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# EFFECT OF THERMOTROPIC PHASE TRANSITIONS OF DIPALMITOYLPHOSPHATIDYLCHOLINE ON THE FORMATION OF MIXED MICELLES WITH TRITON X-100

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## SUMMARY

High-resolution proton magnetic resonance techniques at 220 MHz have previously been employed to show that the addition of the nonionic detergent Triton X-100 to aqueous dispersions of unsonicated egg phosphatidylcholine converts the phospholipid from bilayer structures to mixed micellar structures containing Triton X-100. These techniques have now been employed to follow the transformation of dipalmitoylphosphatidylcholine bilayers to mixed micelles at three different temperatures: 20, 37 and 49 °C. This synthetic phospholipid in aqueous dispersions undergoes thermotropic phase transitions and the results obtained here show that the formation of mixed micelles is more favorable at 37 and 49 °C than at 20 °C where the phospholipid is in a gel phase. An approximate phase diagram for the Triton X-100–dipalmitoylphosphatidylcholine–water system is presented on the basis of the proton magnetic resonance results and a determination of the cloud point of the samples. Reported differences in the activity of the enzyme phospholipase $A_2$ towards egg phosphatidylcholine and dipalmitoylphosphatidylcholine at different temperatures are interpreted in terms of the temperature dependence of mixed micelle formation characterized by the proton magnetic resonance studies reported here.

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We have recently reported results of kinetic studies in which phospholipase $A_2$ acts on substrates consisting of mixed micelles of phosphatidylcholine and the nonionic detergent Triton X-100 [1]. In order to better characterize the physical state of the phospholipid substrate in the mixed micelles, we have developed proton magnetic resonance (PMR) techniques to study the formation of the mixed micelles and we have recently reported preliminary results on the general characteristics of this system using natural phosphatidylcholine isolated from egg yolk [2]. The activity of the phospholipase $A_2$ in the presence of Triton X-100 has been reported to be much greater towards natural phosphatidylcholine than towards its saturated analogue [3, 4] and synthetic dipalmitoylphosphatidylcholine [4, 5]. We have found [1] that the difference in activity towards natural and dipalmitoylphosphatidylcholine occurs only at low assay temperatures and we have suggested [1, 6] that this difference in

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activity can be correlated with the thermotropic phase changes which occur for saturated phosphatidylcholines [7–9], but not for the naturally occurring material in this temperature range. Since dipalmitoylphosphatidylcholine undergoes these phase transitions, we have now used the PMR techniques to investigate the effect of temperature on the formation of mixed micelles of dipalmityolphosphatidylcholine and Triton X-100.

## MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine refers to synthetic dipalmitoyl-L-α-lecithin, Lot X-3415 (Mann Research Labs); the optical purity of this material, which is about 55%, is discussed elsewhere [6]. Thin-layer chromatography of the phospholipid in chloroform–methanol–water (65:25:4; by vol.) on Silica gel G using 0.25 mm × 20 cm × 20 cm glass plates (Brinkmann Instruments) and developed with iodine or the phosphorus reagent of Dittmer and Lester [10] gave one major spot. The dipalmitoylphosphatidylcholine was used without further purification and was assumed to have one bound water molecule and a molecular weight of 752. A phosphorus determination [11] was consistent with this molecular weight. Triton X-100 (Rohm and Haas), which was used without further purification, is a polydisperse preparation of *p*, *tert*-octylphenoxypolyethoxyethanols with an average chain length of about 9.5 oxyethylene units; the hydrophobic portion may also contain some heterogeneity [12]. Concentrations are expressed in terms of its average monomer molecular weight which is 628.

