Mihara, K., & Sato, R. (1978) *Methods Enzymol. 52*, 102–108.
Miller, L. R., Pinkerton, F. R., & Schroepfer, G. J., Jr. (1980) *Biochem. Int. 1*, 223–228.
Munkres, K. O., & Richards, R. M. (1965) *Arch. Biochem. Biophys. 109*, 466–479.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.
Oshino, N., Imai, Y., & Sato, R. (1966) *Biochim. Biophys. Acta 128*, 13–28.
Prasal, M. R., & Joshi, V. C. (1979) *J. Biol. Chem. 254*, 6362–6369.
Rosenstraus, M., & Chasin, L. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 493–497.
Ryan, J., Hardeman, E. C., Endo, A., & Simoni, R. D. (1981) *J. Biol. Chem. 256*, 6762–6768.
Schneider, W. J., Goldstein, J. L., & Brown, M. S. (1980) *J. Biol. Chem. 255*, 11442–11447.
Sinensky, M., Torget, R., & Edwards, P. A. (1981) *J. Biol. Chem. 256*, 11774–11779.
Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B., & Redline, R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4565–4569.
Takeda, Y., Suzuki, F., & Inoue, H. (1969) *Methods Enzymol. 13*, 153–160.
Van Harken, D. R., Dixon, C. W., & Heimberg, M. (1969) *J. Biol. Chem. 244*, 2278–2285.
Wattenberg, B. W., Freter, C. E., & Silbert, D. F. (1979) *J. Biol. Chem. 254*, 12295–12298.

Synthesis of the Diastereoisomers of 1,2-Dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine and Their Stereospecific Hydrolysis by Phospholipases A_2 and C†

George A. Orr,* Curtis F. Brewer, and Gayle Heney

ABSTRACT: A convenient three-step synthesis of the phosphorothioate analogue of phosphatidylethanolamine is described. The reaction pathway involves the conversion of a 1,2-diacyl-*sn*-glycerol to its corresponding thiophosphoric acid dichloride by using $PSCl_3$ in the presence of a tertiary base. Treatment of the dichloride with ethanolamine results in the formation of a cyclic thiophosphoramidate which, upon acidification, undergoes P–N cleavage, giving rise to 1,2-diacyl-*sn*-glycero-3-thiophosphorylethanolamine. ^{31}P NMR reveals that both diastereoisomers are present in equivalent amounts. It is not possible, however, to separate the two isomers by high-pressure liquid chromatography. ^{31}P NMR and high-pressure liquid chromatography are used to show that phospholipases A_2 and C exhibit absolute and opposite stereoselectivity in the hydrolysis of the pair of diastereoisomers.

Phosphorothioate analogues of nucleotides have proved to be invaluable tools for probing the mechanistic basis of enzyme-catalyzed adenyl- and phosphoryl-transfer reactions and also the role that nucleotides perform in complex biochemical processes (Eckstein, 1975, 1979; Yount, 1975). This suggested to us that phosphorothioate analogues of phospholipids may make important contributions toward our understanding of phospholipid metabolism and of the role that phospholipids play in membrane and cellular function. The consequences of replacing one of the nonbridge oxygens in the phosphodiester linkage of a phospholipid by a sulfur atom are 2-fold. First, the phospholipids will exist as pairs of diastereoisomers due to the chiral phosphorus atom, allowing one to probe the stereoselectivity of specific phospholipases for diastereoisomeric pairs of the thiophospholipids. In certain cases, e.g., phospholipases C and D, it may also enable one to determine the stereochemical outcome of the hydrolysis reaction. Second, it may be expected that the sulfur substitution will make the phospholipid analogues more resistant to enzymatic hydrolysis by phospholipases. Since methods are available for incorporating phospholipids into biological membranes by using either phospholipid exchange proteins (Wirtz, 1974; Zilversmit & Hughes, 1976) or liposomes (Papahadjopoulos et al., 1979), the possible increased stability of the thiophospholipids makes them a potential probe for looking at the role of phospholipid turnover to membrane function.

This paper describes a facile three-step synthesis (without purification of intermediates) of the phosphorothioate analogue of phosphatidylethanolamine.[1] Evidence is presented which shows that phospholipases A_2, which selectively removed the fatty acyl group at C_2, and C, which splits off the complete polar head group, show absolute[2] and, moreover, opposite preferences for one of the diastereoisomers of thiophosphatidylethanolamine.

