The stereochemical configuration of lysosomal phosphatidylcholine and phosphatidylethanolamine: comparison with lysobisphosphatidic acid

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Abstract Lysosomal phosphatidylcholine and phosphatidylethanolamine were isolated from liver of rats treated with Triton WR1339 and from cultured BHK-cells. Stereochemical analysis proved that these lipids, in contrast to the lysosomal lysobisphosphatidic acid, were derivatives of *sn*-glycero-3-phosphate.—**Joutti, A., and O. Renkonen.** The stereochemical configuration of lysosomal phosphatidylcholine and phosphatidylethanolamine: comparison with lysobisphosphatidic acid. *J. Lipid Res.* 1979. **20:** 230–233.

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Lysobisphosphatidic acid, known also as bis(monoacylglycero)phosphate, is a phospholipid of unusual structure; its backbone is *sn*-1-glycerophospho-*sn*-1′-glycerol (1–3), a molecule in which the phosphate is esterified with the glycerol residues at C-1. In contrast, all usual phospholipids carry the phosphate group at C-3 of the glycerol moiety (4). Lysobisphosphatidic acid is a lysosomal component; it is not found in other cellular organelles in significant concentrations (5–7). To see whether this odd phospholipid is accompanied by other derivatives of *sn*-glycero-1-phosphate in lysosomes, we have analyzed the stereochemical configuration of lysosomal phosphatidylcholine and phosphatidylethanolamine from liver of rats treated with Triton WR1339 and from BHK-cells.

MATERIALS AND METHODS

Male rats received intraperitoneal injections of 150 mg of Triton WR 1339 dissolved in distilled water over a 20-day period, after which gradient-purified liver lysosomes were obtained according to Franson, Waite, and LaVia (8). Floating lysosomes were isolated from fresh monolayers of cultured BHK-cells as described (6). The lysosomal lipids were extracted (9). The purity of the lysosomal fractions was checked by two-dimensional thin-layer chromatography of phospholipids (10) and by phosphorus analysis (11).

The lysosomal phosphatidylcholine and phosphatidylethanolamine were isolated from the lysosomal lipids by preparative thin-layer chromatography on silica gel G plates (Merck) (3). The liver phosphatidylcholine was obtained from total lipid extract by silicic acid column chromatography (3). Plasmalogens were eliminated from the lipids prior to the stereoanalysis by mild acid methanolysis (12).

Treatment of total phosphatidylcholine with phospholipase A_2 was done according to Hanahan, Brockerhoff, and Barron (13). About 40 μmol of the liver phosphatidylcholine was dissolved in 3 ml of ether and the enzyme solution (500 μg of phospholipase A_2 from *Crotalus adamanteus* in 0.5 ml of imidazole buffer, pH 7.0, containing 1 mg of $CaCl_2$) was added. The reaction was monitored by separation of phosphatidylcholine and lysophosphatidylcholine by thin-layer chromatography. At the end of the reaction the unhydrolyzed phosphatidylcholine was separated from the reaction mixture by preparative thin-layer chromatography.

Stereoanalysis of the isolated phospholipids was carried out as previously described (1–3). The phospholipids (0.5–1 μmol) were degraded with 0.15 M NaOH at 100°C for 24 hr and the hydrolysate was passed through a small column of Dowex 50 W-X8 (NH_4^+). The eluate was taken to dryness, and the residue was dissolved in distilled water. The pH of the hydrolysate was adjusted to 5 with dilute HCl, and the fatty acids were extracted with ether. Total phosphate was analyzed from one aliquot of the water phase, and a second sample of the hydrolysate was oxidized with

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NAD+ and *sn*-glycero-3-phosphate dehydrogenase (14). Another sample of the hydrolysate was subjected to thin-layer chromatography on a cellulose plate (Merck); the solvent used was 1-propanol–ammonia–water 6:3:1 (v/v). This system separated α-glycerophosphate from β-glycerophosphate. The chromatograms were stained with Hanes–Isherwood reagent (15) and the phosphorus content of the α-glycerophosphate and β-glycerophosphate spots was determined.

