Conversion of diphosphatidylglycerol to bis(monoacylglyceryl)phosphate by lysosomes

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Abstract Diphosphatidyl[1′,2′,3′-^{14}C]glycerol (cardiolipin) is converted to bis(monoacylglyceryl)phosphate when incubated in vitro with rat liver lysosomes at pH 4.4. The stereochemical configuration of the product is unknown. This reaction probably takes place via lysophosphatidylglycerol, one of the major products of diphosphatidylglycerol hydrolysis by lysosomes. Phosphatidyl[1′,2′,3′-^{14}C]glycerol was introduced into mitochondrial membranes by incubating mitochondria with [U-^{14}C]*sn*-glycerol-3-phosphate and cytidine diphosphate diacylglycerol. Membrane-bound phosphatidyl[1′,2′,3′-^{14}C]glycerol is also converted to bis(monoacylglyceryl)phosphate when incubated with lysosomes in a reaction that is dependent on the concentration of lysosomal protein and on incubation time. These results support our previous proposal (Poorthuis, B. J. H. M., and K. Y. Hostetler. 1976. *J. Biol. Chem.* **251:** 4596–4602) that bis(monoacylglyceryl)phosphate formation may require the interaction of lysosomes with other membranes that contain the substrates for the reaction. The stereochemistry of bis(monoacylglyceryl)phosphate biosynthesis is discussed.

Supplementary key words phosphatidylglycerol · lysophosphatidylglycerol · lysobisphosphatidic acid · cardiolipin · cytidine diphosphate diacylglycerol

Bis(monoacylglyceryl)phosphate is a phospholipid that is highly enriched in lysosomes of rat liver (1), alveolar macrophages (2), and BHK 21 cells (3). Its absence in other subcellular organelles suggests that it is specific for the lysosomes (4). This phospholipid (also referred to as lysobisphosphatidic acid) accumulates in the liver of patients with Type A Niemann-Pick disease (5) where it has been shown to be associated with multilamellar cytoplasmic bodies which are characteristically found in this lipid storage disease (6).

We have shown previously that rat liver lysosomes are the site of synthesis of bis(monoacylglyceryl)phosphate (7, 8). Both phosphatidylglycerol and lysophosphatidylglycerol are substrates for the reaction (8). Lysosomes also contain phospholipases with acid pH optima that readily hydrolyze diphosphatidylglycerol in the presence of Triton X-100 (9, 10). Lysophosphatidylglycerol is one of the hydrolysis products of these reactions, as shown by Hambrey and Mellors (10). This suggested the possibility that diphosphatidylglycerol might also be an important precursor of bis(monoacylglyceryl)phosphate.

Although lysosomes are the site of synthesis of bis(monoacylglyceryl)phosphate, lysosomal membranes do not contain phosphatidylglycerol or diphosphatidylglycerol, potential precursors of bis(monoacylglyceryl)phosphate (4, 8). We have proposed that the synthesis of this compound may occur via an interaction of lysosomes with other intracellular membranes that contain the substrates for the reaction. In this report, the results of experiments are presented that show that diphosphatidyl[^{14}C]glycerol as well as membrane-bound phosphatidyl[^{14}C]glycerol can be converted to [^{14}C]bis(monoacylglyceryl)phosphate when incubated with lysosomes in vitro.

MATERIALS AND METHODS

Preparation of substrates

Cytidine diphosphate diacylglycerol and phosphatidyl[1′,2′,3′-^{14}C]glycerol were synthesized and purified as described before (6). Diphosphatidyl[1′,2′,3′-^{14}C]glycerol was synthesized by incubating dispersions of phosphatidyl[1′,2′,3′-^{14}C]glycerol and cytidine diphosphate diacylglycerol with gradient-purified mitochondria in the presence of Co^{2+} (11). The lipids were extracted (12) and diphosphatidyl[1′,2′,3′-^{14}C]glycerol was purified by successive preparative thin-layer chromatography in two systems: on silica G plates impregnated with 0.4 M boric acid developed with chloroform–methanol–water–concentrated ammonia 70:30:3:2 (v/v) (system 1) and on silica G plates developed with chloroform–methanol–water 65:25:4 (v/v) (system 2). In some cases final purification was obtained by rechromatography in system 1. The

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radioactive lipids were located by scanning with a Panax thin-layer chromatography scanner.