Experimental Procedures

Materials

Phospholipase A_2 (bee venom, 1200 units/mg), phospholipase C (*Bacillus cereus*, 500 units/mg), 1,2-dipalmitoyl-*sn*-glycerol, and phosphatidylethanolamine were purchased from Sigma. $PSCl_3$ (Alfa) and ethanolamine (Fisher) were distilled before use.

† From the Departments of Molecular Pharmacology (G.A.O., C.F.B., and G.H.) and Microbiology and Immunology (C.F.B.), Albert Einstein College of Medicine, Bronx, New York 10461. *Received January 4, 1982.* This investigation was supported in part by Grants GM28170 and GM28751 from the National Institutes of Health.

[1] Abbreviations: HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; phosphatidylethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine; thiophosphatidylethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[2] Under our assay conditions, once the hydrolysis of the susceptible isomer has finished, there is no observable change in the HPLC profile over a period of 24 h. It is possible, however, that the "so-called" resistant diastereoisomer is being hydrolyzed at a rate undetectable by HPLC.

0006-2960/82/0421-3202$01.25/0

Methods

Thin-layer chromatography (TLC) was carried out on silica gel plates (EM Laboratories), and the solvents used were (A) chloroform/methanol/7 N NH_4OH, 60:35:5 (v/v), and (B) 2-propanol/H_2O/concentrated aqueous NH_4OH, 70:25:5 (v/v). I_2 vapor was used as a general detection method; thiophosphates were visualized by a phosphate spray (Hanes & Isherwood, 1979) (thiophosphates turn blue on heating in contrast to phosphate esters which require a combination of heat and UV light); amino-containing compounds were visualized by ninhydrin.

High-pressure liquid chromatography (HPLC) was performed on a Beckman Model 332 instrument attached to an Altex 165 variable wavelength detector. Thiophosphatidylethanolamine and its lyso derivative were separated on an Ultrasil-NH_2 column (10 μm, 250 × 4 mm). The elution solvent was acetonitrile/methanol/H_2O (66.5:28.5:5 v/v). The rate of elution was 1 mL/min, and the phospholipids were monitored at 206 nm.

^{31}P NMR spectra were recorded at ambient temperature with a JEOL PFT-100 spectrometer operating at 40.8 MHz in the pulsed Fourier-transform mode and equipped with a Nicolet 1080 digital computer for signal accumulation and Fourier transformation. All samples were prepared in $CDCl_3$ in 10-mm tubes. ^{31}P chemical shifts were determined with respect to 85% H_3PO_4 in a coaxial tube.

Synthesis of 1,2-Dipalmitoyl-sn-glycero-3-thiophosphorylethanolamine. 1,2-Dipalmitoyl-*sn*-glycerol (1.5 g, 2.64 mmol) dissolved in trichloroethylene (25 mL) was added dropwise over a period of 30 min to a stirred solution of $PSCl_3$ (0.67 g, 3.95 mmol) and pyridine (0.31 g, 3.95 mmol) in trichloroethylene (10 mL) at 4 °C. After an additional 30 min at this temperature, the reaction was left stirring at room temperature for 4 h. The solution was filtered to remove pyridinium chloride and the solvent removed in vacuo. The oily residue was dissolved in toluene (5 mL) and reevaporated. This procedure was repeated twice. The crude thiophosphoryl dichloride dissolved in tetrahydrofuran (25 mL) was treated at 4 °C with a solution of ethanolamine (0.18 g, 3 mmol) and pyridine (0.95 g, 12 mmol) in tetrahydrofuran (25 mL) added over a period of 30 min. The reaction was left stirring at room temperature for 4 h. The solution was filtered and the organic solvent removed in vacuo. The residue was dissolved in 2-propanol/tetrahydrofuran (50 mL; 1:1 v/v) and treated with 20% acetic acid in H_2O (50 mL). The reaction was left stirring at room temperature for 4 h. The product, which precipitated on standing, was collected by filtration and purified by silica gel chromatography, eluting with solvent A (yield 45%, mp 89–90 °C). This product was pure as judged by (a) HPLC on Ultrasil-NH_2 (elution time = 11 min) and by (b) TLC (silica gel) eluting with solvent A (R_f 0.70). The material was ninhydrin positive. Anal. Calcd for $C_{37}H_{74}NO_7PS$: C, 62.76; H, 10.53; N, 1.98; P, 4.37; S, 4.53. Found: C, 62.61; H, 10.43; N, 1.93; P, 4.33; S, 4.63.

