Phosphorylation of 1-Alkenyl-2-acylglycerol and Preparation of 2-Acylphosphoglycerides*

Hansjörg Eibl† and William E. M. Lands

ABSTRACT: 1-(1′-Alk-1′-enyl)-2-acylglycerol 3-phosphate was prepared by phosphorylation of the alkenylacylglycerol. Relatively pure (99%) 2-acyl isomers of glycerol phosphate, glycerylphosphorylcholine, and glycerylphosphorylethanolamine were prepared in yields of 80–100% based on the initial alkenyl precursor.

Separation of the monoacyl from the diacyl contaminants can be achieved by solvent partition, eliminating the need for pure starting materials or chromatographic resolution of the products. The 2-acyl isomers could be stored for a short time in some solvents without appreciable acyl migration to the 1 position.

Studies of glycerolipid metabolism sometimes require samples of 2-acylphosphoglycerides that are not contaminated with the other, 1-acyl, positional isomer. The existing synthetic procedures require a somewhat involved series of reactions and the blocking groups for the primary hydroxyl are not quantitatively removed under mild conditions. Previous experience with alkenylglycerol derivatives led us to use those naturally occurring glycerolipids as convenient precursors. Early work on the cleavage of the alkenyl group with mercuric acetate or iodine and subsequent purification on silicic acid columns produced a mixture of the two positional isomers (Lands and Merkl, 1963). However, when cleavage with iodine was used without subsequent chromatography, Robertson and Lands (1962) successfully obtained the correct positional isomers, which were then used as intermediates in preparing stereospecifically labeled lecithins. Later, buffered iodine and cold acetone extractions were used to get more consistently pure material (Lands, 1965). Nevertheless, yields were not quantitative and variable amounts of diacyl contaminants produced turbidities during spectrophotometric studies. The need for purer alkenylacyl preparations of the various classes of glycerolipid became increasingly apparent. Attempts to prepare alkenylacylglycerol phosphate with the aid of cabbage phospholipase D did not give the desired product, but did enrich the alkenyl content of the remaining material (Lands and Hart, 1965). Thus, relatively useful preparations of 2-acylglycero-3-phosphorylcholine and 2-acylglycero-3-phosphorylethanolamine have been available for metabolic studies, but not 2-acylglycero-3-phosphate.

Two remaining problems were examined and resolved by the present studies: (1) preparation of relatively pure 2-acylglycerol 3-phosphate and (2) quantitative and reproducible preparation of the 2-acyl derivatives free from diacyl and 1-acyl contaminants.

Materials and Methods

Choline and ethanolamine phosphoglycerides were isolated from beef heart phospholipids and treated with phospholipase D (EC 3.1.4.4) as described earlier (Lands and Hart, 1965) to produce 1-alkenyl-2-acyl-GPC[1] and 1-alkenyl-2-acyl-GPE, respectively. The products still contained some diacyl analogs as indicated by the molar ratios of phosphorus:acyl:alkenyl of 1.0:1.2:0.8 for 1-alkenyl-2-acyl-GPC and 1.0:1.25:0.75 in the case of 1-alkenyl-2-acyl-GPE. 1-Acyl-GPC and 1-acyl-GPE were prepared by selective hydrolysis of diacyl-GPC and diacyl-GPE with phospholipase A_2 (EC 3.1.1.4). Diacyl-GP was formed by the treatment of diacyl-GPC with phospholipase D (EC 3.1.4.4), and 1-acyl-GP was prepared from 1-acyl-GPC using phospholipase D in a variation of the method described by Long *et al.* (1967).

Phospholipase C (EC 3.1.4.3) from *Bacillus cereus* was purchased from Grain Processing Corp. (Muscatine, Iowa) and phospholipase D from Calbiochem. Triglyceride lipase (EC 3.1.1.3) was prepared according to Lands *et al.* (1966). Because the enzymic reagents were used in amounts suitable for quantitative preparative work rather than kinetic analysis, the amounts are expressed in milligrams rather than catalytic units (which vary considerably with reaction conditions).

All solvents used were reagent grade. The composition of all solvent mixtures is presented as volume to volume. The petroleum ether had a boiling point range of 30–60°. All solvents were evaporated under vacuum and in an atmosphere of nitrogen.