[^{3}H]CDP-diacylglycerol was prepared by the New England Nuclear Corporation, Boston, MA, by a catalytic exchange method. The crude [^{3}H]CDP-diacylglycerol was purified by silicic acid column chromatography as described previously (7). [^{3}H,^{14}C] doubly-labeled phosphatidylglycerol and diphosphatidylglycerol were synthesized and purified as described above with [^{3}H]CDP-diacylglycerol substituting for unlabeled CDP-diacylglycerol.

Phosphatidyl[1′,2′,3′-^{14}C]glycerol-labeled mitochondrial membranes were obtained by incubating gradient-purified mitochondria with [U-^{14}C]*sn*-glycerol-3-phosphate and CDP-diacylglycerol in isotonic medium for 1 hr at 37°C as described before for the synthesis of phosphatidyl[1′,2′,3′-^{14}C]glycerol (7). The reaction was stopped by adding ice-cold 0.25 M sucrose containing 5 mM tris(hydroxymethyl)-aminomethane-HCl (pH 7.0). The membranes were spun down at 8,600 *g* for 10 min and the supernatant was discarded. The pellet was resuspended in cold 0.25 M sucrose–5 mM Tris-HCl and centrifuged at 8,600 *g* for 10 min. The pellet was taken up in 0.25 M sucrose–5 mM Tris-HCl (pH 7.0) and stored at −70°C until use. Analysis of the radioactive lipids by thin-layer chromatography with system 1 indicated that 97% of the radioactivity was present in phosphatidylglycerol and 3% was in lysophosphatidylglycerol.

Preparation of mitochondria and lysosomes from rat liver

Sucrose gradient-purified mitochondria and lysosomes were obtained from male Sprague-Dawley rats injected with Triton WR-1339 according to established methods described in a previous publication (8). The lysosomes obtained in this way were approximately 35 times enriched in acid phosphatase over the homogenate.

Assays of enzymatic activity

Acid phosphatase, assayed as described by Bergmeyer (13), was used as a lysosomal marker.

Hydrolysis of diphosphatidylglycerol by lysosomes was measured as follows. Diphosphatidyl[1′,2′,3′-^{14}C]-glycerol or [^{3}H]diphosphatidyl[1′,2′,3′-^{14}C]glycerol was dispersed in a dilute Triton X-100 solution by sonication so that the final concentration of Triton X-100 in the incubation mixture was 0.05 mg/ml. The incubation conditions are described in the respective figure legends. The reaction was stopped by adding 20 volumes of chloroform–methanol 2:1 and the lipids were extracted by the method of Folch, Lees, and Sloane Stanley (12). The radioactive lipid products were analyzed by two-dimensional thin-layer chromatography, as previously described, on 0.25-mm silica gel G plates impregnated with 0.4 M boric acid and developed with chloroform–methanol–water–concentrated ammonia 70:30:3:2 (by volume) in the first dimension and chloroform–methanol–water 65:35:5 (by volume) in the second dimension (14). The labeled lipids were located by radioautography with Kodak No-Screen X-ray film. The areas corresponding to the radioactive lipids were either scraped into scintillation vials for liquid scintillation counting or extracted with chloroform–methanol–water 10:10:1 (by volume) for further analysis. Doubly-labeled diphosphatidylglycerol was used when reaction products were analyzed further by acetolysis.

Bis(monoacylglyceryl)phosphate formation from membrane-bound phosphatidyl[1′,2′,3′-^{14}C]glycerol was assayed by incubating mitochondrial membranes containing phosphatidyl[1′,2′,3′-^{14}C]glycerol with lysosomes as described in the legend to **Table 1**. The incubations were stopped by adding 20 volumes of chloroform–methanol 2:1 (v/v). The lipid extract was washed with 0.73% sodium chloride (11) and analyzed by one-dimensional thin-layer chromatography with system 1. The radioactive lipids were located by thin-layer scanning and the respective areas were scraped off and counted as described below.