PMR spectra were obtained with a Varian HR-220 spectrometer operating at 220 MHz and equipped with a variable temperature probe. Temperatures were obtained from the chemical shift of an ethyleneglycol sample. In general, chemical shift standards were not included in samples in order to avoid the addition of extraneous materials during measurements, and the $^1H^2HO$ peak in $^2H_2O$ was used for tuning purposes and approximate chemical shift localization. The line width of the $^1H^2HO$ peak (about 0.6 Hz) was always constant throughout a given experiment. For the determination of intensities and line widths, spectra were recorded at a sweep rate of 2 Hz/s over a sweep width of 500 Hz at radio frequency power levels below saturation. Peak intensities were determined from peak areas measured with a planimeter on the blown-up spectra so obtained; this was found to be more accurate than the electronic integrator in the spectrometer. The error in the measurements of the peak intensities with the planimeter was in the range of ±5–±10% for individual peaks. Intensities of the phospholipid methyl peak (Peak x) are reported, but less significance is attached to measurements of this peak as the error may be even greater since this peak is small and surrounded by two large peaks. Line widths were measured as the full width at half-height maximum intensity, and the error in the line width measurements is about ±5%, but in the case of very broad peaks the error may be as great as ±15%.

Samples were prepared by drying an aliquot of phospholipid in chloroform under nitrogen in the vessel of a Potter–Elvejhem homogenization apparatus, removing the last traces of chloroform under vacuum, adding either $^2H_2O$ or a solution of Triton X-100 in $^2H_2O$ to the vessel, homogenizing briefly, and transferring 1.00 ml of the resulting dispersion to a PMR sample tube. Control samples of Triton X-100, lacking phospholipid, were processed through the same procedure. When

samples containing little or no Triton were allowed to stand, the phospholipid settled to the top of the sample. Before the PMR sample tubes were placed in the spectrometer probe, they were shaken briefly to form a homogeneous dispersion, but in the spectrometer probe, the rapid spinning of the sample tube acted as a centrifuge yielding, in general, a distinct small upper layer of phospholipid. Since the PMR spectrometer probe detects approximately the bottom 0.3 ml of the sample, the phospholipid layer which floats on the top of the 1.0 ml sample, even with vortexing, is out of range of detection. Thus, PMR spectra of samples containing little or no Triton can be considered to be spectra of only the phospholipid and Triton in the bottom aqueous layer at the temperature of the probe. When a large amount of Triton X-100 is present in samples of phospholipid and Triton, no separation of phospholipid is observed; thus a representative sample of the phospholipid and Triton present is reflected in the PMR spectrum.

## RESULTS

Relevant portions of the 220-MHz PMR spectrum of 10 mM dipalmitoylphosphatidylcholine in the presence of 20 mM Triton X-100 at 37 °C are shown in Fig. 1. This spectrum has the same peaks and intensities as we have shown [2] to occur in Triton X-100 in the absence of phospholipid plus additional peaks expected for dipalmitoylphosphatidylcholine based on its structure, its PMR spectrum in $^2$H-labeled chloroform [13, 14] and [$^2H_4$]methanol [14], and previous assignments in egg phosphatidylcholine [2]. A preparation of dipalmitoylphosphatidylcholine in $^2H_2O$ at 37 °C in the absence of Triton X-100 did not yield a high-resolution spectrum. Although most of the phospholipid in the sample without Triton is presumably in the top layer as described in Materials and Methods, and hence would be out of the range of detection, any phospholipid in the bottom layer or a spectrum of the top layer itself would not be expected to give a well-resolved spectrum, since previously reported PMR spectra of unsonicated phospholipids in $^2H_2O$, where obtainable, show large line broadening [15].

Thus in the experimental setup employed, phosphatidylcholine bilayers* alone do not give the expected peaks, whereas mixed micelles of the phosphatidylcholine and Triton X-100 do. Thus, the conversion of dipalmitoylphosphatidylcholine bilayers to mixed micelles of Triton X-100 and phosphatidylcholine can be quantitated by following the changes of intensity of the principal peaks in the phospholipid and Triton when aliquots of Triton are added to a dispersion of dipalmitoylphosphatidylcholine as shown in Fig. 2. The observed intensities of each of the peaks in control samples of Triton X-100 alone in the concentration range of the experiment are directly proportional to concentration. However, at 37 °C (Fig. 2A), when 10 mM dipalmitoylphosphatidylcholine is present, the principal Triton peaks [*tert*-butyl protons (Peak a) and ethoxy protons (Peaks e and f)] do not show full intensities unless the molar ratio of Triton to phospholipid is greater than about 2:1. Similarly, the principal phospholipid peaks [terminal methyl protons (Peak x) and choline methyl protons (Peak z)], which show only negligible intensity without the presence

* Unsonicated preparations of phospholipid in water have been referred to in the literature as smectic mesophases [16], lamellar phases, liposomes, multilayers, concentric bilayers, etc.; for simplicity, we will refer to these preparations as 'bilayers'.

