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Acyl and Phosphoryl Migration in Lysophospholipids: Importance in Phospholipid Synthesis and Phospholipase Specificity†

Andreas Plückthun‡ and Edward A. Dennis*

ABSTRACT: Three isomeric lysophosphatidylcholines (1-palmitoyl-*sn*-glycero-3-phosphorylcholine, 2-palmitoyl-*sn*-glycero-3-phosphorylcholine, and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine) have been prepared by the action of phospholipase A_2 or lipase on 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine or phospholipase A_2 on 1,3-dipalmitoyl-*sn*-glycero-2-phosphorylcholine. The structures of the lyso compounds have been confirmed by a complete assignment of the polar head groups using ^1H NMR spectroscopy. The product of phospholipase A_2 action on phosphatidylcholine is 1-acyl-*sn*-glycero-3-phosphorylcholine. Acyl migration between this compound and the 2-acyl isomer and phosphoryl migration between this compound and the 2-phosphoryl isomer were followed by ^{31}P NMR. The acyl migration was found to be first order in both lysophospholipid and acid or base with a base-catalyzed second-order rate constant of about 4×10^{-4} M^{-1} s^{-1}. At alkaline pHs, 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 lysophospholipids. At alkaline pHs, no phosphoryl migration was detected in the time scale of acyl migration and hydrolysis. ^{31}P NMR could also directly demonstrate the positional specificity of phospholipase A_2 and lipase, which acts as a phospholipase A_1, by the direct observation of the products formed under conditions where migration was slow. While 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-phosphatidylcholine.

The rearrangement of lysophospholipids via acyl migration has long been recognized as a serious problem that has to be taken into account in the determination of phospholipase specificity (de Haas et al., 1960; de Haas & van Deenen, 1961; van Deenen et al., 1963), synthesis of mixed acyl phospholipids (Gupta et al., 1977; Warner & Benson, 1977), and the elucidation of biosynthetic pathways that lead to mixed acyl phospholipids (van Golde & van den Bergh, 1977) involving lysophospholipid intermediates (Homma et al., 1981). The importance of phosphoryl migration is illustrated by the recent observation that trace amounts of 1,3-diacyl-*sn*-glycero-2-phosphorylcholine produced by phosphoryl migration during the synthesis of 1,2-diacyl-*sn*-glycero-3-phosphorylcholine (Lammers et al., 1978; Ponpipom & Bugianesi, 1980) can increase the stability of sonicated vesicles of the latter by 100-fold (Larrabee, 1979).

There are six different lysophosphatidylcholines consisting of three enantiomeric pairs of positional isomers as shown in Figure 1. The possible acyl and phosphoryl migrations are indicated. The lack of a suitable analytical tool has previously prevented a detailed investigation of the reaction rates and equilibria involved in migration. We have now used ^{31}P NMR to follow the kinetics and determine the equilibria of these reactions.[1]

The phenomenon of acyl migration was first discovered by Fischer (1920) and has subsequently been found to occur frequently in chemical and biochemical systems [reviews: Akahori, 1965; Pavlova & Rachinskii, 1968]. It has been characterized quite extensively in the rearrangement between 1- and 2-monoacylglycerides, because the vicinal diol in 1-monoacylglycerides can be easily quantitated by using a periodate determination (Martin, 1953; van Lohuizen & Verkade, 1960; Wolfenden et al., 1964; Verkade, 1966; Serdarevich, 1967). However, such a reaction is not possible in lysophospholipids. A number of techniques have been used for studies on the rearrangement of diglycerides, such as a thin-layer chromatographic separation (Freeman & Morton, 1966), enzymatic breakdown (Mattson & Volpenhein, 1962), and ^1H NMR spectroscopy (Serdarevich, 1967).

In the case of lysophospholipids, however, it was found to not be possible to separate compounds Ia and IIa by any thin-layer or paper chromatography technique (Slotboom et al., 1963; Eibl & Lands, 1970). Column chromatography on silicic acid or alumina causes rapid equilibration of lysophospholipids (Lands & Merkel, 1963; de Haas & van Deenen, 1965; Eibl & Lands, 1970; Gupta et al., 1977). No chemical methods have appeared which can quantitate mixtures of isomers of lysophospholipids without themselves causing isomerization (Hanahan et al., 1960). The difference in activity of phospholipase A_2 toward Ia and IIa was used in an attempt to qualitatively assess the stability of the isomers (Slotboom et al., 1963; Albright et al., 1973), but this depends on the correct assignment by another, independent method. Another enzymatic technique, which has been employed to distinguish Ia and IIa, used the degradation of lysophospholipids to monoglycerides by phospholipase C (van den Bosch & van Deenen, 1965; Eibl & Lands, 1970). Although the resulting 1- and 2-monoglycerides can be analyzed by a variety of techniques (Hofmann, 1962; Wolfenden et al., 1964; Johnson & Holman, 1966; Satouchi & Saito, 1977; Sugatani et al., 1980; Matsuzawa & Hostetler, 1980), this is rather cumbersome and involves the problem of further acyl migration during the workup procedure. Spectroscopic methods such as IR (Slotboom et al., 1963, 1967; de Haas & van Deenen, 1965), ^{13}C NMR (Schmidt et al., 1977), or ^1H NMR (discussed below) can all detect spectral differences between the

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[1] A preliminary report of some of these results was presented at the International Conference on Phosphorus Chemistry, Duke University, Durham, NC, June 1–5, 1981.

