The stereoconfiguration of bis(monoacylglycero)phosphate synthesized in vitro in lysosomes of rat liver: comparison with the natural lipid

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Abstract A procedure for stereoanalysis of radiochemically labeled glycerophospholipids is described. It is based on the study of the labeled α-glycerophosphate which retains its original configuration when liberated upon alkaline hydrolysis of the lipids. The labeled α-glycerophosphate is oxidized enzymatically with *sn*-3-glycerophosphate dehydrogenase and the product, dihydroxyacetone phosphate, is degraded with alkali to inorganic phosphate. The nonoxidizable α-glycerophosphate (*sn*-1-glycerophosphate), the β-glycerophosphate, and the inorganic phosphate derived from *sn*-3-glycerophosphate are quantitated after separation by thin-layer chromatography. The procedure gave the expected results when applied to [^{3}H]glycerol- and ^{32}P-labeled phosphatidylcholine, bis(monoacylglycero)phosphate, and phosphatidylglycerol from natural sources. Bis(monoacylglycero)phosphate, known also as lysobisphosphatidic acid, was synthesized from [^{32}P]diphosphatidylglycerol and from phosphatidyl[1′,3′-^{3}H]glycerol in lysosomal preparations of rat liver according to Poorthuis and Hostetler (1978. *J. Lipid Res.* **19:** 309–315). Stereoanalysis proved that the product was in both cases a derivate of *sn*-1-glycerophospho-*sn*-1′-glycerol.—**Joutti, A., and O. Renkonen.** The stereoconfiguration of bis(monoacylglycero)phosphate synthesized in vitro in lysosomes of rat liver: comparison with the natural lipid. *J. Lipid Res.* 1979. **20:** 840–847.

Supplementary key words α-glycerophosphate (*sn*-1-glycerophosphate or *sn*-3-glycerophosphate) · β-glycerophosphate · lysobisphosphatidic acid · *sn*-1-glycerophospho-*sn*-1′-glycerol

Bis(monoacylglycero)phospate, known also as lysobisphosphatidic acid, is a derivative of *sn*-1-glycerophospho-*sn*-1′-glycerol. This very uncommon structure has been found in the lipids of BHK cells (1), in pig and rabbit lung, as well as in rat liver (2); recently it was found also in cultured chicken cells.[1] Bis(monoacylglycero)phosphate is found specifically in lysosomes (3, 4). High levels of this lipid are present in the lysosomes in Niemann-Pick disease (5) and in drug-induced lipidoses (6, 7).

When incubated with lysosomal preparations, diphosphatidylglycerol and phosphatidylglycerol can be converted into a lipid that resembles bis(monoacylglycero)phosphate (8–11). However, the stereoconfiguration of the lipid formed in these experiments is not known. In order to see whether the bis(monoacylglycero)phosphate synthesized in vitro is in fact the natural compound, we have set up a procedure that allows the stereoanalysis of labeled glycerophospholipids. The present report describes this procedure and shows that, indeed, the bis(monoacylglycero)phosphate formed in vitro is identical with the natural derivative and has a backbone of *sn*-1-glycerophospho-*sn*-1′-glycerol.

MATERIAL AND METHODS

Preparation of the substrates

Bis(monoacylglycero)phosphate and phosphatidylcholine labeled with [^{3}H]glycerol were synthesized in BHK cells (clone Wi-2) grown on a serum-containing medium in the presence of [1,3-^{3}H]glycerol (5 mCi/2.5 mmol, New England Nuclear Corp.) for 20 hr; the radioactive medium was presented to full monolayers of BHK cells (12). [^{3}H]Glycerol-labeled phosphatidylglycerol was synthesized in *Staphylococcus aureus* grown in the presence of [1,3-^{3}H]glycerol for 10 hr (13).

[^{32}P]Bis(monoacylglycero)phosphate and [^{32}P]phosphatidylcholine were isolated from BHK cells incubated with [^{32}P]P_i (1 mCi/mmol, The Radiochemical Centre,

Abbreviations: BMP, bis(monoacylglycero)phosphate; DPG, diphosphatidylglycerol; α-GP, α-glycerophosphate (phosphate is linked to either of the two primary hydroxyl groups of glycerol); β-GP, β-glycerophosphate (phosphate is linked to the secondary hydroxyl group of glycerol); LPG, lysophosphatidylglycerol; PG, phosphatidylglycerol; P_i, inorganic phosphate.

[1] Somerharju, P. Unpublished observations.

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Amersham) for 60 hr. [^{32}P]Diphosphatidylglycerol was isolated from BHK cells labeled with [^{32}P]P_i for 20 hr. The phospholipids were extracted with chloroform–methanol (14) and isolated by preparative thin-layer chromatography (2).