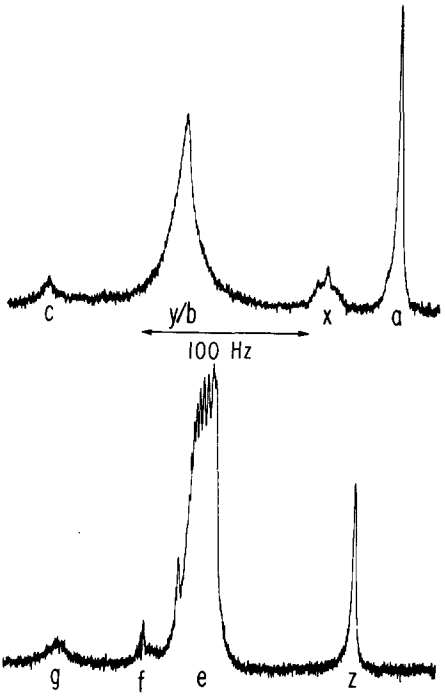
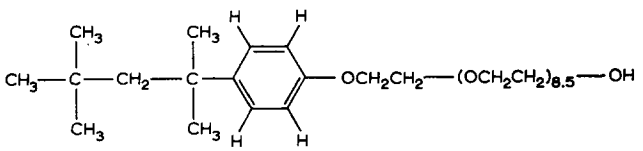
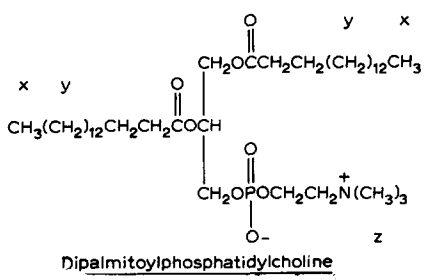



Fig. 1. Portions of the PMR spectrum recorded at 220 MHz and 37 °C of a mixture of 20 mM Triton X-100 and 10 mM dipalmitoylphosphatidylcholine in $^2H_2O$. (The sharp spike in f is a sideband.) The assignment of peaks is given below [2]:




Triton X-100



Dipalmitoylphosphatidylcholine

of Triton, reach their full expected intensity only when Triton is present at a molar ratio greater than about 2:1 Triton to phospholipid. The methylene protons (Peak y) of the phospholipid show similar behavior to the other peaks, but it is not clear at this time if full intensity is reached at a molar ratio of 2:1 Triton to phospholipid. This uncertainty arises because at low molar ratios of Triton to phospholipid, the