Fatty acids were analyzed by quantitative gas chromatography. Phospholipid (0.5–1 μmole) and 0.25 μmole of methyl pentadecanoate as internal standard were treated 5 min with 2 ml of 0.5 N sodium methoxide in methanol to form the methyl esters. Petroleum ether (5 ml; bp 30–60°) was then added, followed by mixing and centrifugation. The upper layer was taken and dried 10 min over solid sodium sulfate–

* From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104. *Received October 7, 1969.*

† Supported in part by a research grant from the U. S. Public Health Service (AM05310) and by a fellowship from the Max Planck Gesellschaft.

[1] The nomenclature is that described in the IUB–IUPAC report (*Biochemistry 6*, 3287 (1967)). The following abbreviations will be used: GP, glycerol 3-phosphate; GPC, glycerol-3-phosphorylcholine; GPE, glycerol-3-phosphorylethanolamine.

sodium bicarbonate (2:1, w/w). The solution was decanted and evaporated in a stream of nitrogen. The residue was dissolved in carbon disulfide and filtered through glass wool. After evaporation with nitrogen, the residue was taken in 25 μl of carbon disulfide and about 5 μl was used for analysis as described by Brandt and Lands (1968).

Phosphorus was determined using the turbidimetric method of Eibl and Lands (1969a). Alkenyl ether content was assayed by measuring the uptake of iodine using the spectrophotometric method of Gottfried and Rapport (1962). Periodate analysis of diols was performed by determining the formation of iodate with the spectrophotometric method of Eibl and Lands (1969b).

Thin-layer chromatography plates were prepared with the thin-layer chromatographic apparatus described by Stahl (1965). Five large plates (25 × 25 cm), 0.37 mm thick, were spread using the following materials and solvents. Silica gel G (40 g, Merck) was shaken 1 min with 90 ml of distilled water and then added to the plates. After 30-min drying in the air the plates were activated at 125° for 2 hr, stored in a closed tank, and used within 1 week.

In a similar way silica gel G plates containing boric acid (Thomas *et al.*, 1965) were prepared by shaking 40 g of silica gel G and 85 ml of 0.5 M boric acid for 1 min and adding to the plates. Thin-layer chromatographic plates of silicic acid (Mallinckrodt reagent, <200 mesh) were prepared by shaking 40 g in a mixture of 68 ml of distilled water and 2 ml of methanol. Activation and storage of the plates was as described above. Microslides, prepared as described by Peifer (1963), were used for a fast check of the fractions when column chromatography was used for separations. The compounds were detected by spraying with molybdate (Dittmer and Lester, 1964) or sulfuric acid.

Experimental Procedures and Results

Preparation of 1-Alkenyl-2-acylglycerol. Crude 1-alkenyl-2-acyl-GPC (900 μmoles; molar ratio of phosphorus:acyl:alkenyl = 1.0:1.2:0.8, indicating the presence of some diacyl-GPC) was dissolved in 60 ml of ether. Then 60 ml of 0.05 M Tris-maleate buffer containing 120 mg of phospholipase C and 120 μmoles of calcium chloride with the pH adjusted to 7.1 were added to the etheral solution. The reaction was followed by thin-layer chromatography and was stopped after 2-hr stirring at room temperature, when the characteristic spot of 1-alkenyl-2-acyl-GPC (R_F 0.4 in chloroform–methanol–water (65:30:4) with silica gel G) had completely disappeared. The reaction mixture was separated into two phases by centrifugation and the ether phase was collected. The water phase was treated two more times with 60 ml of ether and the combined ether extracts were evaporated to dryness.

The residue, a mixture of 1-alkenyl-2-acyl- and 1,2-diacylglycerol, was treated with a solution of 6 ml of 1 M Tris-chloride buffer (pH 7.4), 9 ml of 1 M sodium chloride, and 25 mg of triglyceride lipase in 5 ml of distilled water. The pH was adjusted to 7.1 to minimize migration of the acyl group from the 2 to 3 position. After 1-hr vigorous stirring at room temperature, the reaction was stopped by the addition of 40 ml of chloroform–methanol (1:1). Mixing and centrifuging resulted in two phases. The lower phase was collected. The water phase was treated two more times with 20-ml portions of chloroform and the combined chloroform extracts were taken to dryness.

