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The Action of Lysosomal Lipolytic Enzymes on Alkyl Ether-Containing Phospholipids

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Summary: Alkyl ether-containing phospholipids were subjected to the lipolytic enzymes present in the soluble and membrane fractions of rat liver lysosomes. The following substrates were used:

1) (1-octadecyl-2-linoloyl-glycero-3-phosphoryl)-ethanolamine,
2) (1-octadecyl-glycero-3-phosphoryl)ethanolamine,
3) 1-octadecyl-2-linoloyl-glycero-3-phosphate and
4) 1-octadecyl-glycero-3-phosphate.

Lysosomes are devoid of an alkyl ether-splitting enzyme. The soluble phospholipase A_2 with an acidic pH-optimum (pH 4.5) and the lysosomal membrane bound phospholipase A_2 with alkaline pH-optimum (pH 8.0) hydrolyze the fatty acid in the 2-position. (1-Octadecyl-glycero-3-phosphoryl)ethanolamine is not further degraded by other lysosomal enzymes. 1-Octadecyl-2-linoloyl-glycero-3-phosphate is hydrolyzed by lysosomes to 1-octadecyl-2-linoloyl-glycerol, batyl alcohol and 1-octadecyl-glycero-3-phosphate. The latter is the substrate of an acid phosphatase which yields batyl alcohol. The formation of 1-octadecyl-2-linoloylglycerol indicates that the alkyl ether analog of phosphatidic acid is also the substrate of the lysosomal phosphatidic acid phosphatase.

Die Wirkung von lysosomalen lipolytischen Enzymen auf alkylätherhaltige Phospholipide

Zusammenfassung: Es wurde der Einfluß der löslichen und Membranfraktion aus Rattenleberlysosomen („Tritosomen") in vitro auf folgende Substrate untersucht:

1. (1-Octadecyl-2-linoloyl-glycero-3-phosphoryl)-äthanolamin,
2. (1-Octadecyl-glycero-3-phosphoryl)äthanolamin,
3. 1-Octadecyl-2-linoloyl-glycero-3-phosphat,
4. 1-Octadecyl-glycero-3-phosphat.

Die Lysosomen besitzen kein die Ätherbindung spaltendes Enzym. Die lösliche Phospholipase A_2 mit saurem pH-Optimum (pH 4.5) und die an die lysosomale Membran gebundene Phospholipase A_2 mit alkalischem pH-Optimum (pH 8.0) spalten die 2-ständige Fettsäure. Das gebildete (1-Octadecyl-glycero-3-phosphoryl)äthanolamin kann in den Lysosomen nicht weiter abgebaut werden. 1-Octadecyl-2-linoloyl-glycero-3-phosphat wird zu 1-Octadecyl-glycero-3-phosphat, 1-Octadecyl-2-linoloyl-glycerin und Batylalkohol hydrolysiert. Die Bildung von 1-Octadecyl-2-linoloylglycerin beweist, daß das Alkyläther-Analoge der Phosphatidsäure ebenfalls das Substrat der lysosomalen Phosphatidsäurephosphatase ist.

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Enzymes:

Acid phosphatase, orthophosphoric-monoester phosphohydrolase (acid optimum) (EC 3.1.3.2)
Glycerophosphorylcholine diesterase, L-3-glycerylphosphorylcholine glycerophosphohydrolase (EC 3.1.4.2)
Phosphatidate phosphatase, L-α-phosphatidate phosphohydrolase (EC 3.1.3.4)
Phospholipase A_2, phosphatide 2-acyl-hydrolase (EC 3.1.1.4)
Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3)
Phospholipase D, phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4).
Triacylglycerol lipase, triacylglycerol acyl-hydrolase (EC 3.1.1.3)

Plasmalogens represent a class of widely distributed phospholipids. The 1′-alkenyl ether substituent linked to position 1 of the glycerophosphate backbone is characteristic for these compounds, which represent about 50% of the phosphatidylethanolamine fraction of brain phospholipids, but also occur in numerous other organs and organisms.