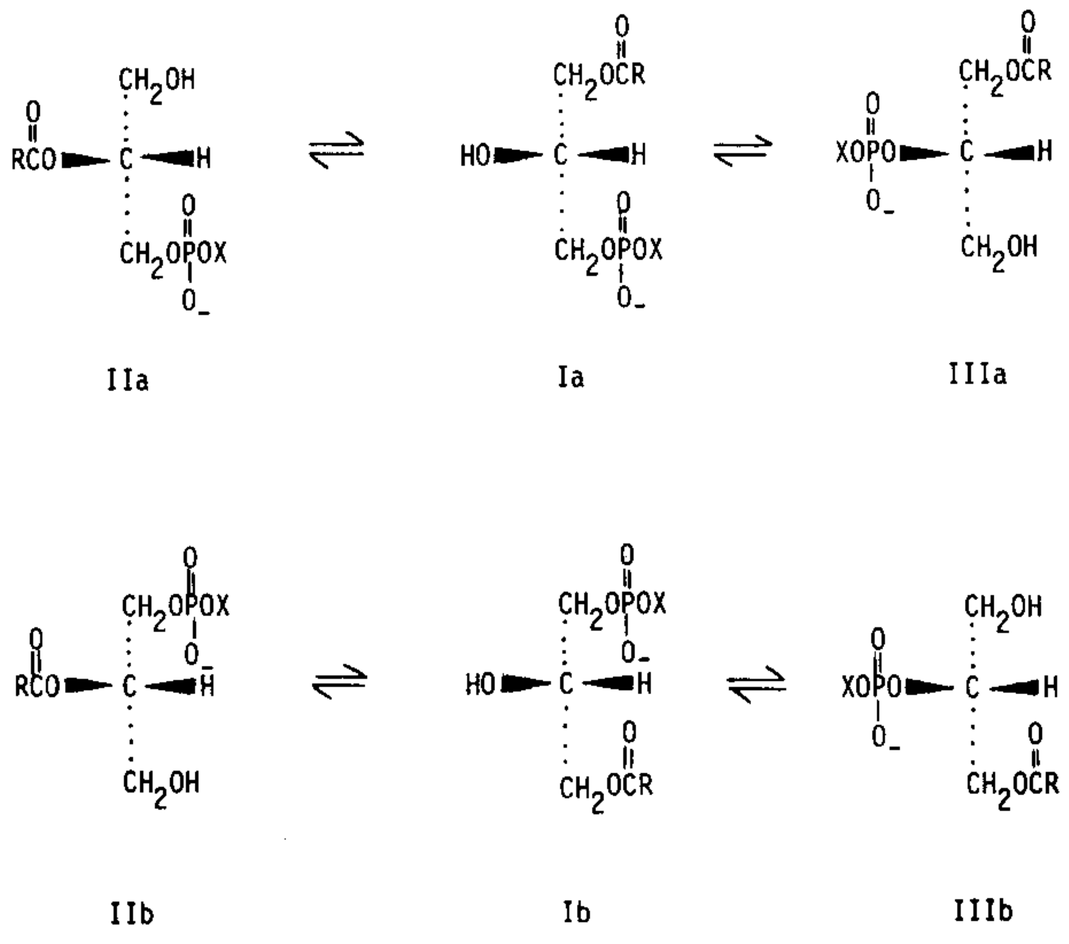

FIGURE 1: Possible interconversions via acyl (I ⇌ II) and phosphoryl (I ⇌ III) migration of lysophospholipids derived from natural phospholipids (a series) and their enantiomorphs (b series). When RCOO– is palmitic acid and X is choline, the structures correspond to the following compounds: 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia), 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa), 1-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIa), 3-palmitoyl-*sn*-glycero-1-phosphorylcholine (Ib), 2-palmitoyl-*sn*-glycero-1-phosphorylcholine (IIb), and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb).

lysophospholipid isomers but are not particularly suitable for kinetic studies. We have found that ^{31}P NMR provides a simple, sensitive, and rapid method to observe the kinetics of acyl and phosphoryl migration in lysophospholipids, and this has now made possible a detailed analysis of these processes which is reported here.

Experimental Procedures

Phospholipids and Detergents. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (dipalmitoylphosphatidylcholine) and 1,3-dipalmitoyl-*sn*-glycero-2-phosphorylcholine were obtained from Calbiochem. 1-Palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb) were prepared by the action of phospholipase A_2 on 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine and 1,3-dipalmitoyl-*sn*-glycero-2-phosphorylcholine, respectively, as described previously (Plückthun & Dennis, 1981). Phospholipase A_2 stereospecifically generates 3-acyl-*sn*-glycero-2-phosphorylcholine (IIIb) from 1,3-diacyl-*sn*-glycero-2-phosphorylcholine (de Haas & van Deenen, 1964, 1965). The specific rotation of IIIb in $CHCl_3$–CH_3OH (9:1 v/v) was $[\alpha]^{28}_D$ + 3.8 (c = 2.5) [literature (de Haas & van Deenen, 1965): $[\alpha]^{20}_D$ + 4.2 (c = 12)]. 2-Palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) was prepared by the action of lipase on 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (Slotboom et al., 1970b) and purified by chromatography on Sephadex LH-20 at 4 °C. The lysophosphatidylcholines were stored as solutions in $CHCl_3$ in a desiccator at −60 °C. Under these conditions no measurable rearrangement occurred over several weeks. Dibutyrylphosphatidylcholine was prepared as described elsewhere (Patel et al., 1979) and purified by column chromatography on silicic acid. All phospholipids were checked for purity by thin-layer chromatography on silica gel (solvent $CHCl_3$–CH_3OH–H_2O, 65:25:4 v/v/v) and were found to give single spots. ^{31}P NMR showed single peaks for all phospholipids, except for 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa), which contained about 20% 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) formed by migration during the lipase reaction and purification. The nonionic detergent Triton X-100 was obtained from Rohm and Haas. Deoxycholate was obtained from Sigma. All other chemicals were of the highest purity available and were used without further purification.

Enzymes. Phospholipase A_2 from cobra venom (*Naja naja naja*), obtained from the Miami Serpentarium, lot no. NNP9STLZ, was isolated and purified as described previously (Deems & Dennis, 1975; Roberts et al., 1977). Lipase (*Rhizopus arrhizus*) was a gift of Dr. Hans Nilsson, Kockums Chemical AB, Malmö, Sweden, or obtained from Boehringer.

^1H NMR Spectroscopy. ^1H NMR spectra were obtained at 360 MHz on a modified Varian instrument equipped with quadrature phase detection and a 1080 FT Nicolet system. The spectra were run at ambient temperature (~22 °C). Samples were generally 30 mM in lysophospholipid in D_2O at pD 5.0 and contained TSP[2] as an internal chemical shift standard. Computer simulations of the ^1H NMR spectra were made by using the ITRCAL program on the Nicolet computer.

^{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 JNM-SD-HC unit was used with a Schomandl ND-100-M frequency generator. The temperature was 40 ± 0.5 °C unless otherwise indicated. The spin–lattice relaxation time (T_1) was measured for Ia and IIa and was found to be identical for both compounds within experimental error (about 2.3 s) as expected for compounds with such similar chemical structures. The NOE for both compounds was also found to be the same within experimental error. Therefore, a delay time between pulses of about 2 s was employed with a 60–70° pulse in order to maximize the signal-to-noise ratio per time unit. Because of the similar T_1 and NOE for each isomer, direct comparison of peak heights could be used for quantitation of rearrangements and equilibria. In fact, during the migration reactions, the sum of all peak heights did remain constant within experimental error. Furthermore, when the values obtained by peak heights were compared to those obtained by using an electronic planimeter (Talos Systems), no difference could be detected. Chemical shifts are reported for 10 mM phospholipid in buffer containing 50 mM Tris-HCl, 10 mM $CaCl_2$, and 30% D_2O at pH 8.0. Triton X-100 (80 mM) was included where indicated. Trimethyl phosphate (5% in D_2O, v/v) was used as an external standard as described previously (Plückthun & Dennis, 1981). Chemical shifts are reported relative to 85% H_3PO_4 (upfield is positive).

Migration Reactions. Buffer, containing 50 mM Tris-HCl or 50 mM citrate and 20% D_2O, was brought to the pH indicated. The pH meter reading was not corrected for D_2O. The lysophospholipid was dried from $CHCl_3$ under a stream of N_2, and the last traces of solvent were removed under high vacuum. The buffer was added to the dry phospholipid and the solution immediately transferred to the NMR tube. The final phospholipid concentration generally was 20 mM. Spectra were accumulated and stored on the computer disk in such a fashion that at least 10 time points per reaction half-time were obtained. Reactions were run either to completion or for 24 h. At pH 1.0, a slow precipitation during the reaction was observed with concomitant spectral broadening. Therefore, Triton X-100 was added in this case to give a detergent:lysophospholipid ratio of 8:1, resulting in a clear solution.

