reprinted from:
E. A. Dennis and A. Plückthun, Phosphorus-31 NMR of phospholipids in micelles. In: Phosphorus-31 NMR, Principles and Applications, D. G. Gorenstein, Ed. (Academic Press Inc., 1984) pp. 423-447.

CHAPTER 14

Phosphorus-31 NMR of Phospholipids in Micelles

Edward A. Dennis
Andreas Plückthun
Department of Chemistry
University of California at San Diego
La Jolla, California

I. Introduction and Perspective 423
II. Spectral Characteristics of Phospholipids in Mixed Micelles with Detergents 425
III. T_1, Nuclear Overhauser Effect, and Quantitative Analysis 431
IV. Solubilization of Phospholipids by Detergents 433
V. Critical Micelle Concentration Determinations and Micellization of Monomeric Phospholipids by Detergents 435
VI. Lysophospholipids: Acyl and Phosphoryl Migration 438
VII. Phospholipases: Specificity and Kinetics 442
References 444

I. Introduction and Perspective

NMR spectra of phospholipids in natural membranes and of synthetic phospholipids in model bilayer membranes are generally characterized by broad lines (see Smith and Ekiel, Chapter 15). However, when the phospholipids are dispersed in mixed micelles with detergents, significant improvement in the resolution of the phospholipid resonances is observed in ^{31}P (London and Feigenson, 1979; Roberts *et al.*, 1979) as well as in ^{1}H NMR (Dennis and Owens, 1973; Ribeiro and Dennis, 1974) and in ^{13}C NMR (Ribeiro and Dennis, 1976). Such spectra can be characterized as high-resolution spectra. Although they are not bilayer membranes, mixed micelles of phospholipids and detergents do provide useful membrane models for examining lipid–protein interactions and the mechanism of action of lipolytic enzymes (Dennis *et al.*, 1981; Plückthun and Dennis, 1982b). ^{31}P-NMR spectra (with ^{1}H decoupling) of mixed micelles have the advantage of allowing resolution and quantitation of individual classes of phos-

PHOSPHORUS-31 NMR
Principles and Applications

ISBN 0-12-291750-2

pholipids in single peaks in the presence of a large variety of detergents (London and Feigenson, 1979; Roberts *et al.*, 1979). For lysophospholipids (Plückthun and Dennis, 1982a) and synthetic phospholipids containing short chain fatty acids (Plückthun and Dennis, 1981), which form micelles in aqueous solution without detergent added, ^{31}P NMR can be used to identify individual isomers, as well as to determine critical micelle concentrations (CMCs)[1] and aggregation states.

This chapter limits itself to ^{31}P-NMR studies on phospholipids and lysophospholipids in monomeric and micellar states and focuses on the identification of species and aggregation states, as well as on the dynamic processes of migration and reaction kinetics. For this purpose, micelles and mixed micelles are defined as dilute isotropic solutions of phospholipids, either with or without detergents that form spontaneously and are at thermodynamic equilibrium; this definition specifically excludes sonicated or small unilamellar vesicles as well as membranes. These are covered by Smith and Ekiel (Chapter 15). This limitation in scope necessarily requires heavy reliance on work from the laboratory of the authors of this chapter.

The ^{31}P-NMR characteristics of phospholipids in micelles and mixed micelles with detergents have not been reviewed previously, but many reviews have appeared that concentrate in full (Seelig, 1978; Yeagle, 1978; Cullis and de Kruyff, 1979) or in part (Bocian and Chan, 1978) on ^{31}P NMR of phospholipids in crystalline or powder form, multibilayers, natural membranes, and sonicated vesicles. These studies have emphasized headgroup orientation, phospholipid conformation, and packing in membranes, as well as the asymmetric distribution of phospholipids in sonicated vesicles (Michaelson *et al.*, 1973; Berden *et al.*, 1975; Nolden and Ackermann, 1976). Interest has also focused on the hexagonal phase (Cullis and de Kruyff, 1976, 1979) of phospholipids such as PE which, like the multibilayers, is not isotropic. In mixtures of certain phospholipids, an isotropic "lipidic particle" phase (presumably consisting of inverted micelles sand-

[1] Abbreviations: PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine, phosphatidylcholine, or lecithin; β-PC, 1,3-diacyl-*sn*-glycero-2-phosphorylcholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine or phosphatidylethanolamine; β-PE, 1,3-diacyl-*sn*-glycero-2-phosphorylethanolamine; PS, 1,2-diacyl-*sn*-glycero-3-phosphorylserine or phosphatidylserine; PI, 1,2-diacyl-*sn*-glycero-3-phosphorylinositol or phosphatidylinositol; PG, 1,2-diacyl-*sn*-glycero-3-phosphorylglycerol or phosphatidylglycerol; PA, 1,2-diacyl-*sn*-glycero-3-phosphate or phosphatidic acid; CL, diphosphatidylglycerol or cardiolipin; SM, sphingomyelin; lyso-PC, 1-acyl-*sn*-glycero-3-phosphorylcholine; 2-lyso-PC, 2-acyl-*sn*-glycero-3-phosphorylcholine; lyso-PE, 1-acyl-*sn*-glycero-3-phosphorylethanolamine; lyso-β-PC, 3-acyl-*sn*-glycero-2-phosphorylcholine; lyso-β-PE, 3-acyl-*sn*-glycero-2-phosphorylethanolamine; P_i, inorganic phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; T_1, spin–lattice relaxation time. For phospholipids in which the identity of the acyl group or its natural source is known, this will be specified.

wiched between the two monolayers of the bilayer) has been suggested from ^{31}P-NMR studies (de Kruyff *et al.*, 1979). Different phospholipids have been resolved in organic solvents and sonicated vesicles (Henderson *et al.*, 1974; Berden *et al.*, 1975), and phospholipids in lipoprotein structures have also been studied and found to give very sharp resonances (Assmann *et al.*, 1974; Glonek *et al.*, 1974; Henderson *et al.*, 1975).

Most of the data summarized herein were originally reported using the ^{31}P-NMR convention of positive chemical shifts designating increasing field strength. All tables and figures reproduced here have been altered to reflect the newer convention that positive chemical shifts are in the direction of decreasing field strength, which has always been used for NMR studies with most nuclei other than phosphorus. When referring to the original literature cited herein, the interconversion of (+) and (−) must be made. Chemical shifts are reported relative to external 85% phosphoric acid but have usually been determined with other secondary standards. Samples usually contain some D_2O as a lock signal. With mixed micelles, data have generally been obtained with broadband ^{1}H decoupling to achieve narrow linewidths.

II. Spectral Characteristics of Phospholipids in Mixed Micelles with Detergents

In 1979 London and Feigenson showed that individual classes of phospholipids in the presence of sodium cholate could be resolved readily by ^{31}P NMR with ^{1}H broadband decoupling as illustrated in Fig. 1. In their studies they included an excess of the detergent, and mixed micelles were presumably formed. The peaks appeared better resolved and the linewidths narrower than in earlier spectra of similar mixtures of phospholipids in organic solvents, such as 2 : 1 chloroform : methanol (which were, however, obtained without ^{1}H broadband decoupling) as reported by Henderson *et al.* (1974). With ^{1}H broadband decoupling, other investigators (Berden *et al.*, 1975) found the linewidths to be as small as 1.5 Hz in organic solvents. London and Feigenson (1979) report linewidths of <1 Hz in mixed micelles. The chemical shifts for the various phospholipids in mixed micelles with cholate as shown in Table I differ from those measured in organic solvents as shown in Table II.

In the same year Roberts *et al.* (1979) also showed that various phospholipids in mixed micelles, in this case with the nonionic surfactant Triton X-100, could be resolved, as well as the corresponding lysophospholipids as illustrated in Fig. 2. ^{31}P-NMR chemical shifts of various phospholipids,

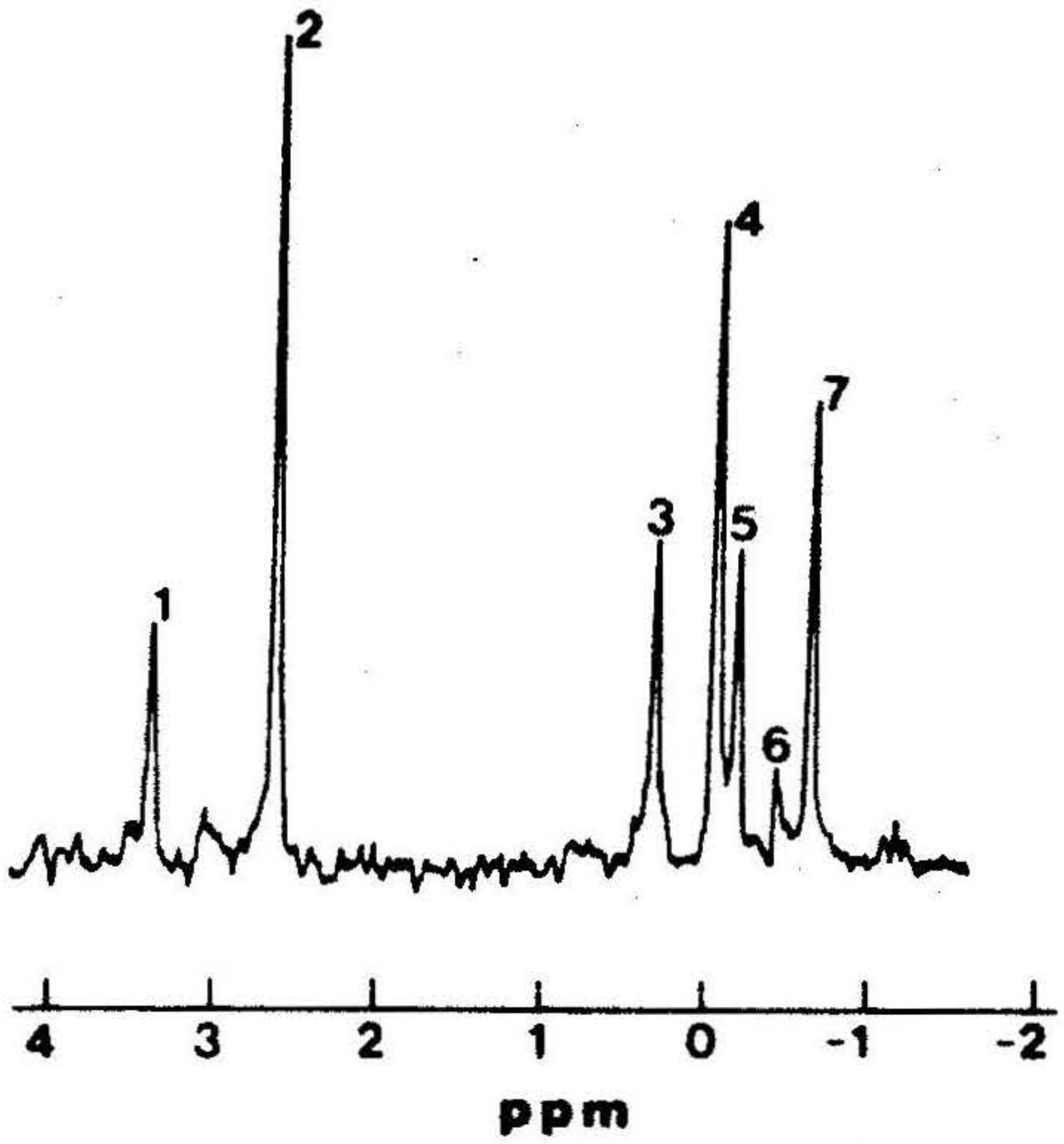

Fig. 1. ^{31}P-NMR spectrum of a phospholipid mixture consisting of 5% w : v cholate, 50 m*M* EDTA, and the following: peak 1, 10 mg of PA; peak 2, 6.6 mg of K_2HPO_4; peak 3, 8 mg CL; peak 4, 13 mg of PE; peak 5, 12.5 mg of PS; peak 6, PI present as an impurity in PS; peak 7, 12.5 mg of dipalmitoyl-PC. Total volume 1 ml, pH ~ 8. A Varian CFT-20 NMR spectrometer operating at 32.19 MHz was employed and spectra were recorded at 40°C with ^{1}H broadband decoupling; 100 transients were collected with an acquisition time of 2 s per transient, no delay between transients, and a filtering time constant of 1 s. From London and Feigenson (1979), but note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

TABLE I

^{31}P-NMR Chemical Shifts of Various Phospholipids in Potassium Cholate at pH ≈ 8[a]

Phospholipid	Chemical shift[b] (ppm)
PC	−0.65
PI	−0.40
lyso-PC	−0.15
PS	−0.12
PE	0.00
SM	0.00
CL	0.31
PG	0.43
PA	3.80

[a] Adapted from London and Feigenson (1979).

