Lanthanide-induced phosphorus-31 NMR downfield chemical shifts of lysophosphatidylcholines are sensitive to lysophospholipid critical micelle concentration

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ABSTRACT Lysophosphatidylcholine (lysoPC) monomers or micelles in water give rise to a narrow, isotropic phosphorus-31 NMR signal (40.6 ppm; $v_{1/2}$ 1.7 Hz; 32.2 MHz). Upon addition of praseodymium ions, the phosphorus signals are shifted downfield. However, the downfield shifts for the longer-chain lysophosphatidylcholines, which exist in the aggregated state, are far greater than those for the shorter-chain homologues, which exist as monomers. At a Pr^{3+}/lysoPC molar ratio of 0.5, the signals of C_{12}lysoPC through C_{18}lysoPC were shifted by 12.1 ppm, whereas the signals of C_6lysoPC and C_8lysoPC were shifted by only 2.26 ppm. This very pronounced difference in lanthanide-induced downfield shifts between micelles and monomers can be utilized to determine with accuracy lysoPC critical micelle concentrations (CMC) from downfield shift-vs.-concentration plots. The CMC values we determined were 57 mM for C_8lysoPC, 5.7 mM for C_{10}lysoPC, and 0.6 mM for C_{12}lysoPC. The shift reagent phosphorus-31 nuclear magnetic resonance technique particularly lends itself to the measurement of CMC values in the millimolar and high micromolar range. The method can equally be used for measuring critical micelle concentrations of short-chain phosphatidylcholines.

INTRODUCTION

Critical micelle concentration (CMC)[1] is a characteristic physical property of amphiphiles, such as lysophosphatidylcholines (lysoPC). Fluorescence methods (Nakagaki et al., 1986), surface tension techniques (van Dam-Mieras et al., 1975; Kramp et al., 1984; Stafford et al., 1989), and equilibrium dialysis (Haberland and Reynolds, 1975) have commonly been used to determine lysoPC critical micelle concentrations. The surface tension technique is quite accurate, but somewhat sensitive to contaminants. The fluorescence method involves insertion of a foreign probe into the micelles.

Earlier nuclear magnetic resonance (NMR) studies from our laboratory had shown that nitrogen-14 spin-lattice relaxations (^{14}N T_1) and ^{13}C-^{14}N quadrupolar couplings (J_{CN}) of choline phospholipids are most sensitive to changes in the nitrogen environment (Murari and Baumann, 1981). We furthermore observed that ^{14}N T_1 and J_{CN} of lysophosphatidylcholines differing in chain length are indicative of whether a lysoPC species exists in the monomeric or in the aggregated state (Kumar and Baumann, 1989). Recently, phosphorus-31 NMR chemical shift measurements have been used to estimate choline lysophospholipid critical micelle concentrations (Stafford et al., 1989).

In the present study, we observed that the paramagnetic ion-induced phosphorus-31 NMR downfield chemical shifts of lysophosphatidylcholines are most sensitive to lysophospholipid aggregation. While the phosphorus spectra (32.2 MHz) of monomeric lysophosphatidylcholines (20 mM; Pr^{3+}/lysoPC ratio 0.5) were shifted downfield by only ~73 Hz (2.26 ppm), the spectra of lysoPC micelles were shifted downfield by as much as 390 Hz (12.1 ppm). This very pronounced difference in lanthanide-induced downfield shifts between lysoPC monomers and lysoPC micelles can be utilized to measure critical micelle concentrations of lysophosphatidylcholines with convenience and accuracy. The shift reagent phosphorus-31 NMR method particularly lends itself to CMC measurements on lysophosphatidylcholines of short and medium chain length which are less accessible by other physical techniques.