[2] Abbreviations: TSP, sodium salt of 3-(trimethylsilyl)tetradeuteriopropionic acid; NOE, nuclear Overhauser effect; Tris, tris(hydroxymethyl)aminomethane; dipalmitoylphosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine.

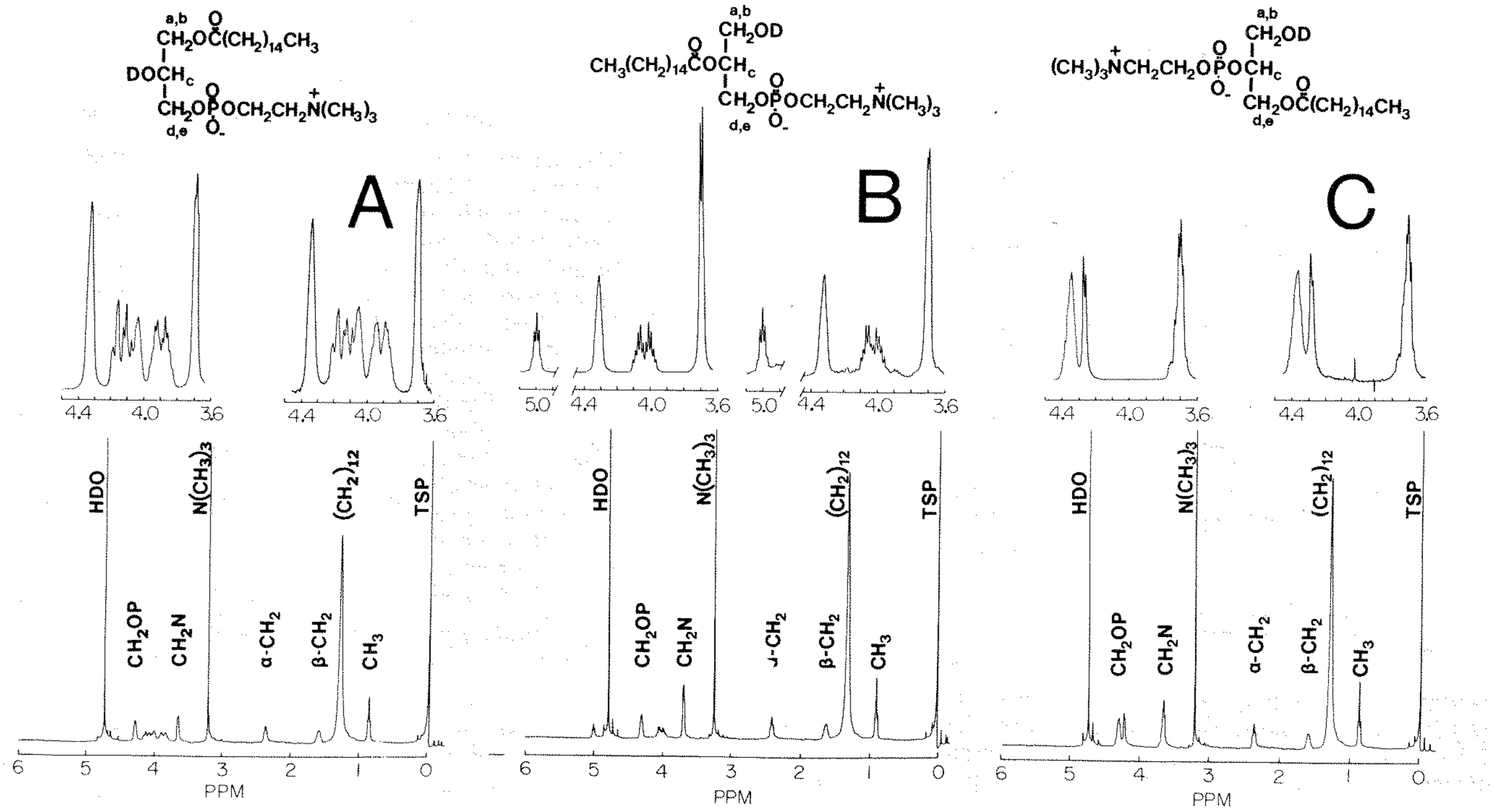

FIGURE 2: ^{1}H NMR spectra of the isomeric lysophosphatidylcholines: (A) 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia); (B) 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa); (C) 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb). On the bottom is the complete spectrum with the assignments indicated. On the top right, an expansion of the polar head group region is shown. On the top left, a computer simulation of the polar head group region is shown by using the parameters listed in Table I.

Table I: ^{1}H NMR Chemical Shifts and Coupling Constants of the Glycerol Backbone of the Three Isomeric Lysophosphatidylcholines

chemical shift (ppm)	coupling constant (Hz)	1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia)	2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa)	3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb)
H_a		4.08	3.69	3.70
H_b		4.15	3.69	3.74
H_c		4.02	5.03	4.37
H_d		3.84	3.99	4.26
H_e		3.91	4.06	4.27
	J_{ab}	11.4		12.2
	J_{ac}	3.4	5.0	6.0
	J_{bc}	6.5	5.0	4.0
	J_{cd}	4.1	5.0	5.0
	J_{ce}	6.0	5.0	5.0
	J_{de}	10.8	10.8	11.5
	J_{P-c}			5.9
	J_{P-d}	6.0	6.0	
	J_{P-e}	5.8	5.8	

Enzymatic Reactions. Enzymatic reactions were run so that the time (indicated in the figures) for completion of the reaction was very short compared to the half-time of the migration reaction under those conditions.

Results

Structural Determination of the Isomeric Lysophosphatidylcholines. The ^{1}H NMR spectra of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia), 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa), and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb) are shown in Figure 2. The assignment of the polar head group of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) is that of Hauser and coworkers (Hauser et al., 1978). When their values for chemical shifts and coupling constants for compound Ia were used, an excellent fit of the computer-simulated spectrum to the observed spectrum was obtained. We also used their values for the coupling constants of the choline methylene protons in the simulations of the other two isomers; however, the chemical shift of the choline methylene groups was found to be slightly different in all positional isomers, and the glycerol backbone of all these compounds gave distinctly different signals. All assignments are summarized in Table I. The *sn*-2-methine proton H_c of the 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) shows a first-order quintet at 5.03 ppm, whereas it is a second-order multiplet at 4.02 ppm for the 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia). This is consistent with it being bound to a $-CR_2O$-acyl group in IIa but to a $-CR_2OH$ group in Ia.

