^{31}P Nuclear Magnetic Resonance Study on the Incorporation of Monomeric Phospholipids into Nonionic Detergent Micelles

Andreas Plückthun and Edward A. Dennis*

Department of Chemistry, University of California at San Diego, La Jolla, California 92093 *(Received: August 5, 1980; In Final Form: December 3, 1980)*

^{31}P nuclear magnetic resonance chemical shifts of monomeric and micellar phospholipids are reported. Micellization of dihexanoylphosphatidylcholine as a function of concentration was followed, and the critical micelle concentration determined. Chemical-shift differences and gel permeation chromatography were employed to follow the solubilization of monomeric phospholipids by the nonionic surfactant Triton X-100 to form mixed micelles. For dihexanoylphosphatidylcholine, a partition coefficient between Triton X-100 micelles and free solution was calculated as a function of the Triton X-100 concentration. Dibutyrylphosphatidylcholine was only sparingly incorporated into the surfactant micelles in contrast to phosphatidylcholine containing long-chain fatty acyl groups, which is completely solubilized.

Introduction

Nonionic surfactants containing the poly(oxyethylene) group have been used widely to solubilize membrane phospholipids.[1,2] The structures of the pure detergent micelles[2-6] and the mixed micelles[7] that are formed from natural phospholipids and the surfactant Triton X-100[8] have been previously characterized. Solubilization by various surfactants in aqueous micellar systems has been reviewed recently by Mukerjee,[9] and equilibria in mixed micelles have been described by Rubingh.[10] Although the physical chemistry of synthetic short-chain phospholipids, which form micelles by themselves, has been studied extensively by Overbeek and co-workers,[11] little is known about the solubilization of these compounds by detergents or about the structure of the resulting mixed micelles. The present study focusses on the incorporation of water-soluble compounds (the short-chain phospholipids) at concentrations below their cmc[12] into detergent micelles.

Preliminary experiments have shown differences in both the ^{1}H and ^{31}P NMR chemical shifts of short- and long-chain phospholipids.[13-15] Phospholipids containing long fatty acyl chains show a characteristic ^{1}H NMR chemical-shift difference of 17–18 Hz between the *sn*-1 and *sn*-2 α-methylene protons in the presence of Triton X-100, whereas monomeric dihexanoylphosphatidylcholine shows a chemical-shift difference of only 7–8 Hz in the absence of detergent. This latter chemical-shift difference increases upon the addition of Triton X-100.[14] Preliminary experiments have demonstrated that the ^{31}P NMR chemical shift of dihexanoylphosphatidylcholine moves upfield upon the addition of Triton and becomes similar to egg phosphatidylcholine,[15] suggesting that incorporation into micelles occurs provided that the Triton concentration is high enough. In the present study, we attempt to test this hypothesis and quantitate the incorporation by using the phase-separation model for mixed micelle formation. Since the changes in chemical shift upon solubilization are very small, several controls had to be carried out to estimate the influence of other parameters on the chemical shift of phospholipids.

Experimental Section

Materials. Egg phosphatidylcholine was prepared from fresh egg yolks by the method of Singleton et al.[16] Egg phosphatidylethanolamine, prepared by transesterification of egg phosphatidylcholine, was obtained from Avanti Biochemicals. Lysophosphatidylethanolamine was also obtained from Avanti Biochemicals. Dibutyryl- and dihexanoylphosphatidylcholine and glycerophosphorylcholine were obtained from Calbiochem or synthesized.[17] Triton X-100 was obtained from Rohm and Haas. Bis(monoacylglyceryl) phosphate was a gift of Dr. Karl Hostetler, University of California at San Diego, and dodecylphosphorylcholine was a gift of Dr. H. S. Hendrickson, Saint Olaf College, Northfield, MN.

Lysophosphatidylcholine was prepared as follows. Egg phosphatidylcholine (1 mmol) in a benzene stock solution

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(7) R. J. Robson and E. A. Dennis, *Biochim. Biophys. Acta*, **573**, 489 (1979).
(8) Triton X-100 is a polydisperse preparation of *p*-(1,1,3,3-tetramethylbutyl)phenoxypoly(oxyethylene) glycols, containing an average of 9.5 oxyethylene units per molecule.
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(12) Abbreviations are as follows: cmc, critical micelle concentration; phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; lysophosphatidylcholine, 1-acyl-*sn*-glycero-3-phosphorylcholine; dibutyrylphosphatidylcholine, 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine; dihexanoylphosphatidylcholine, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; phosphatidylethanolamine, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine; lysobutyrylphosphatidylcholine, 1-butyryl-*sn*-glycero-3-phosphorylcholine; lysophosphatidylethanolamine, 1-acyl-*sn*-glycerol-3-phosphorylethanolamine.
(13) M. F. Roberts and E. A. Dennis, *J. Am. Chem. Soc.*, **99**, 6142 (1977).
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was dried under N_2, taken up in 500 mL of an ether/water mixture (1:2.5, v/v), and then treated with 1.0 mg of cobra venom phospholipase A_2 (*Naja naja naja*). The aqueous phase contained 5 mM Ca^{2+} acetate, 25 mM NaCl, and 50 mM sodium borate buffer, pH 6.8. The mixture was allowed to react to completion as determined by thin-layer chromatography on silica gel (Brinkman) using the solvent system $CHCl_3$–CH_3OH–H_2O (65:25:4, v/v/v). The ether was evaporated, and the mixture was brought to pH 3.0 and extracted 3 times with an equal volume of $CHCl_3$–CH_3OH (2:1, v/v). The organic phases were collected, and the solvent was evaporated. The residue was carefully taken up in the minimum amount of methanol necessary to dissolve it. This solution was then added in portions to 12 test tubes containing 30 mL each of cold ether to precipitate lysophosphatidylcholine. The tubes were centrifuged, and the pellet was washed 3 times with cold ether. It gave a single spot on thin-layer chromatography as described above. Lysobutyrylphosphatidylcholine was prepared in an analogous fashion but without ether. After completion of the reaction, the mixture was lyophilized. The resulting solid was then extracted with $CHCl_3$–CH_3OH (1:1, v/v) and purified by silicic acid chromatography (Unisil, Clarkson Chemical Co.) using a $CHCl_3$–CH_3OH gradient.