[b] Note that the signs of the chemical shifts have been switched from the original report so that positive chemical shifts are now shown in the direction of decreasing field strength.

TABLE II

^{31}P-NMR Chemical Shifts of Phospholipids in Chloroform–Methanol and Analogs in Aqueous Solution[a]

Compound (20 m*M*)	Solvent[b]	Chemical Shift[c] (ppm)
Egg PC	C–M	−0.9
N,N-Dimethyl-PE	C–M	0.3
N-Methyl-PE	C–M	0.0
PE	C–M	0.2
PS	C–M	0.0
Dipalmitoyl-PC	C–M	−0.8
Distearyl-PC	C–M	−0.8
Dioleoyl-PC	C–M	−0.8
Distearyl-PE	C–M	−0.1
Lyso-PC	C–M	−0.2
Lyso-PE[d]	C–M	0.2
Lyso-PS[e]	C–M	0.2
Plasmalogen PE	C–M	0.2
SM	C–M	0.0
PI	C–M	0.2
PA	C–M	2.8
PG	C–M	1.2
Cl		
Low-field band	C–M	1.0
High-field band		0.8
Phosphorylcholine	Water	3.2
Phosphorylethanolamine	Water	3.7
Phosphorylserine	Water	3.7
Glycerophosphorylcholine	Water	−0.1
Glycerophosphorylethanolamine	Water	0.4
Glycerophosphorylserine	Water	0.1

[a] Adapted from Henderson *et al.* (1974). Copyright 1974 American Chemical Society.

[b] C–M is chloroform–methanol 2 : 1 (v : v); water contained 0.2 *M* EDTA, Na^+ ion, pH 7.0.

[c] Note that the signs of the chemical shifts have been switched from the original report so that positive chemical shifts are now shown in the direction of decreasing field strength.

[d] Concentration 7 m*M*.

[e] Concentration 5 m*M*.

lysophospholipids, and related analogs in Triton X-100 or aqueous solution are given in Table III. Chemical shifts for some water-soluble analogs also included in the studies of Henderson *et al.* (1974) are summarized in Table II. The chemical shifts of additional water-soluble analogs are reported by Assmann *et al.* (1974). Relative chemical shifts of PC, PE, monomethyl-PE,

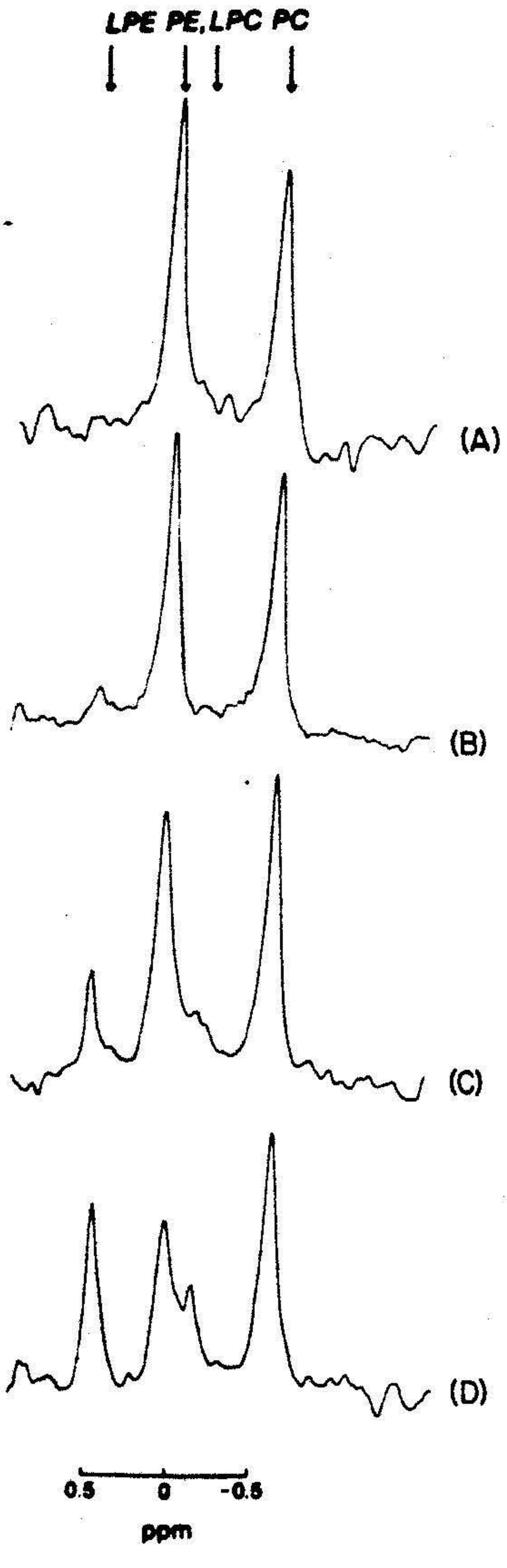

Fig. 2. Cobra venom phospholipase A_2 (0.05 μg) was added to 1.4 ml of solution containing egg PC (2.6 m*M*) and egg PE (3.0 m*M*) in 48 m*M* Triton X-100, 50 m*M* Tris-HCl, and 10 m*M* $CaCl_2$; pH 8.0, 40°C. ^{31}P-NMR spectra are shown at the following times (min) after the initiation of reaction: A, 0; B, 7; C, 27; D, 87. A JEOL PFT-100 NMR spectrometer operating at 40.3 MHz was employed and spectra were recorded with ^{1}H broadband decoupling. Peaks corresponding to lyso-PE (LPE), lyso-PC (LPC), PE, and PC are indicated. From Roberts *et al.* (1979). Copyright 1979 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

and dimethyl-PE and their lyso derivatives with the fatty acid composition of egg PC have also been reported (Roberts *et al.*, 1979), as well as chemical shifts for PC and PE in Triton X-100 (London and Feigenson, 1979) and, within experimental error, do not differ significantly from those given in Table III for the same phospholipids but of defined fatty acid composition. In both organic solvents and mixed micelles with detergents, it appears that

TABLE III

^{31}P-NMR Shifts of Phospholipids and Analogs in Aqueous Solution with Triton X-100[a,b]

Compound	Triton X-100 (48 m*M*)	Chemical shift (ppm)	Reference[c]
Dipalmitoyl-PC	+	−0.86	1,2
1-Palmitoyl-lyso-PC	−	−0.34	2
1-Palmitoyl-lyso-PC	+	−0.38	2
2-Palmitoyl-lyso-PC	−	−0.52	2
2-Palmitoyl-lyso-PC	+	−0.55	2
Dipalmitoyl-β-PC	+	−1.45	2
Palmitoyl-lyso-β-PC	−	−1.13	2
Dibutyryl-PC	−	−0.60	2
Dibutyryl-PC	+	−0.61	1
1-Butyryl-lyso-PC	−	−0.24	2
1-Butyryl-lyso-PC	+	−0.25	1
2-Butyryl-lyso-PC	−	−0.44	2
Dipalmitoyl-PE	+	−0.15	1
Palmitoyl-lyso-PE	+	0.26	1
Dipalmitoyl-β-PE	+	−0.71	3
Palmitoyl-lyso β-PE	+	−0.46	3
Dipalmitoyl-*N*-methyl-PE	+	−0.30	3
Palmitoyl-lyso-*N*-methyl-PE	+	0.10	3
Dipalmitoyl-*N*-methyl-β-PE	+	−0.86	3
Palmitoyl-lyso-*N*-methyl-β-PE	+	−0.60	3
Dipalmitoyl *N*,*N*-dimethyl-PE	+	−0.42	3
Palmitoyl-lyso-*N*,*N*-dimethyl-PE	+	−0.02	3
Dipalmitoyl-*N*,*N*-dimethyl-β-PE	+	−1.02	3
Palmitoyl-lyso-*N*,*N*-dimethyl-β-PE	+	−0.75	3
Glycero-3-phosphorylcholine	−	−0.08	2
Glycero-3-phosphorylcholine	+	−0.09	1
Glycero-2-phosphorylcholine	−	−0.79	4
Glycero-3-phosphate[d]	−	4.3	4
Glycero-2-phosphate[d]	−	3.9	4
Dodecylphosphorylcholine	+	−0.39	1
Bis(monoacylglyceryl)phosphate	+	−0.70	1

[a] Note that the signs of the chemical shifts have been switched from the original report so that positive chemical shifts are now shown in the direction of decreasing field strength.

[b] $CaCl_2$ was generally present. When the compound readily dissolves in water in the absence of detergent, its chemical shift is also indicated. All compounds were analyzed at pH 8.0.

[c] References: 1, Plückthun and Dennis (1981); 2, Plückthun and Dennis (1982a); 3, A. Plückthun, J. de Bony, and E. A. Dennis (manuscript in preparation); 4, A. Plückthun and E. A. Dennis (unpublished).

[d] Contained 10 m*M* EDTA.