MATERIALS AND METHODS

Synthetic lysophosphatidylcholines (1-O-acyl-*sn*-glycero-3-phosphocholines) of defined chain length were obtained from Avanti Polar Lipids (Birmingham, AL); they were pure as judged by thin-layer chromatography (TLC; developing solvent, $CHCl_3/CH_3OH/H_2O$, 65:35:8, by vol). The lysophospholipids were characterized by carbon-13 NMR (Murari et al., 1982), using $CDCl_3/CD_3OD/D_2O$ (50:50:15, by vol) as solvent (Murari and Baumann, 1981). Deuterated solvents ($CDCl_3$, 99.8% *d:* CD_3OD, 99.5% *d,* D_2O, 99.8% *d*) were from KOR Isotopes (Cambridge, MA). For CMC measurements, known quanti-

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[1]*Abbreviations used in this paper:* CMC, critical micelle concentration; lysoPC, lysophosphatidylcholine, 1-O-acyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; TLC, thin-layer chromatography.

ties of lysoPC were taken up in D_2O and were vortexed until the dispersions appeared clear.

Phosphorus-31 NMR spectra were recorded at 32.2 MHz on a model FT-80A pulse Fourier transform instrument (Varian Associates, Inc., Palo Alto, CA) equipped with a broadband probe. Spectra were measured at 37 ± 1°C in 10-mm (o.d.) sample tubes under proton noise decoupled conditions using identical instrument settings (90° flip angle, 4 KHz sweepwidth, 8 K data points). Accumulated free induction decays (FID) of 100–10,000 transients (depending upon the lysoPC concentration) were Fourier transformed by applying 3.2 Hz exponential line broadening. Chemical shifts (ppm) are expressed in absolute terms. Praseodymium-induced downfield shifts are given in Hertz relative to the chemical shifts of the respective lysoPC in the absence of lanthanide ions. Reproducibility of chemical shifts between experiments was within ± 0.4 Hz; all measurements were done at least on two sample preparations.

Proton-decoupled carbon-13 NMR spectra on lysophosphatidylcholines were recorded at 20 MHz. $N(CH_3)_3$ line shapes were measured (~40,000 transients) on 20 mM dispersions in D_2O.

RESULTS AND DISCUSSION

Aqueous dispersions of lysophosphatidylcholines give rise to a narrow, isotropic phosphorus-31 NMR signal near 40.6 ppm. The linewidth at half-height ($v_{1/2}$), which we measured (32.2 MHz) for a series of lysophosphatidylcholines ranging in chain length from C_6 to C_{16}, were quite independent of the length of the acyl chain and averaged 1.7 Hz. In the presence of praseodymium ions, the lysoPC phosphorus signals were shifted downfield in their entirety indicating that in these lysoPC dispersions all polar head groups were accessible to the ions (Kumar and Baumann, 1986; Kumar et al., 1988). However, there were distinct differences in the magnitude of the Pr^{3+}-induced downfield chemical shifts observed.

Fig. 1 *A* shows the Pr^{3+}-induced phosphorus downfield shifts of lysophosphatidylcholines of different chain length (C_6 through C_{18}) as function of Pr^{3+} concentration. Phosphorus downfield shifts (in Hertz) are expressed relative to the chemical shifts of the respective lysophosphatidylcholines in the absence of lanthanide ions. Pr^{3+} concentrations are given as Pr^{3+}/lysoPC molar ratios at a constant lysoPC concentration (20 mM). As one would expect, downfield chemical shifts increased with increasing Pr^{3+}/lysoPC molar ratios. However, the magnitude of the downfield shifts observed did not directly correlate with chain length. The shifts rather fell into three distinct categories. The shorter-chain lysophosphatidylcholines (C_6lysoPC, C_8lysoPC) showed relatively modest but identical downfield shifts which at a Pr^{3+}/lysoPC 0.5 molar ratio reached 73 Hz (2.26 ppm). The series of longer-chain lysophosphatidylcholines (C_{12}lysoPC through C_{18}lysoPC) also responded to Pr^{3+} in identical fashion. However, the magnitude of the shifts observed was more than five times greater (390 Hz; 12.1 ppm) than that for the shorter-chain lysophosphatidylcholines. The lanthanide-induced downfield shifts for C_{10}lysoPC fell between those of the two other groups (Fig. 1 *A, center curve*).