Analytical methods

Acetolysis of labeled lipid products was carried out according to the procedure of Renkonen (15). The incubation time was 4 hr. The acetolysis products were identified by thin-layer chromatography on 0.25-mm silica gel G plates developed with hexane–diethyl ether 60:40 (v/v). Radioactive diphosphatidylglycerol, phosphatidylglycerol, triolein, lysophosphatidylcholine, and glycerol standards were carried through the procedure.

Mild alkaline methanolysis of the radioactive lipid products was done by the method of Dawson (16). The water-soluble products were analyzed by thin-layer chromatography on Baker-Flex cellulose sheets (20 × 20 cm) (G. T. Baker Co., Phillipsburg, N.J.) developed with 1-propanol–conc. ammonia–water 7:3:1 (v/v). Radioactive diphosphatidylglycerol, phosphatidylglycerol, glycerol, and *sn*-glycerol-3-phosphate were carried through the procedure as reference standards.

Liquid scintillation counting was done in a Nuclear-Chicago/Searle Mark III liquid scintillation counter with 0.5% 2,5-diphenyloxazole and 0.04% *p*-bis[2-(5-phenyloxazole)]benzene in toluene–Triton X-100–

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TABLE 1. Conversion of membrane-bound phosphatidyl[1′,2′,3′-^{14}C]glycerol to bis(monacylglyceryl)phosphate, lysophosphatidylglycerol, and aqueous methanol-soluble products by lysosomes

Product	pH 7.0 No Lysosomes	pH 7.0 + Lysosomes	pH 4.4 No Lysosomes	pH 4.4 + Lysosomes
Bis(monoacylglyceryl)phosphate	0.9	15.3	3.6	1015
Lysophosphatidylglycerol	156	169	139	539
Aqueous methanol-soluble	210	214	126	1294

The incubation medium contained 50 mM sodium acetate buffer (pH 4.4) or 50 mM Tris-HCl buffer (pH 7.0); 10 mM 2-mercaptoethanol; 2.5 mg/ml mitochondrial protein containing 6.8 nmol of phosphatidyl[1′,2′,3′-^{14}C]glycerol (sp act 130 mCi/nmol) per mg and either 500 μg/ml or no lysosomal protein in a total volume of 0.200 ml. Incubations were for 60 min at 37°C. Results are given as picomoles of product formed.

water 2:1:0.2 (v/v/v) as counting fluid. Corrections for quenching were made by an external standard method.

Chemicals

Cytidine monophosphate morpholidate and Triton X-100 were obtained from Sigma, St. Louis, MO. *sn*-Glycerol-3-phosphate was purchased from Calbiochem, La Jolla, CA. [U-^{14}C]-*sn*-glycerol-3-phosphate, 130 mCi/mmol and [2-^{3}H]glycerol, 8 Ci/mmol were obtained from New England Nuclear, Boston, MA. [1-^{14}C]Triolein was purchased from Dohm Products, Los Angeles, CA and 1-[1′-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine from Applied Science, State College, PA. Silica gel G and silicic acid 45–270 mesh ASTM were obtained from EM Reagents, through Brinkmann Instruments, Burlingame, CA. All other chemicals were analytical reagent grade and obtained from the usual commercial suppliers. Solvents were redistilled before use.

RESULTS

Conversion of diphosphatidylglycerol to bis(monoacylglyceryl)phosphate by a lysosomal preparation

Fig. 1 shows radioautograms of the radioactive hydrolysis products of diphosphatidyl[1′,2′,3′-^{14}C]-glycerol upon incubation with lysosomes as described in the legend. The identity of the hydrolysis products was established by mild alkaline hydrolysis and acetolysis (not shown). Panel *A* shows a radioautogram of the two-dimensional thin-layer chromatogram of the zero time control showing that only diphosphatidylglycerol and very small amounts of phosphatidyllysophosphatidylglycerol and di(lysophosphatidyl)glycerol are present. Panel *B* shows the products of hydrolysis after 4 hr of incubation. In addition to di(lysophosphatidyl)glycerol, phos-

