STUDIES ON MIXED MICELLES OF TRITON X–100 AND PHOSPHATIDYLCHOLINE USING NUCLEAR MAGNETIC RESONANCE TECHNIQUES

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(Received October 3, 1972; accepted December 15, 1972)

The addition of the nonionic detergent Triton X-100 to aqueous phosphatidylcholine dispersions converts the bilayer structures to mixed micellar structures containing Triton X-100. High-resolution nuclear magnetic resonance spectroscopy at 220 MHz was used to follow this conversion, and the general spectral characteristics of the mixed micelles are presented. The results are discussed in terms of the precise change in structure which occurs as Triton is mixed with the phospholipid bilayers, and it is concluded that, above a molar ratio of about 2:1 Triton to phospholipid, most or all of the phospholipid is in mixed micelles. The relevance of these results to the study of enzymes which require substrate in the form of micelles is discussed.

The nonionic detergent Triton X-100 is used extensively in the assay and purification of enzymes. For example, in our previous work on the membrane-bound enzyme phosphatidylserine decarboxylase, the assay was conducted in the presence of Triton X-100 (1, 2), and this enzyme has now been solubilized and purified using Triton X-100 (3). However, the mode of action of this detergent in the assay systems is not clearly understood. In order to assess the function of Triton X-100 more precisely, we have conducted kinetic studies on the effect of Triton X-100 on the activity of a small, soluble, and highly purified enzyme, phospholipase A_2, where the main role of Triton X-100 appears to be in the alteration of the physical state of the substrate rather than in any specific solubilization of the enzyme. Both factors, however, may be involved in the case of membrane-bound enzymes. We found that the enzyme requires Triton X-100 for activity, but it is inhibited at high detergent concentrations. The kinetic results, which will be reported elsewhere (4), can be interpreted in terms of a requirement of the enzyme for substrate in the form of mixed Triton-phospholipid micelles of a specific composition. This is consistent with studies on phospholipase A_2 where synthetic phospholipids containing short chain fatty acids were used as substrates. These studies suggested that this enzyme prefers substrates which form micelles of a specific geometry to substrates which form either monomers or bilayers (5). This appears also to be the case for pancreatic lipase (6). It is thus of interest to characterize the mixed Triton-phospholipid micelles more precisely in order to understand the role of Triton X-100 in the activation of this and other enzymes.

Triton X-100 is a polydisperse preparation of *p-t*-octylphenoxypolyethoxy-

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Journal of Supramolecular Structure

ethanols (7) with an average chain length of 9 to 10 oxyethylene units and an average molecular weight of 628 (8). In aqueous solution, Triton X-100 forms micelles with a CMC* of about 0.3 mM according to surface tension measurements (9); the CMC decreases slightly in high salt according to ultraviolet difference spectroscopy measurements (10). Measurements of the micelle size by ultracentrifugation (11) and light scattering (12–14) have suggested a molecular weight in the range of 63,000 to 105,000, which corresponds to 100–160 monomers. Phospholipids such as egg phosphatidylcholine when dispersed in water form large aggregated structures in the form of bilayers. Detergents are generally considered to solubilize phospholipids by forming mixed phospholipid-detergent micelles. Mixed micelles of certain ionic detergents such as phosphatidylcholine and sodium cholate have been studied in some detail (15); however, little is known about mixed micelles of Triton X-100 and phospholipid. Because the addition of Triton to phospholipid apparently effects enzymatic activity by converting phospholipid bilayers to micelles (containing Triton X-100), it would be of interest to assess more precisely the change in physical state of the phospholipid molecules as they undergo this transformation and to better characterize the structural geometry of the mixed phospholipid-Triton micelles. Since high-resolution nuclear magnetic resonance spectroscopy (NMR) offers the potential of observing individual nuclei in both the phospholipid and Triton environments and since changes in structure may be reflected in changes in the relaxation time of these nuclei, we have initiated NMR studies on this system. Some preliminary observations are reported here.