The protons H_a and H_b also show a downfield shift in the presence of an adjacent acyl group. They are bound to a >CRO-acyl group in Ia and give multiplets at 4.15 and 4.08 ppm, respectively, whereas for IIa they are bound to an >CROH group and show a multiplet at 3.69 ppm. The assignment of the glycerol portion of 3-palmitoyl-*sn*-glycero-2-phosphorylcholine is only tentative, because the overlap between the choline methylene groups and the glycerol protons obscures the multiplet structure of the latter. The signals at

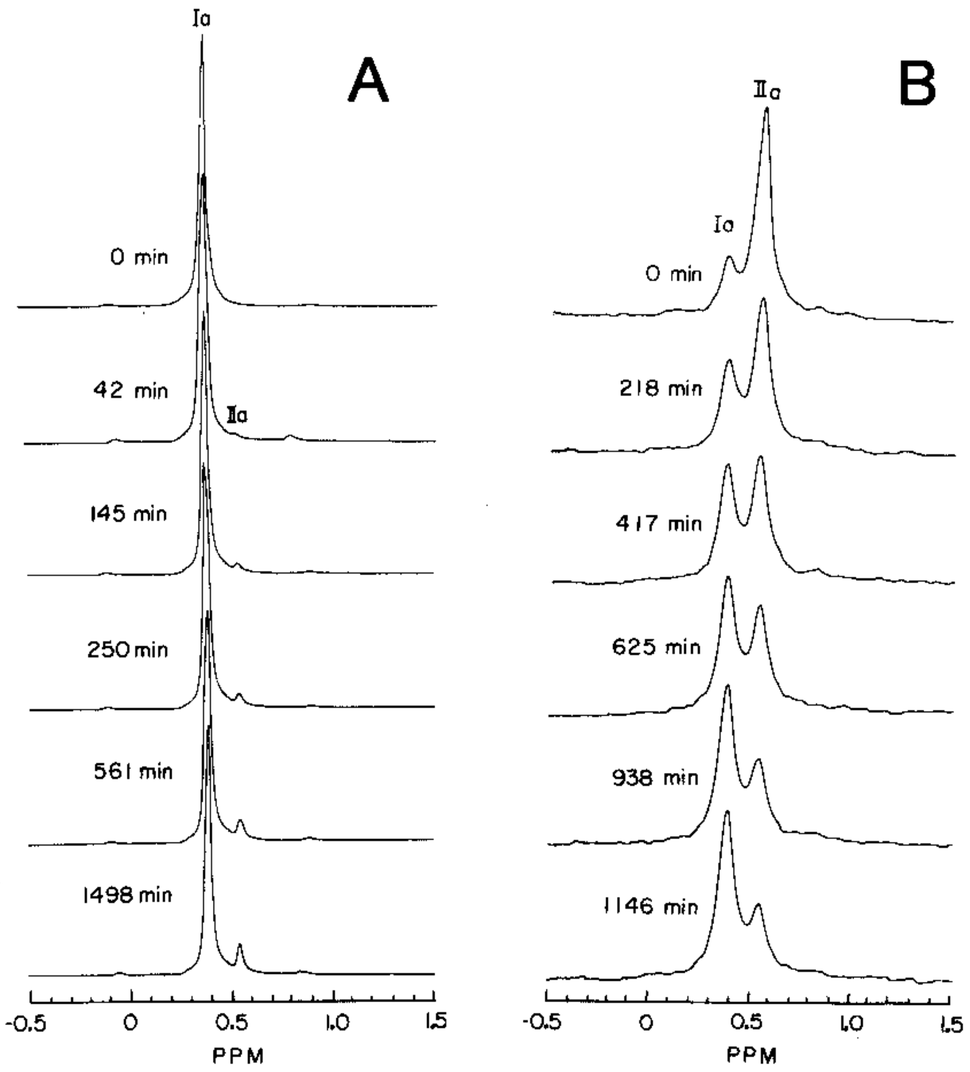

FIGURE 3: ^{31}P NMR spectra obtained after the times indicated of (A) 30 mM 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) at pH 7.0 and (B) 20 mM 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa), of which some had migrated to Ia during its preparation, at pH 7.0.

about 3.72, 4.27, and 4.37 ppm integrate to 4:2:3. By analogy the multiplets at 3.72 and 4.37 ppm should contain the choline methylene protons. When the proton H_c is decoupled, the protons H_a and H_b appear to be an AB quartet with J_{ab} = 12.2 Hz. This might be explained by hydrogen bonding between the hydroxyl group and the vicinal phosphate in IIIb. In contrast, the $-CH_2OH$ group in IIa collapses to a singlet upon decoupling H_c. This equivalence of H_a and H_b in IIa is an indication of free rotation, presumably because of the absence of hydrogen bonding. In both cases, a chemical shift of about 3.7 ppm is consistent with a $-CH_2OH$ group. Because of the broadness of the overlapping choline CH_2OP signal, the multiplet structure of H_d and H_e in IIIb could not be deduced. A satisfactory appearance of the simulated spectrum was, however, obtained with $\delta(H_d)$ = 4.27 ppm, $\delta(H_e)$ = 4.26 ppm, and J_{de} = 11.5 Hz. These shifts are consistent with a $-CH_2O$-acyl group.

Although the above results show that all three positionally isomeric lysophosphatidylcholines can be distinguished by ^{1}H NMR, there are several factors that make this method less suitable for kinetic studies: (i) Because of signal overlap, kinetic measurements would have to rely upon the appearance or disappearance of one distinct signal rather than on ratios. (ii) The peaks of interest are the smallest in the spectrum and are therefore very easily distorted, especially by the presence of a large water peak. The H_c proton in IIa is very close to the spinning side bands of HDO. The ^{1}H NMR spectra serve, however, for a structural determination of the three isomeric phospholipids which together with their different methods of enzymatic preparation allow the ^{31}P NMR spectral data to be assigned unequivocally.

Kinetics of Acyl Migration. Figure 3 shows typical ^{31}P NMR spectra obtained during the acyl migration reaction. Most dramatically, 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa), which had been prepared by the action of lipase, is converted into 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) over time (Figure 3B). Table II gives a summary of the ^{31}P NMR chemical shifts of the lysophosphatidylcholines and other related compounds. In Figure 3A, the rearrangement starting from isomer Ia to give the equilibrium mixture containing Ia and IIa is shown. It is found, as expected, that an

Table II: ^{31}P NMR Chemical Shifts of Phosphatidylcholine and Hydrolysis Products at pH 8.0

compd	Triton X-100[a]	δ (ppm)
1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine	+	0.86
1,3-dipalmitoyl-*sn*-glycero-2-phosphorylcholine	+	1.45
1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia)	−	0.34
	+	0.38
2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa)	−	0.52
	+	0.55
3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIa)	−	1.13
sn-glycero-3-phosphorylcholine	−	0.08
1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine	−	0.60
1-butyryl-*sn*-glycero-3-phosphorylcholine	−	0.24
2-butyryl-*sn*-glycero-3-phosphorylcholine	−	0.44

[a] Triton to phospholipid ratio 8:1 where included.

