Phosphorus NMR analysis of phospholipids in detergents

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Abstract Various detergents can be used to dissolve phospholipids, resulting in very narrow ^{31}PNMR resonances. These resonances are well resolved, allowing identification and quantitative analysis of phospholipids in a mixture. The chemical shift depends strongly on pH, reflecting changes in the state of ionization of the phospholipid headgroup moieties. Samples of phospholipids dissolved in aqueous detergents are conveniently prepared and give narrower ^{31}P resonances than do phospholipids dissolved in organic solvents.—**London, E., and G. W. Feigenson.** Phosphorus NMR analysis of phospholipids in detergents. *J. Lipid Res.* 1979. **20:** 408–412.

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An important aspect of the utility of ^{31}PNMR spectroscopy in studies of biomembranes and sonicated model membrane systems (1–6) is that the headgroup ^{31}P nuclei of the different phospholipids have different chemical shifts. Henderson, Glonek, and Myers (1) measured the ^{31}PNMR chemicals shifts of many phospholipids in organic solvents. In that study and in others, relatively broad resonances of line-width 20–40 Hz were observed in organic solvents (1), in sonicated vesicles (2), and in detergents (3). However, later studies (4, 5) report much sharper resonances of line-width 1–3 Hz in solvents and 7–10 Hz in vesicles. Some of the factors influencing line-width have been identified. Henderson et al. (1) observed that the presence of multivalent cations resulted in broad ^{31}P resonances which could be narrowed by treatment with EDTA. However, these workers still observed broad resonances, evidently because they did not use broadband ^{1}H decoupling. The ^{31}P chemical shift anisotropy was recognized by Berden et al. (7) to be a significant source of line-broadening in vesicle systems at high magnetic field strengths. Berden, Barker, and Radda (5), having obtained sharp resonances, illustrated the usefulness of ^{31}PNMR for analyzing phospholipid asymmetry in vesicles and for detecting the presence of different phospholipids in a membrane extract. However, solvent extraction and pretreatment with EDTA were necessary steps in these preparations. The present study describes a convenient method of sample preparation which results in significantly better resolved phospholipid spectra.

MATERIALS AND METHODS

Materials were obtained as follows: dipalmitoyl PC, Triton X-100, cholic acid, and deoxycholic acid from Sigma; dilauroyl PE from Calbiochem; bovine SM and bovine PS from Applied Science; egg PG from Avanti; plant PI from Lipid Products; bovine PS and bovine CL from GIBCO; crude soybean phospholipid from Associated Concentrates, Inc.; egg yolk PC, dimyristoyl PC, lyso egg yolk PC were prepared according to standard procedures (8, 9). All other chemicals were reagent grade. Sarcoplasmic reticulum (R_1, washed) was prepared by the method of MacLennan (10). Sarcoplasmic reticulum phospholipids were prepared by a Folch extraction (11), followed by removal of acetone-soluble and ether-insoluble impurities (12).

Identity of the phospholipids was confirmed by TLC on silicic acid in two solvent systems: chloroform–methanol–acetic acid–water 25:15:4:2 (v/v), and chloroform–methanol–conc. ammonium hydroxide 65:25:5. Phospholipids were detected with a phosphate-sensitive spray (13) and subsequent charring. Apparent purity of each phospholipid by TLC was >95%. Soy bean phospholipids were separated by two-dimensional TLC. The developing solvent in the first dimension was chloroform–methanol–conc. ammonium hydroxide 65:25:5; the developing solvent in the second dimension was chloroform–methanol–water 65:25:1. Each phospholipid spot was visualized with iodine

Abbreviations: ^{31}PNMR, phosphorus 31 nuclear magnetic resonance; NOE, nuclear Overhauser effect; SDS, sodium dodecyl sulfate; P_i, inorganic orthophosphate; EDTA, ethylenediaminetetraacetate; CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography.

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staining and identified by comparison with standard samples of purified phospholipids. The phospholipid spots were extracted in chloroform–methanol–water 65:25:4 and analyzed for phosphate by standard procedures (14, 15). ^{31}PNMR spectra were recorded at 40°C on a Varian CFT-20 NMR spectometer operating at 32.19 MHz. Except where noted, all spectra were broadband ^{1}H decoupled.