The predominant chains are the hexadecanyl, octadecanyl and 9-octadecenyl residues. Their origin is of interest and has been studied for a long time but recent studies in vivo and in vitro have established the 1-alkyl ethers of glycerophospholipids as their immediate precursors[1–4]. The desaturation of the alkyl ether to the vinyl ether group apparently occurs after the formation of the ether bond. The stereochemistry of the reaction has been established as a *cis*-elimination of the 1*S*, 2*S* prochiral hydrogen atoms of the corresponding glyceryl ether[5]. So far there is insufficient evidence to permit a decision as to whether this desaturation occurs at the level of (2-acyl-1-alkyl-glycero-3-phosphoryl)ethanolamine [6,7], its lyso-derivative[8], or at the level of 2-acyl-1-alkyl-glycero-3-phosphate, of 1-alkyl-glycero-3-phosphate or 1-alkyl-dihydroxyacetonephosphate.

The basic mechanism of the ether formation between long chain alcohols and dihydroxyacetone phosphate or 3-glycerophosphate is also not sufficiently understood.

Microsomal enzyme preparations from mammalian liver[9–11] and from *Tetrahymena pyriformis*[12,13] and dog fish[14] oxidize the *O*-alkyl ether bond, reduced pteridine being required. The reaction product is the corresponding long chain aldehyde[15].

The results presented in this report should further contribute to our understanding of the catabolism of alkylether-containing phospholipids. The function of rat liver lysosomes ("tritosomes")[16] in this process and the characteristics of lysosomal lipolytic enzymes, which act on the four possible structures of the alkyl ether-containing phospholipids has been defined.

In view of studies in vivo it seemed especially important to understand the possibly metabolic fate of intermediates and precursors.

Results

The following alkyl ether-containing phospholipids have been synthesized and used as substrates of soluble lysosomal matrix and lysosomal membrane enzymes:

(2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)ethanolamine,

(1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)-ethanolamine,

2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate and 1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate.

These substrates could be easily solubilized by ultrasonication and formed clear micellar solutions. Lysosomes were prepared as subcellular particles filled with Triton WR-1339[16] and they could be obtained by density gradient centrifugation almost free of contamination by other cell constituents. Our previous studies also demonstrated that the enzyme constituents of "tritosomes" and genuine secondary lysosomes were comparable*. Since our first report on lipolytic enzymes in lysosomes ("tritosomes"), namely phospholipases, lipase and cholesterolester hydrolase[17], numerous reports have contributed further to our present knowledge of the enzymes that hydrolyze complex lipids. Using double labelled diacylglycerophosphorylethanolamine and phosphatidic acid we were able to demonstrate the presence of phospholipase A_1 and A_2 with acidic pH-optima, which are present as matrix enzymes[18]; simultaneously a phospholipase A_2, which has an alkaline pH-optimum and is bound to the lysosomal membrane, has been described[18, 19].

1. (2-Linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)ethanolamine

The substrate was solubilized by ultrasonication in the presence of serum albumin (2 mg/m*l*). Incubations with whole lysosomes as the source of enzyme were carried out under hyposmotic conditions at different pH-values between 3 and 9. The total lipid extracts of the in-vitro incubations were separated by thin-layer chromatography in two successive solvent systems which permit the separation of all possible hydrolysis products. The following results can be derived from Fig. 1.

a) The alkyl ether glycerophospholipid is hydrolyzed by lysosomal enzymes only to the corresponding lyso compound.

b) Since only the alkyl ether group is labelled in the substrate and the only labelled product cochroma-

* Henning, R. & Heidrich, H. J., in preparation.