Phosphatidyl[1′,3′-^{3}H]glycerol was obtained from egg lecithin and [1,3-^{3}H]glycerol with cabbage phospholipase D (15) and purified from the reaction mixture by CM-cellulose column chromatography (16). Lysophosphatidylglycerol was obtained from phosphatidylglycerol with phospholipase A_2 from *Crotalus adamanteus* according to Haverkate, Houtsmuller, and van Deenen (17).

Alkaline degradation of the lipids

The phospholipid was degraded with 0.15 M NaOH (0.2 ml) at 100°C for 24 hr. The alkaline hydrolyzate was passed through a small column of Dowex W50-X8 (H^+) (200–400 mesh) (2 ml, ϕ5 mm). The eluate (3–4 ml) was lyophilized and the neutralized hydrolyzate was dissolved in a small volume of water.

Enzymatic oxidation of the alkaline hydrolyzate and degradation of the dihydroxyacetone phosphate formed

The incubation mixture, pH 9.5, contained 0.1 M hydrazine, 0.5 M NAD^+, 10 μg of rabbit muscle *sn*-3-glycerophosphate dehydrogenase (E.C. 1.1.1.8) (Boehringer), and about 2000 cpm of [^{3}H]glycerol, or [^{32}P]phosphate of the neutralized alkaline hydrolyzate, in a total volume of 0.2 ml. The mixture was incubated for 1 hr at 37°C. Twenty μl of 10 M NaOH was added and the mixture was heated at 100°C for 20 min and was then again passed through a small Dowex 50W-X8 (H^+) column (2–3 ml). The eluate was lyophilized and dissolved in a small volume of water.

Separation of P_i, α- and β-glycerophosphate

The phosphates were chromatographed for 24 hr in propanol–25% ammonia–water 6:3:1 (v/v) on Whatman No. 1 paper treated with 0.4 M borate; alternatively they were chromatographed in methanol–25% ammonia–water 6:3:1 (v/v) on cellulose plates (Merck) sprayed with 0.4 M borate. Unlabeled P_i, α- and β-glycerophosphate were used to mark the location of the radioactive products; the unlabeled phosphates were visualized by the method of Hanes and Isherwood (18). The areas of the chromatograms containing the radioactive phosphates were cut for liquid scintillation counting.

Preparation of the lysosomes from rat liver

Sucrose gradient-purified lysosomes were obtained from male rats injected with Triton WR 1339 according to Franson, Waite, and LaVia (19).

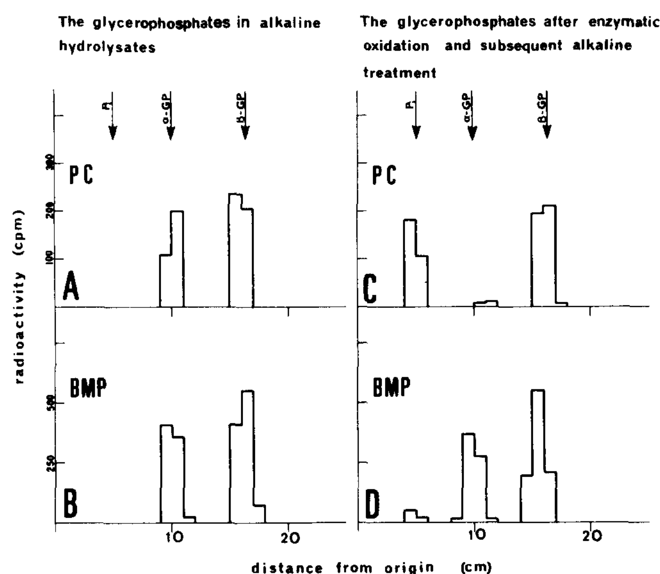

Fig. 1. Thin-layer chromatograms of products obtained after alkaline hydrolysis of lipids (*A* and *B*), and of products obtained after alkaline hydrolysis and enzymatic oxidation of the glycerophosphates and degradation of dihydroxyacetone phosphate (*C* and *D*). Chromatography performed on borate-treated cellulose plates (Merck) in methanol–ammonia–water 6:3:1 (v/v). Bands of 1.0-cm width were scraped from the dried chromatograms for liquid scintillation counting. PC, [^{32}P]phosphatidylcholine of BHK cells; BMP, bis(monoacylglycero)[^{32}P]phosphate of BHK cells; α-GP, α-glycerophosphate; β-GP, β-glycerophosphate.

In vitro synthesis of bis(monoacylglycero)phosphate

The substrate was blown to dryness with a nitrogen stream and the residue was dispersed in 1 ml of 50 mM sodium acetate buffer containing Triton X-100 (50 μg/ml) by sonication with a Branson sonicator for four 30-sec bursts.