METHODS

Egg phosphatidylcholine refers to "highly purified" egg lecithin (Mann Research Laboratories) which was either used as obtained or further purified by alumina chromatography according to the method of Singleton et al. (16) and lyophillized from benzene. Thin-layer chromatography (17) in chloroform/methanol/water (65:25:4) on silica gel G using commercially prepared glass plates (Brinkmann Instruments), and developed with iodine or the phosphorus reagent of Dittmer and Lester (18) gave a single major peak. The molecular weight was based on a phosphorus determination according to a modification of the method of Chen et al. (19). Triton X-100 was obtained from the Rohm and Haas Co. and used without further purification; concentrations are expressed in terms of its monomer molecular weight. D_2O was obtained from the Mallinckrodt Chemical Works and 3-trimethylsilyl tetradeutero sodium proprionate (TSP) was obtained from the Wilmad Glass Co.

NMR spectra were obtained on a Varian HR-220 NMR Spectrometer operating at 220 MHz and 37°. In order to avoid the presence of extraneous reference compounds during the studies reported here, TSP was included only in samples used for chemical shift determination; chemical shifts are reported in ppm downfield from this standard. The spectrometer was in general tuned on the HOD peak present in the D_2O of the sample. For the determination of intensities and line widths, spectra were recorded at a sweep rate of 2 Hz/sec over a sweep width of 500 Hz

*Abbreviations used are: CMC, critical micelle concentration; NMR, nuclear magnetic resonance; phosphatidylcholine, 1, 2-diacyl-*sn*-glycero-3-phosphorylcholine; Triton, Triton X-100; TSP, 3-trimethylsilyl tetradeutero sodium propionate.

and at radio frequency power levels below saturation. Line widths were measured as the full width at half-height maximum intensity, and the error in the measurement of line widths is estimated to be about ± 5%. Intensities were measured using a planimeter to measure the peak areas on the resulting spectra and the error in these measurements is estimated to be about ± 10% (see Discussion Section).

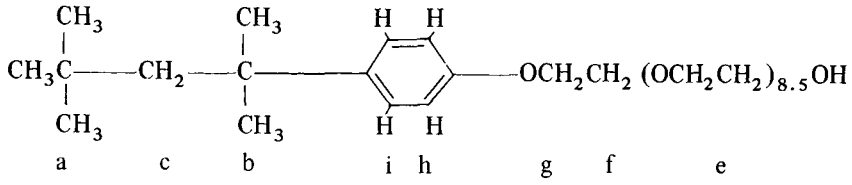
Samples were prepared by drying an aliquot of phosphatidylcholine in chloroform under argon in the vessel of a Potter-Elvejhem homogenization apparatus, adding either D_2O or a solution of Triton X-100 to the vessel, and homogenizing briefly. NMR sample tubes were prepared with 1.00 ml of freshly homogenized material. When the sample tubes containing little or no Triton were allowed to stand, phospholipid often settled to the top of the sample tube since it is less dense than D_2O. Before the tubes were placed in the spectrometer probe, they were shaken briefly to insure a homogeneous sample. As the samples were warmed to the probe temperature, the rapid spinning of the sample tube acted as a centrifuge providing, after equilibration, a distinct, but small, upper layer of phospholipid. Since the spectrometer probe only detects the bottom 0.3 ml of the sample, the phospholipid layer which floats on top of the 1.00 ml sample, even with vortexing, is out of range of detection. Thus, the NMR spectra taken of such samples can be considered to be that of a saturated dispersion of phospholipid (in some cases in the presence of Triton) at the temperature of the probe. When a large amount of Triton X-100 is present, no separation of layers is observed; thus a representative sample of the phospholipid/Triton dispersion is then reflected in the NMR spectrum. For the experiments involving the determination of intensities, aliquots of phospholipid were homogenized with the appropriate amount of Triton X-100 and control samples lacking phospholipid were processed through the same procedure. For the experiment involving the determination of line widths, the phospholipid was dispersed in D_2O and successive aliquots of Triton X-100 (at a concentration 10 times that of the phospholipid) were added to the NMR sample tube between spectra. The molar ratio of Triton to phospholipid in the line width experiment is based on the amount of Triton and phospholipid added to the NMR sample tube and is thus independent of the small increase in volume as the titration progresses; actually the absolute concentration of both the phospholipid and Triton decrease slightly as the titration proceeds (9% at 1:1 molar ratio).