RESULTS AND DISCUSSION

The present experiments describe the analysis of phosphatidylcholine and phosphatidylethanolamine from two lysosomal preparations, Triton WR1339-filled lysosomes of rat liver, and lipid-filled lysosomes of BHK-cells. The purity of the lysosomal preparations was estimated by analysis of their phospholipid compositions, which are given in **Table 1** together with the data of the corresponding homogenates.

The lipid composition of the Triton-filled lysosomes was similar to that described by Wherret and Huterer (16) and by Weglicki, Ruth, and Owens (17), which confirms the purity of the "tritosomes" in our experiments. In comparison to the lipids of the whole liver, the Triton-filled lysosomes had a higher content of lysobisphosphatidic acid. This "lysosomal marker lipid" (18) was enriched about 10-fold in the tritosomes. On the other hand, the tritosomes contained no cardiolipin; this eliminates the possibility of the mitochondrial contaminations.

The amount of lysobisphosphatidic acid in the floating lysosomes of the BHK-cells was lower than in the tritosomes; however, even in this fraction lysobisphosphatidic acid was enriched 3-fold. The concentration of cardiolipin was also decreased. Reflotation of the lysosomes (6) would have increased the purity of the lysosomes of the BHK-cells; in this experiment reflotation was not possible due to the small amount of material.

The lysosomal phosphatidylcholine and phosphatidylethanolamine were extracted from the lysosomal preparations and isolated by two-dimensional thin-layer chromatography. Typical chromatograms are shown in **Fig. 1.**

TABLE 1. Composition of the lysosomal phospholipids from rat liver and from BHK-cells

	Rat Liver		BHK-Cells	
Phospholipids	Homogenate	Triton-Filled Lysosomes	Homogenate (6)	Floating Lysosomes
	% of lipid phosphorus			
Lysobisphosphatidic acid	1.1	12.2	1.7	5.6
Phosphatidylcholine	51.8	45.1	55.1	44.7
Phosphatidylethanolamine	28.0	24.3	25.7	37.3
Cardiolipin	3.1	n.d.[a]	3.2	0.8
Sphingomyelin	7.5	16.2 (Sphingomyelin + Phosphatidylinositol)	6.5	5.8
Phosphatidylinositol	4.5		2.3	4.6 (Phosphatidylinositol + Phosphatidylserine)
Phosphatidylserine	3.3	2.2	4.3	

[a] n.d., not detected.

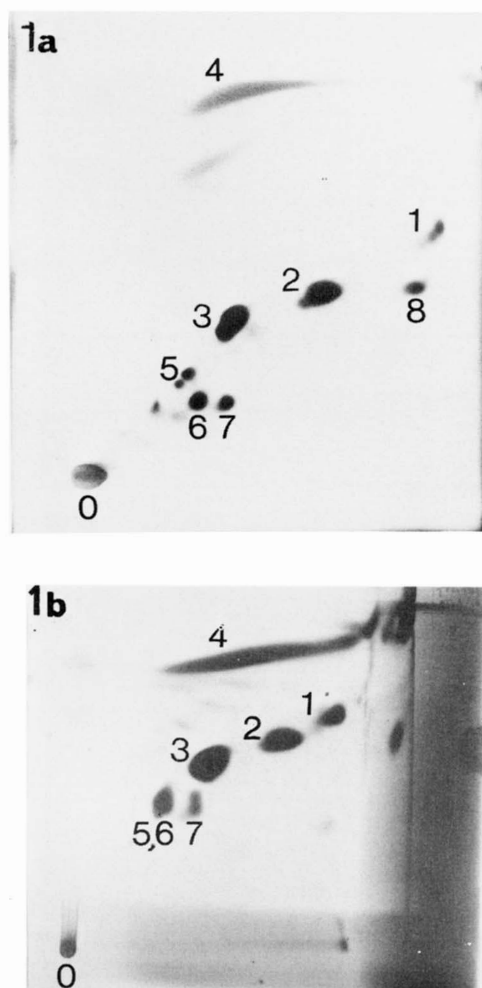

Fig. 1. The phospholipids of (*a*) rat liver and (*b*) Triton WR1339-filled lysosomes analyzed by two-dimensional thin-layer chromatography (10). The lipids were applied to the lower left corner of the plates. The first direction (upwards) was run twice with chloroform–methanol–7 N ammonia 65:20:4 (by vol) and the second direction (from left to right) with chloroform–acetone–methanol–acetic acid–water 50:20:10:10:5 (by vol). Iodine stain—*1*, lysobisphosphatidic acid; *2*, phosphatidylethanolamine; *3*, phosphatidylcholine; *4*, Triton WR1339, *5*, sphingomyelin; *6*, phosphatidylinositol; *7*, phosphatidylserine; *8*, cardiolipin; and *O*, origin.

