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Note

Requirement of an activator for the hydrolysis of sphingoglycolipids by glycosidases of human liver*

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Sphingoglycolipids, widely distributed in the animal tissue, are known to play many important and intriguing biological roles. The accumulation of sphingoglycolipids in a number of lipid-storage diseases has recently drawn attention to the catabolism of these molecules. While studying the degradation of sphingoglycolipids by glycosidases, we found that glycosidases of human tissues, purified by following their activities toward synthetic substrates, have very little activity toward sphingoglycolipids. Recently, we reported that purified β-D-hexosaminidase A of human liver or urine requires a heat stable activator to activate the hydrolysis of 2-acetamido-2-deoxy-D-galactopyranosyl residue from Tay-Sachs ganglioside[1]. In this communication, we report that, in addition to this stimulation of G_{M2}‡ hydrolysis, the partially purified activator also stimulates the hydrolysis of ceramide trihexoside (CTH) and G_{M1} ganglioside by α- and β-D-galactosidase of human liver, respectively. It also activates the hydrolysis of CTH by the α-D-galactosidases A and B isolated from human placenta[2].

G_{M1} and G_{M2} gangliosides were isolated from human brain[3]. Radioactive G_{M1} and G_{M2}, tritium-labeled in the terminal D-galactose and 2-acetamido-2-deoxy-D-galactose residues, respectively, and CTH isolated from human kidney were gifts of Dr. L. Svennerholm of the University of Göteborg (Sweden). CTH, tritium-labeled in the terminal D-galactose residue was a gift of Dr. G. Romeo of the International Laboratory of Genetics and Biophysics, Naples (Italy). The ganglioside mixture isolated from human brain was a gift of Dr. K. Suzuki of the Albert Einstein College of Medicine, New York, N.Y. α-D-Galactosidases A and B, isolated from human placenta[2], were gifts of Dr. E. Beutler of the City of Hope National Medical Center,

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‡Abbreviations: G_{M1}, β-D-Gal-(1→3)-β-D-GalNAc-(1→4)-[α-NeuNAc-(2→3)]-β-D-Gal-(1→4)-D-Glc→Ceramide; G_{M2}, β-D-GalNAc-(1→4)-[α-NeuNAc-(2→3)]-β-D-Gal-(1→4)-D-Glc→Ceramide; G_{M3}, α-NeuNAc-(2→3)-β-D-Gal-(1→4)-D-Glc→Ceramide; CTH, α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc→Ceramide; NeuNAc, N-acetylneuraminic acid.

California. Analytical thin-layer chromatography of gangliosides and neutral sphingoglycolipids was performed as previously described[4]. Protein was determined by the method of Lowry *et al.*[5] with crystalline bovine-serum albumin as the standard.

For convenience, *p*-nitrophenyl glycosides were used as substrates to follow the β-D-hexosaminidase and β-D-galactosidase activities, and 4-methylumbelliferyl α-D-galactopyranoside for the α-D-galactosidase activity; the procedures have been previously described[2,6]. One unit of enzyme was defined as the amount of enzyme that hydrolyzes 1 μmole of *p*-nitrophenyl or 4-methylumbelliferyl glycoside per minute at 37°.

Enzyme isolation was carried out at 0–5°. Normal human liver (500 g) obtained *post mortem* was diced and homogenized with 0.1% (v/v) Triton X-100 (3000 ml) and centrifuged to obtain a clear, brown crude extract, as described previously[1]. Solid citric acid was added with stirring to adjust the pH of the extract to 4.3. After removal of the precipitated protein by centrifugation, the pH of the supernatant was adjusted to pH 7.0, and subsequent fractionation by addition of solid ammonium sulfate gave proteins that precipitated between 30% and 60% of saturation. The precipitated protein was collected and dissolved in 0.1M sodium phosphate buffer (50 ml), pH 7.0,