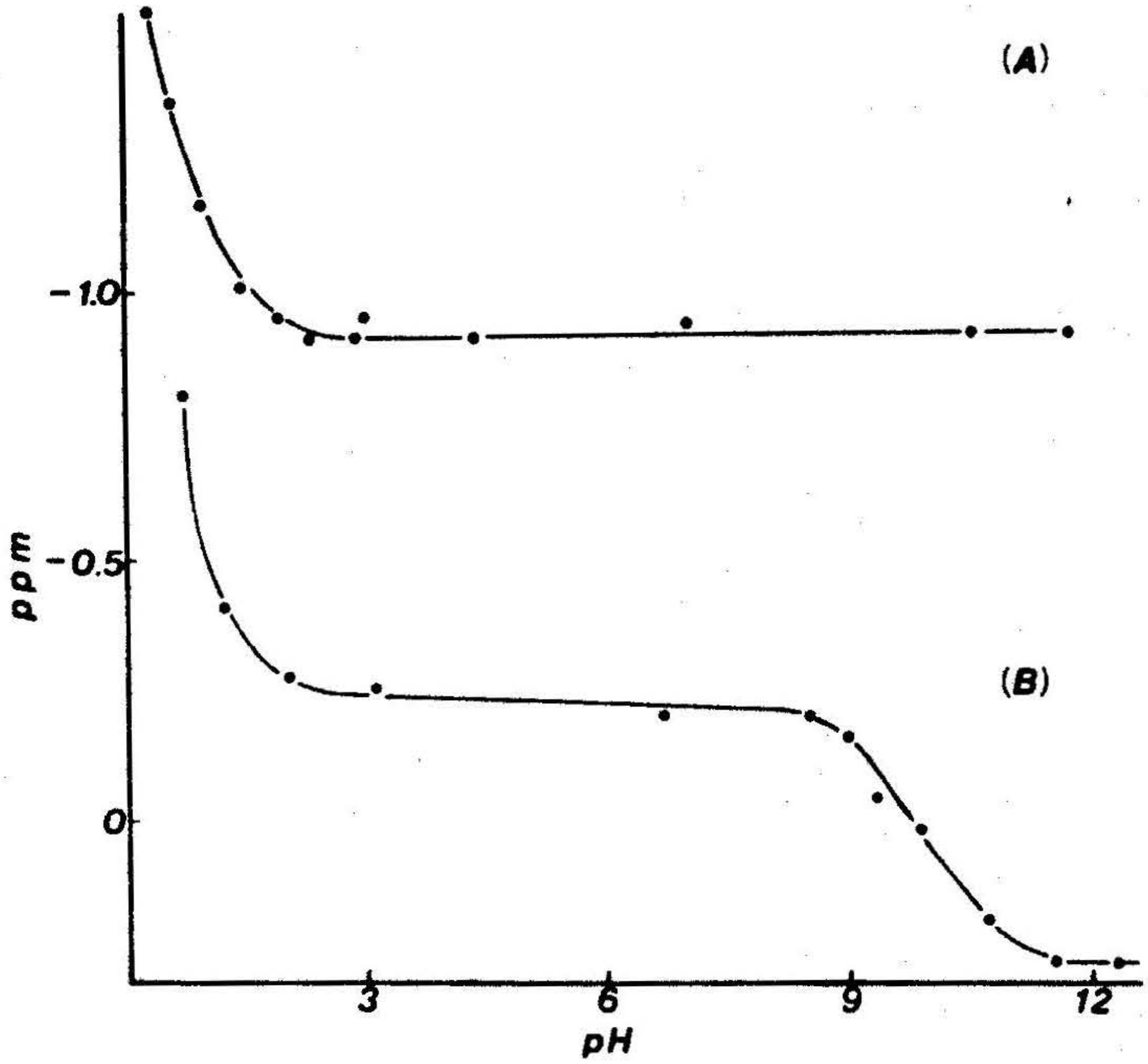

Fig. 3. The pH dependence of the chemical shift of phospholipids dissolved in Triton X-100. (A) 4 mg ml^{-1} dimyristoyl-PC dissolved in 2.5% (w:v) Triton X-100; (B) 2.1 mg ml^{-1} dilauryl-PE dissolved in 2.5% (w:v) Triton X-100. Samples contained an external D_2O field-frequency lock. From London and Feigenson (1979), but note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

the specific fatty acid composition of normal long-chain phospholipids does not affect chemical shifts within experimental error (Henderson *et al.*, 1974; London and Feigenson, 1979). Absolute chemical shifts in these tables are probably only reproducible to ± 0.1 ppm, although relative chemical shifts within a given series of measurements have much less error (Plückthun and Dennis, 1981).

Another advantage of examining ^{31}P-NMR chemical shifts in aqueous solution with detergents is that pK_a values can be obtained readily from chemical-shift changes. As illustrated in Fig. 3, the ionization of the phosphate in PC and PE can be followed readily in Triton X-100 mixed micelles where the apparent pK_a is < 1, as can the ionization of the amino group on PE, where the p$K_a \simeq 9.75$ (London and Feigenson, 1979). Thus ^{31}P NMR is an excellent method for determining pK_a values of phospholipids in mixed micelles. In addition to being pH-dependent, chemical shifts of phospholipids in detergents are temperature-dependent, as illustrated in Fig. 4. Chemical shifts may also depend on the presence of metal ions, especially multivalent cations, if the lipids and/or detergents are ionic. In organic

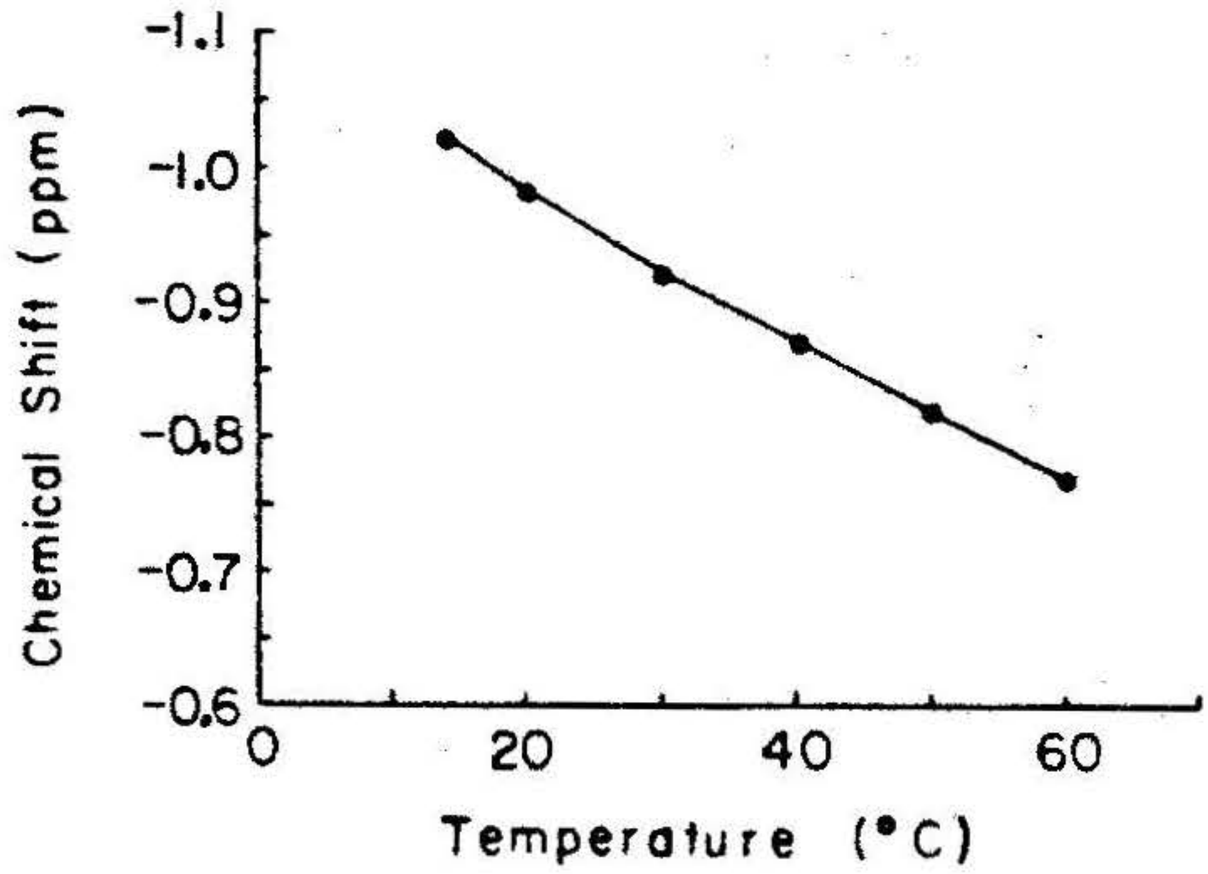

Fig. 4. ^{31}P-NMR chemical shift of 10 m*M* egg PC in mixed micelles with 80 m*M* Triton X-100 as a function of temperature. From Plückthun and Dennis (1981). Copyright 1981 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

solvents the addition of EDTA gives narrower lines and better resolution (Henderson *et al.*, 1974), but a comprehensive study of metal-ion dependence in mixed micelles has not been reported. London and Feigenson (1979) routinely included EDTA in their studies, whereas Dennis and co-workers (Roberts *et al.*, 1979; Plückthun and Dennis, 1981) usually included $CaCl_2$ to obtain data under conditions used in enzymatic studies; this may in certain cases broaden the lines but has a negligible effect on the chemical shift of zwitterionic phospholipids such as PC in Triton X-100. Detergent, phospholipid, and D_2O concentration probably have only small effects on chemical shifts (Plückthun and Dennis, 1981).

III. T_1, Nuclear Overhauser Effect, and Quantitative Analysis

The spin–lattice relaxation time T_1 (determined at 32.2 MHz) for PC, PE, and PA in potassium cholate in the presence of EDTA at pH ≃ 8 was found to be 3.3, 3.2, and 3.3 s, respectively, and shorter than 4.3 s for P_i (London and Feigenson, 1979). In mixed micelles with Triton X-100 in the presence of EDTA, Roberts *et al.* (1979) found the T_1 (determined at 40.3 MHz) for PC and PE to be 2.5 and 2.2 s, respectively. Plückthun and Dennis (1981) found a T_1 of 2.5 s for PE and 12.4 s for the monomeric phospholipid dibutyryl-PC in the presence of Triton X-100 under slightly different experimental conditions; the dibutyryl-PC was not incorporated into the micelles. T_1 for 1-palmitoyl-lyso-PC and 2-palmitoyl-lyso-PC is 2.3 s (Plückthun and Dennis, 1982a), and for egg lyso-PC, T_1 is 2.3 s (Yeagle, 1979).