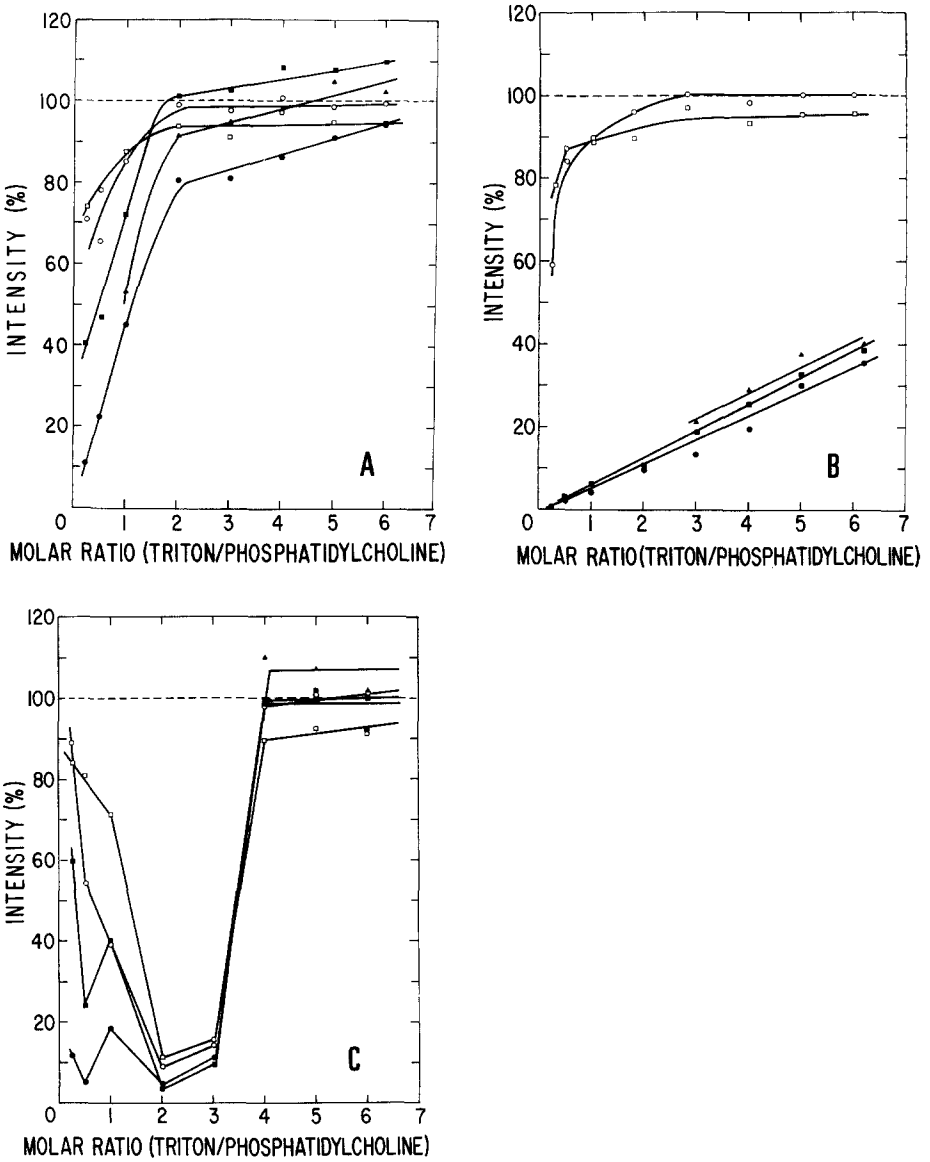



Fig. 2. Observed intensities at 37 °C (A), 20 °C (B) and 49 °C (C) for 10 mM dipalmitoylphosphatidylcholine and various amounts of Triton X-100. Phospholipid peaks are: ▲, terminal methyl (Peak x); ●, methylene (Peak y) and ■, choline methyl (Peak z). Triton peaks are: ○, *tert*-butyl (Peak a) and □, ethoxy (Peaks e and f). The intensities are expressed as a percentage of the expected intensities for each peak relative to the linear least-square values of intensity for the *tert*-butyl peak (Peak a) determined in control samples of pure Triton X-100 at the same concentrations as the mixtures. The expected intensities of the Triton and phospholipid peaks are based on their molecular structures (see Fig. 1). To obtain the intensity of the phospholipid methylene Peak y which overlaps the Triton Peak b, Peak b was assumed to have an intensity equivalent to 2/3 of the observed intensity of the Triton Peak a, and this was subtracted from the measured intensity of the combined Peak y and b.

contribution of Peak b of Triton to this peak is negligible, but at high molar ratios, the contribution becomes significant. Presumably the increase in intensity of the phospholipid peaks with added Triton as shown in Fig. 2A reflects both the increased quantities of phospholipid in the bottom layer in the PMR sample tube and a decreased broadening of the resonance lines of the phospholipid in that layer in the presence of Triton. Changes in intensity at 20 °C (Fig. 2B) and 49 °C (Fig. 2C) were also followed; these will be considered in the Discussion.