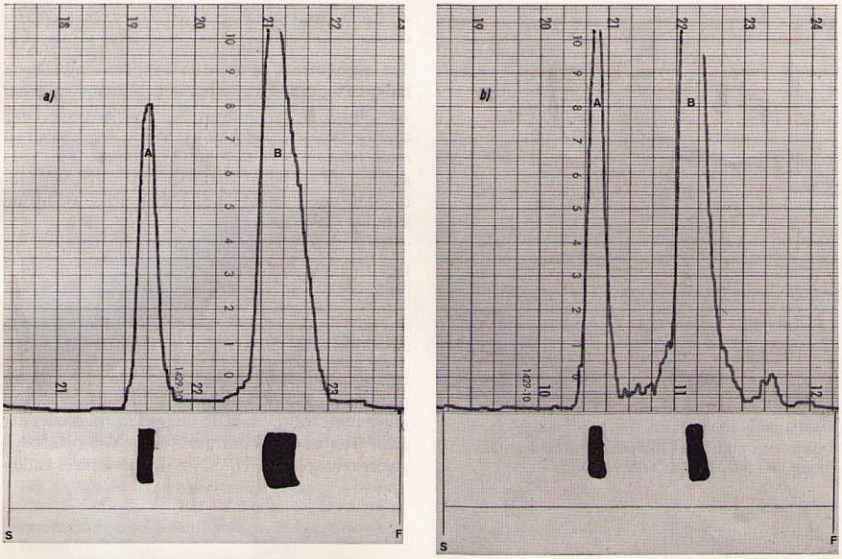


Fig. 1 a). Radiochromatographic analysis of products after incubation of (2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)ethanolamine with rat liver lysosomes.

Total volume: 1 m*l*, containing: 0.1 μmol substrate, 0.1M acetate buffer, pH 4.5, 100 μg lysosomal protein. Incubation for 60 min at 37°C. A = (1-octadecyl-glycero-3-phosphoryl)ethanolamine; B = substrate. Solvent system: chloroform/methanol/water 65:25:4. Spots were visualized with ninhydrin-Zinzadze reagent and by charring.

b) The same conditions as under 1a except (2-linoloyl-1-[9,10^3-H_2]oleoyl-glycero-3-phosphoryl)ethanolamine (0.02 μmol) was the substrate. A = (1-[9,10-$^3H_2$]oleoyl-glycero-3-phosphoryl)ethanolamine; B = substrate.

tographed with (1-alkyl-glycero-3-phosphoryl)ethanolamine it can be concluded, that the alkyl ether bond cannot be cleaved by the lysosome.

c) The pH-optimum is at pH 4.6 (Fig. 2).

d) Neither a phospholipase C which could release 2-linoloyl-1-[9,10-$^3H_2$]octadecylglycerol nor a phosphodiesterase for the hydrolysis of the product (1-octadecyl-glycero-3-phosphoryl)ethanolamine is present in lysosomes.

In order to test whether the phospholipase A_2 activity with the acidic pH-optimum is identical with the enzyme we described before, which hydrolyses the 2-position fatty acids of diacylphosphoglycerides, parallel experiments were carried out with (2-[1'-^{14}C]linoloyl-1-[9,10^{-3}H_2]oleoyl-glycero-3-phosphoryl)ethanolamine as substrate. The optimal pH-value for the hydrolysis was again pH 4.5. The matrix phospholipase A_2 is strongly inhibited by $Ca^{2\oplus}$ ions and Triton W-1339 as demonstrated before[19]. Comparative inhibition experiments with the alkyl ether (Fig. 3a) and diacyl derivatives (Fig. 3b) as substrates showed an identical and almost complete inhibition of the hydrolysis of the two substrates again referring to the same enzyme. The alkyl ether phospholipid molecule is a suitable substrate for the determination of the K_m since only one product is formed; and this product is not further degraded, unlike the diacylphospholipids, which are also attacked by the lysosomal phospholipase A_1. We determined the K_m as 1.5×10^{-4}M.

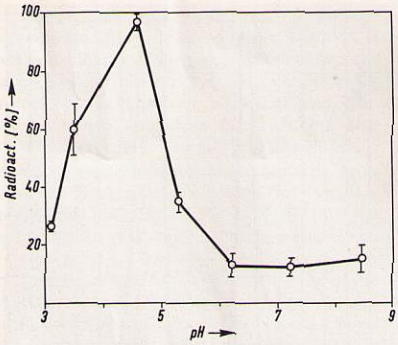


Fig. 2. pH Optimum of lysosomal matrix phospholipase A_2.

Conditions of incubations and analysis were the same as described under 1a. Buffers for pH 3.6 to 5.0 0.1M acetate; pH 5 to 9. 0.1M Tris/maleate.