All phospholipids were routinely checked for purity before use by thin-layer chromatography using the solvent system described above. Mixed micelles were prepared by adding solutions of detergent to dry phospholipids; mixing was achieved by vortexing and then allowing the foam to subside before the sample was used.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained at 220 MHz with a Varian HR-220/Nicolet TT-100 pulse Fourier transform system as described elsewhere.[14]

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were obtained at 40.3 MHz with a JEOL PFT-100 system equipped with a modified Nicolet 1080 computer and disk. The Hetero spin decoupler JMN-SD-HC unit was used with a Schomandl ND 100 M frequency generator. The temperature was 40 ± 0.5 °C unless otherwise indicated. For chemical-shift determinations, a 60–70° pulse was employed, and the delay time between pulses was about equal to T_1 for the phospholipid employed. The T_1 measurements were carried out by using a 180°–τ–90° pulse sequence with broad-band proton decoupling.[18] The delay between pulse sequences was $5T_1$. A value of 2.5 s was found for 10 mM phosphatidylethanolamine and 12.4 s for 10 mM dibutyrylphosphatidylcholine under standard conditions as specified below.

All chemical-shift measurements were carried out in Wilmad 516 CC 10-mm coaxial tubes with 5% trimethyl phosphate in D_2O (v/v) in the outer compartment as a reference standard, but all chemical shifts are reported relative to 85% phosphoric acid (upfield is positive). The chemical shift of the secondary standard was determined as −3.02 ± 0.01 ppm. Unless otherwise indicated, the samples contained Triton and phospholipid in a molar ratio of 8:1, 50 mM Tris-HCl buffer pH 8.0, 10 mM $CaCl_2$, and 30% D_2O (standard conditions). The D_2O is necessary to provide a lock signal. All reported chemical shifts are an average of at least three measurements. The variation between measurements of the same sample was usually less than ±0.02 ppm when the tube was taken out of the spectrometer between the measurements, and after temperature equilibration (10 min) the instrument was readjusted before each determination. Different samples of the same compound prepared under identical conditions in different tubes showed variations up to ±0.04 ppm, although it was usually ±0.02 ppm. The accuracy of chemical shifts reported here is therefore estimated to be on the order of ±0.04 ppm.

Determination of Cmc. The cmc of dihexanoylphosphatidylcholine was determined by using eq 1. Here

$$\nu = x_{mono}\nu_{mono} + x_{mic}\nu_{mic} \quad (1)$$

ν is the observed chemical shift, ν_{mono} is the chemical shift below the cmc, ν_{mic} is the chemical shift of dihexanoylphosphatidylcholine micelles, x_{mono} is the molar fraction of phospholipid in the monomeric state, and x_{mic} is the molar fraction of phospholipid in the micellar state. The values for ν_{mono} and ν_{mic} were estimated from the experimental data, and a value for the cmc was assumed in order to obtain the best fit of the theoretical curve to the experimental data.

Determination of Partition Coefficient. The titration of monomeric phospholipids with Triton X-100 was carried out in the following manner. A solution (3.0 mL) containing 50 mM Tris-HCl pH 8.0, 30% D_2O, and 7.5 mM phospholipid was placed in the inner compartment, and 5% trimethylphosphate in D_2O (v/v) was placed in the outer compartment of a coaxial NMR tube. The chemical shift was measured at 40 °C, and aliquots of Triton X-100 from a 500 or 7.5 mM stock solution were added. The solution was mixed and allowed to reach thermal equilibrium (10 min), and the chemical shift was measured twice. The temperature and pH were checked several times during the experiment and were always found to remain constant.

The fraction of dihexanoylphosphatidylcholine present in Triton X-100 was calculated from eq 1 by using the relationship $x_{mono} = 1 - x_{mic}$, which results in eq 2. A

$$x_{mic} = \frac{\nu - \nu_{mono}}{\nu_{mic} - \nu_{mono}} \quad (2)$$

partition coefficient K can be defined[19] as shown in eq 3.

$$K = X_{mic}/c_{Pmono} \quad (3)$$

Here c_{Pmono} is the concentration of phospholipid in the monomeric state and X_{mic} is the molar fraction of phospholipid in the micellar phase consisting of Triton and phospholipid as defined in eq 4. Here c_{Pmic} and c_{Tmic} are

$$X_{mic} = c_{Pmic}/(c_{Pmic} + c_{Tmic}) \quad (4)$$

the concentrations of phospholipid and Triton, respectively, which are in micelles. The value for c_{Tmic} was calculated from the relation $c_{Tmic} = c_{Ttot} - \mathrm{cmc}_T$, where c_{Ttot} is the total Triton concentration employed and cmc_T is the critical micelle concentration of pure Triton. Using the relations $c_{Pmic} = x_{mic}c_{Ptot}$ and $c_{Pmono} = (1 - x_{mic})c_{Ptot}$, where c_{Ptot} is the total phospholipid concentration, and by combining eq 3 and 4, one can then determine K from the chemical-shift data as shown in eq 5.