Results

The reaction pathway for the synthesis of thiophosphatidylethanolamine is shown in Figure 1. It is a modification of the procedure described by Eibl (1978) for the synthesis of glycerophospholipids. The method involves the conversion of 1,2-diacyl-*sn*-glycerol to its corresponding thiophosphoric acid dichloride by treatment with an excess of $PSCl_3$ in the presence of a tertiary base, e.g., pyridine. Addition of ethanolamine to the crude dichloride, after removal of the excess $PSCl_3$, results in the formation of the cyclic oxaazaphospholidine-2-thione intermediate. Subsequent

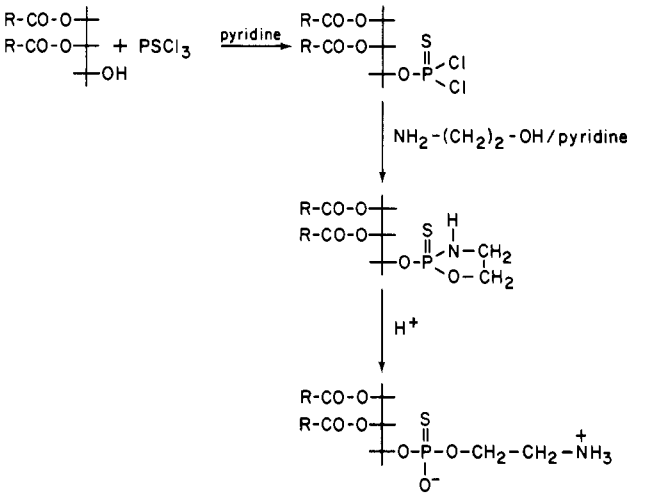

FIGURE 1: Reaction scheme for the synthesis of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine (R = dipalmitoyl).

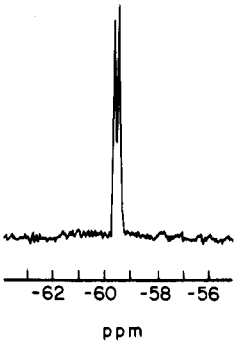

FIGURE 2: Proton-decoupled ^{31}P NMR spectrum of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine. (See Experimental Procedures for details.)

treatment of this cyclic phosphoramidothioate with 10% acetic acid at room temperature results in opening of the ring system by P–N cleavage, giving rise to the desired 1,2-dipalmitoyl-*sn*-glyero-3-thiophosphorylethanolamine. It has previously been observed that cyclic phosphoramidothioates in the presence of acid undergo P–N rather than P–O cleavage (Cooper et al., 1977; Hall & Inch, 1979).

The two expected diastereoisomers of thiophosphatidylethanolamine can be distinguished by proton-decoupled ^{31}P NMR (Figure 2). The two signals occur at −59.61 and −59.47 ppm. Such large downfield chemical shifts from phosphoric acid are characteristic of phosphorothioate mono- and diesters (Eckstein, 1979). We have been unable to resolve the two diastereoisomers by HPLC on Ultrasil-NH_2 or on Ultrasphere-Si (data not shown). On Ultrasil-NH_2, thiophosphatidylethanolamine elutes as a single, symmetrical peak, with a retention time of 11 min (Figures 3A and 4A). The peak eluting at 4 min is chloroform. Mild alkaline hydrolysis of thiophosphatidylethanolamine gave rise to a water-soluble thiophosphate- and amino-containing compound, *sn*-glycero-3-thiophosphorylethanolamine, which had an R_f of 0.42 on silica gel eluting with solvent B.