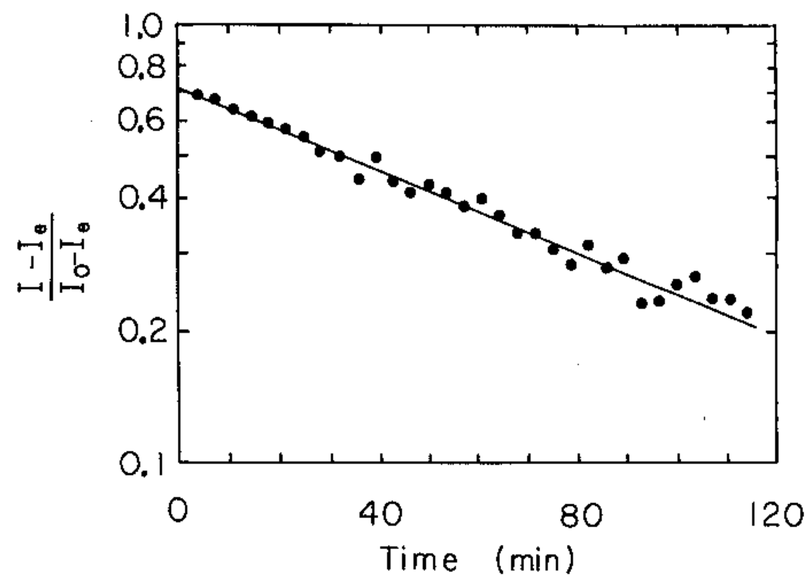

FIGURE 4: Typical time course of the acyl migration reaction. The reaction shown is the rearrangement of 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) to an equilibrium mixture of 90% 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) and 10% 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) at pH 8.0. I and I_e are the intensities of the ^{31}P NMR peak of compound IIa (peak heights) at time t and at equilibrium, respectively. I_0 is the sum of the intensities of peaks Ia and IIa.

identical equilibrium mixture is obtained starting from either compound. The equilibrium mixture was determined between pH 6.2 and 9.0 and was found to be nearly independent of pH within experimental error and to consist of about 90% Ia and 10% IIa. This value was used in rate calculations throughout.

The rates were calculated by assuming a pseudo-first-order reversible reaction A ⇌ B with a forward rate constant k_1 and a backward rate constant k_{-1}. A typical time course is shown in Figure 4. A plot of ln $(I - I_e)/(I_0 - I_e)$ gives a straight line with slope $k_1 + k_{-1}$. Here I is the concentration of one isomer at time t, I_e is its equilibrium concentration, and I_0 is the sum of the concentrations of both isomers. By use of the relation $K_{eq} = k_1/k_{-1}$, k_1 and k_{-1} can then be immediately obtained. At pH greater than 2, no competing reactions such as phosphoryl migration or hydrolysis could be observed on the same time scale. Figure 5 shows a plot of log k_1 vs. pH. The slope in the basic region is about 0.92; we attribute the small difference from unity to experimental error and a possible contribution of general acid/general base catalysis by the buffer. From these data, a second-order rate constant of base-catalyzed acyl migration of about k_2 = 160 M^{-1} s^{-1} can be calculated. The pH–rate profile is similar to that of a typical ester hydrolysis (Kirby, 1972). The second-order rate constant of base-catalyzed acyl hydrolysis of lysophosphatidylcholine is about 1.8 × 10^{-2} M^{-1} s^{-1} (C. R. Kensil and E. A. Dennis, unpublished results), so that the acyl mi-

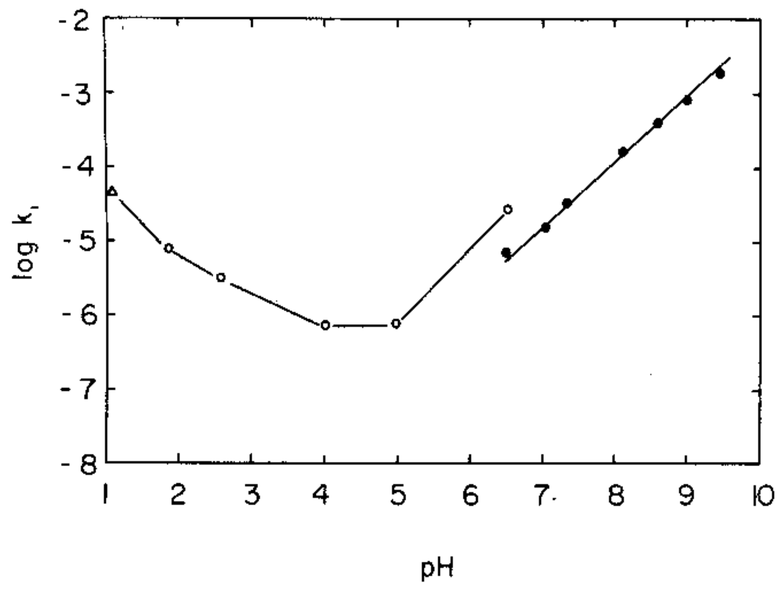

FIGURE 5: pH dependence of the acyl migration. The log of the pseudo-first-order rate constant k_1 for the rearrangement of 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) into 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (Ia) is plotted against the pH buffered with (●) 50 mM Tris-HCl, (○) 50 mM citrate, or (△) 0.1 M HCl alone. At pH = 1.0, Triton X-100 was included.

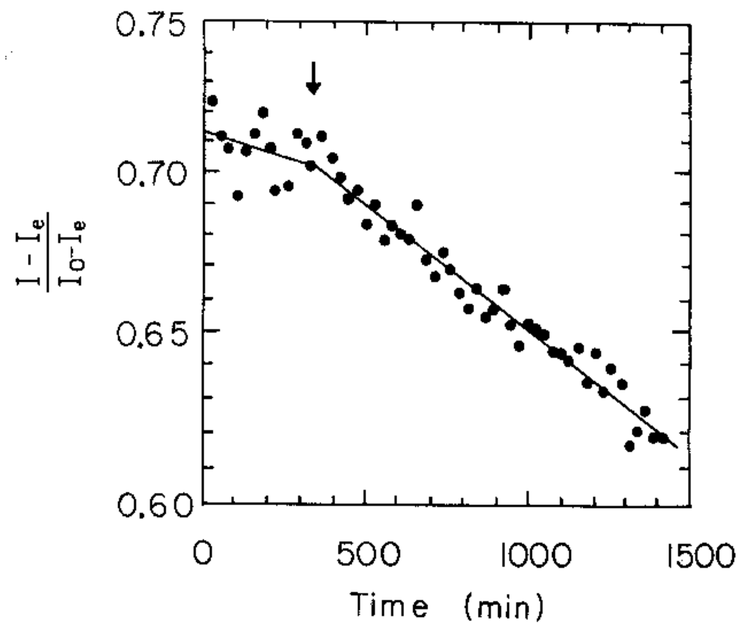

FIGURE 6: Time course of the acyl migration in $CHCl_3$. The solution contained 20 mM 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa). The arrow indicates the time of addition of an equimolar amount of *p*-(dimethylamino)pyridine. In this assay, the temperature was 25 °C. Symbols I, I_e, and I_0 are defined in Figure 4. The vertical axis has a logarithmic scale.

gration is about 8500 times faster than hydrolysis at all basic pH values. A similar value (6500 times faster) was found for the ratio of base-catalyzed acyl migration to hydrolysis of monoacetylglycerol (Wolfenden et al., 1964).