The incubation was carried out essentially according to Poorthuis and Hostetler (9, 10); the actual conditions are described in the figure legends. After incubation the lipids were extracted from the incubation mixture according to Folch, Lees, and Sloane Stanley (14) and the radioactive lipids of the chloroform phase were analyzed by one-dimensional thin-layer chromatography on silica gel G plates (Merck) in chloroform–methanol–ammonia–water 65:20:2:2 (v/v). The labeled lipids were located with authentic unlabeled lipids which were detected according to Hanes and Isherwood (18), and areas corresponding to the radioactive lipids were scraped for liquid scintillation counting or extracted with chloroform–methanol 1:1 (v/v) for further analysis. Alternatively the lipid products were separated by two-dimensional thin-layer chromatography (20) and located by radioautography with Kodak X-ray film.

Liquid scintillation counting

Liquid scintillation counting was carried out in a Wallac 8100 liquid scintillation counter. Triton

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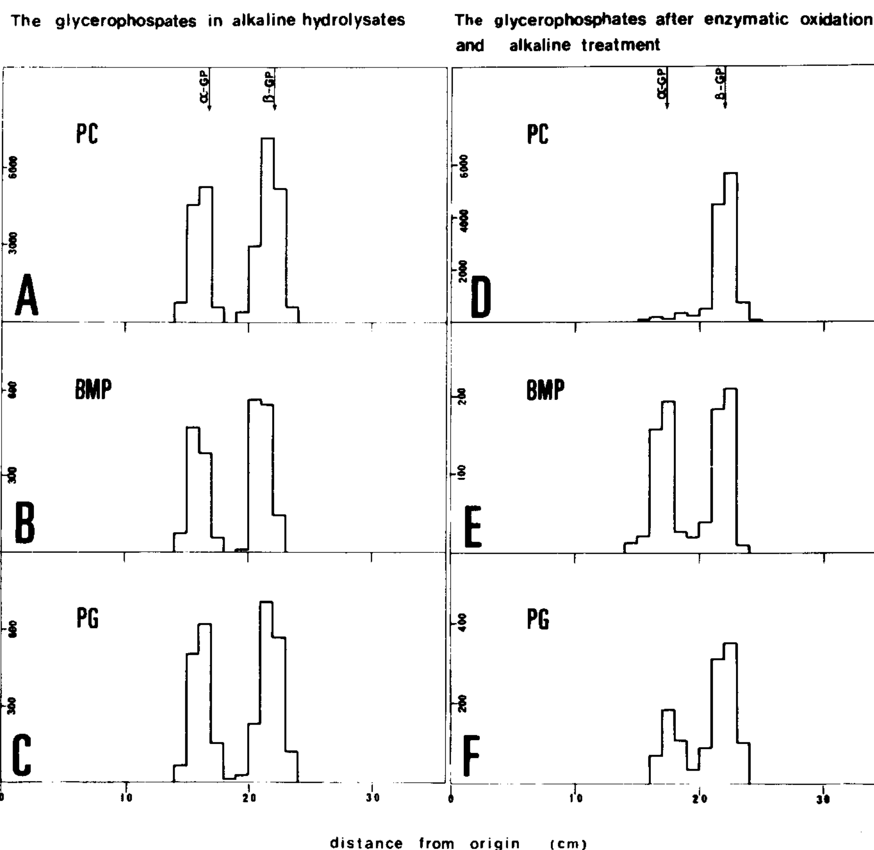

Fig. 2. Paper chromatograms of products obtained after alkaline hydrolysis of lipids (*A–C*), and products obtained after alkaline hydrolysis and enzymatic oxidation of the glycerophosphates and degradation of dihydroxyacetone phosphate (*D–F*). Chromatography was performed in 1-propanol–ammonia–water 6:3:1 (v/v). The chromatograms were cut into 1-cm sections for liquid scintillation counting. PC, [1,3-^{3}H]-phosphatidylcholine of BHK cells; BMP, [1,3,1′,3′-^{3}H]bis(monoacylglycero)phosphate of BHK cells; PG, [1,3,1′,3′-^{3}H]phosphatidylglycerol of *S. aureus*.

X-114-xylene 3:8 (v/v) containing 0.3% Permablend III (Packard) was used as the scintillation fluid for water-soluble components; toluene containing 0.5% Permablend was used for lipid-soluble molecules. Samples from silica gel G plates were measured in the toluene-based scintillation fluid containing 10% methanol. Generally, counting times of 20 min were used.