It was possible to follow the phospholipase A_2 and C catalyzed hydrolysis of the thiophosphate analogue by HPLC. Upon the addition of phospholipase A_2, the disappearance of the thiophosphatidylethanolamine peak was observed with the concomitant increase in a peak eluting after 21 min (Figure 3B–F). Mild alkaline hydrolysis of the material in the new peak gave the same product as thiophosphatidylethanolamine, when analyzed by TLC (silica gel, solvent B). This new peak

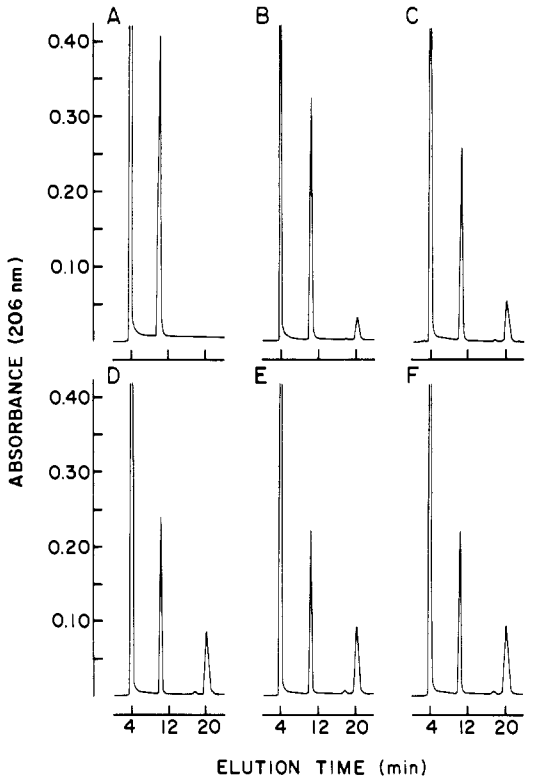

FIGURE 3: HPLC of phospholipase A_2 digestion of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine. Lipid (2 mg) dissolved in $CHCl_3$ (2 mL) was added to 50 mM Tris-HCl, pH 8.4, containing 40 mM $CaCl_2$ (1.5 mL). The reaction was initiated by the addition of phospholipase A_2 (20 units) and the solution vortexed continuously at room temperature. Samples were removed at time intervals and analyzed by HPLC as described under Experimental Procedures: (A) 0, (B) 60, (C) 120, (D) 210, (E) 270, and (F) 360 min. Additional phospholipase (20 units) was added after 120 and 270 min. The peak eluting at 4 min in all the chromatograms is $CHCl_3$.

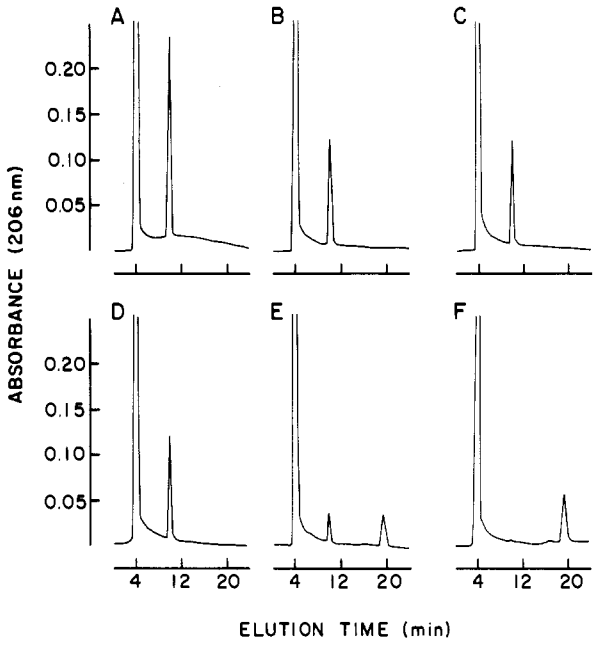

FIGURE 4: HPLC of phospholipase C digestion of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine. Lipid (2 mg) dissolved in $CHCl_3$ (2 mL) was added to 50 mM Tris-HCl, pH 7.9, containing 2 μM $ZnCl_2$ (1.5 mL). The reaction was initiated by the addition of phospholipase C (50 units) and the solution vortexed continuously at room temperature. Samples of the chloroform layer were removed at time intervals and analyzed by HPLC as described under Experimental Procedures: (A) 0, (B) 30, (C) 90, and (D) 150 min. Additional phospholipase C (50 units) was added after 90 min. After 150 min, the aqueous layer was removed and the chloroform layer washed several times with 50 mM Tris-HCl, pH 8.4, containing 40 mM $CaCl_2$. For initiation of the phospholipase A digestion, 1.5 mL of this buffer containing 20 units of enzyme was added to the washed $CHCl_3$ solution: (E) 10 min after initiation of A_2 digestion; (F) 60 min after initiation of A_2 digestion. The peak eluting after 4 min in all the chromatograms is $CHCl_3$.