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TABLE 2. Analysis of glycerophosphates formed in alkaline degradation of the lysosomal phospholipids[a]

Lipids	α-Glycerophosphate[b]	Enzymically Oxidizable Glycerophosphate[c]	
		mol/molP	
sn-Glycero-3-phosphate[d]	1.00	0.99 ± 0.04	(4)[e]
Egg lecithin	0.42	0.40 ± 0.02	(4)
Rat liver lysobisphosphatidic acid	0.42	0.02	
Lysosomal phosphatidylcholine of rat liver	0.41	0.37 ± 0.02	(4)[f]
Lysosomal phosphatidylethanolamine of rat liver	0.41	0.36 ± 0.02	(4)[f]
Lysosomal phosphatidylcholine of BHK-cells	0.44	0.38	(1)[f]
Lysosomal phosphatidylethanolamine of BHK-cells	0.41	0.37	(1)[f]
Phosphatidylcholine of total rat liver unhydrolyzed by phospholipase A_2	0.42	0.38	(1)

[a] The phospholipids were degraded with alkali, and α-glycerophosphate and enzymatically oxidizable glycerophosphate were analyzed in the hydrolysate.

[b] Total phosphate is assumed to equal the sum of α- and β-glycerophosphate which were measured by thin-layer chromatography. One analysis only.

[c] Oxidizable glycerophosphate was measured by oxidation with NAD^+ and *sn*-glycero-3-phosphate dehydrogenase; total phosphate was actually measured here. The results are mean ± SD.

[d] This sample was not treated with alkali before analysis.

[e] The number of experiments.

[f] The difference of 10% between the α-glycerophosphate and the oxidizable phosphate is probably due to different methods used to estimate the total phosphate in the two analyses.

The stereoconfiguration of the phospholipids was analyzed by studying glycerophosphates produced during alkaline degradation of the lipids. This treatment removes the fatty acids and cleaves the phosphate diesters to yield a mixture of α-glycerophosphate and β-glycerophosphate; no other phosphate-containing compounds are believed to be present in significant concentrations (19, 20). The stereochemical configuration of the original lipid is known to be preserved in the α-glycerophosphate formed during alkaline degradation (19, 20).

Analysis of the glycerophosphates formed from diacyl-*sn*-3-glycerophosphocholine (egg lecithin) and from monoacyl-*sn*-1-glycerophospho-*sn*-1′-monoacylglycerol (rat liver lysobisphosphatidic acid) shows two examples of the process (**Table 2**). Both lipids yielded hydrolysates containing α-glycerophosphate (0.42 mol) and β-glycerophosphate (0.58 mol) as the only phosphate-containing compounds visible on thin-layer chromatograms. However, the enzymatic oxidation of the two hydrolysates with NAD^+ revealed a difference. The glycerophosphate mixture obtained from lysobisphosphatidic acid was not oxidized at all; the β-glycerophosphate and the *sn*-1-form of α-glycerophosphate present in the hydrolysate remained intact. In contrast, the glycerophosphates of egg lecithin were oxidized, consuming 0.40 mol of NAD^+. Since the 0.58 mol of β-glycerophosphate did not seem to react, the NAD^+ consumption was due to the α-glycerophosphate.

The lysosomal phosphatidylcholine and phosphatidylethanolamine from rat liver and BHK-cells also gave α- and β-glycerophosphate upon alkaline degradation. The ratio of α-glycerophosphate to β-glycerophosphate, as revealed by thin-layer chromatography, was in all cases 0.4/0.6, which is the same value that has been obtained with several glycerophospholipids (1–3). The data are presented in Table 2 as ratios of α-glycerophosphate to total phosphate, the latter representing the sum of α-glycerophosphate and β-glycerophosphate, the only phosphate-positive components in the hydrolysates. Enzymatic oxidation of the glycerophosphates showed that the lysosomal phosphatidylcholines and phosphatidylethanolamines resembled egg lecithin but differed from lysobisphosphatidic acid; 0.4 mol of the glycerophosphates in each hydrolysate were oxidized and 0.6 mol were not (Table 2). Accordingly, most of the lysosomal phosphatidylcholines and phosphatidylethanolamines were derivatives of *sn*-glycero-3-phosphate.