2. (1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)-ethanolamine

The experiments reported in the preceding section indicate that the lyso compound with an alkylether substituent in position 1 of the glycerol moiety cannot be further degraded either by lysosomal matrix enzymes or membrane enzymes. This was again verified when the lyso derivative was incubated under different pH-conditions, with and without $Ca^{2\oplus}$ ions.

The lysosomal phosphodiesterase is unable to attack this lyso derivative.

3. 2-[1-^{14}C]Linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate

Lysosomes hydrolyze the alkyl analog of phosphatidic acid to acyl-alkyl-glycerol with the loss of phosphoric acid, then to alkylglycerol and fatty acid. The above substrate 2-linoloyl-1-octadecyl-glycerophosphate ($^3H/^{14}C$ = 47) gave three radio-

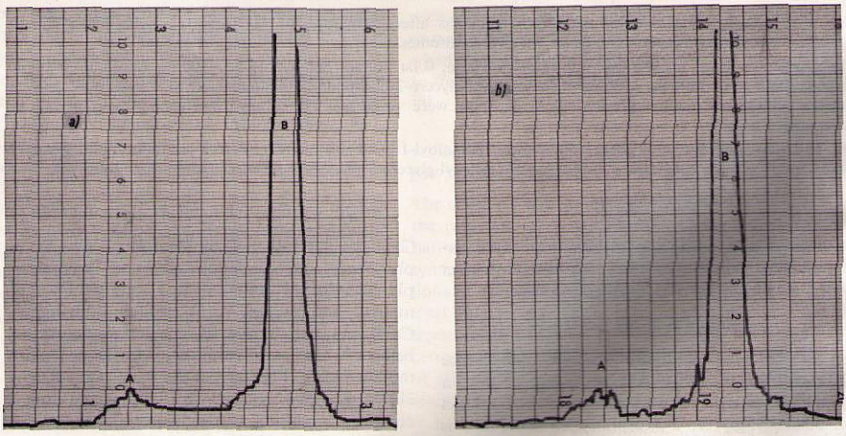
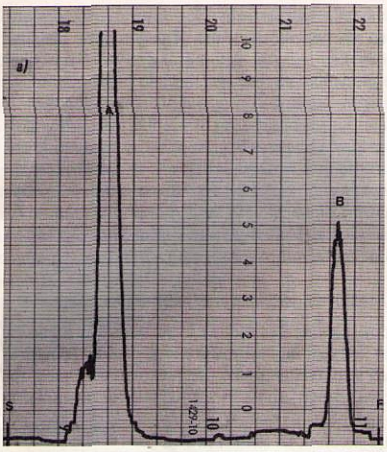


Fig. 3. a) Incubation of lysosomal matrix acidic phospholipase A_2.

Radio thin-layer analysis of products after incubation of (2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)-ethanolamine as described under Fig. 1a except in the presence of 10mM $Ca^{2\oplus}$. A = traces of lyso-compound; B = substrate.

b) Inhibition of lysosomal membrane alkaline phospholipase A_2 in the presence of Triton WR-1339. A = traces of lyso-compound; B = substrate. Conditions and analyses as described for Fig. 1a.



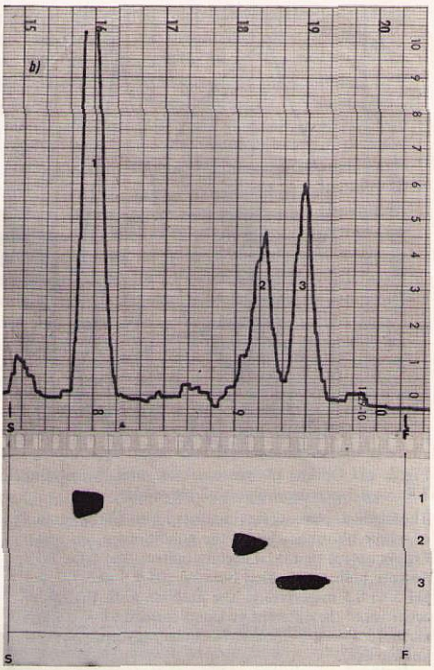


Fig. 4a). Radio thin-layer chromatogram of products after incubation of 2-[1-^{14}C]linoloyl-1-[9,10-$^{3}H_2$]-octadecyl-glycero-3-phosphate with lysosomal enzymes.