RESULTS

Procedure for analysis of stereoconfiguration of labeled glycerophospholipids

The radiochemical procedure used for the stereoanalysis of glycerophospholipids is a modification of the previous method used in our laboratory (1, 2, 21). In the procedure the lipids are subjected to strong alkaline degradation which yields a mixture of α- and β-glycerophosphates. The original configuration is preserved in the α-glycerophosphate (22), and the degraded lipid is analyzed by oxidizing it with *sn*-3-glycerophosphate dehydrogenase (E.C. 1.1.1.8.), which does not react with *sn*-1-glycerophosphate nor with the β-glycerophosphate. The oxidation product, dihydroxyacetone phosphate, is finally degraded with strong alkali to inorganic phosphate. In contrast, the *sn*-1-glycerophosphate and β-glycerophosphate are alkali-stable (23, 24). After chromatographic separation the P_i, the nonoxidizable α-glycerophosphate, and β-glycerophosphate are quantitated; the β-glycerophosphate serves conveniently as an internal standard.

Application of the procedure to model compounds

The products obtained from [^{32}P]phosphatidylcholine (diacylglycero[^{32}P]phosphocholine) of BHK cells after the first alkaline degradation were separated by chromatography as shown in **Fig. 1A;** α-glycero-[^{32}P]phosphate and β-glycero[^{32}P]phosphate were present in the ratio of 0.73. When this reaction mixture was oxidized enzymatically with NAD^+ and treated subsequently with alkali, a reaction mixture which is shown in Fig. 1*C* was obtained. The α-glycero-[^{32}P]phosphate, which was *sn*-3-glycerophosphate in agreement with the known stereoconfiguration of phosphatidylcholine (22), had been destroyed almost

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completely. The ratio of the $^{32}P_i$ to β-glycero[^{32}P]phosphate, 0.70, was nearly the same as the ratio of the α-glycero[^{32}P]phosphate to β-glycero[^{32}P]-phosphate prior to the enzymatic oxidation. Thus, almost all α-glycerophosphate, which had disappeared, had been converted to P_i.

Alkaline hydrolysis of bis(monoacylglycero)[^{32}P]-phosphate gave α- and β-glycero[^{32}P]phosphates in the ratio of 0.77 (Fig. 1*B*). After the oxidation and the alkaline treatment, the reaction mixture shown in Fig. 1*D* was obtained. The labeled α-glycerophosphate, which is mainly *sn*-1-glycerophosphate (1, 2), had resisted the oxidation and the subsequent alkaline treatment, and its relative amount had remained almost unchanged.

The stereoanalysis with [^{3}H]glycerol-labeled phosphatidylcholine and bis(monoacylglycero)phosphate (**Fig. 2**) gave the same information as ^{32}P-labeled compounds. The ratio of the α-[^{3}H]glycerophosphate to the β-[^{3}H]glycerophosphate in the alkaline hydrolyzates of phosphatidylcholine and bis(monoacylglycero)phosphate was 0.74 and 0.80, respectively (Fig. 2*A*, *B*). The enzymatic oxidation and the subsequent alkaline treatment gave ^{3}H-labeled α- and β-glycerophosphates in the ratio of 0.06 and 0.83, respectively, from phosphatidylcholine and bis(monoacylglycero)phosphate (Fig. 2*D*,*E*). Also in this experiment the *sn*-1-glycerophosphate remained intact and the *sn*-3-glycerophosphate was degraded. The third glycerol-labeled reference compound was metabolically labeled bacterial phosphatidylglycerol. The first alkaline hydrolyzate revealed labeled α- and β-glycerophosphate in the ratio of 0.79 (Fig. 2*C*), as expected. The oxidation and the alkaline treatment gave a reaction mixture shown in Fig. 2*F*. The labeled

TABLE 1. Stereoanalysis of bis(monoacylglycero)phosphate formed from [^{32}P]diphosphatidylglycerol

Lipid	α-Glycerophosphate/ β-Glycerophosphate	P_i/ β-Glycerophosphate
After alkaline hydrolysis of the lipid		
Bis(monoacylglycero)phosphate		
exp. 1	0.84	0.01
exp. 2	0.85	0.01
Phosphatidylcholine		
exp. 1	0.83	0.03
exp. 2	0.79	0.01
After enzymatic oxidation and subsequent alkaline treatment of the glycerophosphates		
Bis(monoacylglycero)phosphate		
exp. 1	0.81	0.05
exp. 2	0.77	0.04
Phosphatidylcholine		
exp. 1	0.03	0.80
exp. 2	0.06	0.73