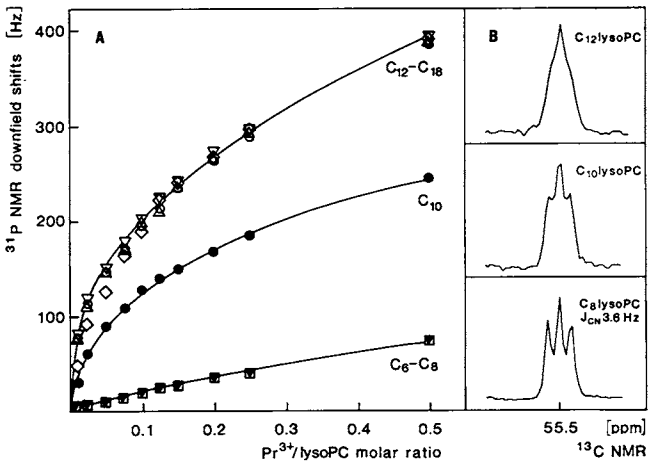

FIGURE 1 (*A*) Pr^{3+}-induced ^{31}P NMR downfield chemical shifts of lysophosphatidylcholines of different chain length (C_6 through C_{18}) in D_2O as function of Pr^{3+} concentration. Phosphorus downfield shifts are expressed in Hertz relative to the chemical shifts of the respective lysophosphatidylcholines in the absence of lanthanide ions. Pr^{3+} concentrations are given as Pr^{3+}/lysoPC molar ratios at a constant lysoPC concentration (20 mM). The symbols represent: □, C_6lysoPC; ▼, C_8lysoPC; ●, C_{10}lysoPC; ◇, C_{12}lysoPC; △, C_{14}lysoPC; ○, C_{16}lysoPC; ▽, C_{18}lysoPC. (*B*) ^{13}C NMR lineshapes of the choline -$N(CH_3)_3$ signals of C_8lysoPC (*bottom*), C_{10}lysoPC (*center*), and C_{12}lysoPC (*top*) in D_2O (20 mM).

Thus, the lanthanide-induced phosphorus downfield shifts of lysophosphatidylcholines do not mirror lysoPC chain length per se. In fact, the shifts are identical within the group of shorter-chain lysophosphatidylcholines on one hand, and within the group of longer-chain homologues on the other. This suggested that the downfield shifts reflect the state of lysophospholipid aggregation, or nonaggregation, in these lysoPC dispersions.

This interpretation of the phosphorus-31 NMR shift data was tested using carbon-13 NMR. Fig. 1 *B* depicts the ^{13}C NMR lineshapes of the choline $N(CH_3)_3$ signals (near 55 ppm) of three representative lysophosphatidylcholines (20 mM). C_8lysoPC (Fig. 1 *B, bottom*) and the shorter-chain lysophosphatidylcholines (not shown) give rise to well-resolved triplets with quadrupolar ^{13}C-^{14}N couplings (J_{CN}) of 3.6 Hz. However, C_{12}lysoPC (Fig. 1 *B, top*) and the longer-chain lysophosphatidylcholines (not shown) produce only a broadened single peak. In case of C_{10}lysoPC (Fig. 1 *B, center*), the triplet is only partially collapsed to a singlet. We had earlier shown that choline phospholipids give rise to well-resolved choline methyl carbon triplets due to quadrupolar ^{13}C-^{14}N couplings only in the monomeric state (Murari and Baumann, 1981). On the other hand, phospholipid aggregation is

indicated by a sudden collapse of the triplets due to a reduction in ^{14}N T_1 caused by a restriction of motion about the choline CH_2-CH_2 linkage (Murari and Baumann, 1981).

The carbon-13 lineshape analyses (Fig. 1 *B*) are consistent with our interpretation of the lanthanide-induced phosphorus-31 NMR chemical shifts observed (Fig. 1 *A*). Both ^{13}C and ^{31}P NMR data support the conclusion that at 20 mM concentration C_8lysoPC and the shorter-chain lysophosphatidylcholines exist as monomers and that C_{12}lysoPC and the longer-chain lysophosphatidylcholines occur as aggregates in water.

