DEGRADATION OF SEMINOLIPID BY A LIPASE IN SECONDARY LYSOSOMES

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Received 7 July 1976

1. Introduction

Sulphatides (*N*-acyl-1-*O*-(β-3′-sulphogalactosyl)-sphingosine) and seminolipid (1-*O*-alkyl-2-*O*-acyl-3-(β-3′-sulphogalactosyl)-glycerol) are both physiological substrates of the sulphatase A (EC 3.1.6.1) [1–3]. For their enzymic degradation in buffers of physiological ionic strength in vitro, either detergents [2–6] or a physiological activator ([7,8], Reiter et al., unpublished results) have to be added. Both the enzyme [9,10] and its activator are localized in the lysosomes [11].

Metachromatic leukodystrophy (MLD) is caused by an inborn deficiency of the lysosomal sulphatase A [12]. This results in an accumulation of sulphatides [13], predominantly in brain and kidney. Seminolipid, however, which is localized in testis [14] and to a smaller extent in brain [15] is not stored in cases of infantile MLD [16]. This may imply either that the deposition of seminolipid in human testis has not been developed in the infantile period (Handa [16]) or that yet another pathway exists for the degradation of seminolipid. In this paper it is shown that iron-loaded secondary lysosomes from rat liver contain a lipase which cleaves the acyl residue of seminolipid thus supporting the second possibility.

2. Materials and methods

2.1. *Isolation and subfractionation of iron-loaded secondary lysosomes*

Iron-loaded secondary lysosomes were obtained from rat liver according to described procedures [17, 18] with minor modifications [11]. The lysosomal pellet was osmotically shocked with 10 ml of distilled water. This dispersion was frozen and stored at −20°C until use, which is referred to throughout this paper as 'total (iron-loaded secondary) lysosomes'.

For subfractionation the dispersion of lysosomes was centrifuged at 100 000 × g_{av}. for 30 min. The pellet was dispersed in the starting volume of distilled water. Without further treatment the supernatant (lysosol) and the dispersed pellet (membranous fraction) were used for the experiments.

2.2. *Enzymic characterization of the lysosomes by their hydrolases with chromogenic substrates*

Acid phosphatase (EC 3.1.3.2) was assayed with *p*-nitrophenylphosphate (Koch-Light Lab. Ltd., Colnbrook, England) [19], arylsulphatase (A and B) (EC 3.1.6.1) with *p*-nitrocatecholsulphate (Sigma Chem. Co., St. Louis, USA) [20,21], β-galactosidase (EC 3.2.1.23) with *p*-nitrophenyl-β-D-galactopyranoside (Koch-Light Lab. Ltd., Colnbrook, England) [19]. Protein was determined by the method of Lowry et al. [22] using bovine serum albumin (Serva, Heidelberg, Germany) as a standard. Specific activities are defined as μmoles of substrate hydrolysed per mg of protein per min under the incubation conditions stated. The values obtained are the average of two experiments with a S.E.M. ⩽ 5%.

2.3. *Assay conditions with ^{35}S-labelled sulphatides [23], seminolipid [24] or 'lysoseminolipid' (prepared according to section 2.4.) as substrates*

The incubation mixtures contained in a total volume of 100 μl: 10 μmol of sodium acetate buffer (pH 4.5), 10 nmol of ^{35}S-labelled lipids and various amounts of lysosomal protein. After incubation periods of 6 to 24 h the total incubation volumes

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were chromatographed on Silicagel plastic sheets (No. 5748, Merck, Darmstadt, Germany) in chloroform–methanol–water (14:6:1 v/v/v). The radioactivity on the plastic sheets was localized with a Berthold radio thin-layer scanner (Wildbad, Germany). The areas representing substrate and products were cut out and the radioactivity was determined in a liquid scintillation analyzer (Mark II; Nuclear Chicago, USA) (fig.1) (Sandhoff, personal communication).