Samples containing phospholipid dissolved in detergent were prepared from dry phospholipid. To achieve solubilization by detergent, an excess by weight of detergent over phospholipid was employed in all samples, with a final concentration of not less than 2% w/v detergent. Samples contained excess (10–125 mM) EDTA and 25–75% D_2O (to provide an internal field/frequency lock) in a total volume of 0.75–1 ml, unless otherwise stated. Alternating short (15 sec) periods of sonication (bath sonicator, Laboratory Supplies Co.) and warming to about 60°C were used to achieve rapid solubilization. Samples can also be prepared by the addition of detergent to multilamellar vesicles (banghasomes) or to unilamellar vesicles prepared by sonication. Sarcoplasmic reticulum samples dissolved immediately upon mixing with detergent.

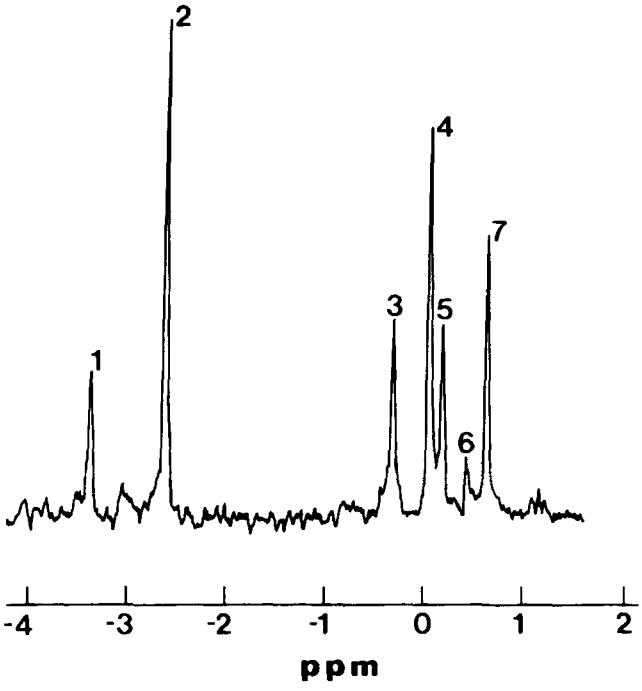

Fig. 1. ^{31}PNMR spectrum of a phospholipid mixture consisting of 5% w/v cholate, 50 mM EDTA and the following: peak *1*, 10 mg of PA; peak *2*, 6.6 mg of K_2HPO_4; peak *3*, 8 mg of CL; peak *4*, 13 mg of PE; peak *5*, 12.5 mg of PS; peak *6*, PI present as an impurity in PS; peak 7, 12.5 mg of dipalmitoyl PC. Total volume 1 ml, pH ~8. 100 transients were collected with an acquisition time of 2 sec per transient, no delay between transients and a filtering time constant of 1 sec.

TABLE 1. ^{31}P Chemical shifts of phospholipids

Species	Chemical Shift from External 86% H_3PO_4 (ppm) in Cholate or [Triton X-100]
PC	+0.65 [+0.9]
PI	+0.40
lyso PC	+0.15
PS	+0.12
PE	+0.00 [+0.25]
SM	+0.00
CL	−0.31
PG	−0.43
external 1 M P_i pH 7.0	−2.1
PA	−3.8

Samples of pH ~8 were prepared in potassium cholate or Triton X-100 as described in Materials and Methods. Upfield shifts are positive. (cf. Henderson et al. (1)).

RESULTS

A spectrum of a mixture of phospholipids is shown in **Fig. 1.** Chemical shifts were assigned using samples containing the phospholipid and external 86% H_3PO_4 or external 1 M P_i at pH 7 as a standard. Line-widths of less than 1 Hz were observed for the phospholipids dispersed in potassium cholate. Sharp phospholipid lines were observed in cholate, deoxycholate, SDS, and Triton X-100. Without broadband ^{1}H decoupling most resonances collapsed into a single peak with a linewidth of about 40 Hz. Cholate and deoxycholate seemed to dissolve lipids somewhat more easily than did SDS or Triton X-100. Furthermore, stable foams did not form as readily with cholate or DOC as they did with SDS and Triton X-100.

