GALACTOSYLGALACTOSYLGLUCOSYLCERAMIDE: GALACTOSYL HYDROLASE IN NORMAL HUMAN PLASMA AND ITS ABSENCE IN PATIENTS WITH FABRY'S DISEASE

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1. Introduction

Fabry's disease is a hereditary defect resulting from the absence of galactosylgalactosylglucosylceramide: galactosyl hydrolase (ceramide trihexosidase); the enzyme is specific for the cleavage of the terminal galactosyl unit of β-galactosyl-(1→4)-β-galactosyl-(1→4)-β-glucosylceramide (GL-3), an intermediate in the catabolism of β-2-acetamido-2-deoxygalactosyl-(1→3)-β-galactosyl-(1→4)-β-galactosyl-(1→4)-β-glucosylceramide (globoside [1]. The consequence is a progressive accumulation of GL-3 in plasma, urine and most tissues of patients with this X-linked disorder of glycosphingolipid metabolism [2–6]. Enzymatic activity has been found to occur in a particulate fraction from normal spleen, small intestine, kidney, brain and liver [7]. The ceramide trihexosidase is absent from small intestinal mucosa of males with Fabry's disease and its concentration was reported to be reduced in tissue from one heterozygous female [1]. Plasma, lymph and other body fluids were not previously examined for enzymatic activity.

This paper reports the occurrence of ceramide trihexosidase activity with a bimodal pH optimum in normal human plasma. The enzymatic activity was not detected in blood from hemizygous patients with Fabry's disease. The level of enzymatic activity in plasma was assayed by a new method with galactose dehydrogenase.

2. Materials and methods

GL-3 was isolated from porcine erythrocytes by the method of Vance and Sweeley [8] and from Fabry kidney [2]. Sodium cholate, NAD^+ and 2-(*N*-morpholine)-ethanesulfonic acid (MES) were purchased from Sigma Chemical Co. (St. Louis). Citrate-phosphate buffer was prepared by the method of Goromi [9]. Galactose dehydrogenase was obtained from Boehringer-Mannheim Corp. (New York) and bovine serum albumin from Pentex Inc. (Kankakee). Protein was determined by the biuret method [10]. Plasma was obtained from freshly drawn blood by centrifugation at 7,700 *g* for 10 min.

The standard reaction mixture for the assay of ceramide trihexosidase consisted of plasma (0.5 ml), from blood collected in heparin (for analyses at pH 7 ± 1) or EDTA (for analyses at pH 5 ± 1); GL-3 (100 nmoles). dispersed in 0.1 ml of aqueous sodium cholate (0.96 mg); 0.1 ml of bovine serum albumin (5.0 mg); 0.1 M MES buffer (pH 4.5) or citrate-phosphate buffer (pH 3.0) to adjust the pH of the incubation mixture to pH 7.2 or 5.4, respectively; and water to a total volume of 0.75 ml. After incubation for 4 hr at 23°, the reaction was terminated by addition of 2.0 ml of chloroform-methanol (2:1). The suspension was mixed thoroughly with 0.2 ml of water and centrifuged to remove protein from the upper phase, which was then further clarified by millipore filtration. The volume of aqueous methanol obtained was 1.2–1.5 ml. A control reaction, in which GL-3 was omitted from the sodium cholate solution, was run with each set of samples.

Galactose liberated from GL-3 in the above reac-

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tion was determined with an end-point assay consisting of 141 μl of 0.1 M tris buffer (pH 8.6); 0.1 μmole of NAD^+; 50 μl of upper phase from the above incubation; and 5.4 μg of galactose dehydrogenase (in 1 M $(NH_4)_2SO_4$) in a final volume of 0.2 ml. The increase in absorbance at 340 nm was measured with a Gilford 2400 Model recording spectrophotometer thermostated at 30°. An absorbance change of 0.01 was equivalent to 0.32 nmoles of galactose in the 50 μl sample. From the observed absorbance change, corrected for the control without added GL-3, galactose in the entire upper phase from the incubation with plasma was calculated.