The residue was dissolved in benzene and chromatographed on a 1-cm diameter column containing 6 g of SilicAR (Mallinckrodt, 100–200 mesh). After washing the column with 20 ml of benzene to remove the free fatty acids, 1-alkenyl-2-acylglycerol was eluted with benzene–acetone (50:1). The purity of the eluates was checked by thin-layer chromatography on silica gel G using a solvent of benzeze–acetone (50:1). 1-Alkenyl-2-acylglycerol had an R_F value of 0.5, whereas R_F values of 0.9 and 0.1, respectively, were measured for fatty acid and monoacylglycerol. The yield of chromatographically pure product with a molar ratio of acyl:alkenyl of 1.0:0.98 was 340 μmoles (54%).

Phosphorylation of 1-Alkenyl-2-acylglycerol. 1-Alkenyl-2-acylglycerol (340 μmoles) was dissolved in 2.5 ml of benzene and added dropwise to a thoroughly stirred, ice-cold mixture of 8 ml of dry chloroform (just before use, the chloroform was washed with 0.1 M sodium hydrogen carbonate, refluxed for 1 hr over phosphorpentoxide, and then distilled), 9.5 ml (66 mmoles) of triethylamine (refluxed for 1 hr over potassium hydroxide and distilled) and 2 ml (22 mmoles) of freshly distilled phosphorus oxychloride. Stirring of the reaction mixture was continued for 1 hr at 0° and for 5 hr at room temperature. The solvent was evaporated at room temperature under reduced pressure (0.1 mm) and in an atmosphere of nitrogen. The reaction product was hydrolyzed by adding an ice-cold solution of 0.5 ml of triethylamine in 100 ml of distilled water and stirring for 30 min in an ice bath. The pH of the solution was then adjusted to 6.5 with glacial acetic acid. The reaction product was extracted with 200 ml of chloroform–methanol (1:1). The lower phase was collected and the extraction was repeated with 100 ml of chloroform. The combined lower phases were evaporated to dryness and the residue was dissolved in 20 ml of benzene–alcohol (5:1). Thin-layer chromatographic analysis of the reaction product in chloroform–methanol–0.8 M ammonia (80:40:4) showed two spots containing phosphate esters with R_F values of 0.3 and 0.9. The lower spot had a R_F value similar to diacyl-GP, was not resolved by cochromatography and was therefore considered to represent 1-alkenyl-2-acyl-GP. A total of 240 μmoles (71%) of 1-alkenyl-2-acyl-GP was formed as determined by the phosphorus analysis.

The crude reaction products were separated by column chromatography (column diameter, 1 cm) on 6 g of Mallinckrodt SilicAR (100–200 mesh). The reaction product was dissolved in 1 ml of chloroform and added to the column. Starting with 20 ml of chloroform, solvent mixtures with increasing amount of methanol and 0.8 M ammonia [(*i.e.*, 20 ml of chloroform–methanol (8:1) and 20 ml of chloroform–methanol–0.8 M ammonia (80:16:1)] were used to elute a contaminating yellow material, with 1-alkenyl-2-acyl-GP remaining on the column. A solvent mixture of chloroform–methanol–0.8 M ammonia (80:40:4) was necessary to finally elute 220 μmoles of 1-alkenyl-2-acyl-GP. The solvent was removed and the 1-alkenyl-2-acyl-GP was dissolved in 20 ml of benzene–methanol (3:2, 11 μmoles/ml) and stored at 0°.

Thin-layer chromatographic analysis of the synthesized 1-alkenyl-2-acyl-GP showed one spot with R_F of 0.3 on silicic acid which was not resolved by cochromatography with diacyl-GP. The molar ratio of phosphorus:acyl:alkenyl was 1.0:0.98:0.97, close to that expected.