The chemical shifts for a variety of phospholipids are compiled in **Table 1.** In general a variation of ±0.08 ppm in chemical shift for individual phospholipids was seen in different sample preparations. The effect of fatty acyl chain unsaturation on the chemical shift of PC was examined by combining in detergent highly unsaturated soy PC (12), moderately unsaturated egg PC (16), and saturated dimyristoyl PC. One sharp resonance was observed.

The values of T_1 and NOE for a mixture of phospholipids solubilized in cholate are compiled in **Table 2.** The values of these parameters determine the conditions of spectral acquisition necessary for quantitative analysis, by ^{31}PNMR, of phospholipid mixtures solubilized in cholate (see Discussion). Table 2 shows that the values of T_1 and NOE are very similar for the different phospholipids tested, but differ significantly from the values for P_i.

The effect of varying pH on chemical shift is illustrated in **Fig. 2.** The observed changes in chemical shift reflect the state of ionization of the phosphate

TABLE 2. T_1 and NOE of phospholipids solubilized in cholate

Species	T_1	NOE
	sec	%
PC	3.3	60
PE	3.2	60
PA	3.3	50
P_i	4.3	5

Sample consisted of 11.7 mg of dimyristoyl PC, 13.75 mg of dilauryl PE, and 7.65 mg of disodium dipalmitoyl PA solubilized in 5% potassium cholate, pH ~8, with 10 mM K_2HPO_4 and 10 mM EDTA. Total volume 1 ml. T_1 was measured by inversion recovery and NOE by the percent increase in signal intensity in a spectrum with gated decoupling over the signal intensity in a spectrum with ordinary broadband decoupling (19). Samples were not deoxygenated.

group of PC and of the phosphate and amino groups of PE (17, 18). The apparent pK_a of the phosphate group is <1 for both PC and PE dissolved in Triton X-100. The apparent pK_a of the amino group is 9.75 for PE dissolved in Triton X-100. PA and PS also exhibited strong pH dependence of chemical shift due to changes in ionization of their headgroup moieties. The reversibility of the pH-dependent chemical shifts upon back titration showed that no lipid decomposition occurred during the titration.

The spectrum of sarcoplasmic reticulum dissolved in detergent is shown in **Fig. 3*A*.** There is no evidence of a broad resonance indicative of enzyme-bound phospholipid. An extract of lipids from sarcoplasmic reticulum gives a spectrum very similar to that of sarcoplasmic reticulum dispersed in detergent (Fig. 3*B*). The phospholipid composition determined from ^{31}PNMR is in good agreement with that previously obtained from TLC (19). **Table 3** shows a comparison of quantitative analysis of two phospholipid mixtures by both ^{31}PNMR and either phosphate or gravimetric analysis. In general there is agreement to within 10%.

DISCUSSION

The ^{31}P chemical shifts of a number of phospholipids are given in Table 1. These shifts are sufficiently different and the peaks are sufficiently sharp to enable quantitative analysis of the phospholipids in a complex mixture, even at the relatively low magnetic field strength at which these spectra were taken. The narrow linewidths observed in detergents result from efficient averaging of chemical shift anisotropy and of dipolar interactions in small micelles. Chemical shifts of phospholipid species are different in cholate, in Triton X-100, and in organic solvents (1). These shifts could be affected by hydrogen bonding, dielectric constant, or ring current shifts in the environment of the phosphorus atom. The concentration of detergent or of phospholipid may have some effect on chemical shift as well.

The pH dependence of chemical shift illustrates that ^{31}PNMR can be useful in determining the pK_a of an ionizable group on a phospholipid. The states of ionization of several phospholipids in a mixture can be determined simultaneously. In addition the pH dependence of chemical shift can be a useful tool in an analysis. If the chemical shifts of two phospholipid species are identical at a particular pH (i.e., as is the case for PE and SM near neutrality), then, by adjusting pH, the individual resonances can be resolved. This is one important advantage of ^{31}PNMR analysis of phospholipids in detergent over ^{31}PNMR analysis of phospholipids in organic solvent.