The conversion of phosphatidylcholine bilayers into mixed micelles of Triton and phospholipid upon the addition of Triton X-100 can also be semiquantitatively followed by observing changes in the line widths of certain 'indicator' peaks as illustrated in Fig. 3. The peak due to the 9 proton singlet arising from the hydrophobic *tert*-butyl protons of Triton (Peak a) becomes greatly broadened at low molar ratios of Triton to phospholipid when compared to its "normal" line width determined in control solutions of Triton lacking phospholipid. The line width then decreases dramatically until a molar ratio of about 2:1, Triton to phospholipid, at which point the line width of Peak a in the presence of phospholipid begins to approach that which occurs in pure Triton as shown in Fig. 3. For the 9 proton singlet arising from the hydrophilic choline protons of the phospholipid (Peak z), line narrowing accompanies the increase in intensity and the line width also appears to level off by a molar ratio of about 2:1, Triton to phospholipid. The line width of the peak due to the hydrophobic methylene protons of the phospholipid (Peak y) cannot be determined precisely because Peak b of Triton has about the same chemical shift. However, at low molar ratios of Triton to phospholipid, the intensity of the 6 proton singlet Peak

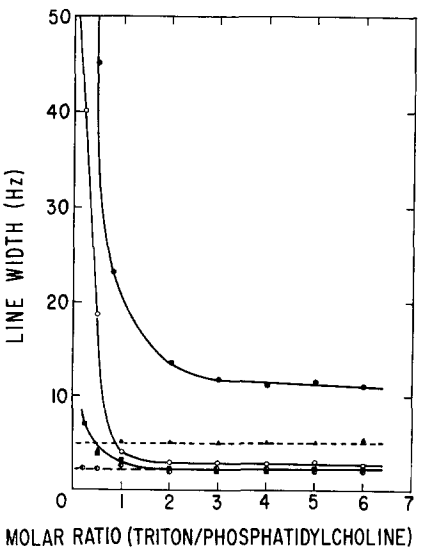



Fig. 3. Line widths of selected Triton and phospholipid peaks at 220 MHz and 37 °C for 10 mM dipalmitoylphosphatidylcholine and various amounts of Triton X-100. Peaks are: ○, *tert*-butyl (Peak a), ■, choline methyl (Peak z) and ●, methylene (Peak y and b). Line widths are also shown for peaks in solutions of pure Triton X-100 at the appropriate concentration; these peaks are: half-filled ○, *tert*-butyl (Peak a) and half-filled ▲, dimethyl (Peak b).

b (two-thirds that of Peak a) is insignificant relative to that of Peak y, and the broad line widths observed presumably reflect the state of the phospholipid. Unfortunately, at high molar ratios of Triton to phospholipid, Peak b of Triton does contribute significantly to the intensity of the observed peak, and the final line width of Peak y cannot be determined accurately from the spectra obtained. Thus, while the exact line width of Peak y cannot be accurately determined with the methods employed at this time, it is clear that its line width does decrease dramatically upon the addition of Triton. It should be noted that the line widths shown are not necessarily true line widths because at low molar ratios of Triton to phospholipid, some of the peak areas may be lost in the baseline and Peak y may represent several chemically shifted and split methylene protons and is effected by Peak b as discussed above; however, the method used does lead to valid qualitative conclusions about the changes in the line widths as Triton is added to the phospholipid. Thus mixed micelles of Triton and phospholipid formed at molar ratios above about 2:1, Triton to phospholipid, give rise to high-resolution PMR spectra with full intensities and relatively narrow line widths.

## DISCUSSION

### *Formation of mixed micelles*

The results obtained here at 37 °C are similar to those obtained in preliminary experiments with egg phosphatidylcholine [2]. The formation of mixed micelles can be considered to occur in the following manner: dilute aqueous dispersions of unsonicated phosphatidylcholine form two phases: a bilayer phase and an aquoues phase. As Triton is added to the mixture of the two phases, Triton monomers are incorporated into the phosphatidylcholine bilayers until the bilayers become saturated with Triton. At that point, additional Triton, which above its critical micellar concentration forms micelles that are perfectly soluble in the aqueous phase, incorporate phosphatidylcholine bilayers (saturated with Triton) to form mixed micelles with a composition of about 2:1, Triton to phospholipid. Thus at intermediate molar ratios, both mixed bilayers and mixed micelles coexist. When the overall ratio of Triton to phospholipid is greater than about 2:1, all of the phospholipid is converted to mixed micelles.