TABLE I: Cleavage of Alkenyl Derivatives with Iodine.[a]

	Alkenyl (nmoles)	Iodine	Phosphorus: Organic Phase (nmoles)	Water Phase: nmoles	Water Phase: % Yield
1-Alkenyl-2-acyl-GP	110	−	96	14	13
		+	5	106	97
1-Alkenyl-2-acyl-GPC[b]	130	−	144	18	14
		+	33	130	100
1-Alkenyl-2-acyl-GPE[c]	126	−	150	16	13
		+	50	125	99

[a] The phospholipids were dissolved in 0.5 ml of petroleum ether and 0.5 ml of 0.1 M Tris-chloride buffer (pH 7.4) were added. Under continuous shaking, 0.1 ml of an iodine solution (saturated solution of iodine in petroleum ether) was added. The reaction mixture was still colored deep red after 5 min, indicating an excess of iodine. Methanol (0.5 ml) was then added, and the shaking was continued for 5 min. The buffer solution was extracted by adding 1 ml of petroleum ether and 1.5 ml of ether. The organic layer was collected after centrifugation and the extraction was repeated twice with 3 ml of petroleum ether–ether (1:1). Total phosphorus was determined in both water and organic phases. [b] alkenyl:P = 0.80. [c] alkenyl:P = 0.75.

TABLE II: Thin-Layer Chromatography Separation of Lipid Derivatives.[a]

Compound	R_F Value
Part A	
Diacyl-GP	0.5
1-Alkenyl-2-acyl-GP	0.5
1-Alkenyl-2-acyl-GP (after iodine treatment in petroleum ether)	0.25, 0.95
1-Acyl-GP	0.25
Palmitic aldehyde	0.95
Part B	
2-Acyl-GP	0.25
1-Acyl-GPC	0.30
2-Acyl-GPC	0.30
1-Acyl-GPE	0.55
2-Acyl-GPE	0.55

[a] A: R_F values of compounds related to 1-alkenyl-2-acyl-GP. The samples (1 μmole of each) were transferred to thin-layer chromatography plates (SilicAR) Mallinckrodt) and developed in a solvent system of chloroform–methanol–0.8 M aqueous ammonia (65:35:4). B: R_F values of compounds after iodine treatment of the 1-alkenyl-2-acyl derivatives of GP, GPC, and GPE and isolation according to the described procedures. The 1-acyl derivatives were also included as a reference; 300 nmoles of each monoacylphosphoglyceride were transferred to the thin-layer chromatography plate.

Formation of 2-Acyl Derivatives. The stability of the 1-alkenyl-2-acyl derivatives of GP, GPC, and GPE was then checked by dissolving 1 μmole of the phospholipid in 1 ml of petroleum ether and then adding 1 ml of 0.1 M Tris-chloride buffer with pH values ranging from 5.2 to 8.0. After 2-hr stirring at room temperature, thin-layer chromatography gave no indication of any decomposition to monoacyl phosphoglycerides. Table I shows the extent of the formation of monoacyl phosphoglycerides by iodine treatment of plasmalogen derivatives. The product was separated by making use of the different solubility of the compounds in water and in ether–petroleum ether. Based on the added amount of plasmalogen, the recovery of monoacylphosphoglyceride in the water phase was more than 95%. Using the conditions described in Table I, we developed the following method for the preparation of 2-monoacyl derivatives of GP, GPC, and GPE.

1-Alkenyl-2-acyl-GP (1–3 μmoles) was dissolved in 1 ml of petroleum ether and 1 ml of a buffer solution, 0.1 M in boric acid and 0.067 M in sodium citrate (pH 5.7), was added. The tube was shaken vigorously and 0.25 ml of iodine (saturated solution in petroleum ether) was added dropwise. The reaction mixture became red due to the excess iodine. After 5 min, 1 ml of methanol was added and shaking was continued for another period of 5 min. The solution (pH 6.5) was then extracted by adding 3 ml of petroleum ether, mixing well, and centrifuging to break the emulsion. The upper layer was discarded and the extraction was repeated three times with 4 ml of petroleum ether. The upper layers were removed and the water phase, which had been adjusted to a pH of 4.4 by adding 0.2 ml of 0.5 M citric acid and 0.5 ml of methanol, was extracted three times with 2.2 ml of chloroform (the chloroform was always treated with 0.1 M boric acid before use). The chloroform layers were combined.

The chloroform fraction was evaporated under nitrogen and the residue was dissolved in 0.1 M boric acid (pH 5.1; 0.75 ml of solution/μmole of starting material). The solution of 2-acyl-GP (about 1 μmole/ml) was water clear. Table IIA shows the effect of the iodine treatment on 1-alkenyl-2-acyl-GP. The original spot of the material disappeared and two new spots appeared with R_F values identical with those for palmitic aldehyde and monoacyl-GP.