At pH 1.0, there were several compounds formed from the starting material 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) on a comparable time scale. We were, however, not able to extract the rate constant of all reactions from the spectra, partly because of signal overlap and partly because of difficulties in signal identification.

From the data in Figure 5, a second-order rate constant for acid-catalyzed acyl migration of about $k_2 = 4 \times 10^{-4}$ M^{-1} s^{-1} can be obtained between pH 1 and 2. This is clearly very much smaller than the base-catalyzed value. The rate constant for water catalysis, obtained at pH 4 and 5 is about 8×10^{-7} s^{-1}. A buffer effect, presumably by general acid/general base catalysis, is suggested by the change in rate between Tris buffer and citrate buffer. Furthermore, the slope in Figure 5 between pH 2 and 4 is smaller than unity. As the mode of this catalysis was not investigated further, the rate constants should be viewed as approximate only.

For determination of the rate constants for phosphoryl migration, analogous experiments were carried out by starting from 3-palmitoyl-*sn*-glycero-2-phosphorylcholine (IIIb), because if it occurred, this compound would rearrange to Ib, which then would undergo rapid acyl migration to form some IIb as well. At pH 5.0, 7.0, and 9.0, there was no noticeable rearrangement taking place over 12 h. At pH 9.0, some hydrolysis of the fatty acyl group was observed. Only at pH 1.0 did several reactions take place, including hydrolysis of the choline group and migration of the phosphate in the product, acyl hydrolysis and migration, and a small amount of phosphoryl migration, but peak overlap and several unidentified peaks prevented us from obtaining a rate constant. At basic pH, however, the phosphoryl migration, if it occurs at all, is clearly very much slower than acyl migration and apparently even slower than acyl hydrolysis.

For studies on the acyl migration under conditions of chemical synthesis, 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (IIa) was subjected to the conditions of the reacylation procedure for lysophosphatidylcholine of Khorana and co-workers (Gupta et al., 1977): $CHCl_3$ as a solvent and a stoichiometric quantity of the basic catalyst *p*-(dimethylamino)pyridine at 25 °C. The results are shown in Figure 6 where it can be seen that the reaction in $CHCl_3$ is rather slow, but the addition of this amount of catalyst increases the rate of migration about 3-fold. Clearly, acylations in the *sn*-1 position without migration are not possible, and acylations in the *sn*-2 position

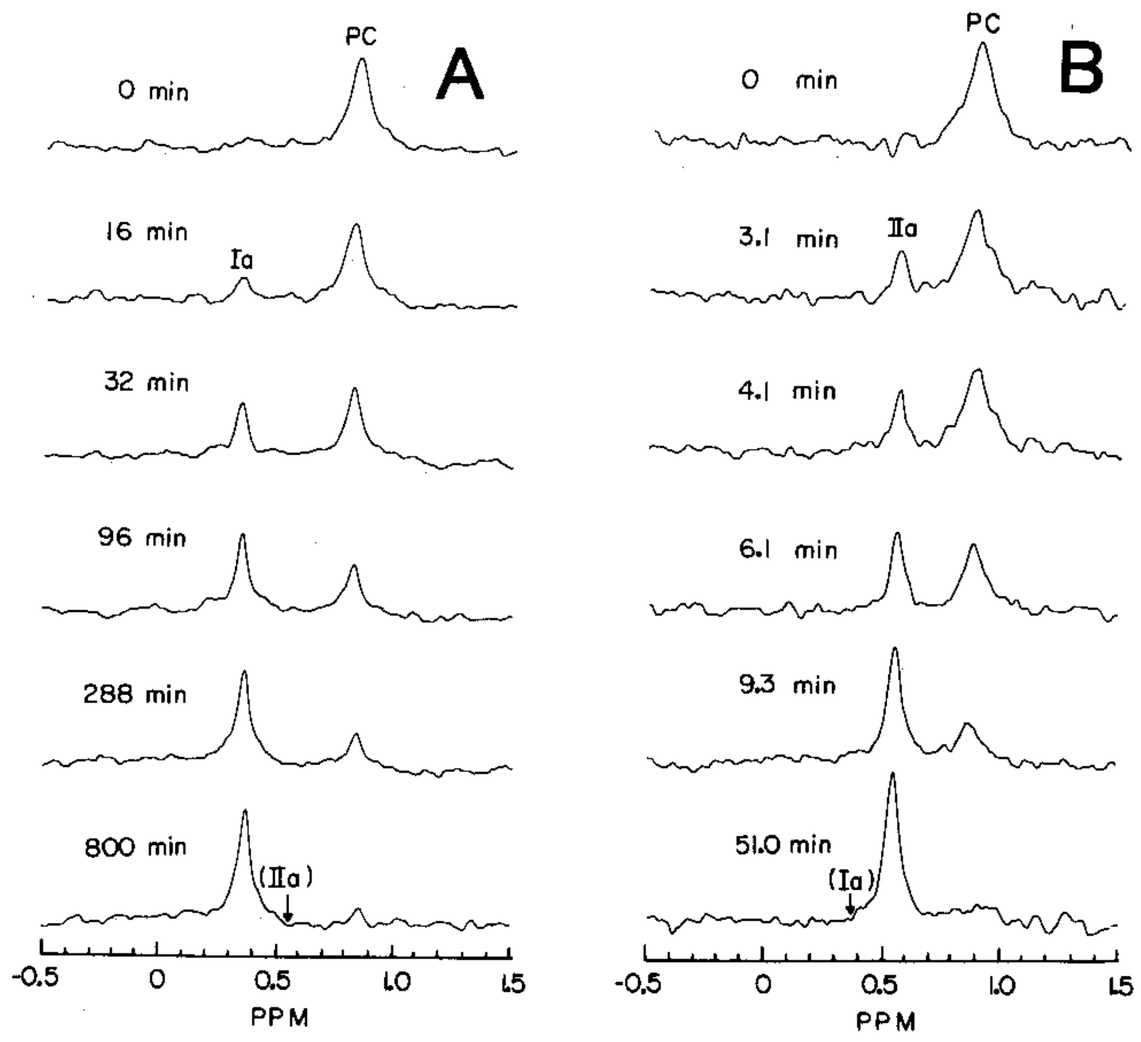

FIGURE 7: (A) Action of phospholipase A_2 on mixed micelles consisting of 15 mM dipalmitoylphosphatidylcholine (PC) and 120 mM Triton X-100. The enzyme concentration was 12.5 μg/mL. The solution contained 10 mM $CaCl_2$, 20% D_2O, and 50 mM Tris-HCl buffer, pH 6.0. (B) Action of lipase (375 μg/mL) under identical conditions.

will also be accompanied by some acyl migration. For example, when Khorana and co-workers (Gupta et al., 1977) acylated compound Ia with this catalyst at twice the concentration employed here (which should double the migration rate), they reported 5% migration in the product. The reaction took 24 h. From the data in Figure 6, 5% migration would be predicted by assuming the same equilibrium mixture as in aqueous solution. Thus the synthesis of mixed acyl phospholipids with absolute positional purity is very difficult to obtain under these conditions.

