 25°C, two phases are present: gel phase phospholipid bilayers and mixed micelles which are quite diluted in phospholipid.

The ratio of rates at 40°C to 25°C for phospholipase A_2 action toward dipalmitoyl phosphatidylcholine at 2:1 Triton/phospholipid is about 7, whereas the ratio is only about 1.8 when dimyristoyl phosphatidylcholine is utilized as substrate under similar conditions (2). Dimyristoyl phosphatidylcholine undergoes a thermotropic phase transition at about 24°C (25, 26), so that at both temperatures, mixed micelles should be present and this ratio should reflect the normal temperature effect on rate. Indeed, when dipalmitoyl phosphatidylcholine is utilized as substrate at a molar ratio of 16:1 Triton/phospholipid, where physical studies would suggest that all of the phospholipid is in mixed micelles, the rate ratio at 40°C to 25°C is about 1.9. Of course, the absolute rates at both temperatures are reduced dramatically due to the surface dilution effect elaborated earlier. Thus, the reduced rate of phospholipase A_2 action toward dipalmitoyl phosphatidylcholine at 25°C compared to 40°C in the presence of 2:1 Triton/phospholipid is due to the normal temperature effect on rate plus the effect of the thermotropic phase transition on mixed micelle formation rather than substrate specificity. It is clear from this example that phase transitions and physical states of phospholipids must be taken into account in studies on the activities and specificities of enzymes that act on or require phospholipids. Furthermore, specificities and physical state effects certainly must be considered carefully in evaluating data on the action of phospholipases toward whole membranes as pointed out by Zwaal et al. (53).

Lipid Phase Separation Model

Phase diagrams for mixtures of saturated phospholipids have recently been determined by Shimshick and McConnell (54) and they have demonstrated that regions exist in which gel phase phospholipid and fluid phase phospholipid coexist. These phase diagrams have led to the hypothesis that lateral phase separations in the surface of membranes can account for the temperature dependence of the rate of sugar transport in certain bacterial systems (13; also, see extensive work by other investigators listed in Ref. 54). Furthermore, it has been suggested on the basis of freeze-fracture results that when lateral phase separation occurs, certain membrane proteins partition with the fluid phase (55).

The phospholipase A_2-dipalmitoyl phosphatidylcholine-Triton X-100 system is an artificial system in which lipid phase separations occur because of the effect of thermotropic phase transitions and these changes in the physical state of the substrate have a marked but analyzable effect on enzymatic activity. This is illustrated schematically

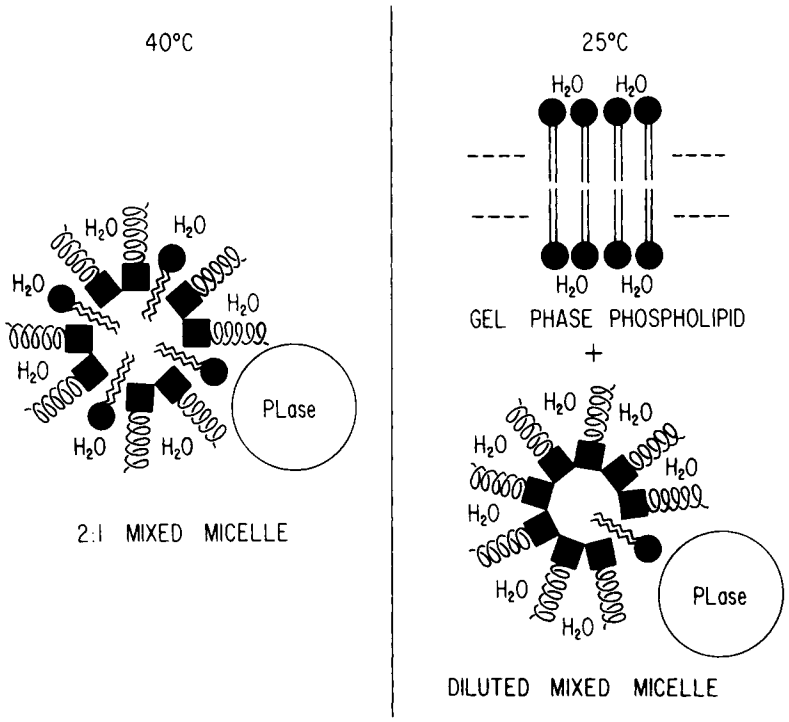

Fig. 6. Schematic representation of the lipid phase separation model for the phospholipase A_2-dipalmitoyl phosphatidylcholine-Triton X-100 system at 40°C and 25°C. It should be noted that at 25°C, the observed stoichiometries are not shown precisely and it is not clear at this time how much, if any, Triton is associated with the gel phase phospholipid.

in Fig. 6. Under saturating concentrations of mixed micelles that are at 2:1 Triton/phospholipid, effectively all of the enzyme is bound at 40°C and the phospholipid is in an appropriate physical state for interaction with the protein. Below the thermotropic phase transition of dipalmitoyl phosphatidylcholine such as at 25°C, lipid phase separation occurs producing gel phase phospholipid bilayers and a more fluid lipid phase (mixed micelles) containing a high molar ratio of Triton/phospholipid. Although this has not been demonstrated, most or all of the soluble enzyme is probably bound to the mixed micelles where the phospholipid can be effectively hydrolyzed by the protein. The rate of reaction on substrate in mixed micelles at 25°C is of course reduced by the surface dilution phenomenon.

This artificial system consists of a pure protein, pure phospholipid, and a surfactant. The enzyme is soluble, rather than membrane-bound, but the enzyme is capable of acting on membranes and the surfactant presumably serves as an inert membrane-like matrix in this system. This system is then useful for studying protein–phospholipid interactions and phenomena such as lipid phase separations and may in the long run serve as a model for studying reconstituted systems in the presence of surfactant. We have conducted studies on a membrane-bound enzyme phosphatidylserine decarboxylase in the presence of saturated phosphatidylserine and Triton X-100 (56) and preliminary data

suggest that the ideas presented herein will be applicable to the analysis of that system (T. G. Warner and E. A. Dennis, unpublished experiments).

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