2-Acyl-GPC and 2-acyl-GPE were prepared following the procedure described for the synthesis of 2-acyl-GP. In these cases, however, 4 ml of a mixture of petroleum ether–ether (1:1) was used for the extractions instead of petroleum ether. The petroleum ether–ether layers were discarded. The water–methanol phase (pH 6.5) was directly extracted with chloroform. The chloroform was evaporated by flushing with nitrogen, and the residue was dissolved in 0.1 M boric acid (1 ml of boric acid was added per μmole of plasmalogen). The solutions of 2-acylphosphoglyceride were also water clear.

Table IIB shows thin-layer chromatographic analysis of the 2-acyl phosphoglycerides, 2-acyl-GP, 2-acyl-GPC, and 2-acyl-GPE. Each showed one spot by visualization with H_2SO_4 with R_F values of 0.25, 0.30, and 0.55 for 2-acyl-GP, 2-acyl-

TABLE III: Preparation of 2-Acylphosphoglycerides According to the Recommended Procedure.[a]

	2-Acyl-GP (Four Preparations)		2-Acyl-GPC (Six Preparations)		2-Acyl-GPE (Three Preparations)	
	Range (nmoles)	Av %	Range (nmoles)	Av %	Range (nmoles)	Av %
Total amounts (P)	2656–2730		6341–6478		2140–2380	
Petroleum ether–ether (1:1)	48–172[b]	4	977–1740	21	648–779	31
Water	528–534	19	117–158	2	41–62	2
Chloroform	2030–2080	77	4580–5240	77	1320–1670	76

[a] The phosphorus distribution among the different fractions is given. [b] Petroleum ether was used instead of petroleum ether–ether (1:1) for the GP derivative.

GPC, and 2-acyl-GPE, respectively, in a solvent system of chloroform–methanol–0.8 M ammonia (65:25:4). The R_F values were identical with those for the corresponding 1 isomers and were not resolved by cochromatography. In addition, the distribution of phosphorus in the three fractions (petroleum ether or petroleum–ether (1:1), water, and chloroform) was examined and the results are summarized in Table III. At pH 4.4 77% of the 2-acyl-GP and at pH 6.5 97% of 2-acyl-GPC and 92% of 2-acyl-GPE were extracted into the chloroform layer.

Hydrolysis with Phospholipases. Isomeric monoacyl phosphoglycerides cannot be resolved by thin-layer chromatography, therefore, we removed the polar groups by enzymatic hydrolysis. Table IV shows R_F values for thin-layer chromatography after formation of monoacylglycerols by treatment with phospholipase C in the case of 2-acyl-GPC and 2-acyl-GPE or with phosphatidic acid phosphatase with 2-acyl-GP as substrate.

Hydrolysis with Phospholipase C. Approximately 1 μmole of 2-acyl-GPC or 2-acyl-GPE was dissolved in 1.75 ml of 0.1 M boric acid, the pH was adjusted to 6.3 with 0.05 M sodium tetraborate and then 0.25 ml of phospholipase C (10 mg/ml of the buffer solution) was added. The pH of the resulting solution was usually between 6.1 and 6.3. Ether (1 ml) was added to this mixture and it was mixed by shaking and then incubated for 2 hr at room temperature. Blanks with enzyme alone were included. After 2 hr, the reaction was stopped by adding 0.2 ml of 0.1 M boric acid, 0.067 M in sodium citrate with the pH adjusted to 5.1. Methanol (2 ml) was then added with mixing. The water–methenol mixture was extracted three times with 2.2-ml portions of chloroform (washed with 0.1 M boric acid). The emulsion was broken by centrifugation and the chloroform layers were collected. The combined extracts of about 7.0 ml were divided, 4 ml was taken for the direct analysis of the 1-acylglycerol content and 2 ml for the total monoglyceride determination as described below. The monoglyceride formation was also determined by measuring the formation of water-soluble phosphate using a variation of our method described by Eibl and Lands (1969a). Free phosphate was formed by digesting each sample in a test tube with 0.6 ml of 6 N sulfuric acid and oxidizing the carbon with two drops of 30% hydrogen peroxide. Then, 5.3 ml of water and 0.06 ml of 1% Triton X-100 were added. After mixing and addition of 0.6 ml of a 2.5% solution of ammonium molybdate, the tubes were mixed again and the absorbance was read at 660 nm after 20 min. The presence of boric acid (at least up to 100 μmoles in the final volume of 6 ml) did not influence the phosphate determination.