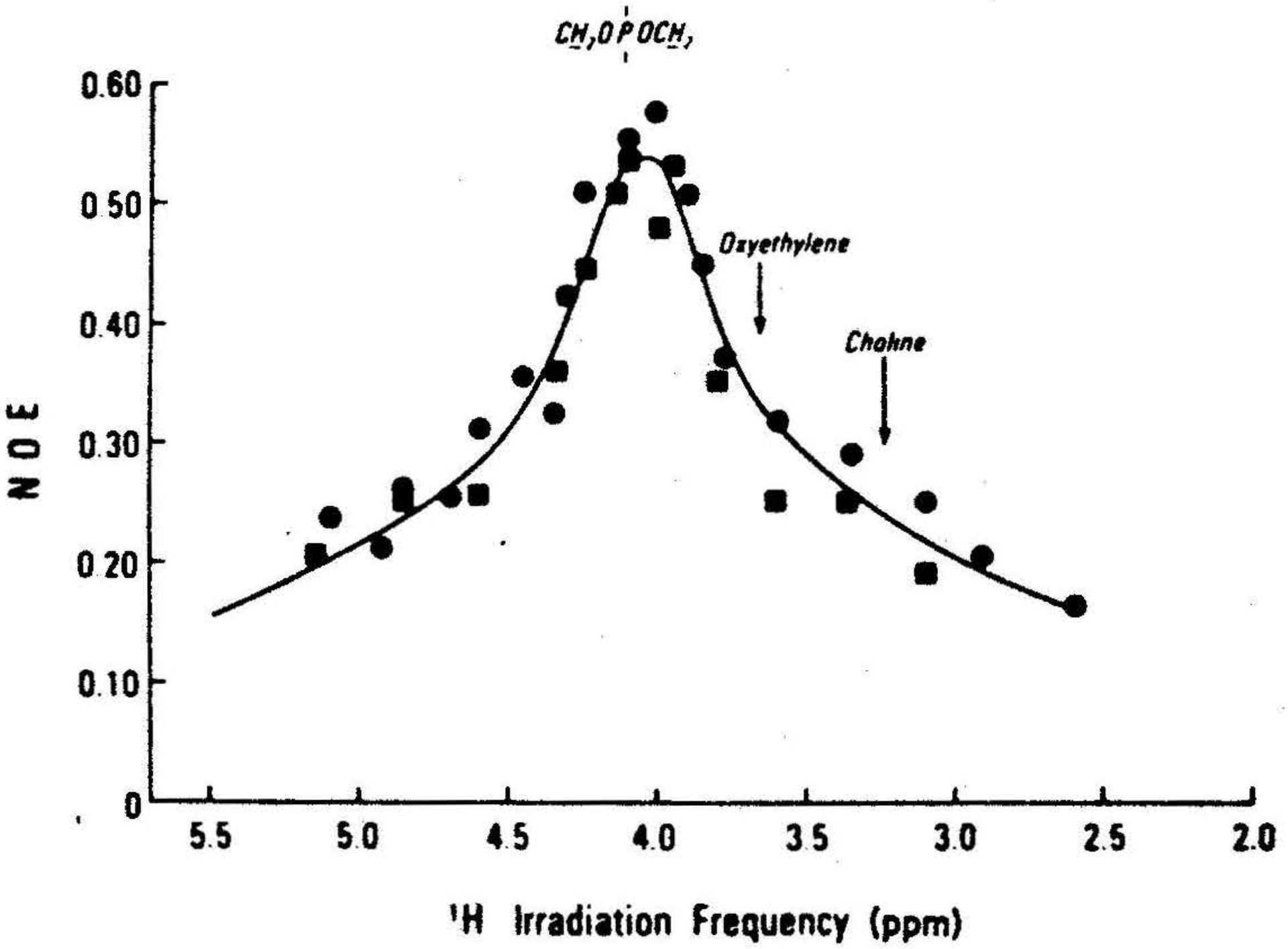

Fig. 5. Frequency dependence of $^{31}P\{^1H\}$NOE of 10 m*M* egg PC (●) and 10 m*M* egg PE (■) in 40 m*M* Triton X-100 at 40°C plotted as a function of the continuous-wave proton-decoupler frequency. The chemical shifts for choline methyl groups, methylene groups adjacent to the phosphate of the phospholipid, and Triton oxyethylene groups are indicated in parts per million from tetramethylsilane. From Roberts *et al.* (1979). Copyright 1979 American Chemical Society.

The nuclear Overhauser effect (NOE) for PC, PE, and PA in potassium cholate was found to be 60, 60, and 50% compared to 5% for P_i. Roberts *et al.* (1979) found the NOEs for PC, PE, lyso-PC, and lyso-PE in mixed micelles to be indistinguishable (60–70%) within experimental error. Yeagle (1979) reported an NOE for lyso-PC of 70%. In mixed micelles with Triton X-100, the NOE arises predominantly from the intramolecular methylene protons adjacent to the phosphate moiety (Roberts *et al.*, 1979) as illustrated in Fig. 5, in contrast to sonicated vesicles where Yeagle *et al.* (1977) found that the choline methyl groups dominate intermolecularly the NOE of both PC and PE in mixed vesicles. Yeagle (1979) found that the NOE of micelles of lyso-PC derived from egg PC is also dominated by intramolecular methylene groups rather than by intermolecular interactions, supporting the contention that in micelles the headgroups are further apart than in vesicles, thereby defeating the intermolecular interactions found in the bilayer. In the case of mixed micelles with detergents, the intermolecular interactions are, in addition, presumably weakened by the separation of phospholipid molecules by detergent (Roberts *et al.*, 1979). On the other hand, it has been suggested that in egg PC–sodium taurocholate mixed micelles, which are thought to be structurally different (Mazer *et al.*,

1980), the NOE is due to intermolecular choline methyl interactions in patches of PC in the mixed micelles (Castellino and Violand, 1979). However, the frequency dependence of the NOE was not determined in the latter case.

With broadband ^{1}H decoupling and pulse intervals of the order of T_1, the similar T_1 and NOE values for various phospholipids in mixed micelles suggest that peak areas or intensities (Plückthun and Dennis, 1982a) can be used reliably for quantitative analysis of the phospholipids present in a mixture. With a significant loss of signal to noise for greater reliability, gated broadband decoupling and longer pulse intervals of the order of $5-10 \times T_1$ can be used; indeed, where comparison of ^{31}P-NMR intensities with weighed mixtures or traditional TLC separation and P_i analysis has been carried out, good agreement is obtained (London and Feigenson, 1979).

IV. Solubilization of Phospholipids by Detergents

Detergents have been widely used to solubilize and purify membrane-bound proteins (Helenius and Simons, 1975; Tanford and Reynolds, 1976; Helenius *et al.*, 1979; Lichtenberg *et al.*, 1983). Central to the effect of detergents is the solubilization of the phospholipid components into mixed micelles. This can be followed by ^{31}P NMR, as illustrated in Fig. 6, where cholate was used to solubilize sarcoplasmic reticulum membranes. In the spectrum, the phospholipids are well resolved and the spectrum is similar to that obtained when the phospholipids are extracted with chloroform–methanol first and then solubilized with cholate (London and Feigenson, 1979). This shows that in the presence of the protein, all of the phospholipids are solubilized as in its absence. Without cholate, the spectrum of the membranes would be quite broad and the individual phospholipid classes would not be resolved.

In principle, ^{31}P NMR can be used to follow the conversion of membranes to mixed micelles by the appearance of sharp peaks for the phospholipids. This would also be the case for model membranes of pure phospholipids. For multibilayers or hexagonal phases (Cullis and de Kruyff, 1979), high-resolution sharp peaks would appear out of the broad baseline; for sonicated vesicles, peaks would become sharper and inside–outside signals would disappear. This is shown in Table IV, where Castellino and Violand (1979) followed the decrease in the linewidth as well as the T_1 and NOE for egg PC vesicles on the addition of sodium taurocholate. The values of the T_1 and NOE in mixed micelles (low phospholipid/detergent ratio) are consistent with those reported in Section III. When only a small amount of detergent is

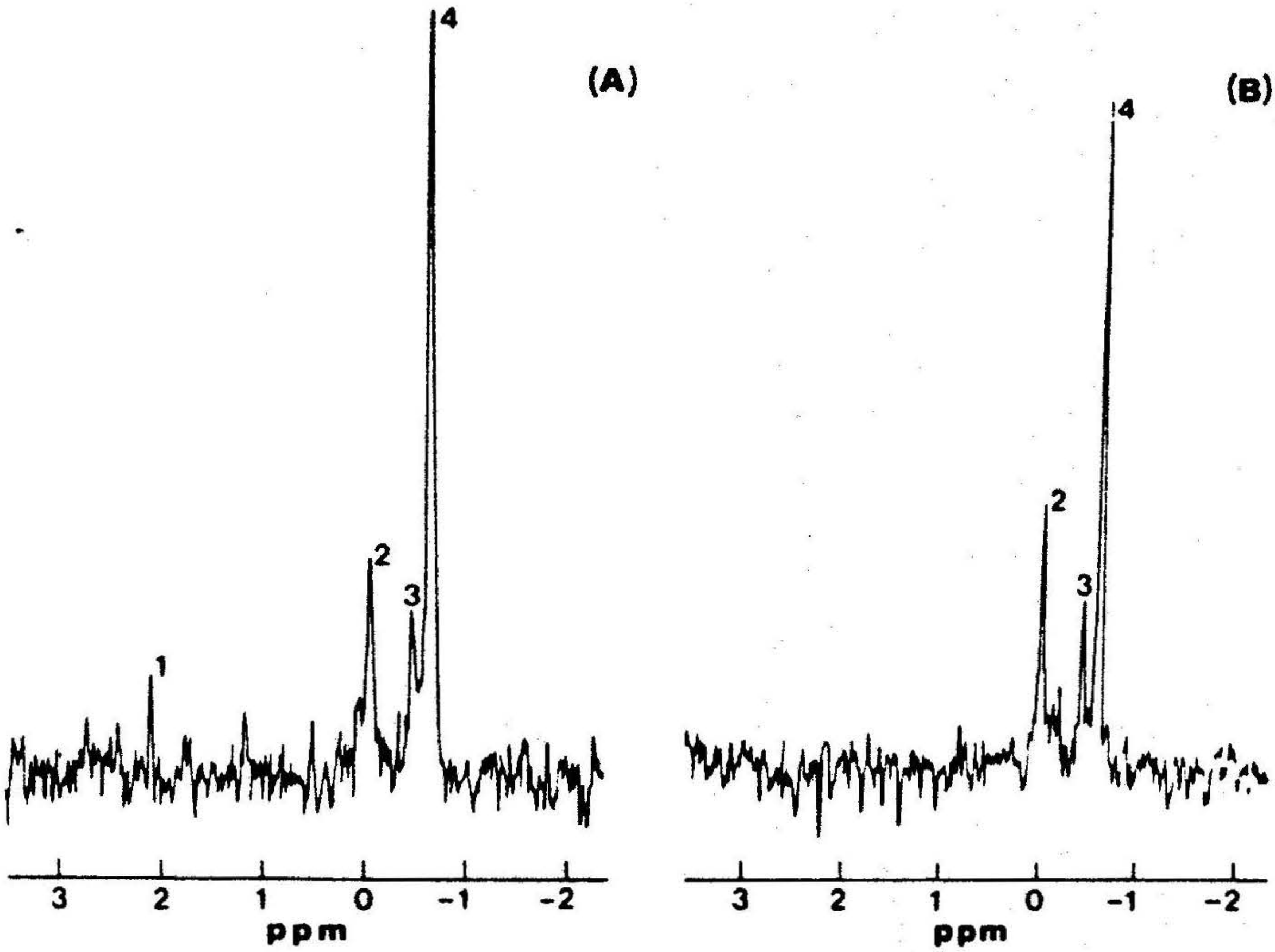

Fig. 6. ^{31}P-NMR spectra of sarcoplasmic reticulum phospholipids using the spectrometer described in Fig. 1. (A) Sarcoplasmic reticulum (12 mg of protein per milliliter) dissolved in cholate, pH ~ 8. Peak assignments: 1, P_i; 2, PE; 3, PI; 4, PC. 6000 transients were collected with an acquisition time of 2 s per transient, no delay between transients, and a filtering time constant of 1 s; (B) lipid extract of sarcoplasmic reticulum (10 mg of lipid per milliliter) dissolved in cholate. Peak assignments as in (A); 600 transients were collected with other spectrometer settings as in (A). From London and Feigenson (1979), but note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

added to the phospholipid, the basic vesicular bilayer structure is retained (Castellino and Violand, 1979), whereas with PE, the addition of small amounts of a variety of detergents can result in the transformation of hexagonal structures into bilayer structures (Madden and Cullis, 1982) but only at detergent/phospholipid ratios below mixed-micelle formation. Jackson *et al.* (1982) have utilized ^{31}P NMR to follow the solubilization of large, unilamellar PC vesicles (which have a very broad spectrum) by octylglucoside to form mixed micelles (with quite narrow lines) and to analyze the solubilization process. ^{31}P-NMR studies of mixtures of PC multibilayers and phosphorus-containing detergents from decyl to hexadecyl phosphonate, in which the signals from both the phospholipid and detergent can be followed, have been carried out by Klose and Hollerbuhl (1981). By examining mixtures at molar ratios when both micellar and lamellar structures are

TABLE IV

^{31}P-NMR Parameters for Egg PC–Sodium Taurocholate Mixtures[a,b]

Mole ratio (egg PC/sodium taurocholate)	Linewidth (Hz)	T_1 (s)	NOE (%)
∞	13	1.7	52
35	13	1.7	50
10	12	1.7	41
4	13	1.8	39
1.3	5.4	2.0	42
0.9	3.8	2.2	43
0.6	2.5	2.2	40
0.1	2.0	2.6	49

[a] Adapted from Castellino and Violand (1979).