Because phosphatidylcholine obtained from egg yolk, which contains a heterogeneous distribution of fatty acid groups, was used in the preliminary experiments [2], it was possible that the partitioning of phospholipid in bilayer form (but containing Triton) between the bilayer and aqueous phases was due to a partitioning of different molecular species of phosphatidylcholine. Since the intensity and line width studies reported herein were conducted on a single molecular species of phospholipid, dipalmitoylphosphatidylcholine, and they gave qualitatively similar results to those obtained with egg phosphatidylcholine (except that the solubility of Triton in the two phospholipids may differ), these results serve to rule out that possibility. While it is most likely that the bilayer structures in the aqueous phase simply represent some fraction of the Triton-containing bilayers (possibly those of a smaller size) that are dispersible in the aqueous phase under the experimental conditions employed, the possibility cannot be ruled out at this time that the Triton X-100, which is polydisperse [12, 17] contributes to the formation of heterogeneous structures. In that case, those structures which on the average are composed of Triton molecules con-

taining larger oxyethylene chains would be expected to be more soluble in the aqueous phase than those composed of molecules containing shorter chains.

*Phase transitions and phospholipase $A_2$ activity*

The previous discussion concerned the formation of mixed micelles at 37 °C. At the three temperatures examined (20, 37 and 49 °C), the phospholipid in the absence of Triton X-100 should be in different phases which have previously been referred to as gel, metastable gel, and lamellar with transition temperatures at 34 and 41 °C [7–9, 18]. The formation of mixed micelles from Triton X-100 and dipalmitoylphosphatidylcholine appears to occur somewhat differently at the three temperatures. At 20 °C, the formation of mixed micelles from the phospholipid and Triton is considerably less favorable than at 37 °C so that even at a molar ratio of 6:1, only about 40% of the phospholipid can be accounted for, while all of the Triton is accounted for even at low molar ratios. Thus, at 20 °C when the phospholipid is in a gel phase, it does not readily form mixed micelles with Triton X-100.

At 49 °C, the Triton peaks show a decrease in intensity until a molar ratio of about 2:1, stay roughly constant between 2:1 and 3:1, and attain full intensity at 4:1 and above. It is not clear what happens to the phospholipid peaks below a molar ratio of 1:1, but their intensities follow those of the Triton peaks at a molar ratio of 2:1 and above. We have demonstrated that Triton X-100 in $^2H_2O$ exhibits a cloud point [19] at about 64 °C and that above that temperature a Triton-rich phase (cloud point phase) is formed and floats on top of the solution [20]. We found that when the Triton sample is contained in a PMR tube and examined under similar experimental conditions to those used in Fig. 2C, the Triton in the cloud point phase is out of the range of detection of the spectrometer probe [20]. Furthermore, we have shown that dipalmitoylphosphatidylcholine lowers the cloud point of Triton X-100 significantly [20]. The simplest interpretation of the results reported here is that the formation of mixed micelles is generally similar at 37 and 49 °C, except that at a molar ratio of Triton to phospholipid of 2:1 to 3:1, the cloud point of Triton is lowered sufficiently to give rise to a separation of most of the mixed micelles into a cloud point phase at 49 °C, but not at 37 °C.

For this reason, the cloud points of the solutions used in the PMR studies reported here were determined and it was found that in fact at 49 °C, the samples containing a molar ratio of Triton to phospholipid of 2:1 and 3:1 were at or above their cloud points, whereas those samples containing a molar ratio of Triton to phospholipid of 4:1 or above were below their cloud points. These results are indicated in Fig. 4 in terms of an approximate phase diagram for the Triton X-100–dipalmitoylphosphatidylcholine–water system derived from the PMR results presented here. The phase diagram for the bile salt–egg phosphatidylcholine–water system has been reported at one temperature [21, 22] and it was found that in the region of excess water and excess bile salt, one isotropic mixed micellar phase occurs. Similarly, we suggest that for 10 mM dipalmitoylphosphatidylcholine dispersions containing Triton X-100 at a molar ratio above about 2:1 and in a defined temperature range centering at about 40 °C, one isotropic mixed micellar phase exists which is shown as M(P+T+W) in Fig. 4. The structure of the mixed micelles in this region is of interest for enzymatic studies.