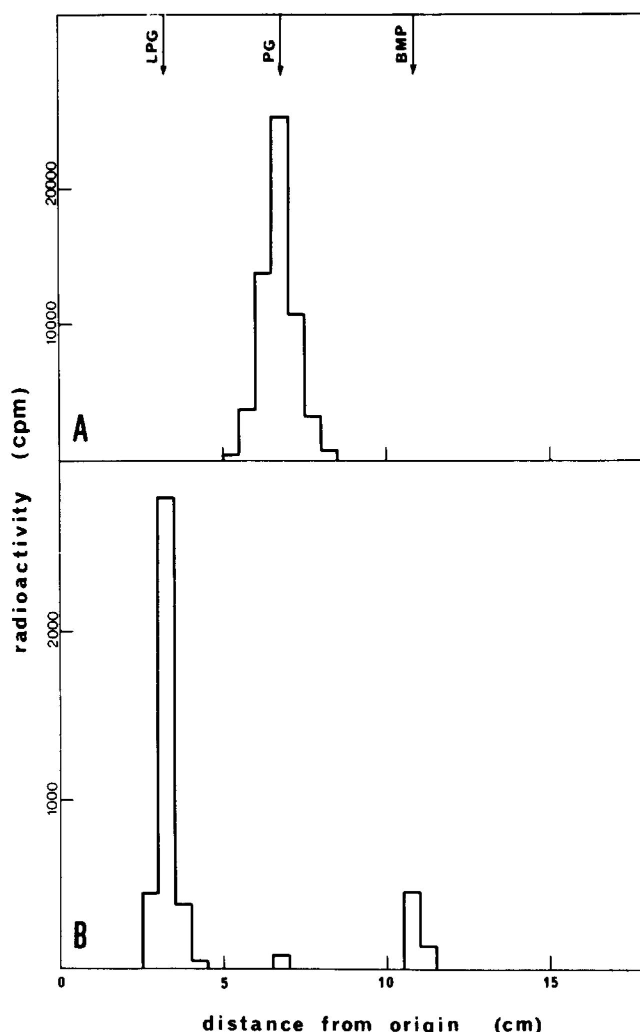

Fig. 3. Thin-layer chromatogram of an aliquot (1%) of phosphatidyl[1′,3′-^{3}H]glycerol used as the substrate (*A*), and of an aliquot (1%) of the radioactive lipids formed from phosphatidyl[1′,3′-^{3}H]-glycerol by lysosomal preparations of rat liver (*B*). The incubation mixture contained 50 mM sodium acetate buffer (pH 4.4), 5 mM $CaCl_2$, 10 mM mercaptoethanol, 1.5 mg of lysosomal protein, Triton X-100 (40 μg/ml), 5 × 10^{6} cpm of phosphatidyl[1′,3′-^{3}H]-glycerol in a final volume of 1.5 ml. Incubation was carried out for 12 hr at 37°C, followed by extraction of the lipids as described in Material and Methods. Thin-layer chromatography was done on a silica gel G plate in chloroform–methanol–ammonia–water 65:20:2:2 (v/v). LPG, lysophosphatidylglycerol; PG, phosphatidylglycerol; BMP, bis(monoacylglycero)phosphate.

α-glycerophosphate, which was an equimolar mixture of *sn*-1- and *sn*-3-glycerophosphates (17) had been destroyed to the extent of 42%.

Stereochemical configuration of bis(monoacylglycero)phosphate formed in vitro

The substrates used for synthesis of bis(monoacylglycero)phosphate in lysosomes of rat liver were metabolically labeled [^{32}P]diphosphatidylglycerol and chemically labeled phosphatidyl[1′,3′-^{3}H]glycerol which was obtained from egg lecithin and [1,3-^{3}H]glycerol in a transphosphatidylation reaction catalyzed by phospholipase D. This preparation is a racemic