is presumed to be the 2-lyso derivative of the thiophosphate analogue. It was observed that the enzyme-catalyzed hydrolysis appeared to proceed to approximately 50% (Figure 3). The extent of reaction was not changed if incubation was carried out for 24 h (data not shown) or if additional enzyme was added at various time intervals (Figure 3). The fact that authentic phosphatidylethanolamine, if added to the apparently complete thiophosphatidylethanolamine reaction, was still hydrolyzed to its lyso derivative (as judged by TLC) indicated that phospholipase A_2 was not inactivated by the thiophosphate analogue. If the hydrolysis reaction of the thiophosphate analogue was followed by TLC, the appearance of a new amino- and thiophosphate-containing material (2-lysothiophosphatidylethanolamine) was seen with an R_f of 0.48 (silica gel, solvent A) while thiophosphatidylethanolamine ran with an R_f of 0.70.

Similar results were observed with phospholipase C (Figure 4A–D) in that (a) enzymic hydrolysis proceeded to approximately 50%, (b) the addition of extra enzyme did not drive the reaction to completion, and (c) the incubation mixture was still capable of hydrolyzing phosphatidylethanolamine after thiophosphatidylethanolamine hydrolysis appeared finished. The water-soluble product of the phospholipase C digestion, presumably thiophosphorylethanolamine, ran as a single spot on TLC (silica gel, solvent B, R_f 0.09) and contained both amino and thiophosphate groups. Since it was possible that the incomplete hydrolysis of thiophosphatidylethanolamine by both phospholipase A_2 and phospholipase C was due to the fact that the enzymes were handling only one of the diastereoisomers, it was important to establish whether each enzyme was hydrolyzing the same or the opposite isomer. As can be seen from Figure 4E,F, the addition of phospholipase A_2 to the complete phospholipase C digestion caused the remaining thiophosphatidylethanolamine to be hydrolyzed to its lyso derivative. This observation is strong evidence that phospholipases A_2 and C are each hydrolyzing the opposite diastereoisomer.

^{31}P NMR was also used to investigate the stereoselectivity of both phospholipases for the two diastereoisomers. In these studies, the hydrolyses were carried out on a semipreparative scale and the chloroform-soluble products analyzed by ^{31}P NMR. No attempt was made to purify the reaction products. After the phospholipase A_2 digestion had gone to completion, as judged by HPLC, two ^{31}P signals were observed (Figure 5A). However, the difference in shift position between the two signals was 17.1 Hz rather than the 5.5-Hz difference observed in the original material (Figure 2). Addition of thiophosphatidylethanolamine to the NMR sample caused the reappearance of the signal from the hydrolyzed diastereoisomer at a position intermediate between that of the resistant isomer and the lyso derivative of the susceptible diastereoisomer (Figure 5B). It would appear, therefore, that the phospholipase A_2 resistant diastereisomer is the one with the furthest downfield chemical shift from 85% phosphoric acid.

Figure 6A shows the spectrum of the chloroform-soluble product of the phospholipase C digestion. Only one signal was observed as would be expected if one of the diastereoisomers was resistant to hydrolysis by this enzyme. Since thio-

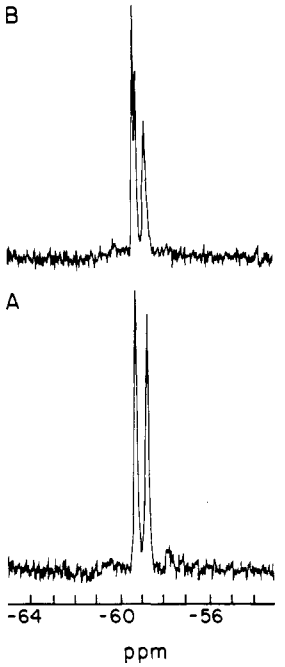

FIGURE 5: ^{31}P NMR spectra of phospholipase A_2 digested 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine. Thiophospholipid (20 mg) was treated with phospholipase A_2 as described in Figure 3 until HPLC indicated that hydrolysis had stopped. The aqueous layer was removed and the chloroform solution extracted with 10 mM EDTA (2 × 5 mL) and H_2O (2 × 5 mL). The chloroform layer was evaporated and the residue dissolved in $CDCl_3$ (1.5 mL). NMR spectra were obtained as described under Experimental Procedures: (A) $CDCl_3$-soluble fraction after phospholipase A_2 teatment; (B) $CDCl_3$-soluble fraction after phospholipase A_2 treatment plus 12.5 mg of thiophosphatidylethanolamine.