Conditions of incubations: substrate: 0.015 μmol, acetate buffer 0.1M, pH 4.6, 50 μg protein. Total volume 0.25 m*l*. 60 min at 37°C. A = substrate; B = reaction product. Solvent system: chloroform/methanol/30% methylamine 65:35:8.

b) Radio thin-layer analysis of front peak B. 1 = batyl alcohol; 2 = 2-linoloyl-1-octadecylglycerol; 3 = fatty acid (linoleic acid). Solvent system: 1) diisopropyl ether/acetic acid 96:4; 2) petroleum ether/ether/acetic acid 90:10:1.

active bands at pH 4.5 (Fig. 4a): two were rather apolar (B) and the third was the substrate (A). When the pooled bands of B were rechromatographed successively in diisopropyl ether/acetic acid 96:4 (10 cm) and petroleum ether/ether/acetic acid 90:10:1 (15 cm) (Fig. 4b) three radioactive bands appeared which were identified as [^{3}H]octadecylglycerol (1), 2-[^{14}C]linoloyl-1-[^{3}H]octadecylglycerol (2) and [^{14}C]linoleic acid (3) by their R_F-values, which were identical with those of authentic compounds, by their isotopes (1 and 2) and by the unchanged isotope ratio (2).

It is remarkable that only fatty acid, batyl alcohol and 2-linoloyl-1-octadecylglycerol and no alkylglycerophosphate (lysophosphatidic acid) can be detected when the enzyme of whole lysosomes act upon the "phosphatidic acid". Maximal production of alkylglycerol is observed at pH 4.5, whereas 2-acyl-1-alkyl-glycerol is the main product at pH 6–7. These data show that phospholipase A_2 and a phosphatase act simultaneously on the substrate. We therefore analyzed the reaction products of incubations of "phosphatidic acid" with the enzymes of whole lysosomes at pH's between 3.5 and 8.5 as described before. The results are summarized in the following diagram (Fig. 5). The curve for 2-linoloyl-1-octadecylglycerol (●) indicates that apparently two phosphatases hydrolyze phosphatidic acid, and acid phosphatase and a phosphatase with a pH optimum around 6 to 7. The maximal formation of batyl alcohol and the concomitant release of linoleic acid around pH 4 to 5 refer to

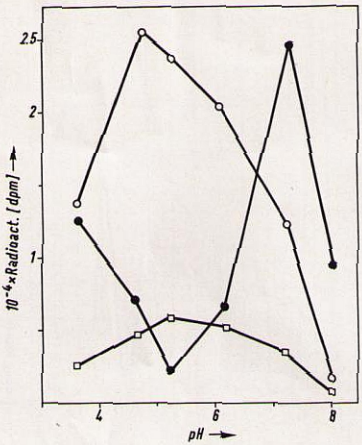


Fig. 5. pH Optima of lysosomal enzymes hydrolyzing 2-linoloyl-1-octadecyl-glycero-3-phosphate.

The labelled substrate was incubated with 100 μg protein of whole lysosomes under the conditions given under Fig. 4a except that 0.1M acetate buffers, and 0.1M Tris/maleate buffer were used between pH 4.0 and 5.0 and pH 5 to 8.5 respectively. The abscissa gives the radioactivities of the different products formed. ○—○ Batyl alcohol; ●—● 2-linoloyl-1-octadecylglycerol; □—□ linoleic acid.

a combined action of phospholipase A_2 and acid phosphatase. This reaction is inhibited by $Ca^{2\oplus}$ ions which permits the conclusion that the same phospholipase A_2 described under 1) also hydrolyzes the acyl-alkyl-glycerophosphate.

Further studies on the action of the enzymes of the lysosomal membrane on phosphatidic acid then proved that phosphatidic acid phosphatase is a membrane-bound enzyme with a pH optimum between pH 6 and 7, a pH interval in which only very little batyl alcohol is observed from the combined hydrolysis by this phosphatase and phospholipase A_2. The main product is linoloyl-octadecyl-glycerol.