Hydrolysis with Phosphatidic Acid Phosphatase. The enzyme (EC 3.1.3.4) was prepared from fresh chicken livers with the procedure described by Smith *et al.* (1957). The chicken liver particulate preparations were stored at −15°.

2-Acyl-GP (700–900 mmoles) was dissolved in 1.9 ml of 0.1 M boric acid and the pH was adjusted to 6.3. Phosphatidic acid phosphatase (0.1 ml of 10 mg/ml in 0.02 M Tris-chloride buffer (pH 8.0) containing 0.001 M EDTA) was added. The pH of the solution was kept between 5.9 and 6.1. Ether (1 ml) was added and the reaction mixture was shaken for 2 hr at room temperature. The reaction was stopped and the extraction and analysis of the products were carried out as described above for the products of 2-acyl-GPC and 2-acyl-GPE.

Resolution of the 1- and 2-acylglycerols was achieved on

TABLE IV: Thin-Layer Chromatography on Silica Gel G Containing Boric Acid.[a]

Sample	R_F Value(s)
Linoleic acid	0.7
1-Stearoylglycerol (90%)	0.4 (0.5)
Diacyl-GPC + phospholipase C and triglyceride lipase	(0.4) 0.5, 0.8
2-Acyl-GPC + phospholipase C	(0.4) 0.5, 0.7, 0.9
2-Acyl-GPE + phospholipase C	(0.4) 0.5, 0.7, 0.9
Phospholipase C	0.7, 0.9
Phosphatidic acid phosphatase	0.7, 0.8, 0.9
2-Acyl-GP + phosphatidic acid phosphatase	(0.4 0.5, 0.7, 0.8, 0.9

[a] The solvent system was chloroform–acetone–acetic acid–methanol (72.5:25:0.5:2). The spots were visualized by iodine vapor and by heating to 400° after spraying with 50% H_2SO_4. Weaker spots, not visible with iodine but visible with sulfuric acid, are indicated in parentheses.

TABLE V: Final Structural Proof of the 2-Acylphosphoglycerides as the Monoacylglycerol Derivatives.[a]

Monoacyl Derivative	Total Amount	Phosphorus (Water Phase)	Hydrolysis (%)	Total Monoglyceride (Glycerol)	1-Acylglycerol (%)[b] (Direct Diol)
1-Acyl-GPC	880	834	95	840	97.1 ± 0.6 (7)
2-Acyl-GPC	1250	1226	98	1252	0.0 ± 0.3 (17)
2-Acyl-GPE	882	855	97	882	0.8 ± 0.4 (8)
2-Acyl-GP	748	720	96	760	0.5 ± 1.3 (5)

[a] The amounts described are nanomoles. 1-Acyl-GPC was used as a references compound. [b] The average and standard deviation for the number of preparations shown in parentheses.

silica gel G containing boric acid. The monoacylglycerols derived from the 2-acylphospholipids gave one spot upon visualization with iodine. Digestion after spraying with 55% H_2SO_4 always showed a small additional spot in the area of 1-acylglycerol as indicated in parentheses in Table IV.

Analysis of Vicinal Diol. The final structural proof of the 2-acylphosphoglycerides was the quantitative analysis of the monoacylglycerols obtained with phosphohydrolases. As shown in Table V, the enzymic hydrolysis was more than 95% complete after 2 hr. The extent of hydrolysis was followed by measuring the nonextractable phosphorus in the water phase. The total monoglycerides were analyzed spectrophotometrically (Eibl and Lands, 1969b) after deacylation with sodium methoxide to form glycerol, and the results are presented in Table V. The measured amount of total monoacylglycerol corresponded closely in each case with that of water-soluble phosphorus. Samples (0.1–1 μmole) were treated with 0.2 ml of 0.5 N sodium methoxide in methanol. After 10 min, 0.2 ml of a periodate solution (37 mM; 40 mg of sodium periodate dissolved in 5 ml of 1 N sulfuric acid) was added and shaken, and the mixture was kept in the dark. Aliquots of 0.1 ml were taken after 30 and 60 min and transferred to 6.3 ml of distilled water in a test tube. The amount of glycerol produced was determined essentially as described previously for the determination of vicinal diols (Eibl and Lands, 1969b).