phosphorylethanolamine, one of the products of the phospholipase C digestion, is water soluble, a signal due to this component was not observed. The addition of thiophosphatidylethanolamine to the NMR sample caused the appearance of a second ^{31}P signal (Figure 6B). In this case, it was not possible to determine unambiguously which diastereoisomer was hydrolyzed, since we found that the chemical shifts were sensitive to solvent composition and to concentration of the added thiophosphatidylethanolamine. However, in view of the HPLC and the above phospholipase A_2 NMR results, it would appear that the signal nearest to 85% phosphoric acid corresponds to the phospholipase C resistant isomer.

Discussion

This paper describes, to the best of our knowledge, the first synthesis of a phosphorothioate analogue of a phospholipid. The reaction procedure, based on the method devised by Eibl (1978) for the synthesis of glycerophospholipids, is such that it will be feasible to synthesize thiophospholipids with a variety of polar head-group substituents. The key intermediate in this scheme is the diacylglycerolthiophosphoric acid dichloride which can react with a variety of vicinal amino alcohols (ethanolamine, *N*-methylethanolamine, carboxyl-protected serines) or diols, yielding the corresponding cyclic intermediate. Mild acid hydrolysis affords the appropriate thiophospholipid. Moreover, recent work in this laboratory has shown that a 1,2-diacyl-*sn*-glycero-3-thiophosphoric acid bromoethyl ester can be prepared by treatment of the cyclic intermediate derived from ethylene glycol with sodium bromide (M. L. Brown and G. A. Orr, unpublished experiments). Direct amination of this bromoethyl ester with trimethylamine (Diembeck & Eibl, 1979) will yield the thiophosphate analogue of phosphatidylcholine.

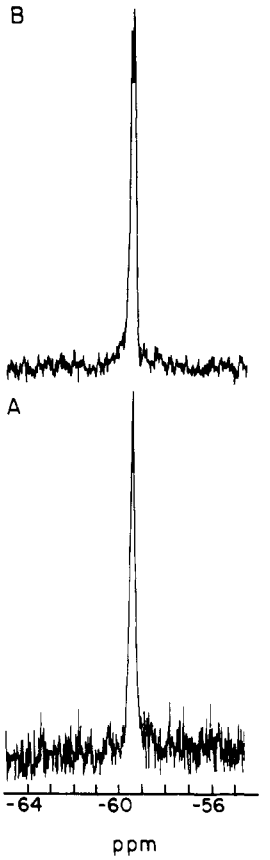

FIGURE 6: ^{31}P NMR spectra of phospholipase C digested 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine. Thiophospholipid (15 mg) was treated with phospholipase C as described in Figure 4, until HPLC indicated that the hydrolysis reaction had stopped. The $CDCl_3$-soluble material of the reaction was obtained as described in Figure 5, and the NMR spectra were obtained as described under Experimental Procedures: (A) $CDCl_3$-soluble fraction after phospholipase C treatment; (B) $CDCl_3$-soluble fraction after phospholipase C treatment plus 20 mg of thiophosphatidylethanolamine.

As expected, sulfur substitution generates the diastereoisomeric pair of thiophosphatidylethanolamines which can be resolved by ^{31}P NMR. We have been unable to separate the diastereoisomers by chromatographic techniques; nevertheless, it is possible to effect their separation by enzymatic digestion. Under our assay conditions, both phospholipases A_2 and C show absolute preference for a single, and opposite, diastereoisomer, leaving the other intact. Phospholipase A_2 digestion would appear to be the more efficient method for separating the diastereoisomers since the lyso derivative can be isolated by chromatographic techniques and subsequently reacylated. The absolute configuration at phosphorus of these diastereoisomers is presently unknown. This will be most readily obtained by X-ray structural analysis of the deacylated derivative, i.e., glycerylthiophosphorylethanolamine.