In accordance with our earlier finding this phosphatidic acid phosphatase is almost completely inhibited by Triton WR-1339 at a concentration of 2 mg/m*l*.

The inhibition of the degradation of this phosphatidic acid by $Ca^{2\oplus}$ ions makes it likely that the first step is hydrolysis by the phospholipase A_2; and since no lysophosphatidic acid could be detected this is also the rate determining step, followed by the phosphatase reaction to yield batyl alcohol. It therefore seemed appropriate to determine the K_m of the phospholipase A_2 at pH 4.6 with 2-linoloyl-1-octadecyl-glycero-3-phosphate, by measuring the batyl alcohol formed at increasing substrate concentration. A K_m of 2×10^{-4}M was obtained, which is almost identical with the K_m of phospholipase A_2 with (2-acyl-1-alkyl-glycero-3-phosphoryl)ethanolamine as substrate.

4. 1-[9,10-$^3H_2$]Octadecyl-glycero-3-phosphate

From the aforementioned experiments it could be expected that this substrate would rapidly be hydrolyzed to batylalcohol by the phosphatase which was shown to participate in the hydrolytic degradation of phosphatidic acid. The only product formed is batyl alcohol, as shown in Fig. 6.

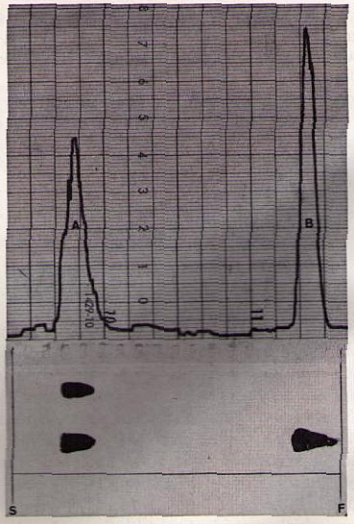


Fig. 6. Radio thin-layer analysis of products after hydrolysis of 1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate (0.05 μmol), with 200 μg lysosomal protein in 0.05M acetate buffer, pH 4.6.

Total volume: 1 m*l*, 37°C, 1 h. A = substrate; B = batyl alcohol. Solvent system: chloroform/methanol/30% methylamine 65:35:8.

Again whole lysosomes, the lysosomal membrane and matrix preparations were incubated with 1-octadecyl-glycero-3-phosphate at different pH values. Whole lysosomes clearly gave optimal hydrolysis of the substrate to batyl alcohol at three distinct pH values at 4.6, 6.2 and 8.2.

When lysosomal membranes were analyzed, a minor activity could be demonstrated at pH 4.6 but the major phosphatidic acid phosphatase activity was manifested around pH 7.5 to 8.5. Repeated washing of the membranes at higher ionic strength released the small activity present at pH 4.6.

The soluble (matrix) fraction exhibited two main phosphatase activities, one at pH 4.6 and one with a broad pH optimum around 6.0. Only the phosphatase with pH optimum at 4.6 is inhibited completely by tartrate, whereas the membrane-bound phosphatase with a pH optimum at 7.8 to 8.0 is completely inhibited by Triton WR-1339. On the other hand the soluble phosphatase activity at pH 6.2 is inhibited by neither tartrate nor Triton WR-1339.

The K_m values of the matrix enzymes and the membrane-bound phosphatidic acid phosphatase with 1-octadecyl-glycero-3-phosphate was determined at pH 4.6, 6.2 and 8.2. The membrane-bound phosphatase had a K_m of 5×10^{-4}M at pH 8.2; at pH 4.6 the K_m of the acid phosphatase was 4×10^{-4}M, and at 6.2 the K_m was 2×10^{-4}M.