Specificity of Phospholipases. Figure 7 shows the reaction of phospholipase A_2 on dipalmitoylphosphatidylcholine in mixed micelles with Triton X-100. It can be seen that a peak corresponding to Ia is generated. This resonance is slightly shifted upfield compared to the previous data because of the presence of detergent. Titration experiments have shown that at a Triton:lysophosphatidylcholine ratio of 8:1, the chemical shift of phosphorus moves about 0.04 ppm upfield. This reaction was run with a high enzyme concentration in order to bring it to completion before isomerization occurs. In order to only test for the absence of IIa, fewer spectra with a better signal-to-noise ratio can be taken. In Figure 7B the reaction

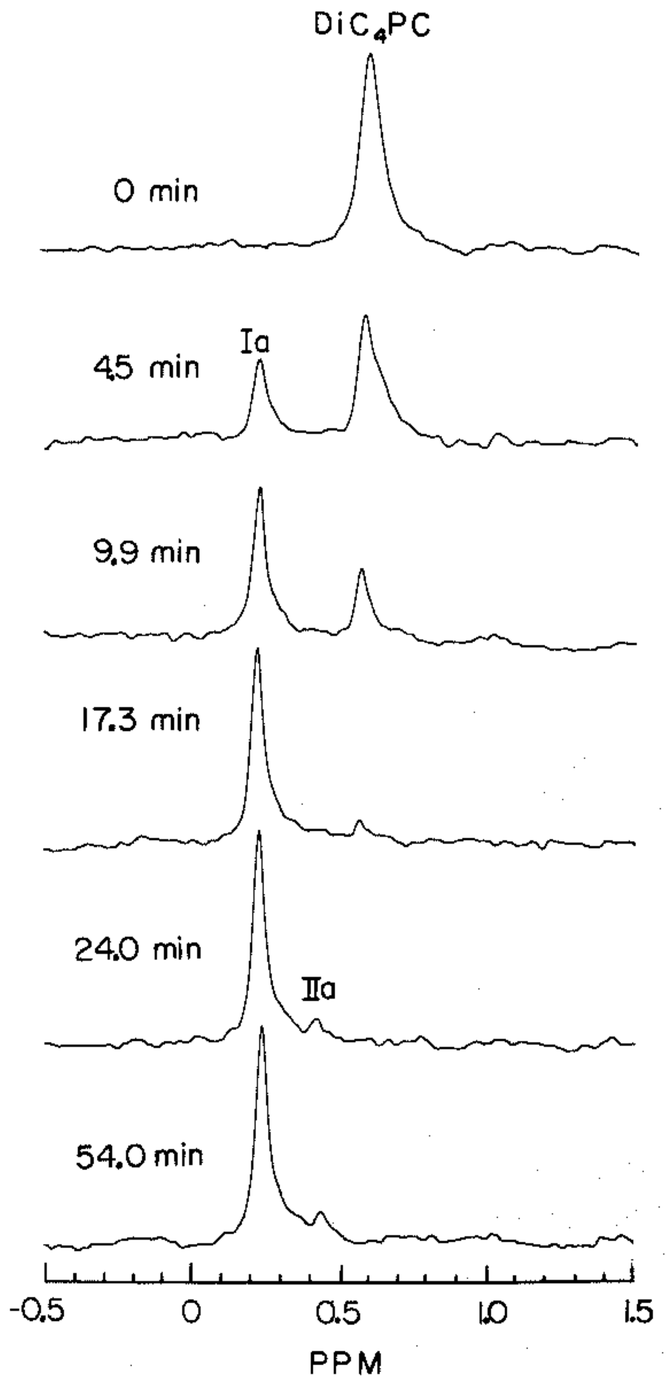

FIGURE 8: Action of phospholipase A_2 on dibutyrylphosphatidylcholine (DiC_4PC). Substrate concentration was 20 mM, and the enzyme concentration was 500 μg/mL. The solution contained 10 mM $CaCl_2$, 20% D_2O, and 50 mM Tris-HCl buffer, pH 7.0. Ia and IIa are the structures shown in Figure 1 where RCOO– is the butyryl group.

of lipase (*Rhizopus arrhizus*) on an identical substrate mixture can be seen. Lipase has been shown to selectively hydrolyze esters of fatty acyl groups with a primary hydroxyl group in phospholipids (de Haas et al., 1965; Slotboom et al., 1970a,b; Fischer et al., 1973). In this study we have also confirmed the structure of the product as IIa by ^1H NMR. Again the reaction was run under conditions such that is was complete before noticeable acyl migration took place.

The specificity of phospholipase A_2 toward the monomeric phospholipid dibutyrylphosphatidylcholine was also examined. As can be seen in Figure 8, initially only one peak is generated, and only after the reaction is complete did a new small resonance between the chemical shifts of dibutyrylphosphatidylcholine and 1-butyryl-*sn*-glycero-3-phosphorylcholine appear at 0.44 ppm. A peak at this position is also generated by the action of lipase on the same compound. Furthermore, both peaks are found if the lysophatidylcholine fraction from a silicic acid column of the products from dibutyrylphosphatidylcholine synthesis is examined. Unfortunately, 2-butyryl-*sn*-glycero-3-phosphorylcholine could not be obtained in pure form, because lipase works on dibutyrylphosphatidylcholine so poorly that even at enzyme concentrations of 5 mg/mL the enzymatic reaction was slower than the acyl migration. Therefore, we could not obtain a quantitative dependence of the migration rate on chain length. It can be estimated qualitatively, however, that the migration is faster for the shorter butyryl moiety, similar to observations in monoglycerides (Serdarevich, 1967).

Discussion

Mechanism of Acyl and Phosphoryl Migration. The data in Figures 4 and 5 show that the acyl migration is a reaction first order in lysophospholipid and base or acid. This is consistent with an intramolecular rearrangement as demonstrated previously for the acyl migration in monoacylglycerides for both the acid-catalyzed (Doerschuk, 1952; Bevan et al., 1953; van Lohuizen & Verkade, 1960) and base-catalyzed cases (Wolfenden et al., 1964). It is probable that this reaction also follows the mechanism proposed originally by Fischer (1920) (Figure 9).