Several variables must be controlled in order that quantitative information be obtained. In particular, differences in spin-lattice relaxation times (T_1) and in the nuclear Overhauser effect (NOE) can result in the lack of correspondence between observed peak area and molarity among phospholipid species. If broadband ^{1}H decoupling is used, the NOE must be measured for each phospholipid in the sample. To circum-

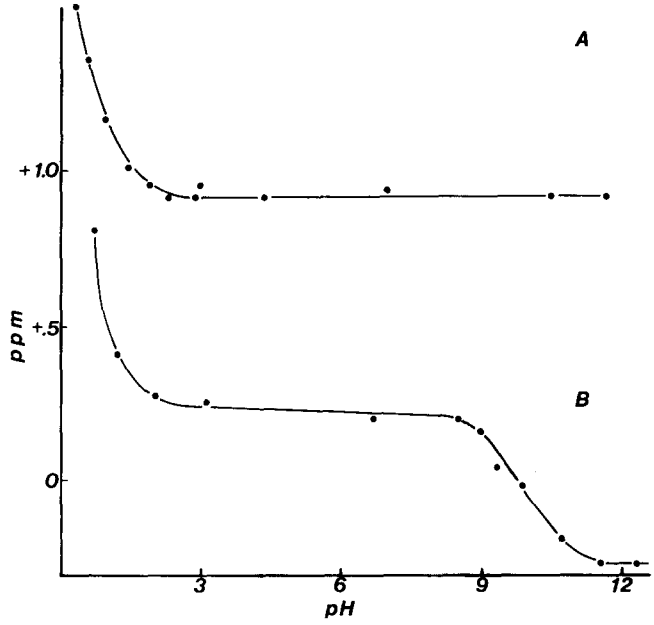

Fig. 2. pH dependence of the chemical shift of phospholipids dissolved in Triton X-100. *A*, 4 mg/ml dimyristoyl PC dissolved in 2.5% (w/v) Triton X-100. *B*, 2.1 mg/ml dilauryl PE dissolved in 2.5% (w/v) Triton X-100. Samples contained an external D_2O field/frequency lock.