$$K = \frac{1}{x_{mic}c_{Ptot} + (c_{Ttot} - \mathrm{cmc}_T)} \frac{x_{mic}}{1 - x_{mic}} \quad (5)$$

Gel-Permeation Chromatography. The partitioning of dihexanoylphosphatidylcholine into Triton X-100 micelles was measured by the equilibrium gel filtration technique of Hummel and Dreyer.[20] A Sephadex G-100 column (1.5 × 50 cm), thermostated at 40 °C, was equilibrated with monomeric dihexanoylphoshatidylcholine solutions

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TABLE I: ^{31}P NMR Chemical Shifts of Phospholipids (6 mM) in the Presence of Triton X-100

phospholipid	chemical shift, ppm
phosphatidylcholine	0.86
lysophosphatidylcholine	0.37
dibutyrylphosphatidylcholine	0.60
lysobutyrylphosphatidylcholine	0.25
glycerophosphorylcholine	0.09
dodecylphosphorylcholine	0.39
phosphatidylethanolamine	0.15
lysophosphatidylethanolamine	−0.26
bis(monacylglyceryl) phosphate	0.70

(0.5–0.75 mM) in 50 mM Tris-HCl buffer, pH 8.0. Samples (1.0 mL) of Triton X-100 (50–200 mM) were eluted with the same buffer. The Triton concentration in the eluate was measured by its absorbance at 276.5 nm while the phospholipid concentration was determined by phosphorus analysis.[21] The molar fraction of phospholipid in the micellar phase consisting of phospholipid and detergent was obtained as shown in eq 6, where n_{Pmic} and n_{Tmic} are

$$X_{mic} = n_{Pmic}/(n_{Pmic} + n_{Tmic}) \quad (6)$$

moles of phospholipid or detergent in the micelles, respectively. Both can be taken directly from the elution profile using an average of the peak and trough for the determination of bound phospholipid. The phospholipid monomer concentration, c_{Pmono}, is the concentration of monomeric phospholipid in the elution buffer. An apparent partition coefficient K was calculated according to eq 3.

Results

^{31}P NMR Chemical Shifts. The ^{31}P NMR chemical shifts of various phospholipids determined under standard conditions are reported in Table I. The aim of the study was to follow the incorporation of monomeric phospholipids into detergent micelles, but, since the change in chemical shift upon solubilization is small and several parameters change slightly during this titration because of dilution, the influence of these parameters on the chemical shift was first investigated.

The chemical shift of egg phosphatidylcholine at a constant Triton-to-phospholipid molar ratio of 8:1 varied from 0.85 ppm at 3 mM to 0.88 ppm at 37 mM, and this is within the error limit of the measurement. Therefore, it is not very critical for reproducibility to maintain the same absolute concentrations of phospholipid as long as the phospholipid-to-detergent ratio remains constant. Also for 7 mM dihexanoylphosphatidylcholine, in a mixture with Triton X-100 at a detergent-to-phospholipid ratio of 8:1, the chemical shift changed from 0.73 to ~0.72 ppm upon dilution with an equal volume of H_2O, 100 mM buffer, or 100 mM EDTA. Similar results were obtained at other detergent-to-phospholipid ratios. The D_2O concentration is also diluted with H_2O during the titration of the monomeric lipids with detergent. It was found that this has no measurable effect on the chemical shift of dihexanoylphosphatidylcholine. Even for phosphatidylethanolamine (6 mM), the observed change is within the range of the error limit (from 0.17 ppm at 10% D_2O to 0.13 ppm at 90% D_2O). This phospholipid shows a pH dependence of the chemical shift[22] with a pK_a of 9.75. At pH 8.0, however, the difference in degree of protonation

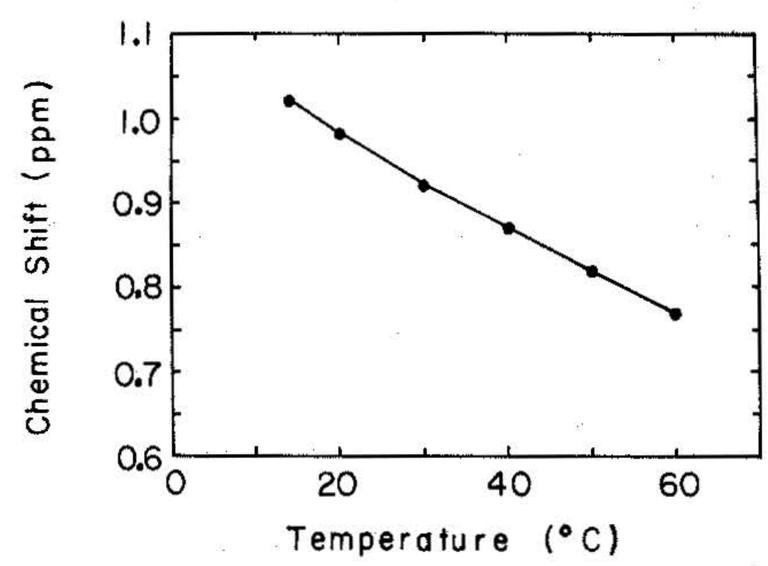

Figure 1. ^{31}P NMR chemical shift of 10 mM phosphatidylcholine in mixed micelles with Triton X-100 under standard conditions as a function of temperature.