RESULTS

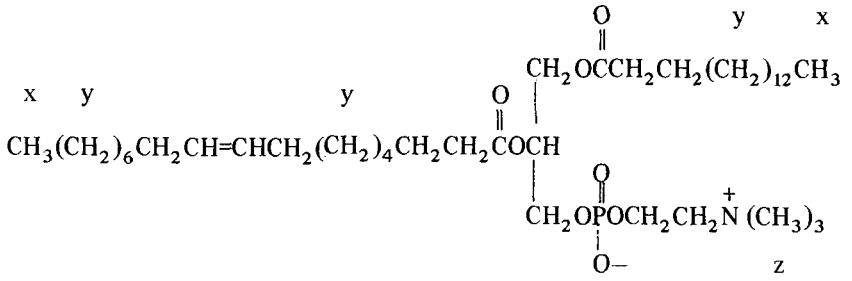
The 220 MHz NMR spectrum of 200 mM Triton X-100 in D_2O is shown in Fig. 1. (Spectrum A) and the assignment of peaks is given in Table I. The intensities of the peaks are consistent with the assignments and with the manufacturer's specification of an average oxyethylene chain length of about 9.5 units (8).

A preparation of unsonicated phosphatidylcholine in D_2O obtained as indicated in the Methods Section gave Spectrum B. Although most of the phospholipid is presumably in the top layer, which would account for the lack of phospholipid peaks in the spectrum obtained, any phospholipid in the aqueous layer or a spectrum of the top layer itself would not be expected to give a well resolved spectrum, since previously reported NMR spectra of unsonicated phospholipid in D_2O, where resolved, show large line broadening (21). A mixture of 40 mM phosphatidylcholine

TABLE I: CHEMICAL SHIFTS



Triton X-100



1-Palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphorylcholine[a]

Peak	Protons	Chemical Shift of Non-Exchangeable Protons[b]	
		Triton	Triton + Phospholipid
a	9	0.70	0.70
b	6	1.26	∿ 1.28
c	2	1.63	1.65
e	34	∿ 3.64	∿ 3.64
f	2	overlaps e	overlaps e
g	2	4.00	4.02
h	2	6.78	6.79
i	2	7.15	7.17
x	6		0.91
y	43[a]		∿ 1.30
z	9		3.24

[a]Major molecular component of egg phosphatidylcholine (16,20); expected intensity of peaks calculated on the basis of 2/3 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphorylcholine and 1/3 1-stearyl, 2-linoleoyl-*sn*-glycero-3-phosphorylcholine.
[b]Experimental conditions indicated in Figure 1.

and 200 mM Triton X-100 in D_2O, which did not give any separation of layers, gave the results shown in Spectrum C. This spectrum shows the same peaks and intensities as Triton X-100 alone in D_2O (Spectrum A) plus additional peaks expected for phosphatidylcholine based on the structure of the phospholipid and its NMR spectrum in $DCCl_3$ (22, 23). The principal peaks of the phosphatidylcholine are indicated in Table I.

Under the experimental conditions employed, the phosphatidylcholine bilayers do not show the expected peaks (Spectrum B) whereas Triton micelles