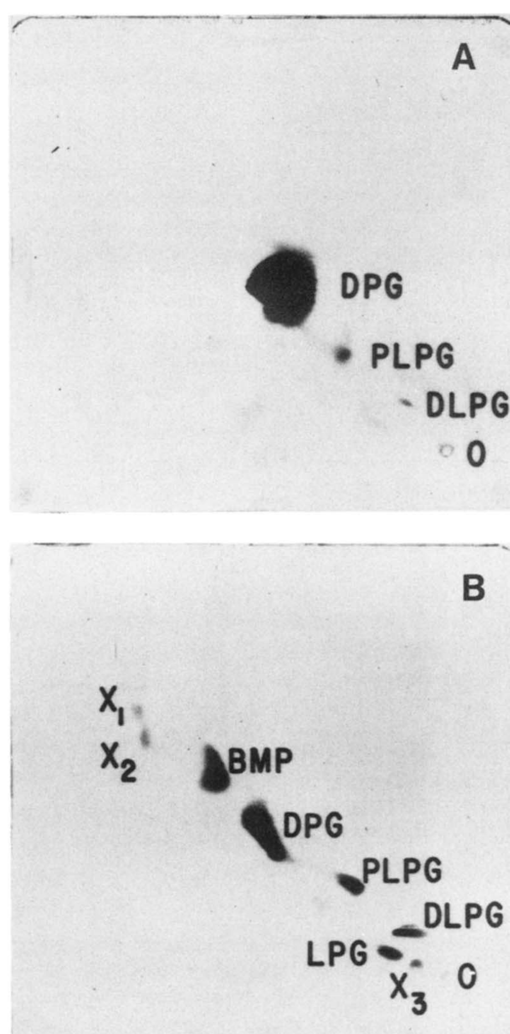

Fig. 1. Radioautogram of lysosomal hydrolysis products of diphosphatidyl[1′,2′,3′-^{14}C]glycerol. The incubation medium contained 50 mM sodium acetate buffer, pH 4.4; 10 mM 2-mercaptoethanol; 5 mM $CaCl_2$; 2×10^{-4}M diphosphatidyl[1′,2′,3′-^{14}C]-glycerol, sp act 1.25 mCi/mmol; 0.05 mg/ml Triton X-100 and 1.25 mg/ml lysosomal protein in a final volume of 0.250 ml. The incubations were done for zero (panel *A*) and 4 hr (panel *B*) at 37°C. Abbreviations used: BMP, bis(monoacylglyceryl)phosphate; DPG, diphosphatidylglycerol; PLPG, phosphatidyllysophosphatidylglycerol; DLPG, di(lysophosphatidyl)glycerol; LPG, lysophosphatidylglycerol; 0, origin.

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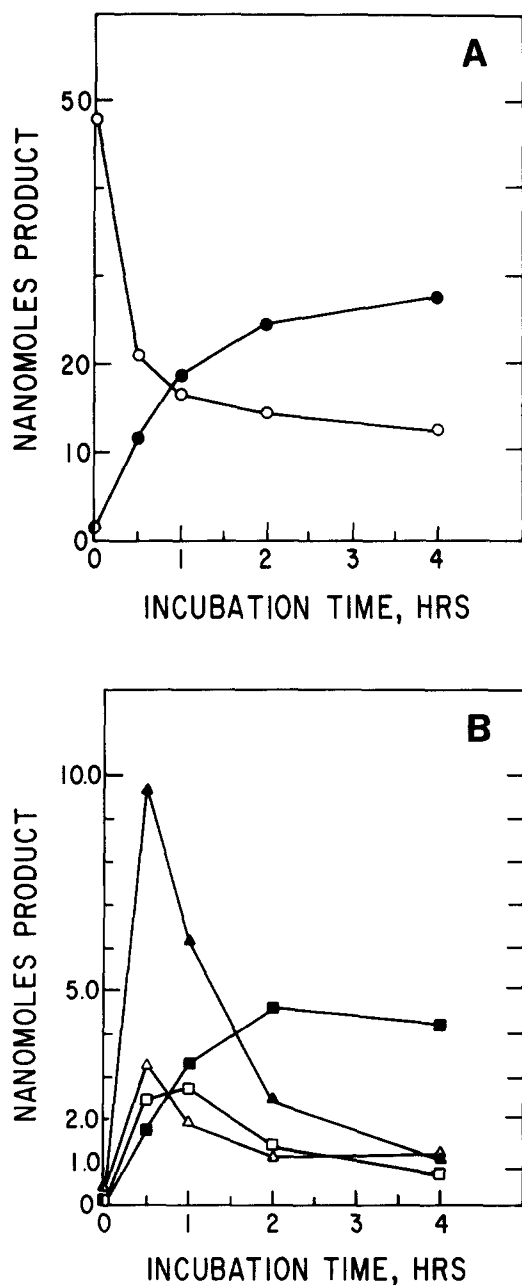