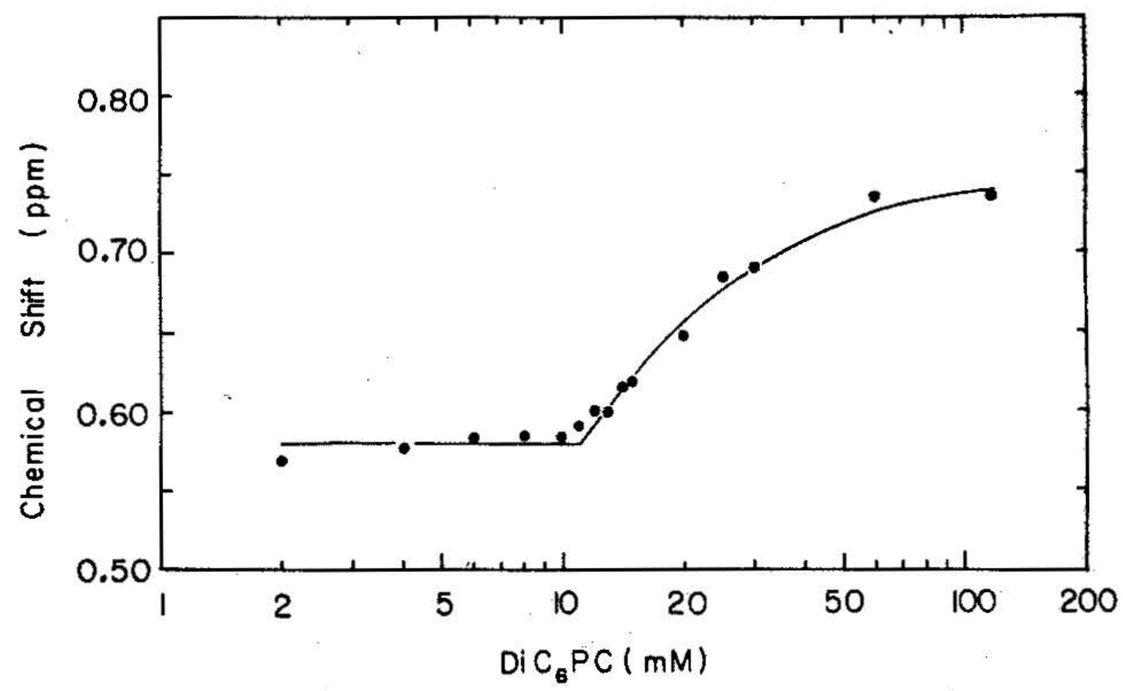

Figure 2. ^{31}P NMR chemical shift of dihexanoylphosphatidylcholine (DiC_6PC) in D_2O as a function of concentration. The solution contained no further additives. The best-fit curve is calculated by using eq 1 and assigning ν_{mono} = 0.58 ppm, ν_{mic} = 0.76 ppm, and cmc = 11 mM.

in H_2O or D_2O is presumably very small but might account for the slight change in chemical shift observed.

The chemical shift of egg phosphatidylcholine relative to H_3PO_4 depends on temperature, as shown in Figure 1, so that all titrations were carried out at a constant temperature.

The effect of Ca^{2+} concentration on the chemical shift of phosphatidylcholine was found to be less than the error limit of shift determinations for a sample of 10 mM egg phosphatidylcholine in going from 10 mM EDTA to 10 mM Ca^{2+}. Similar results were found for dihexanoylphosphatidylcholine. The anionic bis(monoacylglyceryl) phosphate, however, showed a significant dependence on Ca^{2+} concentration. In the presence of 10 mM Ca^{2+}, the chemical shift went from 0.72 to 0.39 ppm. This change was found to be reversible.

Micellization of Dihexanoylphosphatidylcholine. The change in ^{31}P NMR chemical shift was used to follow the micellization of dihexanoylphosphatidylcholine in D_2O in the absence of detergent, as shown in Figure 2. The signal remains a sharp singlet and changes only its position upon change in concentration. Therefore the exchange between monomers and micelles must be rapid compared to the NMR time scale. This is consistent with the results of Hershberg et al.[23] who employed ^1H NMR to study the same compound. The curve is drawn by using their assumption of a constant chemical shift and constant concentration of the monomers above the cmc and eq 1.