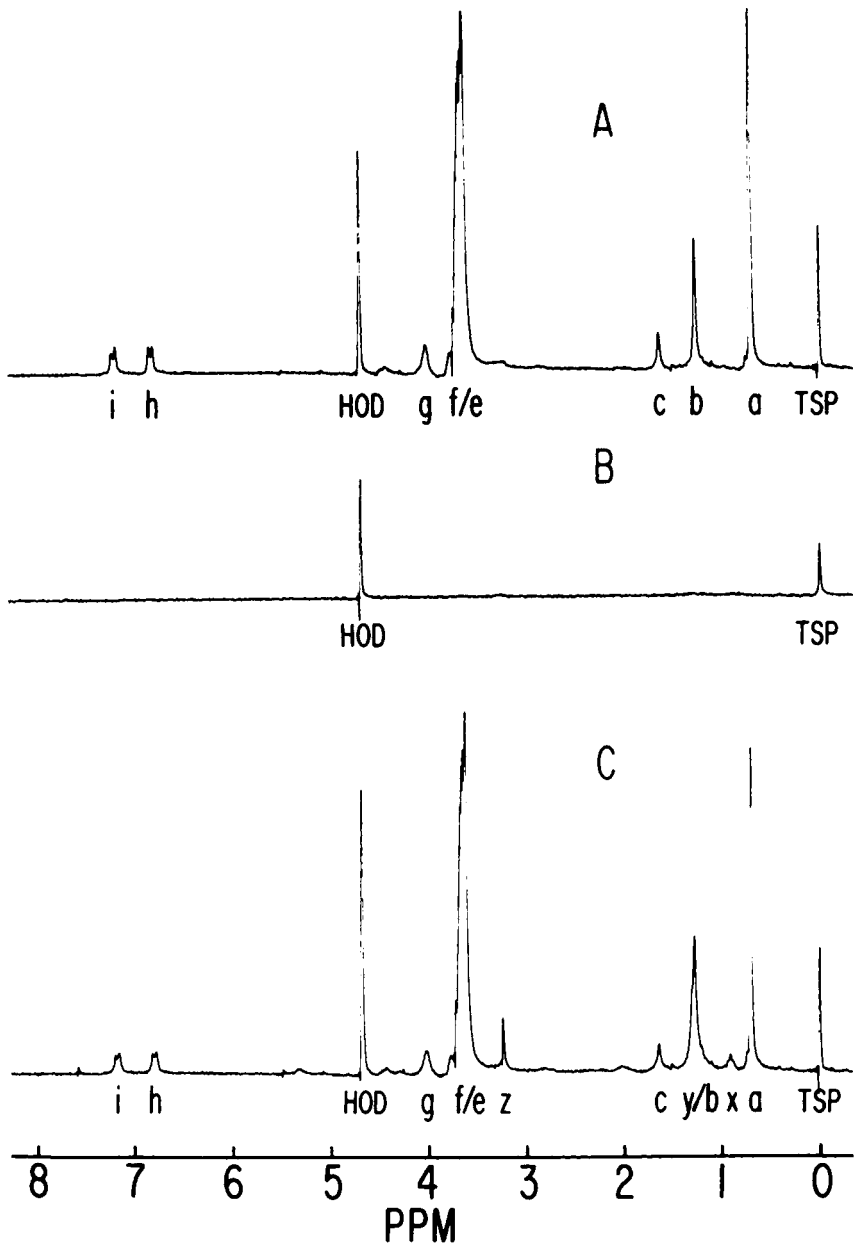


Fig. 1. NMR spectra recorded at 220 MHz and 37° of A. 200 mM Triton X-100 in D_2O; B. the bottom layer of a dispersion of 40 mM phosphatidylcholine in D_2O; and C. 200 mM Triton X-100 plus 40 mM phosphatidylcholine in D_2O.

(Spectrum A) and mixed micelles of Triton and phospholipid at a molar ratio of 5:1 Triton/phospholipid (Spectrum C) do. In Fig. 2, the observed intensities of the principal peaks of both the phospholipid and the Triton are shown for a dispersion of 40 mM phosphatidylcholine and various amount of Triton X-100. The observed intensities of each of the peaks of Triton X-100 alone in the concentration range of this experiment, 40 mM to 200 mM, are directly proportional to concentration, but when 40 mM phosphatidylcholine is present, the principal Triton peaks [*t*-butyl protons (peak a) and ethoxy protons (peak e/f)] do not show full