The rate of galactose formation from GL-3 was proportional to the amount of plasma used in the assay. Although we have no information on the contribution of lactosylceramide: galactosyl hydrolase activity to the observed rate, its maximal effect if present in plasma would be an apparent doubling of the ceramide trihexosidase activity.

3. Results and discussion

Ceramide trihexosidase has been found in normal human serum and plasma. It is stable for at least 24 hr at 0° in an unfrozen state, but activity is completely lost on freezing. The enzyme can be recovered, without loss of activity after precipitation, at 25% of saturation with $(NH_4)_2SO_4$.

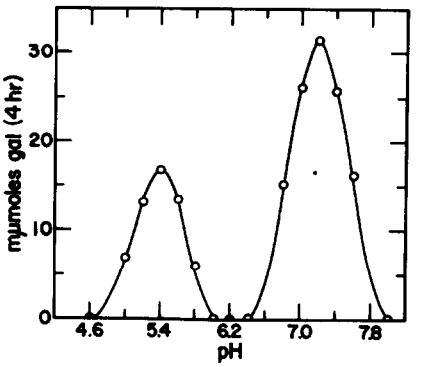

Fig. 1. Effect of pH on ceramide trihexosidase activity in normal plasma. The conditions of incubation and assay are described in the text.

Fig. 1 shows the curve of pH optima, which was similar with whole plasma and the fraction obtained after $(NH_4)_2SO_4$ precipitation. Citrate-phosphate buffer was used for assays between pH 4.6 and 6.2, whereas MES buffer was used between pH 6.2 and 8.2. The bimodal pH curve could also be demonstrated with citrate-phosphate buffer for assays between pH 4.0 and 7.5.

The enzymatic activities of ceramide trihexosidase in normal and Fabry plasma are given in table 1. No enzymatic activity could be detected in blood from the Fabry patients, within the limits of the method of assay, even when 1 ml of plasma was used in the incubation, and 0.1 ml of upper phase was assayed

Table 1
Ceramide trihexosidase activity in normal and Fabry plasma.

Donor plasma	pH 5.4		pH 7.2	
	Total units*	Specific activity**	Total units*	Specific activity**
Normal				
M.B.	16.2	0.11	30.5	0.20
J.H.	16.0	0.11	31.5	0.23
R.D.	15.7	0.13	31.4	0.27
W.K.	16.1	0.12	30.1	0.22
Fabry				
A.G.	< 1.5	<0.01	< 1.5	<0.01
R.L.	< 1.5	<0.01	< 1.5	<0.01
D.L.	< 1.5	<0.01	< 1.5	<0.01
C.B.	< 1.5	<0.01	< 1.5	<0.01

* Total units: nanomoles of galactose liberated per 4 hr per 0.5 ml of plasma.
** Specific activity: nanomoles of galactose liberated per hr per mg of protein.

for galactose liberated from GL-3. Thus the upper limit of enzymatic activity was less than 2% of that found in normal plasma. The assay of ceramide trihexosidase in blood can therefore be used for the biochemical diagnosis of hemizygotes.

The finding of two pH optima for the ceramide trihexosidase activity might be explained by an arrangement of amino acid residues in the enzyme that allows a bimodal pH optimum. An alternative and more attractive explanation might be the occurrence of two ceramide trihexosidase isozymes, one that has a pH optimum of 5.4 and is similar to that previously found in tissues (Form A), and one that has an optimum at pH 7.2 (Form B). The latter hypothesis is consistent with recent findings on the presence of two forms of *N*-acetylhexosaminyl hydrolase in blood and various tissues, one of which is absent in another hereditary glycosphingolipidosis, Tay-Sachs disease [11]. Similarly, in Hurler's syndrome, there is a deficiency of a specific galactosyl hydrolase that is one of two forms with different pH optima [12].

There are clear clinical implications of the finding that ceramide trihexosidase is present in normal human serum and absent in Fabry patients: the level of GL-3 in circulating plasma, which is elevated considerably above normal in Fabry patients [5], may be able to be controlled by enzyme replacement in the blood.

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