The cmc of dihexanoylphosphatidylcholine in pure D_2O obtained from Figure 2 is 11 mM. This is in good agreement with values obtained from ^1H NMR (10 mM),[23] ^{13}C NMR (10.5 mM),[24,25] and refractive-index measurements

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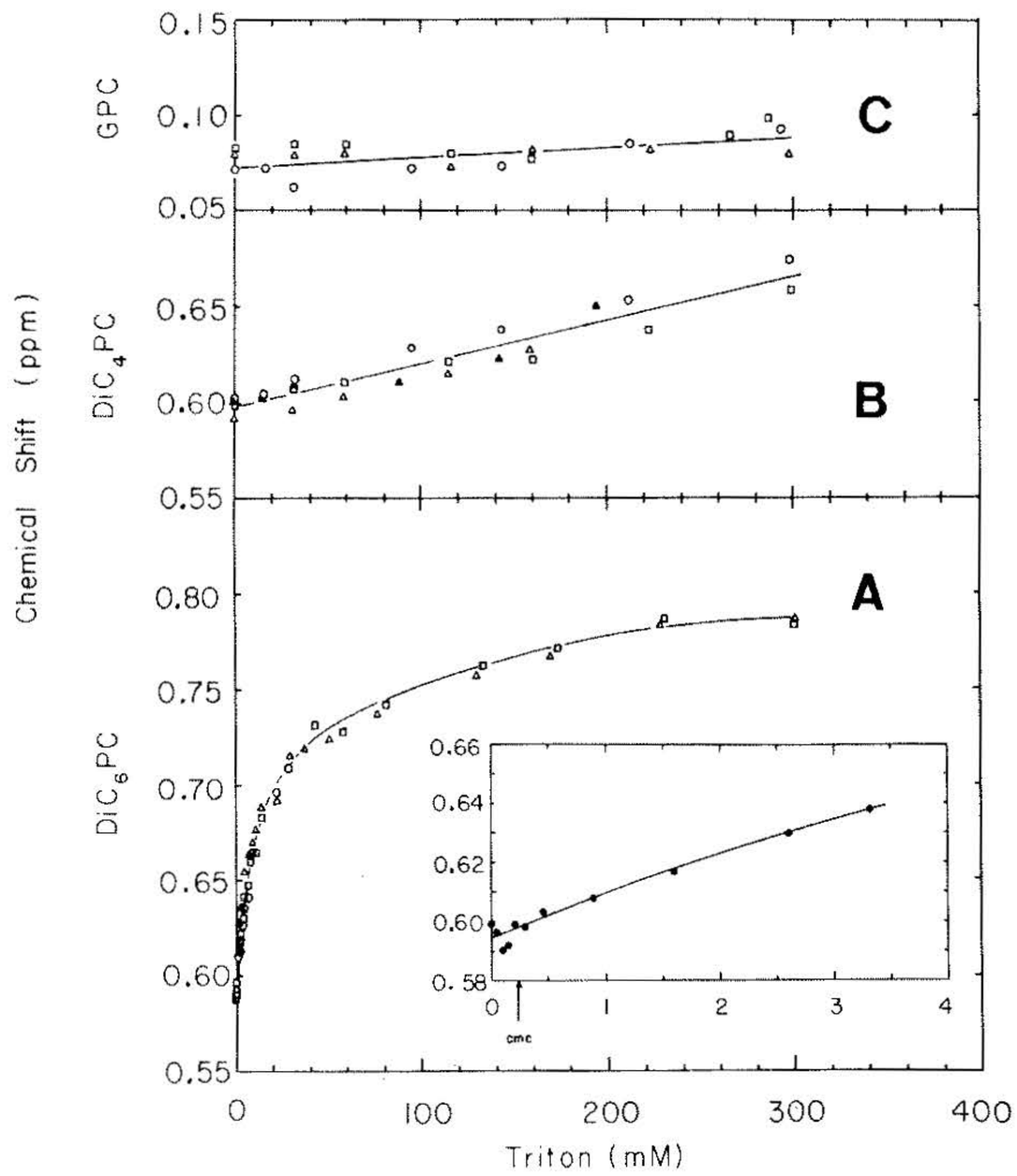

Figure 3. ^{31}P NMR chemical shift of (A) dihexanoylphosphatidylcholine (DiC$_6$PC), (B) dibutyrylphosphatidylcholine (DiC$_4$PC), and (C) glycerophosphorylcholine (GPC) as a function of the concentration of Triton X-100. The initial concentration of phospholipid or GPC was 7.5 mM. The titration was carried out with 500 mM detergent except for the insert to A in which 7.5 mM Triton X-100 was employed. In each panel, the results of three experiments are plotted (O, □, Δ). In B, the titration was also carried out with mixed micelles (▲) consisting of Triton X-100 (500 mM) and phosphatidylethanolamine (125 mM).

(11 mM),[26] in the range of values obtained from dye incorporation (9.5–13.8 mM)[27-30] and slightly lower than those from light-scattering (13.8 mM)[11] and surface-tension measurements (14.6 mM).[11] Experimental conditions such as temperature, buffer, and D_2O content vary slightly between the reported values. With our methods, we could not determine whether there is a change in the micellar properties at a concentration of ~4 times the cmc as proposed by Hershberg et al.[23] and Allgyer and Wells.[24]

Titration of Monomeric Phospholipids with Triton X-100. The chemical-shift change upon the addition of Triton X-100 to monomeric dihexanoylphosphatidylcholine is shown in Figure 3A. In the insert to Figure 3A, the result of a titration of the same amount of phospholipid with a dilute Triton X-100 solution (7.5 mM) is shown. The latter titration was carried out to investigate the behavior of the mixture in the vicinity of the cmc of Triton. The changes in chemical shift around the cmc of Triton X-100 are, however, so small that no conclusions about differences in solubilization of the phospholipid above and below the cmc of Triton can be drawn. In a similar fashion, the incorporation of dibutyrylphosphatidylcholine was investigated (Figure 3B). The incorporation is clearly very much smaller than for dihexanoylphosphatidylcholine