When the 2-acyl-GP was treated directly with sodium methoxide and periodate without phosphatase treatment, the diol–phosphate ratio was found to be 1:1.07. This suggests that the phosphate introduced during the earlier synthesis was located at the primary position as expected. Also, the diol content of the acylglycerol from 1-acyl-GPC equalled the amount of water-soluble phosphorus. However, when the enzymically produced acylglycerols from 2-acylphospholipids were treated with periodate, less than 1% was reactive. This evidence for the absence of 1-acylglycerol contamination was obtained with samples stored in chloroform or in aqueous boric acid (pH 5.1) for more than 40 hr at 0°. Less acidic conditions, however, were not as satisfactory for storage of 2-acyl-GPC as shown in Figure 1, so that most samples requiring storage for any period of time were maintained at slightly acidic pH values.

Discussion

The selective removal of an ester from the secondary hydroxyl of diacylphosphoglycerides by phospholipase A_2 of venom allows a relatively easy preparation of the 1-acyl derivatives (VanDeenen and DeHaas, 1966). Phospholipase A_1 activity, which would produce 2-acyl derivatives by removing the primary ester, has been reported to occur in liver (VandenBosch and VanDeenen, 1965), adrenal (Winkler *et al.*, 1967), and brain (Gatt, 1968), and may be an added activity of some triglyceride lipases from pancreas (DeHaas *et al.*, 1965) and mold (Slotboom, 1968). These sources of phospholipase A_1, however, have not been available in sufficient quantities as a pure reagent to be generally used in preparative work. Methods for the chemical synthesis of 2-acylphosphoglycerides, recently reviewed by Slotboom (1968), are limited to species containing saturated fatty acids because of unsuitable protecting groups for the primary hydroxyl.

The alkenyl group has been useful as a masking agent for the primary hydroxyl. Although Siggia and Edsberg (1948) indicated that treatment with methanolic iodine gave the iodoacetal as the end product, we found that cleavage occurred in the presence of water to yield the free primary alcohol (Robertson and Lands, 1962). The other products, HI and an iodoaldehyde, are easily removed from the mono-

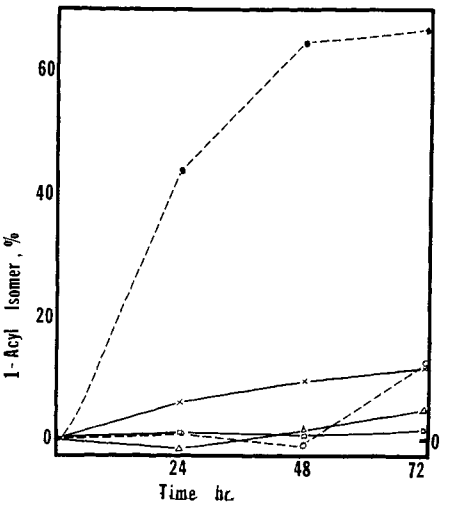

FIGURE 1: Stability of 2-acyl-GPC in various solvents. The 2-acyl isomer was stored at 0° in chloroform (□—□); water (×—×); 0.1 M boric acid, pH 5.1 (△—△); 0.1 M borate, pH 6.3 (○---○); and 0.1 M borate, pH 7.9 (●---●).

acylphospholipid, but the starting materials often contained appreciable amounts of diacyl derivatives that remained unchanged in the reaction mixture. While studying the 1- and 2-positional isomers of monoacylphosphoglycerides as substrates for acyltransferase reactions, the contaminants formed turbid dispersions in water and made the spectrophotometric assays less reliable. In general, the monoacyl derivatives are more soluble than the diacyl in water whereas they were less soluble than the diacyl in ether. These facts were used to develop a selective solvent treatment that provided a purer monoacyl product. The success of the method is indicated by results in Table III showing a transfer of the monoacyl derivative from the diacyl through an aqueous phase and then into chloroform. This purification technique provides isomerically pure derivatives without the acyl migration that has previously been observed with silicic acid columns (Lands and Merkl, 1963; DeHaas and VanDeenen, 1965). Also, it eliminates the need to obtain pure alkenylacyl derivatives as precursors.