The effect of the phase transitions on mixed micelle formation reported here

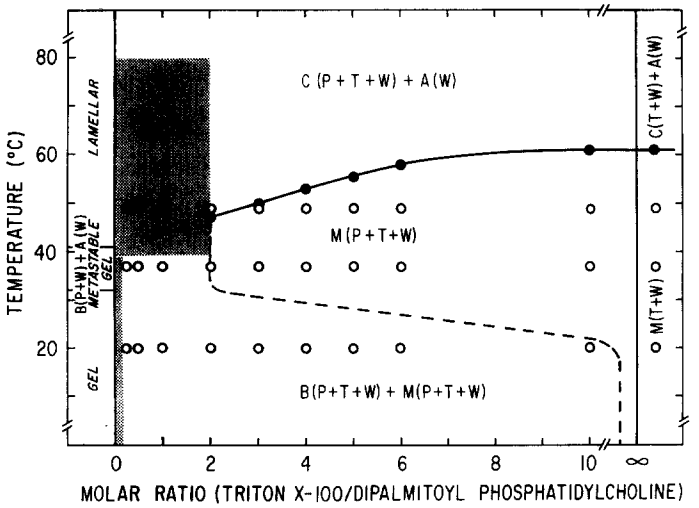



Fig. 4. Schematic diagram of the Triton X-100–dipalmitoylphosphatidylcholine–water system. On the left is shown the reported thermotropic phase transitions [18] of unsonicated dispersions of dipalmitoylphosphatidylcholine (P) in the presence of excess water (W) which forms a bilayer phase (B) and an aqueous phase (A). On the right is indicated the reported thermotropic phase separation [19, 20] of concentrated solutions of Triton X-100 (T) in water from a micellar phase (M) to a cloud point phase (C) plus an aqueous phase. The contribution of free molecules (monomers) of phospholipid and Triton, which would be negligible for the concentrated samples under consideration, is not indicated. The samples used to obtain the PMR results shown in Fig. 2 are indicated (○) in this diagram at the three temperatures employed for 10 mM dipalmitoylphosphatidylcholine in $^2H_2O$ and the various molar ratios of Triton to phospholipid used as well as for a control sample of 100 mM Triton X-100. The cloud point of these samples was determined visually [20] and is indicated (●). The resulting diagram indicates the phases inferred to be present on the basis of the PMR studies and can be considered to be an approximate phase diagram for this system under defined experimental conditions. The situation in the shaded area is not clear at this time; no cloud point was observable for the mixtures at low molar ratios and presumably there is a region containing B(P+T+W)+A(W).

are in agreement with previous reports that the phase change plays a large role in the interaction of dipalmitoylphosphatidylcholine with other compounds such as steroids [23], cholesterol [24] and benzyl alcohol [25]. Since the phase changes in saturated phosphatidylcholines can influence mixed micelle formation, this phenomenon provides a reasonable explanation for the apparent anomaly found in enzymatic studies on the rates of hydrolysis of different phospholipid substrates by phospholipase $A_2$ in the presence of Triton X-100. Salach *et al.* [3,4] using cobra phospholipase $A_2$ in an assay at 25 °C reported that saturated egg phosphatidylcholine reacted at 1/6 the rate of natural egg phosphatidylcholine (and as low as 1/30 for some forms of the enzyme) and that dipalmitoylphosphatidylcholine reacted at 1/2–1/8 the rate of egg phosphatidylcholine; similarly, Tu *et al.* [5] in an assay at 23 °C using sea snake phospholipase $A_2$ presented results that suggested that dipalmitoylphosphatidylcholine reacted at about 1/12 the rate of egg yolk phosphatidylcholine. This is in contrast to the approximately equal rates for dipalmitoylphosphatidylcholine and egg phosphatidylcholine which we [1] found in an assay conducted at 40 °C using cobra phospholipase $A_2$. Presumably, the rate of hydrolysis of the phospholipids is depen-

dent on the presence of mixed micelles [1]; the results reported here show that formation of mixed micelles from gel phase dipalmitoylphosphatidylcholine is unfavorable relative to the formation of mixed micelles at higher temperatures, and this accounts for the slower enzymatic rates observed for the various saturated substrates at room temperature. The effect of the thermotropic phase transitions on mixed micelle formation delineated herein has now been taken into account in detailed kinetic studies on phospholipase $A_2$ action; these results are presented separately [6].

ACKNOWLEDGMENTS

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