Previous studies have shown that enzymatic preference for a specific diastereoisomer of a phosphorothioate diester is not an uncommon occurrence (Eckstein, 1979). For example, hexokinase (Stahl et al., 1974) and nucleoside diphosphokinase (Eckstein & Goody, 1976) utilize only one of the diastereoisomers of adenosine 5′-*O*-(1-thiotriphosphate) as a substrate while snake venom phosphodiesterase hydrolyzes one of the diastereoisomers of a dinucleoside monophosphorothioate (5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate) approximately 3 orders of magnitude faster than the other isomer (Burgers & Eckstein, 1979). In this study, the preference of phospholipase A_2 for a single diastereoisomer of thiophosphatidylethanolamine is unusual in that the susceptible

ester linkage is distant from the chiral phosphorus atom.

The stereoselectivity by both phospholipases A_2 and C could be accounted for in at least two ways. First, sulfur substitution could prevent binding of the resistant diastereoisomer to the enzyme. Second, both diastereoisomers could bind equally well to the enzyme, but the sulfur substitution, in the resistant diastereoisomer, causes misalignment of the susceptible bond at the active site so that hydrolysis cannot occur. It is expected that kinetic analysis of the binding of each diastereoisomer to the phospholipases may clarify this issue.

Since it is becoming increasing clear that phospholipids, as well as providing the structural matrix for the cell, play a dynamic role in the activity of various membrane-bound enzyme and transport activities (Fourcans & Jain, 1974; Sanderman, 1978), the possible increased stability of the phosphorothioate analogues of phospholipids will be of potential interest in those situations where turnover of phospholipid is important for cellular function. Currently, we are developing two methods for the incorporation of the thiophosphate analogues into biological membranes. In *Escherichia coli*, we are making use of the specific transport system for glycerol phosphate, the precursor of all glycerophospholipids in this cell, to effect entry of *sn*-glycero-3-phosphorothioate (GSP) into the organism and eventually into phospholipid (Hammelburger et al., 1980). Studies so far reveal that $^3H/^{35}S$-labeled GSP can be taken up by *E. coli* and incorporated into chloroform-extractable material. Interestingly, GSP is bacteriocidal to *E. coli* cells with a functioning glycerol phosphate transport system at concentrations approximating its K_m for uptake. A second method for incorporation into membranes will make use of the chemically synthesized thiophospholipids and phospholipid exchange proteins (Wirtz, 1974; Zilversmit & Hughes, 1976) or liposomes (Papahadjopoulos et al., 1979) to selectively incorporate the analogues into the lipid bilayer.

Added in Proof

A nonstereospecific chemical synthesis of the phosphorothioate analogue of phosphatidylcholine has been reported (Vasilenko et al., 1982). The material has been used in a ^{31}P NMR study of membrane lipid structure. The authors appear to be unaware that this compound may exist as a pair of diastereoisomers, making any interpretation of their data ambiguous.

References

Burgers, P. J. M., & Eckstein, F. (1979) *Biochemistry 18*, 592–596.

Cooper, D. B., Hall, C. R., Harrison, J. M., & Inch, T. D. (1977) *J. Chem. Soc., Perkin Trans. 1*, 1969–1980.

Diembeck, W., & Eibl, H. (1979) *Chem. Phys. Lipids 24*, 237–244.

Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160–166.

Eckstein, F. (1979) *Acc. Chem. Res. 12*, 204–210.

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.

Eibl, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4074–4077.

Fourcans, B., & Jain, M. K. (1974) *Adv. Lipid Res. 12*, 147–226.

Hall, C. R., & Inch, T. D. (1979) *J. Chem. Soc., Perkin Trans. 1*, 1646–1655.

Hammelburger, J., Heney, G., & Orr, G. A. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1982.

Hanes, C. S., & Isherwood, F. A. (1949) *Nature (London) 164*, 1107–1112.

Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–121.

Sanderman, H., Jr. (1978) *Biochim. Biophys. Acta 515*, 209–237.

Stahl, K. W., Schlimme, E., & Eckstein, F. (1974) *FEBS Lett. 40*, 241–246.

Vasilenko, I., De Kruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 685*, 144–152.

Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta 344*, 95–117.

Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 1–56.

Zilversmit, D. B., & Hughes, M. E. (1976) *Methods Membr. Biol. 7*, 211–259.