Fig. 2. Time course of lysosomal hydrolysis of diphosphatidyl-[1′,2′,3′-^{14}C]glycerol. The incubation conditions were the same as those described in Fig. 1. Incubation time was varied as noted. Panel A: ○, DPG; ●, aqueous methanol-soluble products. Panel B: ▲, PLPG; △, DLPG; □, LPG; ■, BMP. (See legend to Fig. 1.)

phatidyllysophosphatidylglycerol and lysophosphatidylglycerol, which are the expected products (10), bis(monoacylglyceryl)phosphate was found. X_3, which was present in amounts too small to be analyzed, probably represents traces of acyl bis(glycerylphosphoryl)glycerol which is the direct precursor of lysophosphatidylglycerol (10). This material is usually found primarily in the aqueous phase of the extraction (10). X_1 and X_2 could not be identified with certainty.

Fig. 2 shows the time course of lysosomal hydrolysis of diphosphatidyl[1′,2′,3′-^{14}C]glycerol. With sodium acetate buffer at pH 4–5, Triton X-100 has been shown to be required for hydrolysis of diphosphatidylglycerol by lysosomal phospholipases (9, 10). However, we previously found that Triton X-100 inhibits the formation of bis(monoacylglyceryl)-phosphate in lysosomes (8). For this reason a low concentration of Triton X-100 was selected for these experiments; otherwise the conditions were similar to those of Hambrey and Mellors (10). Sixty percent of the diphosphatidyl[1′,2′,3′-^{14}C]glycerol initially present was converted to aqueous methanol-soluble products after 4 hr of incubation (Fig. 2*A*). Fig. 2*B* shows that the levels of radioactive phosphatidyllysophosphatidylglycerol, di(lysophosphatidyl)glycerol, and lysophosphatidylglycerol were highest at 30–60 min and declined thereafter. However, bis(monoacylglyceryl)phosphate radioactivity increased with time, reached a maximum at 2 hr, and did not decrease significantly between 2 and 4 hr. Bis(monoacylglyceryl)phosphate formation accounted for 12% of the diphosphatidyl-[1′,2′,3′-^{14}C]glycerol hydrolyzed in the 4-hr incubation. This is the first demonstration of in vitro conversion of diphosphatidylglycerol to bis(monoacylglyceryl)phosphate.

Conversion of membrane-bound phosphatidylglycerol to bis(monoacylglyceryl)phosphate

Table 1 shows the results of an incubation of mitochondrial membranes containing phosphatidyl-[1′,2′,3′-^{14}C]glycerol with and without lysosomal protein at pH 7 and pH 4.4. The mitochondrial membranes were virtually free of lysosomal contamination as judged by acid phosphatase activity (not shown). Very little bis(monoacylglyceryl)phosphate synthesis was observed at pH 7 in the absence of added lysosomal protein but when lysosomes were added a definite increase was observed. At pH 4.4 bis(monoacylglyceryl)phosphate synthesis was stimulated 280-fold by the addition of lysosomes to the incubation mixture. The formation of bis(monoacylglyceryl)phosphate in the presence of lysosomes was 66 times greater at pH 4.4 than at pH 7.0, confirming our previous results with an aqueous dispersion of radioactive phosphatidylglycerol (8). Bis(monoacylglyceryl)phosphate formation from membrane-bound phosphatidylglycerol increased as a function of the concentration of lysosomal pro-

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tein to 0.75 mg/ml and was linear with time to 30 min.