2.4. *Identification of the degradation product of seminolipid after incubation with the lipase*

The acyl group of seminolipid was removed by mild alkaline hydrolysis under the same conditions as described for the deacylation of 1,2-diacyl-3-(3′-sulphogalactosyl)-glycerol [25]. The crude acyl-free 'lysoseminolipid' was purified by column chromatography on Silicagel 60 (no. 7729, Merck, Darmstadt, Germany) using chloroform–methanol–water (65:25:4 v/v/v) as the solvent system. Its identity with the enzymically obtained degradation product was shown by co-chromatography on Silicagel 60 plates (No. 5721, Merck) in two different chromatographic systems: chloroform–methanol–water (14:6:1 v/v/v) and chloroform–methanol–conc. ammonia–water (70:25:4:1 v/v/v/v) (fig.1).

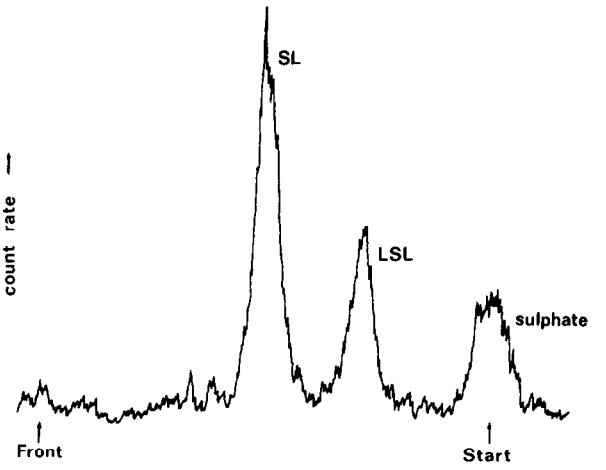

Fig.1. Scan of a thin-layer radiochromatogram showing the degradation of 10 nmol of ^{35}S-labelled seminolipid by the supernatant of secondary lysosomes to give 'lysoseminolipid'. The same experiment was run in two solvent systems with addition of chemically-obtained ^{35}S-labelled 'lysoseminolipid' to ensure identity of the enzymic reaction product. For further details see text. Seminolipid (SL); 'Lysoseminolipid' (LSL).

3. Results

3.1. *Characterization of the lysosomal subfractions by means of the acid hydrolases*

After osmotic shock combined with freezing and thawing the iron-loaded secondary lysosomes were separated by centrifugation into a supernatant (lysosol) and sediment (membranous fraction). To compare the distribution of acid hydrolases in these two subfractions the sediment was suspended in the original volume of distilled water. The distribution of enzyme activities between sediment and supernatant was similar to that reported by Mraz et al. [11]. The specific activities (relative to the protein content (mg/ml) of the total lysosomes) are given in table 1.

Table 1
Distribution of acid hydrolases in isolated secondary lysosomes and their subfractions

		Arylsulphatases		Acid phosphatase		β-Galactosidase	
	Protein mg/ml	Specific activity	Total[a] activity	Specific activity	Total[a] activity	Specific activity	Total[a] activity
Total lysosomes	4.2	0.086	3.3	0.105	4	0.045	1.7
100 000 × *g* supernatant (lysosol)	2.44	0.119	2.6	0.09	2	0.065	1.4
100 000 × *g* sediment (membranous fraction)	1.8	0.04	0.7	0.122	2	0.02	0.32

[a] In a total volume of 10 ml.

3.2. *Degradation of seminolipid and sulphatides by iron-loaded secondary lysosomes*

The degradation of the two lipid substrates, seminolipid and sulphatides, was examined as previously described (see Materials and methods) using 105 or 210 μg of protein per incubation. Figure 2 shows the time dependency of the enzymic hydrolysis of either the sulphate group of sulphatide and seminolipid or the acyl group of seminolipid. The degradation by sulphatase A of seminolipid is slightly higher than that of sulphatides. The amount of radioactive product formed in both cases was virtually independent of the amount of protein added, perhaps due to inhibition. The degradation of seminolipid by the lipase depended on the amount of protein added. The degradation product was identified to be the acyl-free 'lysoseminolipid' (1-*O*-alkyl-3-(β-3′-sulphogalactosyl)-glycerol) (see Materials and methods). The amount of product formed by the lipase reaction was much higher than that obtained by the sulphatase reaction.