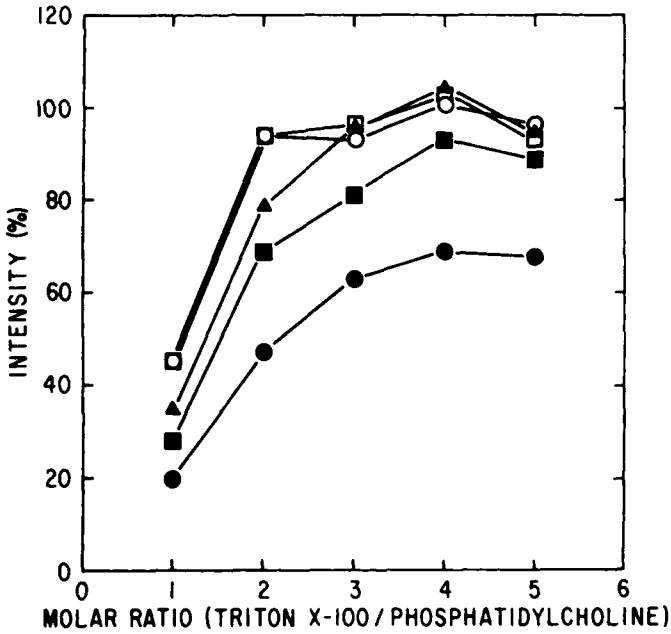


Fig. 2. Observed intensities for 40 mM phosphatidylcholine and various amounts of Triton X-100. The intensities are expressed as a percentage of the expected intensities relative to a control sample of pure Triton X-100 at the same concentration. Phospholipid peaks are: ▲ terminal methyl (peak x), ● methylene (peak y), and ■ choline methyl (peak z). Triton peaks are: ○ *t*-butyl (peak a) and □ ethoxy (peak e/f). 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 peak a and this was subtracted from the measured area of the combined peak y/b (see Table I).

intensitites unless the molar ratio of Triton to phospholipid is greater than about 2:1 as shown in Fig. 2. Similarly, the principal phospholipid peaks [terminal methyl protons (peak x) and choline methyl protons (peak z)], which show only negligible intensity without the presence of Triton, reach their full expected intensity only when Triton is present at a molar ratio greater than about 2:1 Triton to phospholipid, although the point of complete solubilization in the aqueous phase may not be sharp due to the molecular heterogeneity of the fatty acid chains on the phospholipid. The methylene protons (peak y) of the phospholipid show similar behavior to the other peaks, but it is not clear at this time whether this peak reaches all or most of the expected intensity above about 2:1 Triton to phospholipid due to the experimental difficulties involved in determining intensities accurately and the overlap of peak b of Triton. As described in the Methods Section, the rapid spinning of the NMR sample tube for samples containing 40 mM phospholipid and little or no Triton results in the formation of a lipid layer on the top of the sample and the NMR spectrum is actually only of material in the bottom layer, whereas at higher molar ratios of Triton to phospholipid no separation of layers occurs. Presumably, the increase in intensity observed with added Triton as shown in Fig. 2 reflects both the increased quantities of phospholipid in the bottom layer and the decreased line widths of the phospholipid peaks in that layer in the presence of Triton.