Although the structure of most of the lysosomal phosphatidylcholines from rat liver appeared to be settled, the possibility remained that some of them could have been derivatives of *sn*-1-glycerophosphate; the oxidizable glycerophosphate appeared to correspond to 90% of the α-glycerophosphate of this lipid. The following experiment was designed to search for small amounts of *sn*-glycero-1-phosphate in liver lecithins. The bulk, 99.3%, of the liver lecithins of Triton WR1339-treated rats was degraded with phospholipase A_2 of *Crotalus adamanteus* in a reaction stopped before completion. The fraction of phosphatidylcholines remaining intact after the treatment of this strictly stereospecific enzyme (21) should have contained all derivatives of *sn*-1-glycerophosphate initially present. Therefore this fraction of the surviving liver lecithins should have been a richer source of the derivatives of *sn*-1-glycerophosphate than the tritosomal lecithins. This notion is based on the following arguments. The lysobisphosphatidic acid content of total liver phospholipids was 1.2% (Table 1); all of this lysobisphosphatidic acid is lysosomal (5, 6). The amount of phosphatidylcholine in the tritosomes was four times higher than the amount of lysobisphosphatidic acid (Table 1). Therefore about 5% (=4 × 1.2%) of total phospholipids of liver should have

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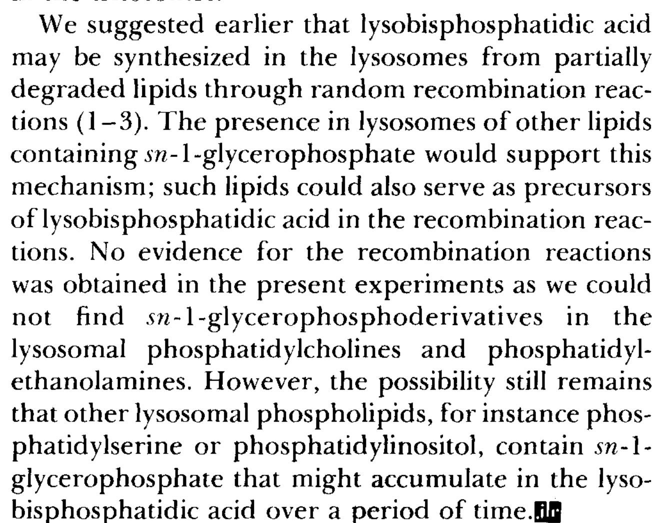
been tritosomal lecithins. Accordingly, about 10% of the total lecithins of the liver were tritosomal. This is a much larger fraction of the total lecithins than the fraction surviving the phospholipase A_2 treatment in our experiment, yet both should contain the same absolute amount of *sn*-1-glycerophosphate-based lecithins if these would be present. The fraction of the surviving lecithins after the phospholipase A_2 treatment was finally degraded with alkali and the glycerophosphates were analyzed. As shown in Table 2, the resulting glycerophosphates were quite similar to those of the lysosomal lecithins; most of the α-glycerophosphate was oxidizable with NAD^+. This suggests that no *sn*-1-glycerophosphate lecithins were present in the tritosomes.



We suggested earlier that lysobisphosphatidic acid may be synthesized in the lysosomes from partially degraded lipids through random recombination reactions (1–3). The presence in lysosomes of other lipids containing *sn*-1-glycerophosphate would support this mechanism; such lipids could also serve as precursors of lysobisphosphatidic acid in the recombination reactions. No evidence for the recombination reactions was obtained in the present experiments as we could not find *sn*-1-glycerophosphoderivatives in the lysosomal phosphatidylcholines and phosphatidylethanolamines. However, the possibility still remains that other lysosomal phospholipids, for instance phosphatidylserine or phosphatidylinositol, contain *sn*-1-glycerophosphate that might accumulate in the lysobisphosphatidic acid over a period of time.

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