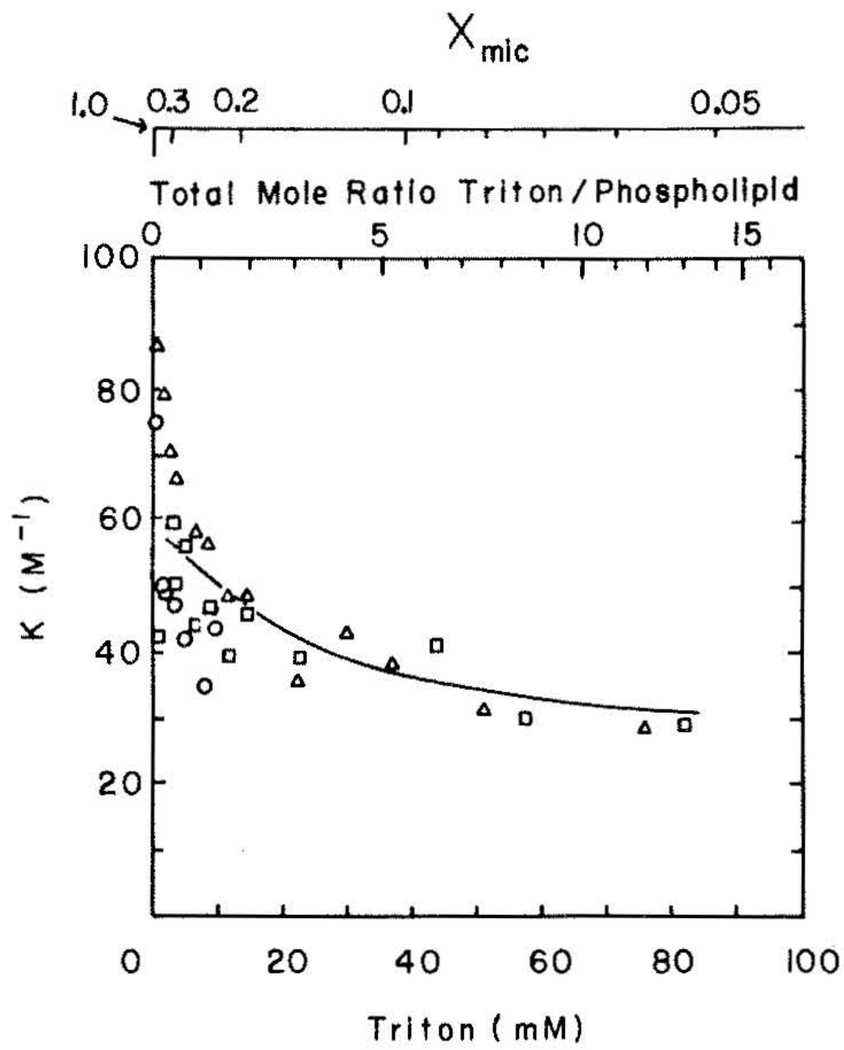

Figure 4. Apparent partition coefficient K of dihexanoylphosphatidylcholine as a function of Triton X-100 concentration. K is determined from the chemical-shift measurements shown in Figure 3A for three experiments (O, □, Δ) as described in the Results. A scale for the total mole ratio Triton/phospholipid is also shown. This scale is not linear with respect to Triton concentration because of the dilution during the titration. The initial phospholipid concentration at 0 mM Triton was 7.5 mM. The uppermost scale shows the molar fraction of phospholipid in the micellar phase, $X_{mic} = c_{Pmic}/(c_{Pmic} + c_{Tmic})$, calculated for the average line shown.

under the same conditions, and at low Triton X-100 concentrations this compound is nearly completely in the monomeric state. When the titration of dibutyrylphosphatidylcholine was carried out with a mixture of Triton and phosphatidylethanolamine at a molar ratio of 4:1, the incorporation of this short-chain phospholipid into the micelles was not enhanced (Figure 3B). As an additional control to monitor effects on the chemical shift during the titration other than solubilization, a titration of glycerophosphorylcholine was carried out analogously. As can be seen in Figure 3C, the chemical shift does not change significantly.

The formation of detergent micelles can be treated quantitatively by a phase-separation model, and the mixed micelles can be considered as a pseudophase.[31-35] In the calculation of a partition coefficient, eq 2 was solved by setting ν_{mono} equal to 0.59 ppm (which is the average value for 7.5 mM dihexanoylphosphatidylcholine in the absence of Triton X-100 in Figure 3A), and ν_{mic} was set at 0.80 ppm (which was estimated from the values at high Triton X-100 concentrations). This calculation is based on the assumption that the electronic environment for phospholipid molecules in the mixed micelles changes only negligibly when going from mixed micelles with high phospholipid content to those with low phospholipid content, so that all micellar dihexanoylphosphatidylcholine molecules show the same ν_{mic}. Because in all titrations the NMR signal remained a sharp singlet, the exchange between monomer and micellar phase is rapid compared to the NMR time