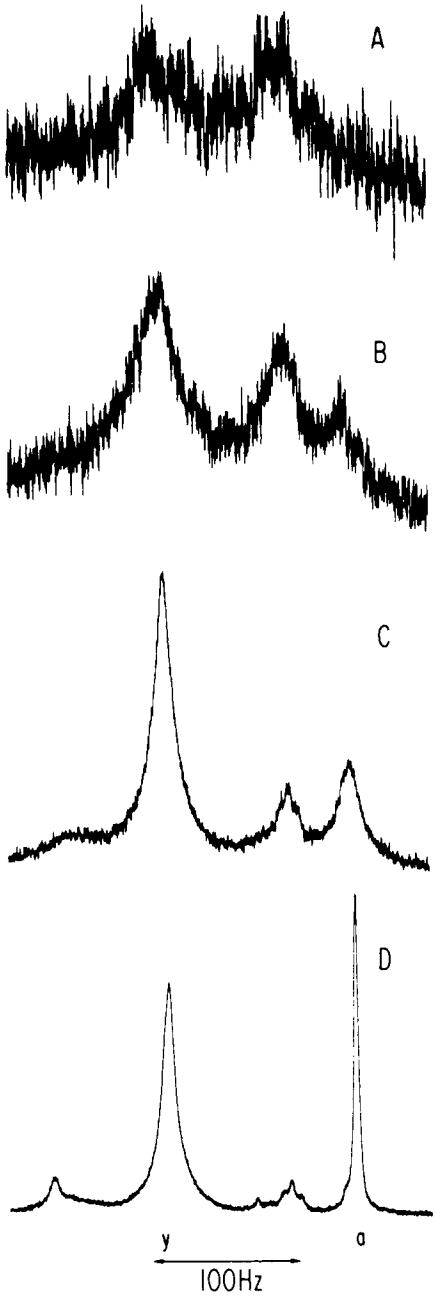


Fig. 3. NMR spectra recorded at 220 MHz and 37° of phospholipid (peak y) and Triton (peak a) indicator peaks for a dispersion of 10 mM phosphatidylcholine at the following molar ratios of Triton X-100 to phospholipid: A. 0; B. 0.4; C. 1.0; and D. 3.0. Note that the spectrum amplitude was decreased with increasing molar ratio in order to obtain peaks of similar size, and that the full recorded baseline is not shown.

The main peak in the hydrophobic region of Triton X-100 is the singlet representing 9 protons which arises from the *t*-butyl protons (peak a) and the main peak in the hydrophobic region of the phospholipid arises from the methylene protons (peak y), which would represent 43 protons at full intensity. The conversion of phospholipid bilayers to mixed micelles can be followed by observing the change of these two "indicator" peaks upon the addition of Triton X-100 micelles to the phospholipid bilayers as illustrated in Fig. 3. As the spectra show, the phospholipid indicator peak (peak y) is very broad and almost totally lacking

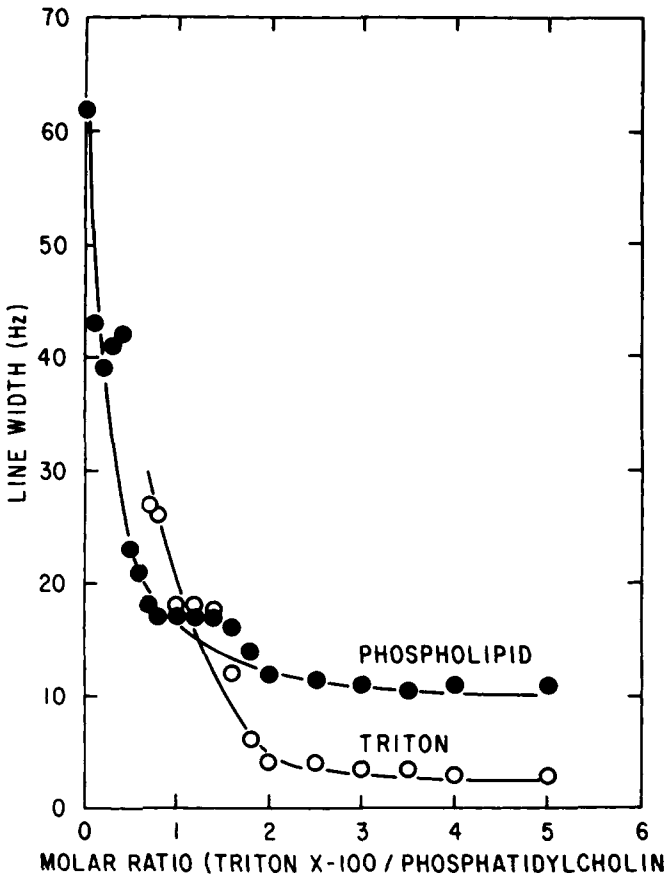


Fig. 4. Line width of Triton and phospholipid indicator peaks for a dispersion of 10 mM phosphatidylcholine titrated with 100 mM Triton X-100.