[b] The egg PC was in the form of sonicated vesicles to which the detergent was added. All values are ± 10%.

present as a function of time after mixing, the process of equilibration and the equilibrium state can be determined.

V. Critical Micelle Concentration Determinations and Micellization of Monomeric Phospholipids by Detergents

Phospholipids can be synthesized with short fatty acid chains to make them water soluble, and the physical properties of such phospholipids have been studied extensively by Tausk *et al.* (1974a,b,c). Their critical micelle concentration (CMC) depends on chain length and has been determined by a variety of methods for a number of PC derivatives. It was found that the ^{31}P-NMR signal is chemically shifted in going from the monomeric form to the micellar form of dihexanoyl PC (Roberts *et al.*, 1979). This difference can be used (Plückthun and Dennis, 1981) to determine the CMC according to

$$\nu = x_{\text{mono}}\nu_{\text{mono}} + x_{\text{mic}}\nu_{\text{mic}} \tag{1}$$

where ν is the observed chemical shift, ν_{mono} the chemical shift below the CMC, ν_{mic} the chemical shift of dihexanoyl PC micelles, x_{mono} the molar fraction of phospholipid in the monomeric state, and x_{mic} the molar fraction of phospholipid in the micellar state. The values for ν_{mono} and ν_{mic} can be estimated from the experimental data, along with the CMC, and all three

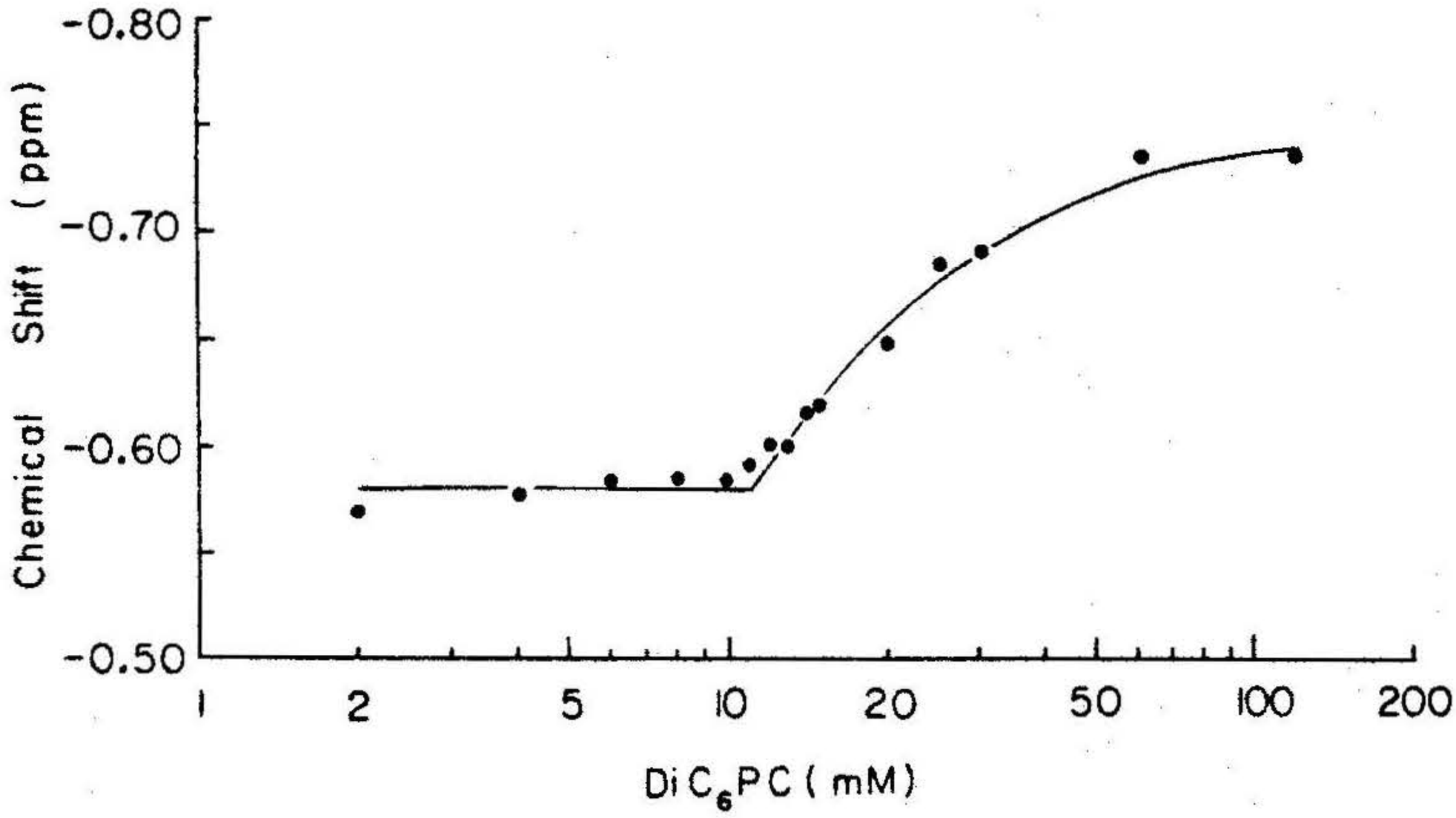

Fig. 7. ^{31}P-NMR chemical shift of dihexanoyl-PC (DiC_6PC) in D_2O as a function of concentration. The solution contained no further additives. The best-fit curve is calculated by using Eq. (1) and assigning $\nu_{mono} = 0.58$ ppm, $\nu_{mic} = 0.76$ ppm, and CMC = 11 m*M*. From Plückthun and Dennis (1981). Copyright 1981 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

varied to obtain the best fit of the theoretical curve to experimental data. Experimental results are shown in Fig. 7. The resulting CMC is in agreement with that determined by other methods, including ^{1}H and ^{13}C NMR. The ^{31}P-NMR method can be used for other phospholipids or to determine if a given phospholipid is monomeric or micellar under certain experimental conditions. ^{31}P-NMR chemical-shift changes similarly have been used to follow micelle formation by decyldimethyl phosphine oxide by Kresheck and Jones (1980). These workers plotted the observed chemical shift ν against the inverse of the concentration of surfactant, which has the advantage of graphically giving ν_{mic} and the CMC.

Similarly, incorporation of monomeric phospholipids by detergents into mixed micelles results in a chemical-shift change in ^{31}P NMR, and this can be used to follow the micellization process. This is illustrated in Fig. 8, where the micellization of dihexanoyl-PC by titration with Triton X-100 was followed by changes in the ^{31}P-NMR chemical shift. In contrast, with the same amount of Triton X-100, dibutyryl-PC is hardly micellized at all. A control titration of the completely water-soluble analog glycerophosphorylcholine is also included. For the dihexanoyl-PC, a partition coefficient between Triton X-100 micelles and free solution was calculated as a function of the Triton X-100 concentration, using the phase-separation approximation for mixed-micelle formation and considering the mixed micelles to be a pseudo-phase. With the data in Fig. 8, the fraction of dihexanoyl-PC

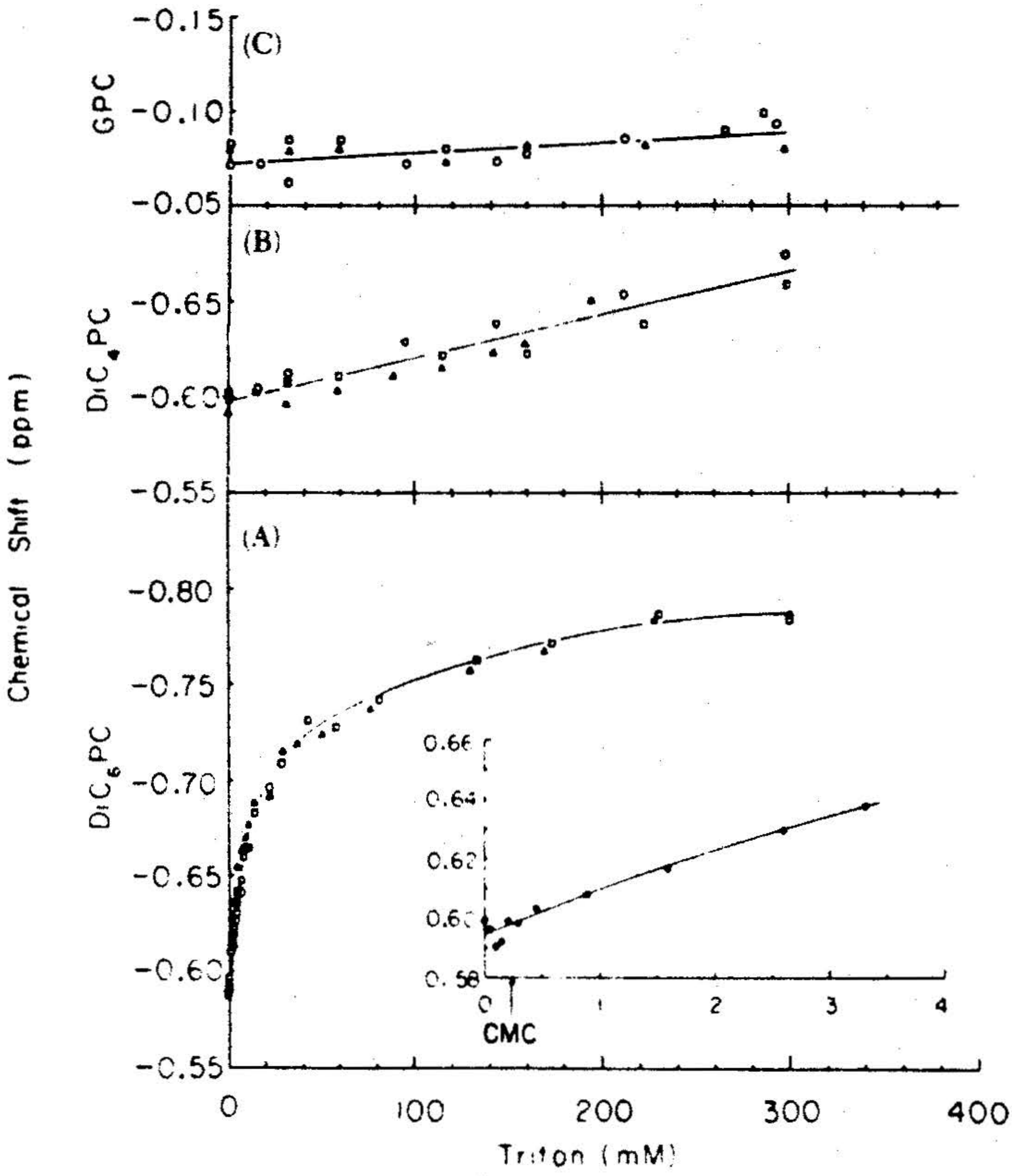

Fig. 8. ^{31}P-NMR chemical shift of (A) dihexanoyl-PC (DiC_6PC), (B) dibutyryl-PC (DiC_4PC), and (C) glycerophosphorylcholine (GPC) as a function of the concentration of Triton X-100. The initial concentration of phospholipid or GPC was 7.5 m*M*. The titration was carried out with 500 m*M* detergent except for the insert to (A) in which 7.5 m*M* Triton X-100 was employed. In each panel, the results of three experiments are plotted (○, □, △). In (B), the titration was also carried out with mixed micelles (▲) consisting of Triton X-100 (500 m*M*) and PE (125 m*M*). From Plückthun and Dennis (1981). Copyright 1981 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

present in Triton X-100 was calculated by Plückthun and Dennis (1981) from Eq. (1) by using the relationship $x_{\text{mono}} = 1 - x_{\text{mic}}$, which results in

$$x_{\text{mic}} = (\nu - \nu_{\text{mono}})/(\nu_{\text{mic}} - \nu_{\text{mono}}) \tag{2}$$

A partition coefficient K can be defined as shown in

$$K = X_{\text{mic}} c_{\text{Pmono}} \tag{3}$$

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

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

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

$$K = \frac{1}{x_{\text{mic}}c_{\text{Ptot}} + (c_{\text{Ttot}} - cmc_{\text{T}})} \frac{x_{\text{mic}}}{1 - x_{\text{mic}}} \tag{5}$$

The partition coefficient K was found to be $\sim 40\ M^{-1}$ at 50 mM Triton X-100 and varied somewhat with Triton concentration (Plückthun and Dennis, 1981). The chemical shift of the short-chain phospholipids incorporated into mixed micelles approaches that of long-chain "normal" phospholipids in the micelles, suggesting similar structures for the mixed micelles.