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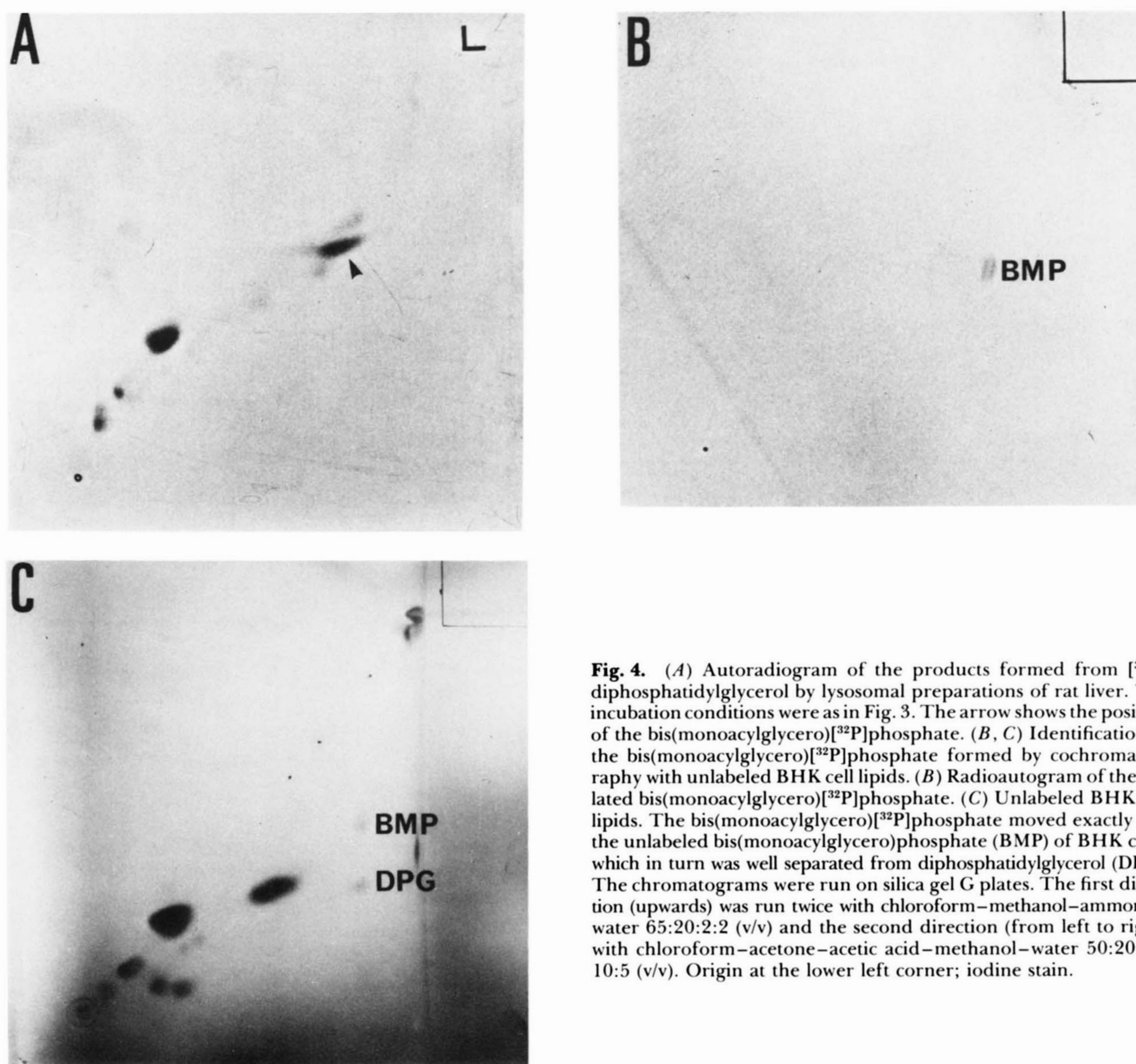

Fig. 4. (*A*) Autoradiogram of the products formed from [^{32}P]-diphosphatidylglycerol by lysosomal preparations of rat liver. The incubation conditions were as in Fig. 3. The arrow shows the position of the bis(monoacylglycero)[^{32}P]phosphate. (*B*, *C*) Identification of the bis(monoacylglycero)[^{32}P]phosphate formed by cochromatography with unlabeled BHK cell lipids. (*B*) Radioautogram of the isolated bis(monoacylglycero)[^{32}P]phosphate. (*C*) Unlabeled BHK cell lipids. The bis(monoacylglycero)[^{32}P]phosphate moved exactly like the unlabeled bis(monoacylglycero)phosphate (BMP) of BHK cells, which in turn was well separated from diphosphatidylglycerol (DPG). The chromatograms were run on silica gel G plates. The first direction (upwards) was run twice with chloroform–methanol–ammonia–water 65:20:2:2 (v/v) and the second direction (from left to right) with chloroform–acetone–acetic acid–methanol–water 50:20:10:10:5 (v/v). Origin at the lower left corner; iodine stain.

mixture of *sn*-3-phosphatidyl-*sn*-1′-glycerol and *sn*-3-phosphatidyl-*sn*-3′-glycerol (25).

Phosphatidyl[1′,3′-^{3}H]glycerol (5×10^6 cpm) was incubated with rat lysosomes for 12 hr in the presence of Triton X-100 exactly as described by Hostetler and Poorthuis (9, 10). After the incubation the lipids were extracted by the method of Folch et al. (14); about 5×10^5 cpm of radioactivity was obtained in the chloroform phase. The lipid extract was subjected to one-dimensional thin-layer chromatography on silica gel G plates (**Fig. 3*B***). Comparison with the chromatogram obtained from the substrate (Fig. 3*A*) suggests that almost all phosphatidylglycerol had disappeared; the major lipid-soluble product was lysophosphatidylglycerol. Bis(monoacylglycero)phosphate accounted for about 1% of phosphatidylglycerol initially present.