when no Triton is present (Spectrum A), but it increases in intensity and becomes considerably narrower upon the addition of Triton (Spectra B–D). When a small amount of Triton is present (Spectrum B), the Triton indicator peak (peak a) is also very broad, but as additional Triton is added, this peak also becomes narrow (Spectrum D). This is shown semi-quantitatively in Fig. 4 where the changes in line width of these indicator peaks are shown for the titration of 10 mM phosphatidylcholine with Triton X-100. For both peaks, the line width decreases dramatically as the molar ratio of Triton to phospholipid is increased until about 2:1 Triton to phospholipid when the line widths of the two peaks begin to level off. The line width of the Triton indicator peak begins to approach that of pure Triton at this point, but since peak b of Triton overlaps the phospholipid indicator peak, the final line width of peak y cannot be determined accurately using these methods. At low molar ratios of Triton to phospholipid, when the *t*-butyl peak of Triton is broadened, there is no evidence for the superimposition of a broad and narrow component; it is therefore reasonable to assume at this time that, if the Triton molecules present in the aqueous phase are in more than one type of structure, the molecules are rapidly exchanging.

DISCUSSION

Phospholipids dispersed in water give high-resolution NMR spectra characterized by low intensities and large line broadening. Considerable controversy has surrounded the determination of the dominant relaxation mechanisms involved (21, 24–28) and there is no general agreement at this time on the role of spin diffusion (26–29). However, it now seems clear that, at least in unsonicated egg phosphatidylcholine bilayers, the motion of the hydrocarbon chain is sufficiently slow to lead to incomplete averaging of the dipole-dipole interactions, although the end of the chain may be considerably more mobile than the rest of the chain (26). Several investigators have now determined the relaxation time using both continuous-wave and pulse methods of various nuclei in sonicated vesicles which serve as artificial membranes and give less line broadening than the unsonicated bilayers (21, 28, 30–32) and bile salt-phospholipid systems have been characterized using NMR techniques (33). Our experiments are not aimed at elucidating the mechanism of line broadening in unsonicated bilayers, but rather are aimed at following the transformation of the phospholipid from bilayer form (with broad lines) to micellar form upon the addition of Triton X-100 and to characterize the mixed micelles.

The results reported here show that the conversion of phospholipid bilayers to mixed phospholipid-Triton micelles can be followed using continuous-wave NMR techniques and that the phospholipid in mixed micelles gives rise to high-resolution peaks. The NMR spectrum of the mixed micelles at a molar ratio of 5:1 Triton to phospholipid is delineated in Table I. The spectral characteristics due to the Triton in the mixed micelles are similar to those in pure Triton micelles, and this is consistent with the idea that the structure of the mixed micelles is generally similar to that of pure Triton micelles. The presence of the peaks due to the phospholipid at full or nearly full intensity is consistent with the assumption that the phospholipid in the mixed micelles has a physical state vastly different that that in the phospholipid bilayers. The intensity and line width studies suggest that the dramatic change in structure from bilayers to mixed micelles occurs by a molar ratio of about 2:1 Triton to phospholipid; further addition of Triton presumably produces mixed micelles of generally similar structure, but of decreasing phospholipid content.

While both the phospholipid and Triton are polydisperse substances and may form heterogeneous structures, the detailed changes which occur in the conversion of phospholipid bilayers to 2:1 mixed micelles upon the addition of Triton X-100 can be examined. The experimental results reported here are consistent with the following explanation: Dilute aqueous dispersions of phosphatidylcholine (10–40 mM) form two phases, a bilayer phase and an aqueous phase. When Triton X-100 is added to the phospholipid dispersion, monomers of the Triton are incorporated into the phospholipid bilayers, thereby maintaining the free Triton concentration in the aqueous phase below the CMC until such point as sufficient Triton has been added so that all of the phospholipid bilayers are saturated with Triton. At that point, the further addition of Triton allows the concentration of free Triton to rise above the CMC and form Triton micelles. These micelles are able to dissolve phospholipid bilayers (already saturated with Triton) forming mixed micelles containing a molar ratio of about 2:1 Triton to phospholipid. Hence, when the overall ratio of Triton to phospholipid is greater than about 2:1, all of the phospholipid is dissolved.