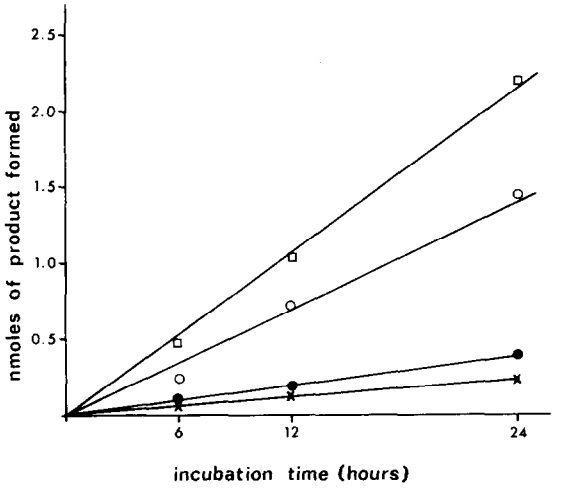

Fig.2. Time dependency of the degradation of sulphatides and seminolipid by isolated iron-loaded secondary lysosomes. Sulphate cleavage from 10 nmol of sulphatides (X—X) or seminolipid (•—•). No difference between the amount of products formed was detectable when 105 or 210 μg of protein were used. Acyl cleavage from 10 nmoles of seminolipid using 105 μg (○—○) or 210 μg (□—□) of protein. Each value is the average of two experiments with a S.E.M. ≤ 5%. For details see Materials and methods.

3.3. *Comparison of the sulphatase and lipase activities in isolated total secondary lysosomes and in the subfractions*

Both the sulphatase and the lipase activities in the lysosomal subfractions were compared with those in the total lysosomes. With different protein concentrations in the incubation mixtures the amount of product formed was measured. Figure 3 shows the degradation of sulphatides, seminolipid and 'lysoseminolipid' by sulphatase A. The specific activities in relation to both substrates (sulphatides and seminolipid) were highest when the supernatant was used. This is in good agreement with the distribution of sulphatases tested with the chromogenic substrate (see table 1). In comparison to the sulphatides the seminolipid and 'lysoseminolipid' are desulphated much better by the supernatant.

The distribution of lipase activities in the subfractions related to the protein concentration in the incubation differs from that of sulphatase A. The activities in the total lysosomes and in the sediment are of the same order of magnitude (fig.4) but higher than in the supernatant. The difference might not be as high as shown in fig.4 since it is probable that a significant percentage of the formed 'lysoseminolipid' is further degraded by the sulphatase A, whose activity is highest in the supernatant.

4. Discussion

These experiments show that seminolipid is degraded in two different ways by enzymes in secondary lysosomes, one occurs through desulphation by sulphatase A, the other by a lipase resulting in deacylation, thus forming 'lysoseminolipid'. The latter reaction is analogous to the phospholipase A_2 reaction with phospholipids [26,27] (or sulphodiglycerides) [28]. Both pathways could also be used to degrade other sulphogalactoglycerolipids such as 1,2-diacyl-3-(3′-sulphogalactosyl)-glycerol, recently detected in mammalian brain [25].

In MLD which is caused by a sulphatase A deficiency [12], only the accumulation of sulphatides was observed [13]. These lipids are exclusively degraded by sulphatase A. The 'lysoseminolipid', though also degraded by sulphatase A, was not detected (and therefore apparently not accumulated)