Discussion

Alkyl ether-containing phospholipids, particularly ethanolamine-containing species, have gained increasing interest since the demonstration that they not only occur together with their desaturated analogous (2-acyl-1-*O*-1′-alkenyl-glycero-3-phosphoryl)ethanolamines (plasmalogens), but also represent intermediates and precursors of these rather ubiquitously occurring lipids. Compared to their diacyl analogs, the metabolic changes of the alkyl ether phospholipids have been scarcely investigated. Not only is the mechanism of the formation of the alkyl ether bond from long-chain alcohols and a glycerol derivative unknown, but many questions regarding the catabolism and enzymatic modifications of these phospholipids remain to be answered. It has been demonstrated that the oxidative cleavage of the alkyl ether bond is catalyzed by a biopterin dependent microsomal enzyme; the product is a long-chain aldehyde which corresponds to the substituent alkyl chain[9–15]. A number of reports exist on the hydrolysis of plasmalogens by snake venom phospholipases A (see ref.[20,21]). Phospholipases C from *Clostridium perfringens*[22–24] and *Bacillus cereus*[25] and cabbage leave phospholipase D[26] and *O*-alkyl ether containing phospholipids have been studied[27]. So far, experiments on the susceptibility of 1-*O*-alkyl ether-containing phospholipids to phospholipases of the mammalian cell are lacking. Our previous studies on lypolytic enzymes of the lysosome (tritosome) were extended to this ether-containing group of phospholipids. In the present study the metabolic role of the lysosome in the degradation of 1-*O*-alkyl ether-containing phosphatidylethanolamine, of its lyso derivative, of 1-*O*-alkyl-2-acyl-glycero-3-phosphate and of 1-*O*-alkyl-glycero-3-phosphate has been studied. It was demonstrated with labelled substrates, that neither the lysosomal membrane nor the matrix contains an alkyl ether-cleaving enzyme activity.

(2-Linoloyl-1-*O*-octadecyl-glycero-3-phosphoryl)-ethanolamine was hydrolyzed by the phospholipase A_2 of the lysosomal matrix, which is characterized by its pH optimum at pH 4.5 and the lysosomal membrane phospholipase A_2 with a pH optimum around 8.

The phospholipases A_2 of the lysosomal matrix and the membrane could be completely inhibited by $Ca^{2\oplus}$ and Triton WR-1339, respectively. Control experiments with labelled (diacylglycerophosphoryl)ethanolamine showed that the hydrolysis proceeded at the same rate and was also inhibited completely by $Ca^{2\oplus}$ and Triton WR-1339. These observations suggest that the activity of the two lysosomal phospholipases A_2 is not influenced by the substituent in position 1.

The reaction product 1-octadecylphosphorylethanolamine cannot be further hydrolyzed by phosphodiesterases of the lysosome. This naturally raises the question of the further metabolic fate of these lyso compounds.

The 1-*O*-alkyl ether analogs of phosphatidic acid are attacked by a number of lysosomal matrix and membrane enzymes. Again, the two phospholipases A_2 yield lysophosphatidic acid 1-octadecyl-glycero-3-phosphate, which in turn is further hydrolyzed to batyl alcohol by phosphatases. The lysosomal phosphatidic acid phosphatase also leads to the intermediate 2-acyl-1-alkyl-glycerol, which can be hydrolyzed by a lysosomal lipase to yield again batyl

alcohol. The higher reaction rates and K_m values of the lysosomal phospholipases A_2, compared to the phosphatidic acid phosphatase, however, suggest that the acyl residue might be hydrolyzed first, followed by the attack of the acid phosphatase on the 1-alkyl-glycero-3-phosphate.

It is noteworthy that three pH optima of phosphatase activity could be observed, one due to the membrane-bound phosphatidic acid phosphatase with a pH optimum around 8 (which again could be completely inhibited by Triton WR-1339), and two matrix phosphatase activities with pH optima at 4.6 and 6. Only the pH 4.6 enzyme is inhibited by tartrate.

The experiments reported demonstrate that 1-*O*-alkyl ether phospholipids are readily degraded by lysosomal matrix and membrane phospholipases in a similar manner to their diacyl analogs; however, the lyso-derivative of the phosphodiester remains unhydrolyzed, and when 2-acyl-1-*O*-alkyl- and 1-*O*-alkyl-3-glycerophosphates are substrates of the matrix and membrane enzymes, batyl alcohol is the main reaction product.

Finally, it should be pointed out that results of studies in vivo (intravenous or intracerebral injections) and with homogenates, with these four potential precursors of plasmalogens, must be interpreted with caution, since these substrates are rapidly altered by lysosomal enzymes.