The pronounced difference in Pr^{3+}-induced phosphorus downfield shifts between lysophosphatidylcholine monomers and micelles appears to offer itself as a unique tool to gain information on the state of lysophospholipid aggregation.

Fig. 2 depicts the Pr^{3+}-induced downfield shifts of the ^{31}P NMR signals of C_8lysoPC (*left panel*), C_{10}lysoPC (*center panel*), and C_{12}lysoPC (*right panel*) as a function of lysophospholipid concentration. In all instances, the Pr^{3+} concentration was kept constant at 3 mM. At the point of initial micellization, the chemical shift-vs.-concentration plots show a sharp break, followed by a steep incline with increasing micellar contribution. The plots eventually plateau as the contribution from monomers becomes negligible. It is noteworthy that the Pr^{3+}-induced relative downfield shifts of all monomers are identical (19 Hz) indicating that the monomer shifts are quite independent of lysoPC chain length or lysoPC concentration. However, the plateaus in the chemical shift-vs.-concentration plots of C_8lysoPC (66 Hz), C_{10}lysoPC (148 Hz), and C_{12}lysoPC (191 Hz) are reached at different levels, which implies that the downfield shifts of lysoPC micelles are greatly Pr^{3+}/lysoPC molar ratio dependent.

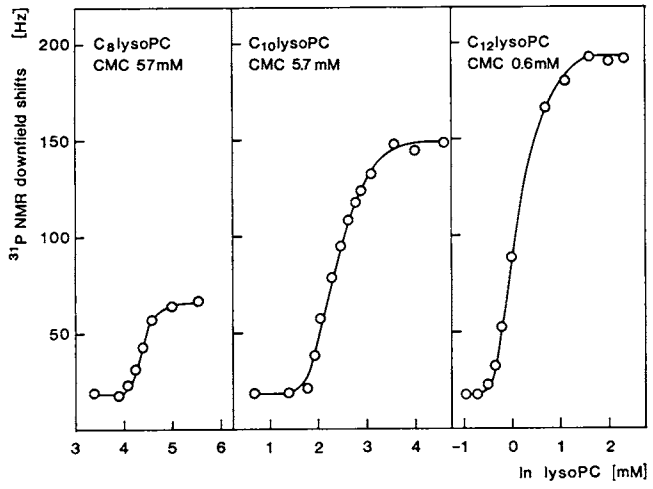

FIGURE 2 Pr^{3+}-induced ^{31}P NMR relative downfield chemical shifts of lysophosphatidylcholines in D_2O as function of lysoPC concentration with the Pr^{3+} concentration being kept constant at 3 mM. (*Left panel*) C_8lysoPC; CMC, 57 mM. (*Center panel*) C_{10}lysoPC; CMC, 5.7 mM. (*Right panel*) C_{12}lysoPC; CMC, 0.6 mM. CMC values were determined from the intercepts of the best fitted two straight lines which extend the concentration dependence of the downfield shifts of the monomers (*horizontal*) and the increase in downfield shift with increasing micellar contribution (*slope*). The linearity of the horizontals toward lower concentrations was ascertained for C_8lysoPC and C_{10}lysoPC beyond the points depicted.

The critical micelle concentration of a given lysophosphatidylcholine is readily obtained from the intercept of the best fitted straight lines which reflect (*a*) the Pr^{3+}-induced downfield shifts of the monomers (horizontal line) and (*b*) the increase in downfield shifts with increasing micellar contribution (*slope*). The CMC values thus determined were 57 mM for C_8lysoPC, 5.7 mM for C_{10}lysoPC, and 0.6 mM for C_{12}lysoPC.