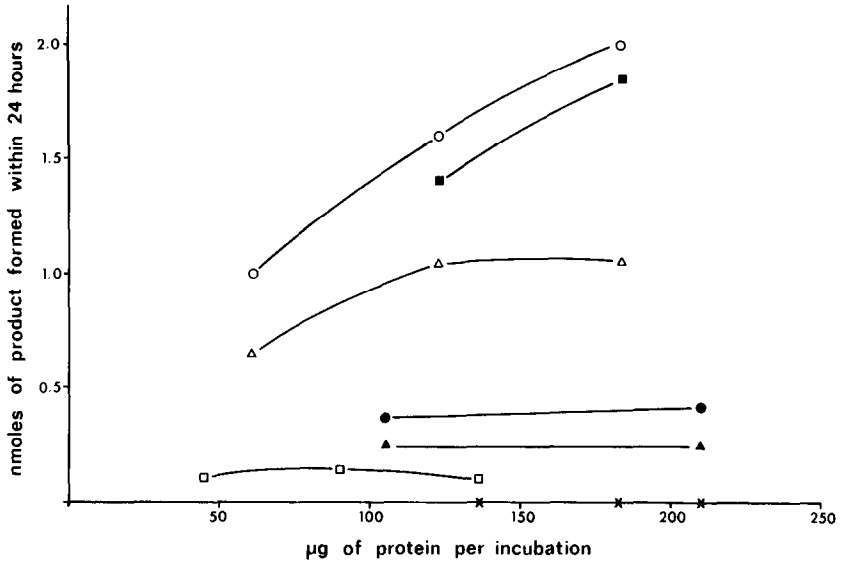

Fig.3. Degradation of sulphatides and seminolipid by sulphatase A in total lysosomes or in lysosomal subfractions. Sulphate cleavage from 10 nmol of sulphatides by total lysosomes (▲—▲), supernatant (△—△) and sediment (□—□), from 10 nmol of seminolipid by total lysosomes (●—●), supernatant (○—○), and sediment (□—□) or from 10 nmol of 'lysoseminolipid' by supernatant (■—■). Heat-inactivated subfractions and total lysosomes (15 min at 95°C) did not show any enzyme activity (X—X). Each value is the average of two experiments with a S.E.M. ≤ 5%. For details see Materials and methods.

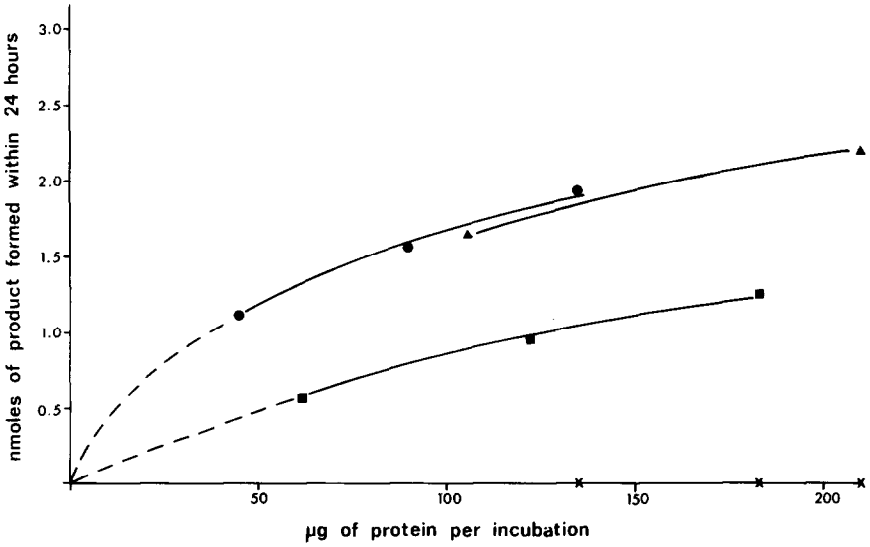

Fig.4. Degradation of seminolipid by the lipase in total lysosomes and in lysosomal subfractions. Cleavage of the acyl group from 10 nmol of seminolipid by total lysosomes (▲—▲), supernatant (■—■), and sediment (●—●). Heat-inactivated subfractions and total lysosomes (15 min at 95°C) did not show any enzyme activity (X—X). Each value is the average of two experiments with a S.E.M. ≤ 5%. For details see Materials and methods.

in MLD brains. Further experiments are necessary to clarify this point.

Acknowledgement

Seminolipid as a reference substance was a generous gift of Prof. T. Yamakawa, Tokyo.

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