The bis(monoacylglycero)phosphate formed in vitro from phosphatidylglycerol was subjected to the stereoanalysis. Alkaline degradation gave α- and β-glycerophosphate in the ratio of 0.82 (exp. 1) and 0.81 (exp. 2) as expected. The oxidation and the subsequent alkaline treatment of this lipid gave nonoxidizable α-glycerophosphate and β-glycerophosphate in the ratio of 0.72 (exp. 1) and 0.75 (exp. 2). Thus the product of the in vitro synthesis appeared to be a derivative of *sn*-1-glycerophospho-*sn*-1′-glycerol.

The in vitro synthesis of bis(monoacylglycero)-phosphate using [^{32}P]diphosphatidylglycerol as the substrate was carried out likewise exactly according to Poorthuis and Hostetler (10): 4×10^5 cpm of [^{32}P]diphosphatidylglycerol was incubated with rat

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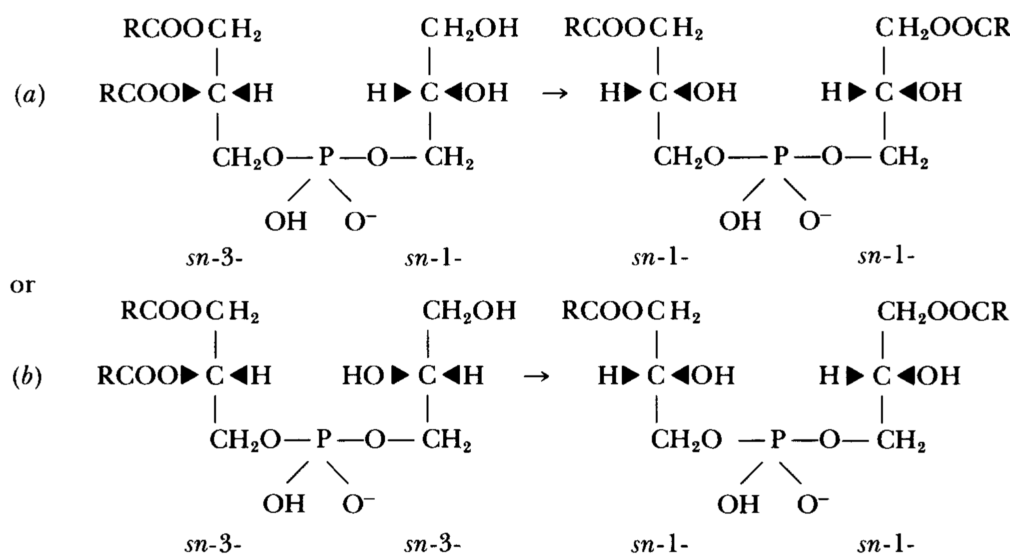
liver lysosomes for 12 hr. After incubation the radioactive lipid products were extracted as above and the chloroform phase (4×10^4 cpm) was subjected to two-dimensional thin-layer chromatography. The radioautogram (**Fig. 4*A***) shows that all diphosphatidylglycerol had disappeared, and in addition to lysocompounds, bis(monoacylglycero)phosphate was formed (about 7% of diphosphatidylglycerol initially present).

The bis(monoacylglycero)phosphate was isolated, and its identity was ascertained by cochromatography with unlabeled BHK cell lipids (Fig. 4*B* and 4*C*). In stereochemical analysis of this lipid, the ratio of α-glycero[^{32}P]phosphate to β-glycero[^{32}P]phosphate was 0.85 after alkaline hydrolysis (**Table 1**); practically no inorganic phosphate was liberated. After the enzymatic oxidation and the subsequent alkaline treatment, the ratio of the nonoxidizable α-glycero-[^{32}P]phosphate to β-glycero[^{32}P]phosphate was nearly the same, 0.79, and the ratio of the inorganic phosphate liberated from dihydroxyacetone phosphate to β-glycero[^{32}P]phosphate was 0.05. Thus the bis(monoacylglycero)phosphate formed from diphosphatidylglycerol was a derivative of *sn*-1-glycerophospho-*sn*-1′-glycerol. The reference compound, [^{32}P]phosphatidylcholine, gave similar results as in the experiment shown in Fig. 1.