Experimental

Substrates: (2-Linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)ethanolamine was synthesized by the following route: selachyl alcohol was catalytically reduced in an atmosphere of tritium gas to yield [9,10-$^3H_2$]batyl alcohol. Acylation of [^{3}H]batyl alcohol with linoloyl chloride yielded 2,3-dilinoloyl-1-[^{3}H]octadecyl-glycerol. Treatment with pancreatic lipase released linoleic acid from the 3-position. The diglyceride was purified by silicic acid chromatography and condensed with *N*-(β,β-β-trichloroethoxycarbonyl)-2-aminoethylphosphoryl chloride according to Pfeiffer *et al.*[28]. Following this procedure the protecting group was hydrolyzed to (2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphoryl)-ethanolamine which was purified by silicic acid chromatography, specif. radioactiv. 4.1 μCi/μmol.

(1-[9,10-$^3H_2$]Octadecyl-glycero-3-phosphoryl)ethanolamine was obtained in quantitative yield by phospholipase A_2 (*Crotalus-adamanteus* toxin) treatment, specif. radioactiv. 4.1 μCi/μmol. Phospholipase D (Boehringer Mannheim GmbH) hydrolysis[29] released 2-linoloyl-1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate, which was purified by silicic acid column chromatography, specif. radioactiv. 4.1 μCi/μmol. Linoleic acid was released from this phosphatidic acid by phospholipase A_2 treatment yielding 1-[9,10-$^3H_2$]octadecyl-glycero-3-phosphate. This "lysophosphatidic acid" was purified by preparative thin-layer chromatography, specif. radioactiv. 4.1 μCi/μmol.

Solvent systems for thin-layer chromatography of

1) 1-octadecyl ether of phosphatidylethanolamine and lysophosphatidylethanolamine: chloroform/methanol/water 65:25:4,

2) phosphatidic acid and lysophosphatidic acid: chloroform/methanol/30% methylamine[30] 65:25:8,

3) fatty acids, batyl alcohol, 2-linoloyl-1-octadecyl-glycerol: petroleum ether/ethyl ether/acetic acid 70:30:1 and diisopropyl ether/acetic acid 96:4 to a distance of 10 cm, drying under N_2 followed by chromatography in petroleum ether/ethyl ether/acetic acid 90:10:1 to a distance of 15 cm[31].

Preparation of lysosomes (tritosomes), lysosomal membrane and matrix fraction

Lysosomes filled with Triton WR-1339 (tritosomes) were prepared according to Wattiaux *et al.*[16] by density gradient centrifugation. The lysosomes were osmotically ruptured by dialysis against distilled water, and their membranes pelleted by centrifugation at 40000 × *g* for 30 min; the supernatant contained all the matrix enzymes. It was concentrated by Millipore filter ultrafiltration or lyophilization. The procedure has been described previously[18].

Substrates were solubilized by two one minute sonication periods at 80 Watts using the Branson sonifier microtip. Incubations were carried out at 37°C. They were stopped by the addition of a chloroform/methanol 2:1 (v/v) mixture. The lower chloroform phase was separated and the upper aqueous phase twice extracted with chloroform/methanol/30% methylamine 65:25:8 when the incubation contained phosphatidic acid and lysophosphatidic acid. Incubations with the 1-octadecyl ether of phosphatidylethanolamine and lysophosphatidylethanolamine were reextracted with chloroform/methanol 2:1 and chloroform. Combined extracts were concentrated and dissolved in chloroform/methanol 2:1 for the analysis on thin-layer plates (silica gel H, 0.25 mm). Radioactive bands were monitored and localized in a Packard radiochromatogram scanner. They were scraped off the plate and the silica gel extracted with chloroform/methanol/water/formic acid 97:97:4:2[32] or chloroform depending on the polarity of the products. Radioactivities were measured in a Tricarb scintillation spectrometer, model 3380 using Bray's solution[33].

0.1M Acetate buffers were used between pH 3.6 and 5.0 and 0.1M Tris/maleate buffers for pH's between 5 and 9. Protein was determined according to Lowry *et al.*[34].

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