Interestingly, Burns *et al.* (1983) have reported that micelles formed by short-chain phospholipids can themselves be used as detergents to solubilize triglycerides containing short fatty chains to form microemulsion particles in which the ^{31}P-NMR linewidths are narrower than for the pure phospholipid micelles. These particles serve as models for lipoproteins.

VI. Lysophospholipids: Acyl and Phosphoryl Migration

Lysophospholipids lack one of the acyl groups on phospholipids and form micelles by themselves without added detergent. The spectral characteristics of lyso-PC, both alone and in the presence of detergents, have been considered in preceding sections along with normal phospholipids. Lysophospholipids possess the ability to rearrange via migration of either the acyl or phosphoryl group and the sensitivity of the ^{31}P-NMR chemical shift to the resulting isomers has been particularly useful in following these migration reactions (Plückthun and Dennis, 1982a). Phosphoryl and acyl migration of lysophospholipids must be taken into account when evaluating phospholipase specificity, the chemical synthesis of mixed acyl phospholipids, and the biosynthesis of phospholipids, so that the determination of the kinetics of

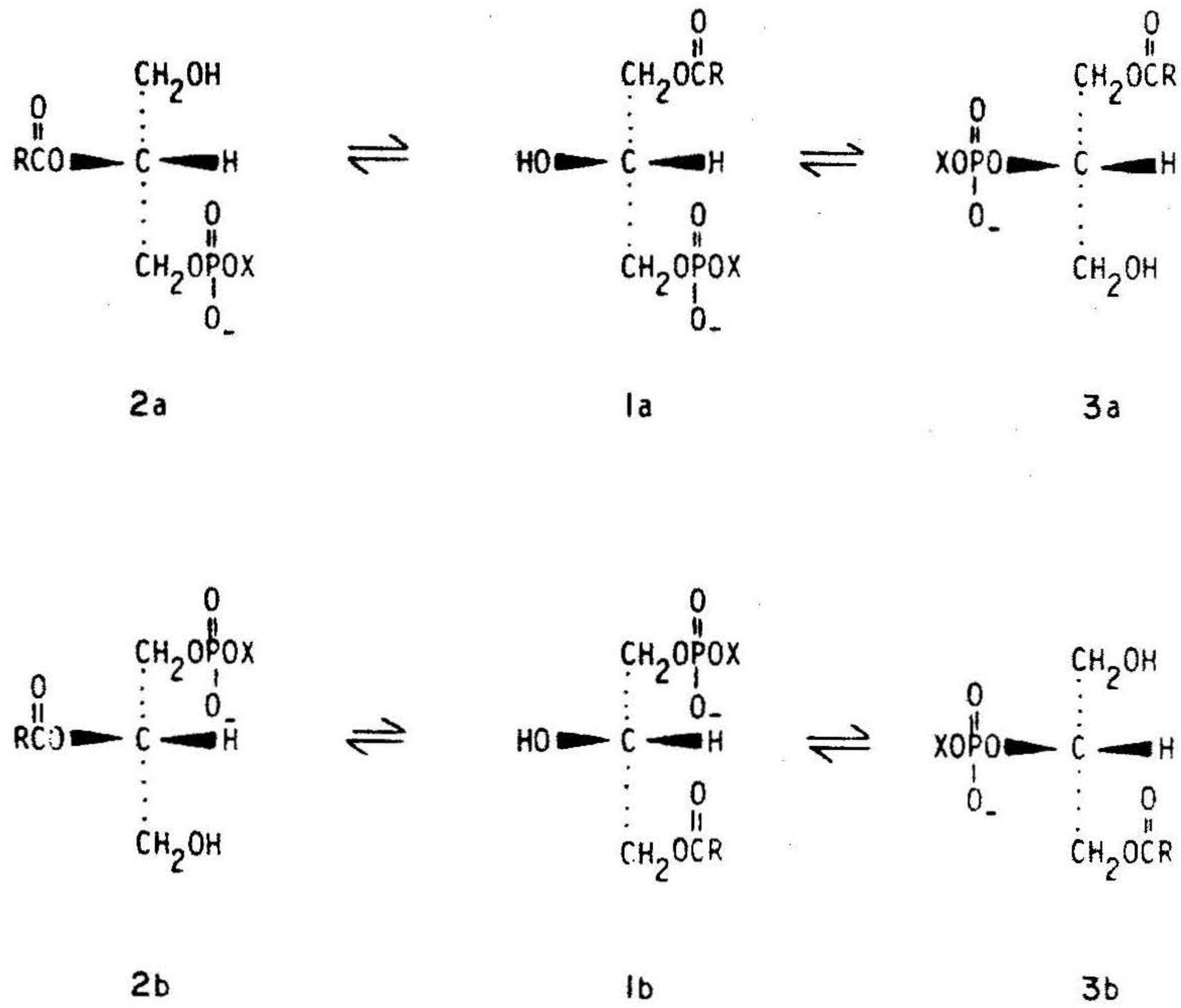

Fig. 9. Possible interconversions via acyl (1 ⇌ 2) and phosphoryl (1 ⇌ 3) migration of lysophospholipids derived from natural phospholipids (a series) and their enantiomorphs (b series). When RCOO is palmitic acid and X is choline, the structures correspond to the following compounds: 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (1a), 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (2a), 1-palmitoyl-*sn*-glycero-2-phosphorylcholine (3a), 3-palmitoyl-*sn*-glycero-1-phosphorylcholine (1b), 2-palmitoyl-*sn*-glycero-1-phosphorylcholine (2b), and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (3b). From Plückthun and Dennis (1982a). Copyright 1982 American Chemical Society.

such processes has been important. The lack of a suitable analytical tool prior to the use of ^{31}P NMR probably prevented an earlier detailed evaluation of these processes. There are six different possible lyso-PCs consisting of three enantiomeric pairs of positional isomers as shown in Fig. 9.

Migration of the acyl group in the 1 position of lyso-PC (**1a**) to the 2 position (**2a**) as well as from **2a** to **1a** is shown in Fig. 10 as a function of time. From data of this type, the rate constant for acyl migration can be determined. The migration was found to be first order in both lysophospholipid and acid or base with a base-catalyzed, second-order rate constant of ~160 M^{-1} s^{-1}. The pH dependence of the migration reaction is shown in Fig. 11. At alkaline pH values, the equilibrium mixture contains about 90% of the 1-acyl and about 10% of the 2-acyl isomer. A slow acyl migration also occurs in organic solvents, most notably in the presence of basic catalysts used in common acylation procedures for the synthesis of phospholipids from

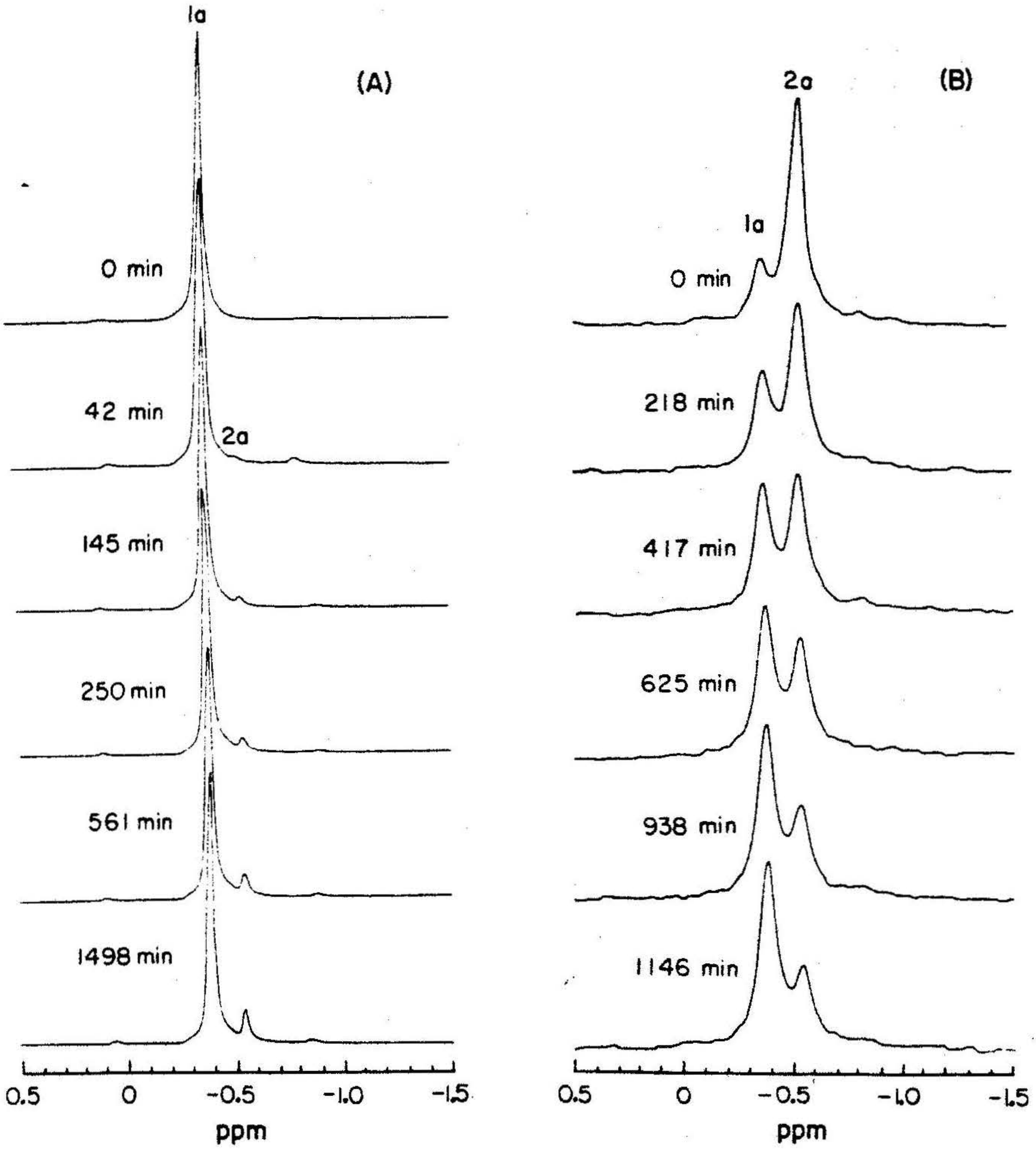

Fig. 10. ^{31}P-NMR spectra obtained using the spectrometer described in Fig. 2, after the times indicated, of (A) 30 m*M* 1-palmitoyl-lyso-PC (**1a**) at pH 7.0 and (B) 20 m*M* 2-palmitoyl-lyso-PC (**2a**), of which some had migrated to **1a** during its preparation, at pH 7.0. From Plückthun and Dennis (1982a). Copyright 1982 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

lysophospholipids. At alkaline pH, no phosphoryl migration was detected in the time scale of acyl migration and hydrolysis, although in acid, phosphoryl migration does occur. Because of competing hydrolysis reactions in acid, the determination of the precise rate constants for phosphoryl migration is quite