Phosphoryl migration must be very much slower than acyl migration at alkaline pH as none was observed, although our experiments do suggest that some phosphoryl migration does occur at acidic pH. In previous observations of phosphoryl migration of phosphate diesters of ribose only acid-catalyzed, but no base-catalyzed, migration was found (Brown et al., 1956). Experiments on phosphoryl migration and hydrolysis of glycerophosphorylcholine (Baer & Kates, 1948) were interpreted in terms of phosphoryl migration of the phosphorylcholine group catalyzed by both acid and base, but the rearranged unhydrolyzed product was never isolated. More detailed experiments by Serdarevich (1967) suggested that migration does not occur in the unhydrolyzed glycerophosphorylcholine in either acid or base as only rearranged glycerophosphate was observed. With lysophosphatidylcholine in acid, the loss of the choline group appears to be most favorable in analogy to the case with glycerophosphorylcholine,

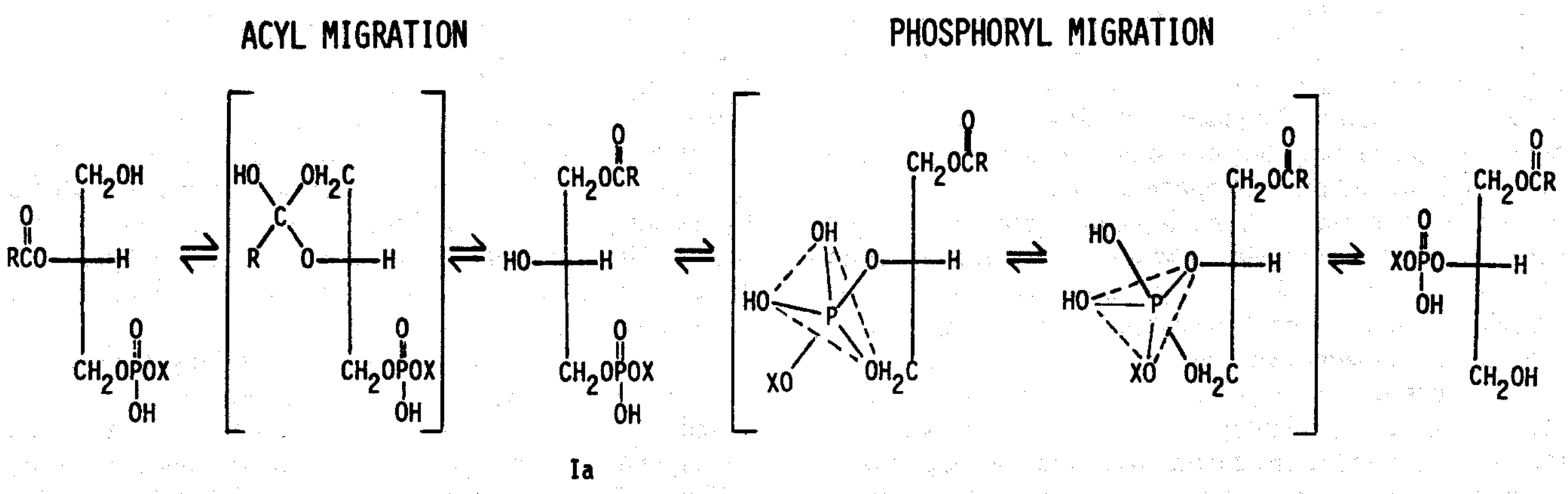

FIGURE 9: Mechanism for the acyl migration and phosphoryl migration in lysophospholipids. For simplicity, the acid-catalyzed reactions with fully protonated intermediates are shown. The phosphoryl migration has to involve pseudorotation of the trigonal bipyramidal intermediate as indicated. Although the OX group is shown in an apical position after initial attack of the hydroxyl group of Ia, 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 remves 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.

but there does appear to be some phosphorylcholine migration. Further work would be required to sort out the rate constants for formation of all of the possible hydrolysis and migration products at acidic pH. When phosphoryl migration has been studied, it has been shown to occur intramolecularly via a cyclic intermediate (Baer & Kate, 1948; Hawthorne & Kemp, 1964). From a mechanistic viewpoint, acid catalysis but not base catalysis would be expected for phosphoryl migration because even if formation of the trigonal bipyramidal intermediate occurs in base, pseudorotation of it (see Figure 9) could be expected to be energetically unfavorable since it would bring an oxyanion from an equatorial to an apical position (Dennis & Westheimer, 1966; Holmes, 1980).

Importance for Phospholipase Specificity and Phospholipid Synthesis Studies. There are principally two methods to determine the specificity of phospholipases, namely, structural determination of the products or the use of specifically labeled substrates. Both methods directly depend on a quantitative knowledge of isomerization rates of lysophospholipids and their equilibria: in the first technique, to assess the validity of the measured isomer distribution in the product and, in the second, to quantitate the migration of the label during substrate synthesis. After the failure of a chemical product determination (Hanahan et al., 1960, and references therein), there have been no further studies to establish phospholipase specificities by product structure determination, although a pure isomer may be identified by ^{1}H NMR as shown here and suggested by Chang & Lo (1975) or by oxidation with O_2 (Thiele, 1968). The complete chemical synthesis of specific mixed acylphospholipids was used by de Haas and van Deenen (de Haas et al., 1960; de Haas & van Deenen, 1961; van Deenen et al., 1963) to establish the specificity of pancreatic and snake venom phospholipase A_2. For differentiation of the residual activity against the second fatty acid, rather pure phospholipids must be used. Since current syntheses of phospholipids start from lysophospholipids generated from naturally derived phospholipids, optimum conditions for synthesis were studied.

The stability of lysophosphatidylcholine against acyl migration is highest between pH 4 and 5. Both acid and base catalyze the acyl migration. In order to obtain very pure isomers, high concentrations of enzyme should be used to generate the lysophosphatidylcholines before significant isomerization can take place, and an optimum pH should be chosen accordingly. We have found in ^{31}P NMR a simple and rapid method to check the purity of the resulting lyso compound directly. If the specificity of phospholipases is investigated, again care should be taken to run the reaction under conditions so that the migration is much slower than the enzymatic hydrolysis. If radiolabeled phospholipids are employed, the fact that lysophospholipids are also substrates of phospholipases, however, much poorer (Slotboom et al., 1963), must be taken into account. Therefore an absolutely specific enzyme can appear to liberate the "wrong" fatty acids by two mechanisms: (i) The substrate was not absolutely specifically labeled, or (ii) migration of the acyl group of the lysophospholipid may give rise to a new substrate at very long reaction times or at high pH. However, both difficulties are overcome by the use of ^{31}P NMR to determine the specificity. A disadvantage of the method is, however, that fairly high enzyme concentrations have to be used.

The positional specificity of lysophospholipases or of phospholipases A_1 and A_2 working on lysophospholipids is a rather difficult problem, because in the absence of direct observation of the isomerization and product formation, it cannot be distinguished whether the hydrolysis reaction goes exclusively via one isomer and rearrangement or via both isomers (van den Bosch et al., 1968). When ^{31}P NMR is used, the occurrence of the other isomer can be directly observed, provided its concentration is not too low.

Previous ^{1}H NMR experiments in this laboratory have shown that there are significant differences in the conformation of the *sn*-1 and *sn*-2 acyl chains in phospholipids, both in micelles (Roberts et al., 1978) and vesicles (De Bony & Dennis, 1981), however, not necessarily in monomers (Roberts et al., 1978; Burns & Roberts, 1980). The arrangement in the interface is such that the α-CH_2 group of the *sn*-2 chain and the carbonyl group are oriented at the interface and the α-CH_2 group and carbonyl group of the *sn*-1 chain are somewhat less exposed. For determination of whether this affects the specificity of the enzyme, the ^{31}P NMR technique was used with the monomeric substrate dibutyrylphosphatidylcholine. The result obtained showing that only the *sn*-2 fatty acid chain was hydrolyzed demonstrates that the specificity for this chain is not due to the particular conformation preferred in aggregated lipids.

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