Attempts were made to prepare mitochondrial membranes labeled only with diphosphatidyl[^{14}C]-glycerol. However, it was not technically possible to drive the reaction of phosphatidyl[^{14}C]glycerol with CDP-diacylglycerol to completion in vitro. This resulted in the presence of both phosphatidyl[^{14}C]-glycerol and diphosphatidyl[^{14}C]glycerol in the membranes. Thus, it was not possible to determine if membrane-bound diphosphatidyl[^{14}C]glycerol can also be converted to bis(monoacylglyceryl) in vitro, although this would seem to be highly probable.

DISCUSSION

These studies show for the first time that lysosomes convert diphosphatidylglycerol to bis(monoacylglyceryl)phosphate. Lysophosphatidylglycerol is an important intermediate in the lysosomal hydrolysis of both phosphatidylglycerol and diphosphatidylglycerol, and it seems likely that it is an important precursor of bis(monoacylglyceryl)phosphate. The following reactions are proposed as major pathways leading to the formation of bis(monoacylglyceryl)-phosphate in these experiments.

In the sequence above, PG refers to phosphatidylglycerol, AGPGPG to acyl bis(glycerophospho)glycerol. The other abbreviations are given in Fig. 1. Pathway *1* is the deacylation of phosphatidylglycerol to lysophosphatidylglycerol with subsequent conversion by transacylation to bis(monoacylglyceryl)-phosphate. The reaction is identical to the Pathway *1* in our previous publication (8). Pathway *2* represents the sequential deacylation of diphosphatidylglycerol by lysosomal phospholipases to acyl bis-(glycerophospho)glycerol. Although this compound can be further deacylated to bis(glycerophospho)-glycerol, the most important pathway has been shown to be the hydrolysis of acyl bis(glycerophospho)-glycerol by a phosphodiesterase to give lysophosphatidylglycerol and glycerol phosphate (10). Lysophosphatidylglycerol formed in this way is the substrate for the step leading to bis(monoacylglyceryl)-phosphate.

The stereochemical configuration of the bis(monoacylglyceryl)phosphate formed in the experiments described above is not known. Bis(monoacylglyceryl)-phosphate formed from phosphatidylglycerol might retain the original 3-*sn*-glycerophospho-1′-*sn*-glycerol configuration; diphosphatidylglycerol could give rise to a mixture of 3-*sn*-glycerophospho-1′-*sn*-glycerol and 3-*sn*-glycerophospho-3′-*sn*-glycerol upon hydrolysis by lysosomes. In our experiments, it has not been possible to determine the stereochemical configuration of the product due to the small amount of material formed. However, recent studies have shown that bis(monoacylglyceryl)phosphate from BHK 21 cells (17) and from pig and rabbit lung and rat liver (18) is mainly a derivative of 1-*sn*-glycerophospho-1′-*sn*-glycerol, a very unusual stereochemical configuration in nature. Up to 20% of the bis(monoacylglyceryl)phosphate was found to be a derivative of either 3-*sn*-glycerophospho-1′-*sn*-glycerol or 3-*sn*-glycerophospho-3′-*sn*-glycerol (18).

A key feature of the reaction sequence that leads to bis(monoacylglyceryl)phosphate is the transfer of an acyl ester group to lysophosphatidylglycerol (19). Our studies have shown that a lysosomal enzyme can transfer an acyl group to the glycerol-1-phosphate moiety of lysophosphatidylglycerol (19). This reaction represents the only currently known mechanism for the acylation of a glycerol-1-phosphate derivative. If the compound formed in the experiments described above is a derivative of 1-*sn*-glycerophospho-1′-*sn*-glycerol, the step that would have to be explained is the mechanism of formation of the glycerolipid backbone, 1-*sn*-glycerophospho-1′-*sn*-glycerol. Several possibilities come to mind that envision 3-*sn*-glycerophospho-1′-*sn*-glycerol as an intermediate. 3-*sn*-(Diacyl)glycerophospho-1′-*sn*-glycerol (naturally occurring phosphatidylglycerol; Fisher projection shown in **Fig. 3**, I) can be deacylated to 3-*sn*-(monoacyl)-glycerophospho-1′-*sn*-glycerol (Fig. 3, II). The glycerol-1-phosphate moiety of this compound can be acylated by this lysosomal enzyme to give 3-*sn*-(monoacyl)glycerophospho-1′-*sn*-(monoacyl)glycerol,(bis(monoacylglyceryl)phosphate, Fig. 3, III). This compound might be deacylated by a lysosomal phospholipase to 3-*sn*-glycerophospho-1′-*sn*-(monoacyl)glycerol (a "reverse" lysophosphatidylglycerol, Fig. 3, IV). This might undergo isomerization to 1-*sn*-glycerophospho-1′-*sn*-(monoacyl)glycerol (Fig. 3, VI) which could accept an acyl group by the mechanism previously suggested (8, 19, and above). Alternately, 3-*sn*-glycerophospho-1′-*sn*-(monoacyl)glycerol might undergo transesterification with monoacylglycerol to form 1-*sn*-(monoacyl)glycerophospho-1′-*sn*-(monoacyl)glycerol (Fig. 3, V) directly. The latter compound might accumulate in lysosomes if it is resistant to lysosomal hydrolysis relative to its stereoisomer, 3-*sn*-(monoacyl)glycero-