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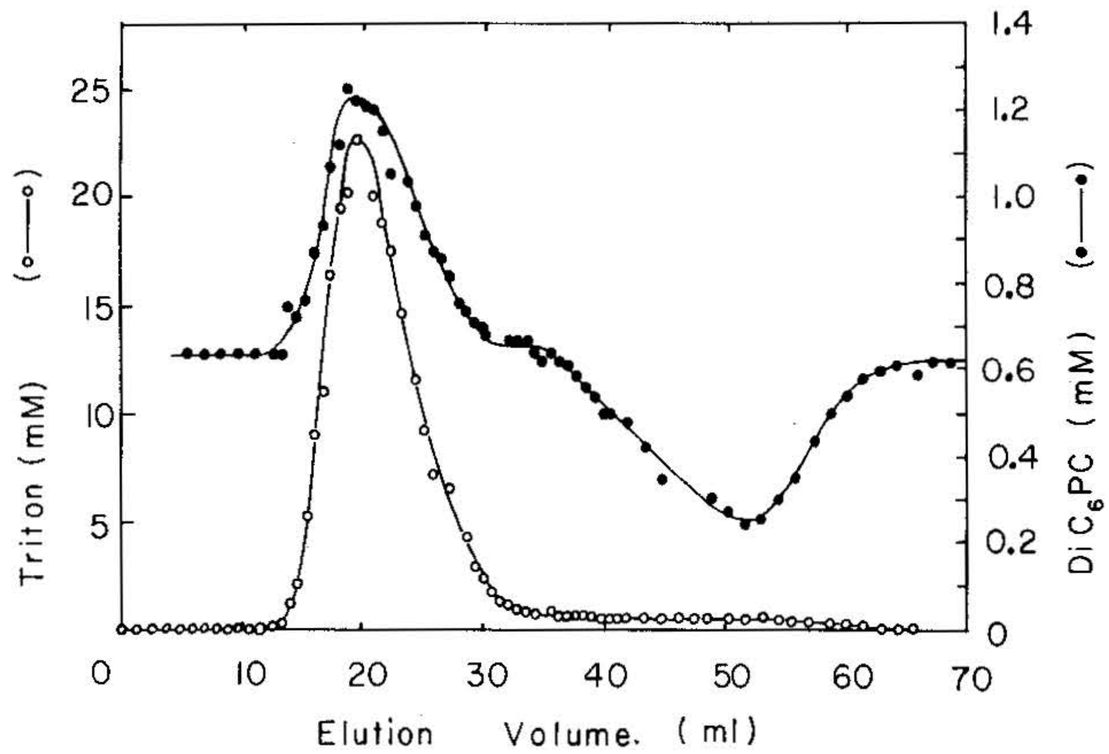

Figure 5. Elution profile of 195 μmol of Triton X-100 on a Sephadex G-100 column preequilibrated and eluted with buffer containing 0.62 mM dihexanoylphosphatidylcholine (DiC_6PC) and 50 mM Tris-HCl, pH 8.0.

TABLE II: Apparent Partition Coefficient for Dihexanoylphosphatidylcholine between Triton X-100 Micelles and Water as Determined by Sephadex G-100 Gel Chromatography

Triton X-100 applied to column, μmol	Triton X-100 elution peak, mM	Triton X-100 av across peak, mM	K, M^{-1}
25	1.8	0.8	90[a]
70	9	4	58
100	15	5	60
195	23	10	45

[a] The very low amount of bound phospholipid introduces a large uncertainty in this value.

scale allowing the use of eq 1.

K was calculated according to equation 5 and plotted in Figure 4 as a function of Triton concentration and Triton-to-phospholipid mole ratio. Also shown in Figure 4 are the approximate micelle compositions, expressed as molar fraction of phospholipid and calculated according to eq 4. The Triton monomer concentration in this titration is not known but was assumed to be 0.25 mM, which is the cmc of pure Triton.[36] This cmc would not be expected to be significantly different in the presence of the buffer employed.[36] The influence of the formation of mixed micelles on the Triton monomer concentration or deviations from the phase-separation model, which assumes constant monomer concentration, are not known. Although in principle it should be possible to devise a method to determine c_{Tmic} directly, for all but the lowest two Triton concentrations employed, the error introduced in c_{Tmic} by uncertainty in the monomer concentration is negligible.

Gel-Permeation Chromatography. A typical elution profile is shown in Figure 5. The amount of phospholipid bound to Triton X-100 micelles was calculated according to the method of Hummel and Dreyer[20] using an average of the peak and trough for this determination. The resulting values of K that can be calculated from these results are shown in Table II. To allow comparison with the NMR data in Figure 4, we have given both the average Triton concentration across the elution peak and the peak Triton concentration. Whichever Triton concentration is used for comparison, the K obtained is in quite good agreement with that obtained by NMR, but it should be noted that, with gel chromatography at the very low Triton concentrations, the measurement of the small amount of bound phospholipid is probably accurate to only ±25%, and thus there is a larger error in the determination of K.

^1H NMR Spectroscopy. For 10 mM dibutyrylphosphatidylcholine in D_2O, the chemical-shift difference between the *sn*-1 and *sn*-2 α-CH_2 groups was determined in the absence and the presence of Triton X-100 at a detergent-to-phospholipid ratio of 8:1. The chemical-shift difference remained constant at 6–7 Hz. This value is consistent with monomeric phospholipid.[14]

Discussion

The solubilization of compounds that are essentially water insoluble was first followed by NMR by Nakagawa and Tori[37] and Eriksson and Gillberg.[38] In the experiments reported here, the incorporation of water-soluble dihexanoylphosphatidylcholine at a concentration below its cmc by Triton micelles was followed by recording the change in the ^{31}P NMR chemical shift upon the addition of Triton X-100, and a partition coefficient was calculated. The ^{31}P NMR results are in quite good agreement with the gel filtration equilibrium binding experiments. This shows that the assumptions used in the calculation of a phase partition coefficient from ^{31}P NMR data are justified as a first approximation.