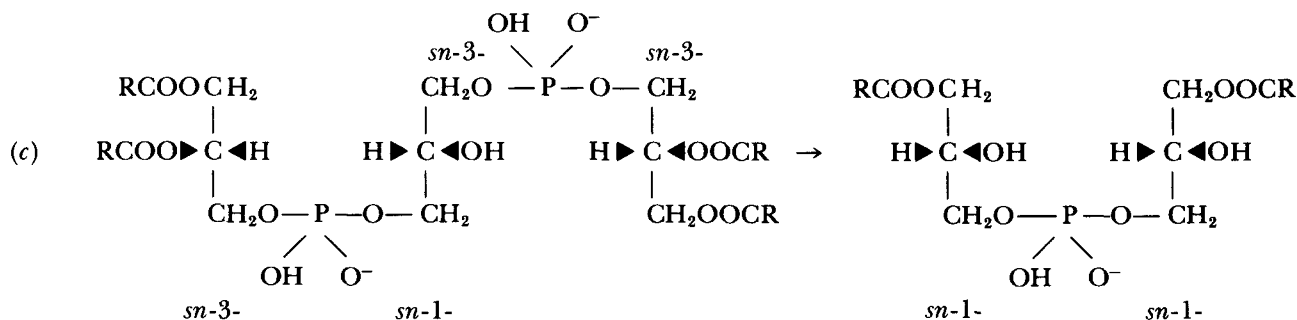
DISCUSSION

The present results confirm the findings of Poorthuis and Hostetler (9, 10) showing that phosphatidylglycerol and diphosphatidylglycerol can be converted in rat lysosomes into bis(monoacylglycero)phosphate. Our data show further that the lipid formed is a derivative of *sn*-1-glycerophospho-*sn*-1′-glycerol, the same backbone that is found in the natural bis(monoacylglycero)phosphate. The phosphatidylglycerol used as precursor was a mixture of *sn*-3-phosphatidyl-*sn*-1′-glycerol and *sn*-3-phosphatidyl-*sn*-3′-glycerol. Therefore the overall reaction was either



or both. At least the reaction (*a*) may have taken place. Poorthuis and Hostetler (9, 10) used pure *sn*-3-phosphatidyl-*sn*-1′-glycerol as the substrate and were able to generate the bis(monoacylglycero)phosphate. Exactly the same conditions were used in their experiments and in ours. Therefore some bis(monoacylglycero)phosphate must have been formed even in our experiments from *sn*-3-phosphatidyl-*sn*1′-glycerol.

With diphosphatidylglycerol as substrate the overall reaction is:



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A key feature of all reaction sequences ($a-c$) leading to the bis(monoacylglycero)phosphate is a change in the substrate's original backbone into *sn*-1-glycerophospho-*sn*-1′-glycerol. Another feature is the transfer of an acyl group to the *sn*-1-glycerophosphate moiety. The individual steps of the reaction sequences are not known, but our data show that the nonacylated, *sn*-1-phosphorylated glycerol residue of phosphatidylglycerol and the phosphate moiety of the diphosphatidylglycerol are transferred to the final product.

The yields of bis(monoacylglycero)phosphate from the in vitro incubations were rather low, lower even than in the experiments of Poorthuis and Hostetler (9, 10). The low yields may have resulted from ongoing metabolism of bis(monoacylglycero)phosphate in the lysosomes; our incubations were longer than those of Poorthuis and Hostetler and may have been longer than required for maximal yields. It is therefore possible that the bis(monoacylglycero)phosphate which was analyzed in our experiments represents a slowly degraded component in an amount less than that synthesized. In fact, we have found that the bis(monoacylglycero)phosphate that is formed initially is rather rich in *sn*-3-glycerophosphate residues whereas, at later stages of the synthesis, the bis(monoacylglycero)phosphate is rich in *sn*-1-glycerophosphate residues[2] (26).

Like the method previously used in our laboratory (1, 2, 21), the micromethod set up in the present study is based on the enzymatic oxidation of the α-glycerophosphate formed in the alkaline degradation of the glycerophospholipid. This new method is suitable for the analysis of the glycerophospholipids labeled with [^{14}C]glycerol, [^{3}H]glycerol, and [^{32}P]phosphate. In the analysis of structures yielding small amounts of *sn*-3-glycerophosphate among *sn*-1-glycerophosphate, the present procedure is most sensitive for ^{32}P-labeled phospholipids, because the $^{32}P_i$ liberated from *sn*-3-glycerophosphate via dihydroxyacetone phosphate is more easily detected and measured than the small loss of ^{3}H- and ^{14}C-labeled α-glycerophosphate. In the search for small amounts of lipids containing the *sn*-1-glycerophosphate moiety, the quantitation of the labeled α-glycerophosphate resistant to the enzymatic oxidation should prove particularly sensitive and advantageous.

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