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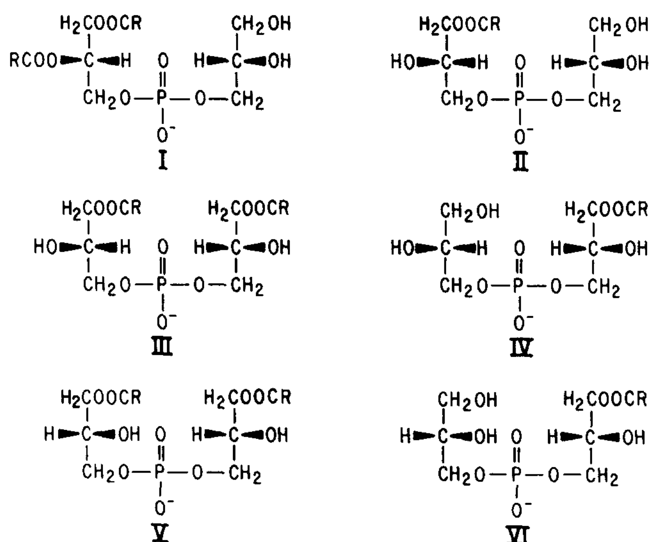

Fig. 3. Stereochemical configuration of suggested intermediates in the biosynthesis of bis(monoacylglyceryl)phosphate. (Fisher projections). I, 3-*sn*-(diacyl)glycerophospho-1′-*sn*-glycerol; II, 3-*sn*-(monoacyl)glycerophospho-1′-*sn*-glycerol; III, 3-*sn*-(monoacyl)-glycerophospho-1′-*sn*-(monoacyl)glycerol; IV, 3-*sn*-glycerophospho-1′-*sn*-(monoacyl)glycerol; V, 1-*sn*-(monoacyl)glycerophospho-1′-*sn*-(monoacyl)glycerol; VI, 1-*sn*-glycerophospho-1′-*sn*-(monoacyl)glycerol.

phospho-1′-*sn*-(monoacyl)glycerol (Fig. 3, III) (17, 18, 20). Reactions of the types suggested above would not have been observed in our experiments with the radiolabeled substrates employed. Further studies will be necessary to resolve this complex problem.

In conclusion, these studies have demonstrated that diphosphatidyl[^{14}C]glycerol can be converted to bis(monoacylglyceryl)phosphate by lysosomes in vitro. In addition, the experiments with membrane-bound phosphatidyl[^{14}C]glycerol indicate that lysosomes can utilize a membrane-bound substrate for bis(monoacylglyceryl)phosphate formation although the orientation of phosphatidylglycerol in this study might not be identical to that found in the mitochondrial membrane in vivo. Diphosphatidylglycerol is found only in the mitochondrial inner membrane (21) and phosphatidylglycerol is found in many organelle membranes including mitochondria and microsomes (8) and the plasma membrane (22). However, neither of these lipids is present in the lysosome (3, 8), the site of synthesis of bis(monoacylglyceryl)phosphate. These findings strongly support the concept that membrane–membrane interaction is required for the formation of bis(monoacylglyceryl)phosphate. The very presence of this compound in secondary lysosomes may be a reflection of extensive prior interaction of lysosomes with other membranes during endocytosis or autophagy.

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