Our attempts to produce alkenylacylglycerol phosphate from phospholipase D reaction mixtures were not successful (Lands and Hart, 1965), although Slotboom (1968) indicated that the reaction can occur to the extent of 40% in 20 hr with freshly prepared enzyme. A partial synthesis of this compound by phosphorylating alkenylacylglycerol was successful, however, and paralleled the approach reported by Slotboom (1968) for partial synthesis of alkenylacylglycerolphosphorylcholine. The principal concern that dimers of the substituted glycerol phosphate might occur was minimized by slowly adding the alkenylacylglycerol to a large excess of phosphorous oxychloride.

The use of boric acid to stabilize acylglycerols has been reviewed by Serdarevich (1967). Since we obtained more consistent results in preliminary studies with borate than without, we routinely added it in subsequent work. The instability of the 2-acyl isomer at pH 7.9 agrees with the findings of Mattson and Volpenhein (1962) for monoolein in lysine–histidine buffer. On the other hand, the expectation that solutions more acidic than pH 6.0 "undoubtedly would result in higher rates of isomerization" did not occur in boric acid (pH 5.1).

References

Brandt, A., and Lands, W. E. M. (1968), *Lipids 3*, 178.

DeHaas, G. H., Sarda, L., and Roger, J. (1965), *Biochim. Biophys. Acta 106*, 638.

DeHaas, G. H., and VanDeenen, L. L. M. (1965), *Biochim. Biophys. Acta 106*, 315.

Dittmer, J. C., and Lester, R. L. (1964), *J. Lipid Res. 5*, 126.

Eibl, H., and Lands, W. E. M. (1969a), *Anal. Biochem. 30*, 51.

Eibl, H., and Lands, W. E. M. (1969b), *Anal. Biochem.* (in press).

Gatt, S. (1968), *Biochim. Biophys. Acta 159*, 304.

Gottfried, E. L., and Rapport, M. M. (1962), *J. Biol. Chem. 237*, 329.

Lands, W. E. M. (1965), *J. Am. Oil Chemists' Soc. 42*, 465.

Lands, W. E. M., and Hart, P. (1965), *Biochim. Biophys. Acta 98*, 532.

Lands, W. E. M., and Merkl, I. (1963), *J. Biol. Chem. 238*, 898.

Lands, W. E. M., Pieringer, R. A., Sr., Slakey, P. M., and Zschocke, A. (1966), *Lipids 1*, 444.

Long, C., Odavic, R., and Sargent, E. J. (1967), *Biochem. J. 102*, 221.

Mattson, F. H., and Volpenhein, R. A. (1962), *J. Lipid Res. 3*, 281.

Peifer, J. J. (1963), *Mikrochim. Acta* 529.

Robertson, A. F., and Lands, W. E. M. (1962), *Biochemistry 1*, 804.

Serdarevich, B. (1967), *J. Am. Oil Chemists' Soc. 44*, 381.

Siggia, S., and Edsberg, R. L. (1948), *Anal. Chem. 20*, 762.

Slotboom, A. J. (1968), Ph.D. Thesis, Rijksuniversiteit te Utrecht.

Smith, S. W., Weiss, S. B., and Kennedy, E. P. (1957), *J. Biol. Chem. 228*, 915.

Stahl, E. (1965), Thin-Layer Chromatography, New York, N. Y., Springer-Verlag.

Thomas, A. E., Scharoun, J. E., and Ralston (1965), *J. Am. Oil Chemists' Soc. 42*, 789.

VanDeenen, L. L. M., and DeHaas, G. H. (1966), *Ann. Rev. Biochem. 35*, 157.

VandenBosch, H., and VanDeenen, L. L. M. (1965), *Biochim. Biophys. Acta 106*, 326.

Winkler, H., Smith, A. D., DuBois, F., and VandenBosch, H. (1967), *Biochem. J. 105*, 38c.