Fig. 3 shows the Pr^{3+}-induced phosphorus downfield shifts of the three representative lysophosphatidylcholines as function of lysoPC concentration, but in this case at constant Pr^{3+}/lysoPC molar ratios. The data were gathered to determine whether and how the critical micelle concentrations measured by the present shift reagent ^{31}P NMR technique would be affected by the prevalent paramagnetic ion concentration. The lower curves show the respective shift-vs.-lysoPC concentration plots at a Pr^{3+}/lysoPC ratio of 0.1, the upper curves at a ratio of 0.5. Because the Pr^{3+} concentrations increase with increasing lysoPC concentrations, the

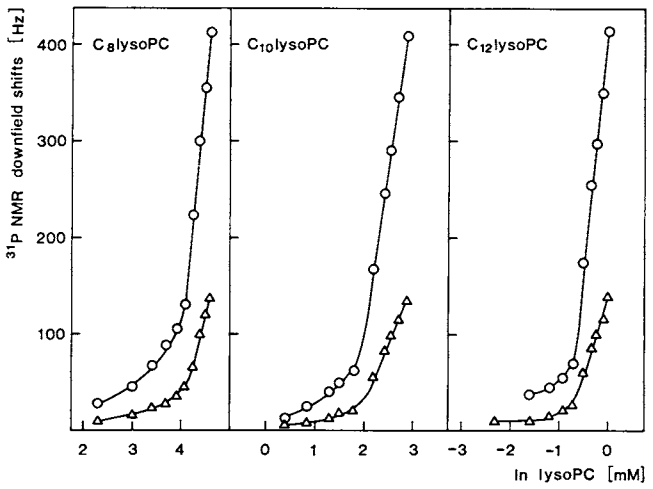

FIGURE 3 Pr^{3+}-induced ^{31}P NMR relative downfield chemical shifts of lysophosphatidylcholines in D_2O as function of lysoPC concentration with the Pr^{3+}/lysoPC molar ratio being kept constant at either 0.1 (△) or 0.5 (○). Respective CMC values at the two Pr^{3+}/lysoPC ratios were identical. They also agreed, within experimental error, with those determined at constant Pr^{3+} concentration (see Fig. 2).

curves differ from those measured at a constant Pr^{3+} level (Fig. 2). For example, unlike in Fig. 2, the chemical shifts of the monomers increase with increasing lysoPC concentration, and the plots do not exhibit the characteristic plateaus which are observed at constant Pr^{3+} levels (Fig. 2). Nevertheless, the breaks in the shift-vs.-lysoPC concentration plots occur at identical lysoPC concentration at either Pr^{3+}/lysoPC ratio, and they match the breaks observed at constant Pr^{3+} concentration (Fig. 2).

Taken together, our data show that lysophosphatidylcholine critical micelle concentrations can readily be obtained from Pr^{3+}-induced phosphorus downfield shift-vs.-concentration plots and that, within practical ranges, the CMC values measured are quite independent of the praseodymium concentration. In our experience, a Pr^{3+} concentration corresponding to a Pr^{3+}/lysoPC molar ratio of 0.5 at the critical micelle concentration appears to be favorable for typical CMC measurements.

The lysophosphatidylcholine critical micelle concentrations which we measured by the present shift reagent phosphorus NMR method are in reasonably good agreement with those reported in the literature. In Table 1, we compare our lysoPC CMC values with data that were obtained by alternate physical techniques. In Fig. 4, we have plotted our CMC values as function of chain length, and the data points are fitted by least square analysis. Also plotted are literature values.

Although the lysoPC CMC data reported in the literature are of similar magnitude (see Fig. 4), the values that have been obtained by different techniques vary somewhat dependent on the study (see Table 1). Thus, for example, the CMC values measured for C_{16}lysoPC by surface tension techniques were 4 μM (Kramp et al., 1984) and 7 μM (Stafford et al., 1989), by equilibrium dialysis 7 μM (Haberland and Reynolds, 1975), and by fluorescence 8.3 μM (Nakagaki et al., 1986). The CMC values that have been reported for other lysophosphatidylcholines can also vary by as much as a factor of 2 (Table 1). It appears noteworthy that *no one technique* gives consistently low or consistently high values. The accuracy of a CMC determination rather seems to depend on the accuracy with which a change in observed characteristic physical property can be pinpointed upon micellization. Hence, the *magnitude* of the effective change observed between monomers and micelles is of crucial importance for the determination of critical micelle concentrations with accuracy and confidence.