The 2:1 mixed micelles are completely soluble in the aqueous phase, and both the phospholipid and Triton molecules in them give rise to high-resolution NMR signals of full intensity and narrow line widths. Although the phospholipid bilayers have little solubility in the aqueous phase, their dispersibility is increased with increasing Triton content. Thus, as Triton is added to the dispersions, some phospholipid bilayers (containing Triton) begin to appear in the aqueous layer and give rise to NMR signals. These signals for the hydrophobic indicator peaks in both the Triton and phospholipid molecules in the bilayers reflect the bilayer structure (broad signals) modified by the Triton present (somewhat narrower signals). When all of the bilayers have become saturated with Triton and mixed micelles begin to form, the NMR spectra reflect the proportion of Triton-saturated bilayers and mixed micelles present in the aqueous phase. This explanation specifically accounts for the broadened *t*-butyl peak of Triton at molar ratios below 2:1 in Figs. 3 and 4.

While the results reported here reflect the general behavior of this system, the large line broadening observed for both the phospholipid and Triton indicator peaks for the structures at low molar ratios of Triton to phospholipid is difficult to quantitate precisely at this time due to the complicated nature of the system and experimental limitations. In particular, the several methylene groups in the fatty acid chains may each have slightly different line widths, and the different parts of the chain may be effected differently as Triton is added. Fortunately, except for the methylene peak, the principal peaks of interest (terminal methyl, choline methyl, *t*-butyl, and ethoxy) do not overlap any other peaks. In the case of the methylene protons (peak y), peak b of Triton overlaps this peak, but at low molar ratios of Triton to phospholipid, the contribution of the Triton peak (6 protons) to that of the phospholipid peak y (43 protons at full intensity) should be negligible. Furthermore, the intensity of peak b can be considered to be proportional to the intensity of peak a so that its contribution can be taken into account at high molar ratios. Thus, while the overlap of peak b does not interfere with the general conclusions reported here, precise information on the behavior of the methylene protons during the course of the titration is complicated by the presence of this additional peak.

In addition, the large spectral broadening at low molar ratios of Triton to phospholipid makes determination of the baseline extremely difficult and this can lead to large errors in the determination of intensities and line widths. Therefore, the measured intensities and line widths reported here reflect lower limits on their true values under our experimental conditions, and, while the changes observed reflect the conversion of bilayers to micelles, they do not reflect true intensities and line widths of the species involved until an excess of Triton is present. The line widths of the peaks in the mixed Triton-phospholipid micelles could be expressed as spin-spin relaxation times (T_2) (34), but more meaningful measurements of the relaxation times can be obtained by using pulsed Fourier transform techniques to measure spin-lattice relaxation times (T_1) (35) now that the conditions for forming the mixed micelles and their spectral characteristics are established; these measurements are in progress.

The intensity experiment (Fig. 2) was carried out on 40 mM phospholipid and the line width experiment (Figs. 3 and 4) was carried out on 10 mM phospholipid. In both cases, the results suggest that, above a molar ratio of about 2:1 Triton to phospholipid, all or most of the phospholipid is in mixed micelles and that the

physical state of the phospholipid in the mixed micelles is significantly different than in pure phospholipid bilayers. From these experiments and those at other concentrations, it seems clear that the molar ratio of Triton to phospholipid, rather than absolute concentration, is the major factor governing the formation of mixed micelles. Interestingly, in our experiments on the dependence of phospholipase A_2 activity on the presence of Triton X-100 conducted at a similar temperature and concentration of phospholipid as the studies reported here, we found that maximal enzymatic activity was obtained at a molar ratio of about 2:1 Triton to phospholipid (4). Thus, it appears that the formation of mixed micelles, which is the kinetically active form of the substate for the enzyme, can be followed by the NMR techniques described here. Using these NMR techniques, it should now be possible to follow the formation of these mixed micelles under various other conditions relevant to the enzymatic studies.

ACKNOWLEDGMENT

We thank Dr. Karol J. Mysels, Dr. Robert L. Vold, and Dr. Regitze R. Shoup for helpful discussions on the interpretation of these results and Dr. John Wright for aid in the operation of the HR-220 Spectrometer. Financial support was provided by the National Science Foundation Grant #GB-19056, the National Institutes of Health Grant #NS-09326, the American Cancer Society Institutional Research Grant #IN-93, and a Merck Company Foundation "Faculty Development Award."

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