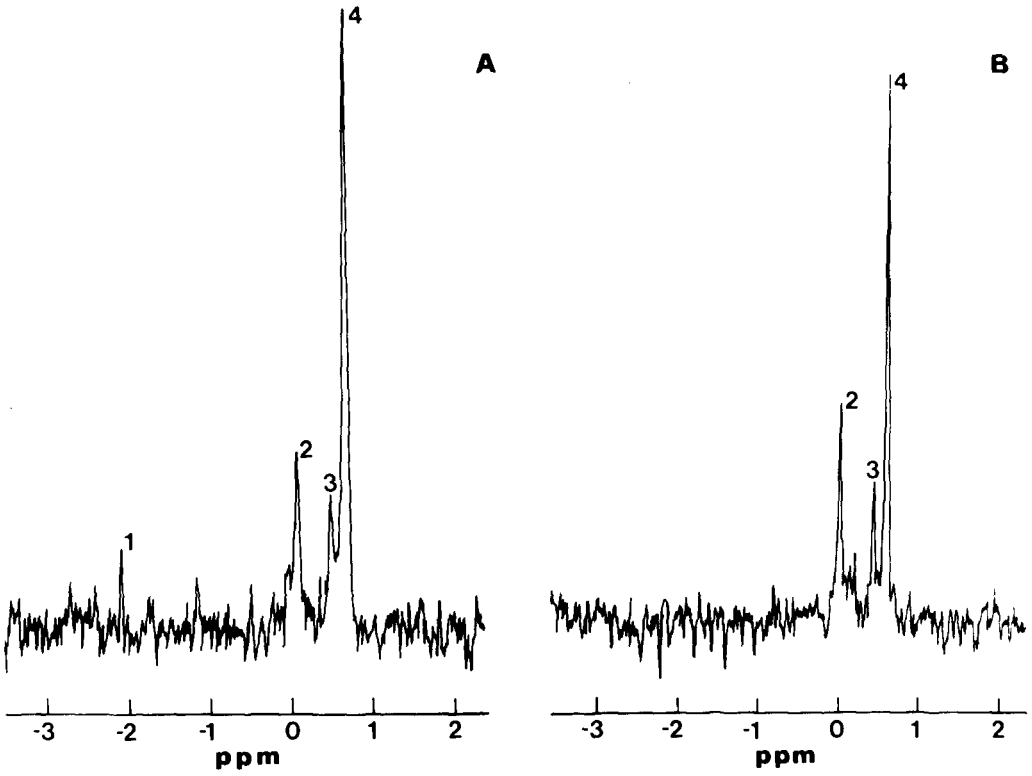

Fig. 3. ^{31}PNMR spectra of sarcoplasmic reticulum phospholipids. *A*, sarcoplasmic reticulum (12 mg protein/ml) dissolved in cholate, pH ~8. Peak assignments: (*1*) P_i, (*2*) PE, (*3*) PI, (*4*) PC. 6000 transients were collected with an acquisition time of 2 sec per transient, no delay between transients and a filtering time constant of 1 sec. *B*, lipid extract of sarcoplasmic reticulum (10 mg lipid/ml) dissolved in cholate. Peak assignments as in *A*. 600 transients were collected with other spectrometer settings as in *A*.

vent the necessity of measuring the NOE in every sample that is broadband ^{1}H decoupled, one may use the gated decoupling method (20), though at some sacrifice in signal-to-noise.

As shown in Table 2, the T_1 values are very similar among the different phospholipids as are the NOE values (these samples were not deoxygenated). These similarities mean that the relative spectral intensities for the phospholipids measured using pulse intervals on the order of T_1 and using ordinary broadband proton decoupling will be very close to the true relative intensities as measured with pulse intervals of approximately 5 to 10 × T_1 and using gated broadband proton decoupling (21). However, it is quite possible that for some samples the T_1 values are different among the phospholipids or the NOE values are different. In these cases rapid pulsing and ordinary broadband decoupling can result in significant errors in the measured relative spectral intensities of the phospholipids.

One factor limiting the utility of this method for analysis is the relatively low sensitivity of ^{31}PNMR.

TABLE 3. Quantitative analysis of phospholipids by ^{31}PNMR

Sample	Species	Relative Amount (mol/mol) (PC ≡ 1.00)	
		By Chemical or Gravimetric Analysis	By ^{31}PNMR
A	PC	1.00	1.00
	PE	1.37	1.25
	P_i	0.66	0.66
	PA	0.60	0.64
B	PC	1.00	1.00
	PI	0.48	0.60
	PE	0.95	1.06
	PA	0.26	0.31

Sample A. See Table 2 for composition. Relative amounts determined from measured weight. For ^{31}PNMR analysis a spectrum was recorded utilizing 300 transients, with 30 sec between successive radiofrequency pulses with gated decoupling.

Sample B. Soybean phospholipid analyzed by two-dimensional TLC followed by chemical P_i analysis (see Materials and Methods). ^{31}PNMR analysis was performed on a sample consisting of 45 mg/ml lipid dispersed in 1.1 ml of 4.5% potassium cholate, pH ~8, with 70 mM EDTA. The spectrum consisted of 2500 transients with 15 sec between successive radiofrequency pulses with gated decoupling.

However, the narrow line-width of the phospholipid resonance compensates for this and we have been able to detect as little as 0.2 μmol of phospholipid by signal averaging. We have found that to detect 10% impurities in 10 μmol of phospholipid at a signal-to-noise ratio of 5/1 requires about 30 min of spectra acquisition, thus allowing rapid assays when more material is available for analysis (e.g., in chemical syntheses of phospholipids).

Because the individual phospholipid resonances are well resolved in detergent, certain features of phospholipid behavior can be monitored simultaneously for a number of different phospholipids in a complex mixture. A potential use is to monitor the action of phospholipases on a complex mixture by ^{31}PNMR. We are currently investigating the binding of different phospholipids to the sarcoplasmic reticulum Ca^{2+} ATPase using these techniques.

Because many lipids are purified using silicic acid thin-layer plates and/or columns there is a need to cross-check purity by a method other than silicic acid chromatography. We have shown that ^{31}PNMR is suitable for this as suggested by Berden et al. (5). Analysis of phospholipids of biomembranes by conventional means requires extraction into solvent, thin-layer chromatographic separation of phospholipids and subsequent phosphorus analysis, or counting radioactivity for quantitation. ^{31}PNMR analysis in aqueous detergent avoids these steps and, in addition, has several advantages over ^{31}PNMR analysis in an organic solvent. The ability to manipulate pH in aqueous detergent systems adds some flexibility by making it possible to shift certain peaks that might otherwise overlap. Membrane samples can be dispersed directly in detergent. The convenience and accuracy of this method makes ^{31}PNMR analysis of phospholipids a powerful tool in studies of lipids or biomembranes.

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