In the present study we found that the Pr^{3+}-induced phosphorus-31 NMR relative downfield chemical shifts of lysophosphatidylcholines are most sensitive to lysophospholipid aggregation. While in the absence of lanthanide ions, micellization of lysoPC is indicated by a minute *upfield* shift of ~0.045 ppm (at 80.99 MHz; Stafford et al., 1989), the difference in observed Pr^{3+}-induced *downfield* shifts between lysoPC monomers and micelles at a Pr^{3+}/lysoPC 0.5 molar ratio is as high as 9.85 ppm (317 Hz). This very pronounced effect makes it possible to measure critical micelle concentrations with the accuracy desired.

The present shift reagent phosphorus NMR method very well lends itself to the measurement of CMC values of lysophosphatidylcholines. The method is particularly well suited for lysophosphatidylcholines of short and

TABLE 1 Critical micelle concentrations of lysophosphatidylcholines

Lysophosphatidylcholines	Critical micelle concentrations (CMC)	
	Shift reagent ^{31}P NMR method	Literature values obtained by alternate physical techniques
C_8lysoPC	57 *mM*	—
C_{10}lysoPC	5.7 *mM*	6 *mM**; 7 mM‡
C_{12}lysoPC	0.6 *mM*	0.4 *mM**; 0.43 *mM*§; 0.5 *mM*‖; 0.7 *mM*‡
C_{14}lysoPC	(61 μ*M*)¶	43 μ*M*§; 63 μ*M*‖; 70 μ*M*‡
C_{16}lysoPC	(6.1 μ*M*)¶	7 μ*M***; 4 μ*M*§; 8.3 μ*M*‖; 7 μ*M*‡
C_{18}lysoPC	(0.61 μ*M*¶	0.4 μ*M*§

*van Dam-Mieras et al. (1975); surface tension equilibrium measurements.

‡Stafford et al. (1989); CMC values of C_{10}lysoPC and C_{12}lysoPC were obtained by surface tension (bubble technique) and ^{31}P NMR chemical shift measurements; CMC values of C_{14}lysoPC and C_{16}lysoPC were obtained by surface tension measurements only.

§Kramp et al. (1984); surface tension equilibrium measurements.

‖Nakagaki et al. (1986); 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) fluorescence measurements.

¶Values were obtained by extrapolation of the least square fits of the CMC values of C_8lysoPC through C_{12}lysoPC obtained by the present shift reagent ^{31}P NMR method (see Fig. 4).

**Haberland and Reynolds (1975); equilibrium dialysis.

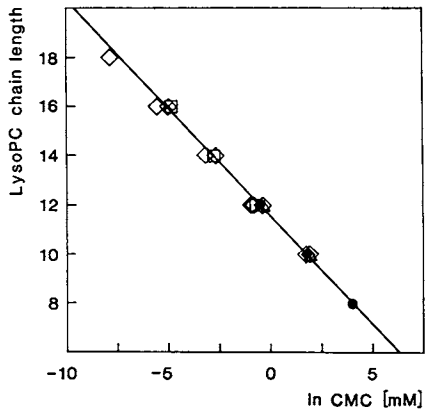

FIGURE 4 Effect of lysophosphatidylcholine chain length on lysoPC critical micelle concentration. The CMC values determined by the present shift reagent ^{31}P NMR method (●) are fitted by least square analysis and are compared with CMC data obtained by surface tension methods (◇), equilibrium dialysis (×), ^{31}P NMR shift measurements (△), and fluorescence techniques (□). The literature data are those listed and referenced in Table 1.

medium chain length with CMC values in the millimolar and high micromolar range. Longer-chain lysophosphatidylcholines, because of their very low critical micelle concentrations, require larger sample volumes and longer accumulation times.

In our hands, the shift reagent NMR method has proven equally useful for measuring CMC values of other micelle forming phospholipids, such as short-chain phosphatidylcholines. The downfield shift-vs.-concentration plots also produced distinct breaks at the CMC. The CMC values we determined, for example, for dihexanoyl PC (15.2 mM) and diheptanoyl PC (1.45 mM) are in excellent agreement with those obtained by surface tension techniques (Tausk et al., 1974).

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