During the titration, the combined effects of the dilution of phospholipid, buffer, and D_2O and a possible change in bulk diamagnetic susceptibility must be very small, because glycerophosphorylcholine shows little change in chemical shift during an analogous titration (Figure 3C). This compound has no hydrophobic residues and should therefore monitor all effects other than solubilization that lead to a chemical-shift change during the titration. Furthermore, the control experiments with egg phosphatidylcholine show that neither these dilution effects nor small changes in temperature could cause large quantitative errors.

In contrast to dihexanoylphosphatidylcholine, the ^1H and ^{31}P NMR results presented here show that dibutyrylphosphatidylcholine is only sparingly incorporated into Triton X-100 micelles, even at high detergent concentrations. This is consistent with previous gel-chromatography studies.[15] Since ν_{mono} is the same for both dibutyryl- and dihexanoylphosphatidylcholine, if it is assumed that ν_{mic} is also the same for both lipids, the fraction of dibutyrylphoshatidylcholine solubilized as a function of Triton concentration can be estimated from the data in Figure 3. For example, at Triton concentrations below 50 mM, at most 5% of the dibutyrylphosphatidylcholine could be present in the micellar phase. Also, the solubilization is not improved even if the titration is carried out in the presence of a long-chain phospholipid (Figure 3B) that is present in the mixed micelles. Therefore dibutyrylphosphatidylcholine can serve as a monomeric reference compound, and egg phosphatidylcholine, which is completely solubilized by Triton X-100,[39] can serve as a micellar reference compound. Dihexanoylphosphatidylcholine is in an intermediate position between these two extremes depending on the detergent concentration as shown by the ^{31}P NMR, ^1H NMR, and gel-chromatography studies reported here.

The concentration of monomeric phospholipid c_{Pmono} was calculated on the basis of the total volume rather than the volume of only the water phase, but the difference between

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the two approaches should be negligible. When the micelle volume (calculated from the known partial molar volumes of Triton X-100[40,41] and of phospholipid[11]) is taken into account and even if a hydration of 1 g of H_2O per g of Triton X-100 is assumed[3] so that c_{Pmono} increases accordingly, the value of K at 50 mM Triton X-100 decreases by only 6.5%. At lower Triton concentrations, the effect is even less and within the experimental error of the K determination.

The apparent phase partition coefficient changes relatively little over at least a 50-fold change in detergent concentration. This can be taken as further evidence that a simple phase-separation model can be used to describe the solubilization behavior of dihexanoylphosphatidylcholine with reasonable accuracy, although an ideal solubilization behavior can clearly not be expected[10] from two substances with such different structures as the phospholipid and Triton X-100.

Usually the observed partition or distribution coefficient is replaced by a thermodynamic equilibrium constant, and activity coefficients are introduced. The activity coefficients can be taken as unity either for the pure solubilized component as the standard state or for the solubilized component in infinite dilution in the detergent micelle as the standard state. In the present case, however, the uncertainty in the observed partition coefficient increases tremendously for the very high and very low concentrations of phospholipid in the detergent micelles. This is due to the fact that in both extreme regions very small differences from a plateau region are measured. It was also not possible to deduce the limiting behavior of the partition coefficient by extrapolation with sufficient accuracy in either region. We therefore report only the apparent partition coefficients.

A trend in the solubilization behavior of dihexanoylphosphatidylcholine can be seen in Figure 4. Its solubility in the detergent micelles increases with the mole fraction of phospholipid already in the micelles, if the micelles are concentrated in phospholipid. On the other hand, it stays nearly constant if the mole fraction of phospholipid in the micelle phase is below 0.1. There are several possible explanations for this phenomenon. (1) A cooperativity in the phospholipid binding to the micelles would suggest that at least at high phospholipid-to-detergent ratios the phospholipid is not randomly dispersed but might be arranged in smaller aggregates. (2) A structural change of the mixed micelle could occur with increasing phospholipid ratio. This might cause phospholipid to be bound more tightly by micelles already having a high phospholipid content. A gradual structural change is consistent with our data; however, there is no evidence for a sudden transition at a certain Triton-to-phospholipid ratio. At low Triton-to-phospholipid molar ratios, the mixed micelles might be better pictured as phospholipid micelles with some Triton intercalated. A coexistence of small phospholipid aggregates, Triton micelles, and mixed micelles in different proportions at different Triton-to-phospholipid ratios cannot be excluded. (3) The apparent cooperativity is also consistent with a simple effect of the size or shape of the detergent micelles on solubilization, caused by an increase in concentration of detergent during the titration, independent of the detergent-to-phospholipid ratio. At the present time we cannot distinguish between these models or a combination of them. This will only be possible when more structural data on this mixed micelle system become available.

Acknowledgment. We thank Dr. Karol J. Mysels and Dr. John C. Wheeler for helpful discussions on micelle solubilization and Dr. Thomas G. Warner for suggesting the procedure employed in the preparation of the lysophosphatidylcholine. Support for these studies was provided by National Science Foundation Grant PCM 79-22839 and National Institutes of Health Grant GM-20,501. A.P. was the recipient of Fulbright and ITT Fellowships and a Studienstiftung des Deutschen Volkes Fellowship for graduate studies.

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