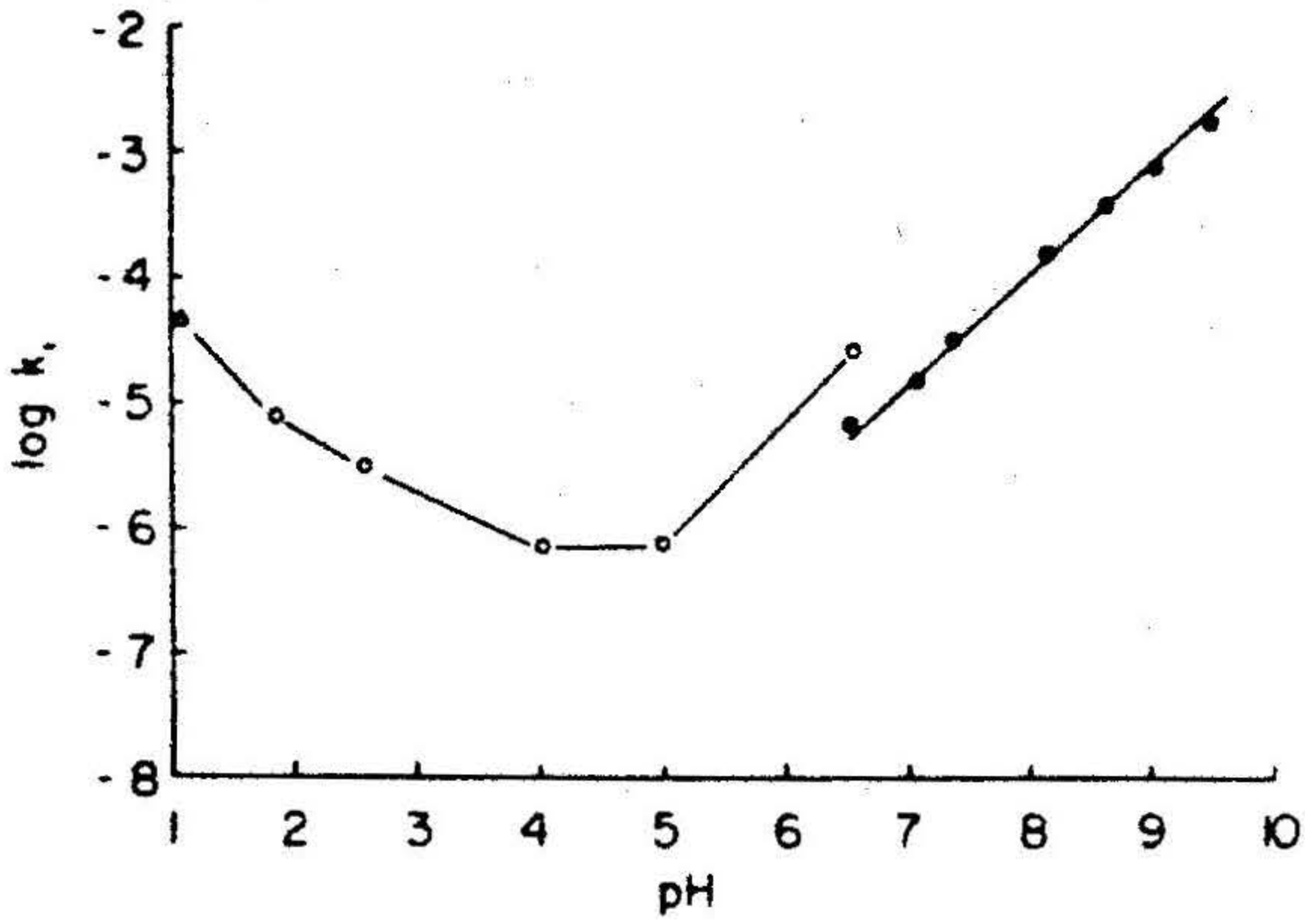

Fig. 11. The pH dependence of the acyl migration. The log of the pseudo-first-order rate constant k_1 for the rearrangement of 2-palmitoyl-lyso-PC (**2a**) into 1-palmitoyl-lyso-PC (**1a**) is plotted against the pH buffered with (●) 50 m*M* Tris-HCl, (○) 50 m*M* citrate, or (△) 0.1 *M* HCl alone. At pH 1.0, Triton X-100 was included. From Plückthun and Dennis (1982a). Copyright 1982 American Chemical Society.

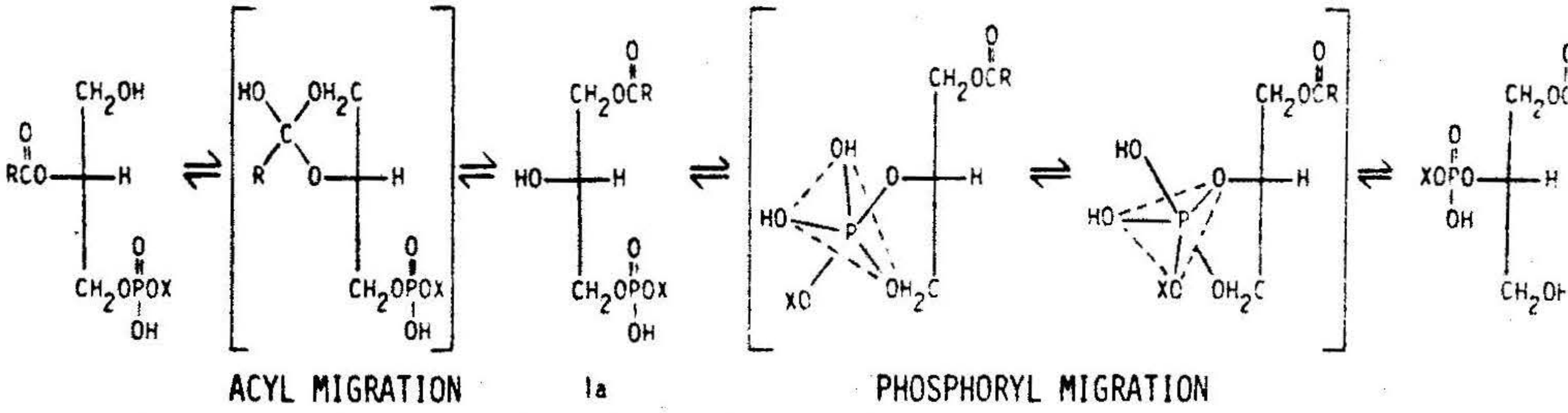

Fig. 12. Mechanism for the acyl migration and phosphoryl migration in lysophospholipids. For simplicity, the acid-catalyzed reactions with fully protonated intermediates are shown. The phosphoryl migration has to involve pseudorotation of the trigonal bipyramidal intermediate as indicated. Although the OX group is shown in an apical position after initial attack of the hydroyxl group of **1a**, the hydroxyl group of the phosphate could instead initially occupy the apical position. Pseudorotation cannot occur in base because it would bring an oxyanion from an equatorial to an apical position. Pathways leading to hydrolysis of the OX group and formation of a tetracoordinated cyclic phosphate diester followed by hydrolysis and migration of the phosphate group are not included in this figure. The acyl migration probably goes through a cyclic ortho ester intermediate. The basic catalyst probably partially removes the proton of the glycerol hydroxyl group and facilitates the attack at the carbonyl group. Under these conditions, the phosphate group bears a negative charge, and the attack of the glycerol oxyanion on the phosphorus atom would be expected to be slow. From Plückthun and Dennis (1982a). Copyright 1982 American Chemical Society.

complex (A. Plückthun and E. A. Dennis, unpublished), and the rate constants have not been determined. The mechanisms of acyl and phosphoryl migration are summarized in Fig. 12.

VII. Phospholipases: Specificity and Kinetics

Phospholipases are enzymes that hydrolyze phospholipids (Dennis, 1983). Among the products of hydrolysis are lysophospholipids, phosphatidic acid (PA), and glycerophosphorylcholine and glycerophosphorylethanolamine. Because these can be resolved readily in the presence of the phospholipid substrates by ^{31}P NMR (Section II), this method provides a ready assay that in many cases has advantages over more traditional methods. Of the phospholipases, the most well-studied and characterized is phospholipase A_2. The substrate phospholipid is most advantageously studied when it is solubilized in mixed micelles with detergents such as Triton X-100 or deoxycholate; for the enzyme from pancreas, its physiological substrates are mixed micelles of bile salts and phospholipids. The usefulness of ^{31}P NMR in following the hydrolysis of PC and PE in Triton X-100 mixed micelles was shown in Fig. 2, where both PC, PE, and their lyso products can be resolved. In this case, following the hydrolysis of phospholipid mixtures would be particularly laborious by traditional methods, and mechanistic studies required carrying out kinetic studies in the presence of mixtures of phospholipids (Roberts *et al.*, 1979). Phosphorus-31 NMR has been employed to follow the kinetics of phospholipase A_2 from a variety of sources in mixed micelles with a large number of types of detergents and with a range of different phospholipids and analogs (Adamich *et al.*, 1979; Roberts *et al.*, 1979; Plückthun and Dennis, 1982b). Phosphorus-31 NMR is particularly advantageous for kinetic studies on mixtures of normal phospholipids in mixed micelles with detergents and synthetic short-chain phospholipids such as dibutyryl-PC, where ^{31}P NMR can at the same time show this compound to be in a monomeric state (Section V; Plückthun and Dennis, 1982b). Implications for the results of these studies are beyond the scope of this chapter (Dennis *et al.*, 1981; Dennis, 1983).

Because 1-lyso- and 2-lyso-PC can be resolved by ^{31}P NMR (Section VI), ^{31}P-NMR can also be used to demonstrate directly the positional specificity of phospholipase A_2 and lipase, which acts as a phospholipase A_1, by the direct observation of the lyso products formed under conditions where migration is slow, as shown in Fig. 13. Historically, the specificities of phospholipases have been determined by utilizing specifically radiolabeled phospholipids, which were synthesized either chemically or by using

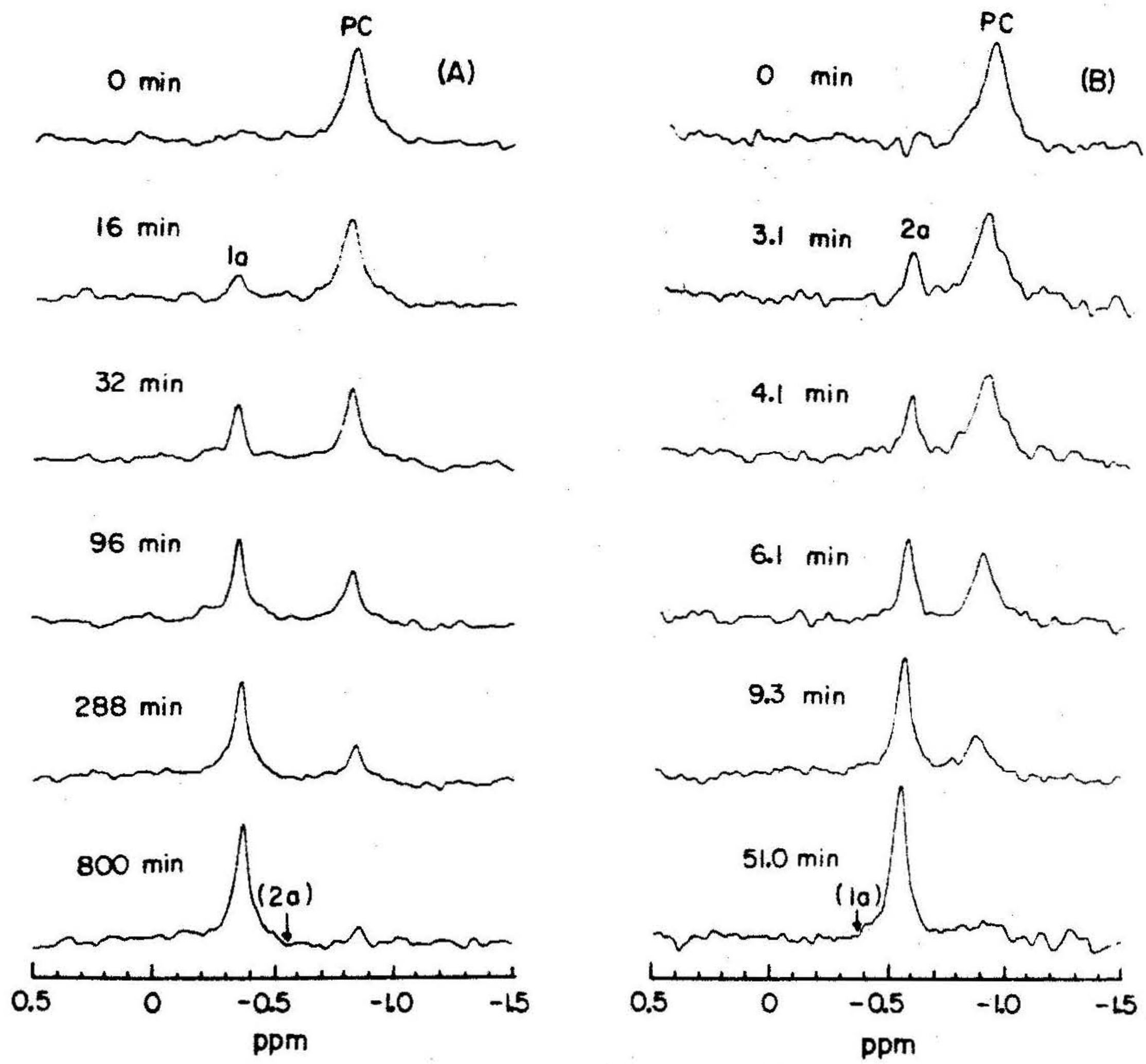

Fig. 13. (A) Action of cobra venom phospholipase A_2 on mixed micelles consisting of 15 m*M* dipalmitoyl-PC (PC) and 120 m*M* Triton X-100. The enzyme concentration was 12.5 μg ml^{-1}. The solution contained 10 m*M* $CaCl_2$, 20% D_2O, and 50 m*M* Tris-HCl buffer, pH 6.0. (B) Action of *Rhizopus arrhizus* lipase (375 μg ml^{-1}) under identical conditions. Spectra were obtained using the spectrometer described in Fig. 2. From Plückthun and Dennis (1982a). Copyright 1982 American Chemical Society. Note that the original figure has been altered to now show positive chemical shifts in the direction of decreasing field strength.

known phospholipases. The ^{31}P-NMR procedure allows one now to make a direct structural determination of phospholipase products that results in a truly independent specificity determination. Whereas it is well-known that phospholipase A_2 is specific for the *sn*-2 position of phospholipids in micelles and bilayer membranes, it was demonstrated by this technique that this specificity also holds for the monomeric phospholipid dibutyryl-PC (Plückthun and Dennis, 1982a).

Phosphorus-31 NMR has also been used to follow phospholipase A_2 hydrolysis in substrate forms other than micelles and mixed micelles, such as lipoproteins (Brasure *et al.*, 1978) where the major phospholipid is PC. In

these studies on lipoproteins, the linewidths of the PC and lyso-PC peaks were ~7.5 Hz, and the small amount of sphingomyelin (SM) present was not resolved from the lyso-PC product, although it could be easily taken into account in the kinetic analysis. In this case, as well as when red blood cells were treated with various phospholipases (Van Meer *et al.*, 1980), ^{31}P NMR was used to identify the physical state of the resulting lysophospholipid product, for if it is dissociated from the lipoprotein particles or the membranes, it should give rise to a sharp resonance indicative of its monomeric or micellar state. For both cases (Brasure *et al.*, 1978; Van Meer *et al.*, 1980), the lysophospholipid product after phospholipase A_2 digestion stays associated with the particle because such a peak does not appear.

An interesting use of ^{31}P NMR in the study of phospholipases A_2 and C is in the resolution of the two diastereomers of the phosphorothioate analog of PE (Orr *et al.*, 1982). The racemic phospholipid gives rise to two peaks (separated by 0.14 ppm) in its ^{31}P-NMR spectrum in chloroform; hydrolysis by phospholipase A_2 gave rise to the loss of one of the peaks, and hydrolysis by phospholipase C gave rise to the loss of the other. This allowed the preparation and identification by ^{31}P NMR of the pure diastereomers, although the absolute configuration of each could not be determined. ^{31}P NMR has also been used with the diastereomers of PE containing one ^{18}O in the phosphate moiety to establish the stereochemical course of the transphosphatidylation reaction catalyzed by phospholipase D under certain experimental conditions (Bruzik and Tsai, 1982; Chapter 6, Tsai). The use of ^{31}P NMR in studying enzymes that can differentiate phosphate-containing compounds that are chiral at phosphorus is receiving attention as discussed elsewhere in this volume, and the identification of diastereomers of phospholipids by ^{31}P NMR should lead to increased study of the phospholipases (Dennis, 1983); perhaps resolution of the diastereomers will be aided by incorporating them into mixed micelles.

Acknowledgment

Support for this work was provided by National Science Foundation grant PCM 82-16963.

References

Adamich, M., Roberts. M. F., and Dennis, E. A. (1979). *Biochemistry* **15**, 3308–3314.

Assmann, G., Sokoloski, E. A., and Brewer, H. B., Jr. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 549–553.

Berden, J. A., Barker, R. W., and Radda, G. K. (1975). *Biochim. Biophys. Acta* **375**, 186–208.
Bocian, D. F., and Chan, S. I. (1978). *Annu. Rev. Phys. Chem.* **29**, 307–335.
Brasure, E. B., Henderson, T. O., Glonek, T., Pattnaik, N. M., and Scanu, A. M. (1978). *Biochemistry* **17**, 3934–3938.
Bruzik, K., and Tsai, M-D. (1982). *J. Am. Chem. Soc.* **104**, 863–865.
Burns, R. A., Donovan, J. M., and Roberts, M. F. (1983). *Biochemistry* **22**, 964–973.
Castellino, F. J., and Violand, B. N. (1979). *Arch. Biochem. Biophys.* **193**, 543–550.
Cullis, P. R., and de Kruyff, B. (1976). *Biochim. Biophys. Acta* **436**, 523–540.
Cullis, P. R., and de Kruyff, B. (1979). *Biochim. Biophys. Acta* **559**, 399–420.
de Kruyff, B., Verkley, A. J., Van Echteld, C. J. A., Gerritsen, W. J., Mombers, C., Noordam, P. C., and de Gier, J. (1979). *Biochim. Biophys. Acta* **555**, 200–209.
Dennis, E. A. (1983). "The Enzymes" (P. Boyer, ed.), 3rd ed., Vol. XVI, pp. 307–353. Academic Press, New York.
Dennis, E. A., and Owens, J. M. (1973). *J. Supramol. Struct.* **1**, 165–176.
Dennis, E. A., Darke, P. L., Deems, R. A., Kensil, C. R., and Plückthun, A. (1981). *Mol. Cell. Biochem.* **36**, 37–45.
Glonek, T., Henderson, T. O., Kruski, A. W., and Scanu, A. M. (1974). *Biochim. Biophys. Acta* **348**, 155–161.
Helenius, A., and Simons, K. (1975). *Biochim. Biophys. Acta* **415**, 29–79.
Helenius, A., McCaslin, D. R., Fries, E., and Tanford, C. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 56, pp. 734–749. Academic Press, New York.
Henderson, T. O., Glonek, T., and Myers, T. C. (1974). *Biochemistry* **13**, 623–628.
Henderson, T. O., Kruski, A. W., Davis, L. G., Glonek, T., and Scanu, A. M. (1975). *Biochemistry* **14**, 1915–1920.
Jackson, M. L., Schmidt, C. F., Lichtenberg, D., Litman, B. J., and Albert, A. D. (1982). *Biochemistry* **21**, 4576–4582.
Klose, G., and Hollerbuhl, T. (1981). *Stud. Biophys.* **83**, 35–40.
Kresheck, G. C., and Jones, C. (1980). *J. Colloid Interface Sci.* **77**, 278–279.
Lichtenberg, D., Robson, R. J., and Dennis, E. A. (1983). *Biochim. Biophys. Acta* **737**, 285–304.
London, E., and Feigenson, G. W. (1979). *J. Lipid Res.* **20**, 408–412.
Madden, T. D., and Cullis, P. R. (1982). *Biochim. Biophys. Acta* **684**, 149–153.
Mazer, N. A., Benedek, G. B., and Carey, M. C. (1980). *Biochemistry* **19**, 601–615.
Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1973). *Biochemistry* **12**, 2637–2645.
Nolden, P. W., and Ackermann, T. (1976). *Biophys. Chem.* **4**, 297–304.
Orr, G. A., Brewer, C. F., and Heney, G. (1982). *Biochemistry* **21**, 3202–3206.
Plückthun, A., and Dennis, E. A. (1981). *J. Phys. Chem.* **85**, 678–683.
Plückthun, A., and Dennis, E. A. (1982a). *Biochemistry* **21**, 1743–1750.
Plückthun, A., and Dennis, E. A. (1982b). *Biochemistry* **21**, 1750–1756.
Ribeiro, A. A., and Dennis, E. A. (1974). *Biochim. Biophys. Acta* **332**, 26–35.
Ribeiro, A. A., and Dennis, E. A. (1976). *J. Colloid Interface Sci.* **55**, 94–101.
Roberts, M. F., Adamich, M., Robson, R. J., and Dennis, E. A. (1979). *Biochemistry* **15**, 3301–3308.
Seelig, J. (1978). *Biochim. Biophys. Acta* **515**, 105–140.
Tanford, C., and Reynolds, J. A. (1976). *Biochim. Biophys. Acta* **457**, 133–170.
Tausk, R. J. M., Karmiggelt, J., Oudshoorn, C., and Overbeek, J. T. G. (1974a). *Biophys. Chem.* **1**, 175–183.
Tausk, R. J. M., van Esch, J., Karmiggelt, J., Voordouw, G., and Overbeek, J. T. G. (1974b). *Biophys. Chem.* **1**, 184–203.

Tausk, R. J. M., Oudshoorn, C., and Overbeek, J. T. G. (1974c). *Biophys. Chem.* **2,** 53–63.
Van Meer, G., de Kruyff, B., Op Den Kamp, J. A. F., and Van Deenen, L. L. M. (1980). *Biochim. Biophys. Acta* **596,** 1–9.
Yeagle, P. L. (1978). *Acc. Chem. Res.* **11,** 321–327.
Yeagle, P. L. (1979). *Arch. Biochem. Biophys.* **198,** 501–505.
Yeagle, P. L., Hutton, W. C., Huang, C.-H., and Martin, R. B. (1977). *Biochemistry* **16,** 4344–4349.