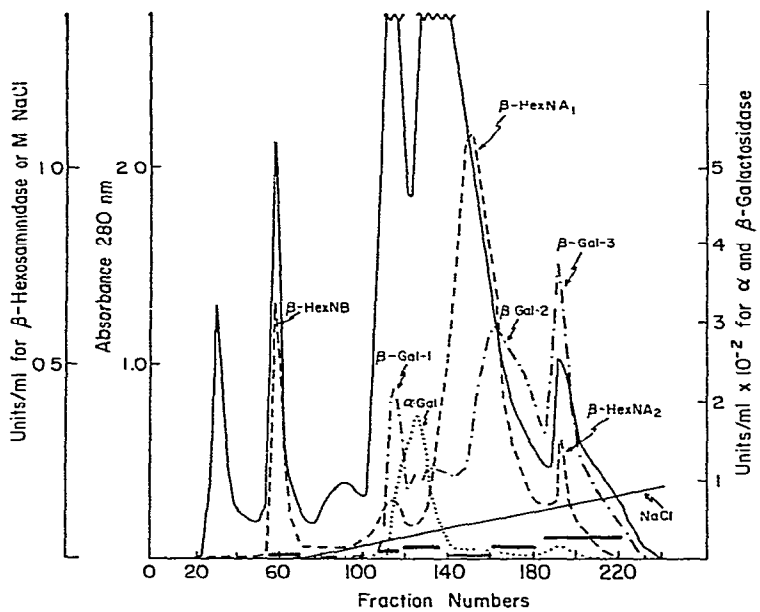

Fig. 1. Separation of various glycosidases of human liver by DEAE-cellulose chromatography. Enzyme solution (55 ml containing 5.5 g protein) obtained after zinc sulfate treatment (see text) was applied to a DEAE-cellulose column (2.5 × 70 cm) that had been previously equilibrated with 0.01M sodium phophate buffer, pH 6.0. The column was eluted at the flow rate of 40 ml per hour; 7 ml per fraction were collected. For detailed condition, see text. α-Gal, α-D-galactosidase; β-Gal-1, β-D-galactosidase-1; β-Gal-2, β-D-galactosidase-2; β-Gal-3, β-D-galactosidase-3; β-HexNA$_1$, *N*-acetyl-β-D-hexosaminidase A$_1$; β-HexNA$_2$, *N*-acetyl-β-D-hexosaminidase A$_2$, β-HexNB, *N*-acetyl-β-D-hexosaminidase B.

and was designated as crude glycosidase fraction. For further purification, the crude glycosidase fraction was diluted with 0.1M sodium phosphate buffer, pH 7.0, to give a protein concentration of approximately 10 mg per ml. To this solution (25 parts), M zinc sulfate (1 part) was added dropwise while stirring. The precipitated protein, which did not contain appreciable enzyme activities, was removed by centrifugation and discarded. Proteins in the supernatant were precipitated by addition of solid ammonium sulfate to reach 70% of saturation, and were dissolved in 0.01M sodium phosphate buffer (40 ml), pH 6.0. After exhaustive dialysis against 0.01M sodium phosphate buffer, the enzyme was applied to a DEAE-cellulose column (2.5 × 75 cm) equilibrated with the same buffer. The column was first eluted with 0.01M sodium phosphate buffer to wash off the unadsorbed protein, and then with 1000 ml of a linear salt gradient from 0 to 0.3M sodium chloride in the same buffer. As shown in Fig. 1, β-D-hexosaminidase was eluted in three peaks (β-D-hexosaminidase B, A_1, and A_2) and β-D-galactosidase in three peaks (β-D-galactosidase-1, -2, and -3). One major peak was found for α-D-galactosidase. β-D-Hexosaminidases A_1 and A_2 were found to overlap with β-D-galactosidase-2 and -3, respectively. The glycosidases in the different peaks were pooled as indicated by the *horizontal bar* in Fig. 1, precipitated by addition of solid ammonium sulfate to 70% of saturation, collected by centrifugation, and dissolved in 0.05M sodium phosphate buffer (5 ml), pH 7.0. These preparations were used for subsequent studies.

For partial purification of the activator, human liver (250 g) was homogenized with distilled water (1500 ml). The homogenate, divided into 500 ml-portions, was heated in a boiling water-bath for 5 min, and the precipitated protein was removed by centrifugation. The supernatant was evaporated to dryness with a rotary evaporator, dissolved in distilled water (50 ml), and dialyzed exhaustively against 0.05M sodium phosphate buffer, pH 7.0. The material that precipitated in the retentate after dialysis was removed by centrifugation and discarded. A 25-ml portion of the clear retentate containing 2 g of protein was applied to a DEAE-Sephadex A-50 column (2 × 30 cm), which had been equilibrated with 0.05M sodium phosphate buffer, pH 7.0. After being washed with the starting buffer to remove the unadsorbed proteins, the column was eluted with 0.05M sodium citrate buffer, pH 6.0, containing 0.05M sodium chloride. Two protein peaks were eluted by this buffer and the second was found to contain an activator that stimulated the hydrolysis of G_{M1}, G_{M2}, and CTH by β-D-galactosidase, β-D-hexosaminidase A, and α-D-galactosidase of human liver, respectively.

Hydrolysis of nonradioactive as well as radioactive sphingoglycolipids by glycosidases was carried out as described previously[1]. Fig. 2 shows thin-layer chromatograms illustrating the effect of the activator on the hydrolysis of CTH and G_{M1} by α-D-galactosidases and β-D-galactosidases, respectively. Quantitative enzymic hydrolysis of various radioactive sphingoglycolipids is shown in Table I. As shown in Fig. 2 and Table I, the activator clearly stimulates the hydrolysis of CTH by α-D-galactosidases, G_{M1} by β-D-galactosidases, and G_{M2} by *N*-acetyl-β-D-hexosaminidase A_1. In the absence of the activator, very little cleavage of sphingoglycolipids by these

TABLE I

EFFECT OF ACTIVATOR ON THE HYDROLYSIS OF SPHINGOGLYCOLIPIDS[a]

Enzymes[b]	*Units of enzyme used*	*Substrate*	*Incubation time (h)*	*Hydrolysis (%)*	
				With Activator	*Without Activator*
α-D-Galactosidase	0.02	CTH	6	40	1
β-D-Galactosidase-1	0.64	G_{M1}	4	39	0
β-D-Hexosaminidase-A_1	6.0	G_{M2}	20	20	3

[a]Radioactive sphingoglycolipids (20 nmoles, 9500 c.p.m. for G_{M1}, 6900 c.p.m. for G_{M2}, and 7500 c.p.m. for CTH) tritium-labeled at the terminal D-galactopyranose or 2-acetamido-2-deoxy-D-galactopyranose residues were incubated with the enzyme in the presence or absence of the activator (100 μg), at 37°, according to the conditions previously described[1]. [b]For a characterization of the various enzymes, see Fig. 1.

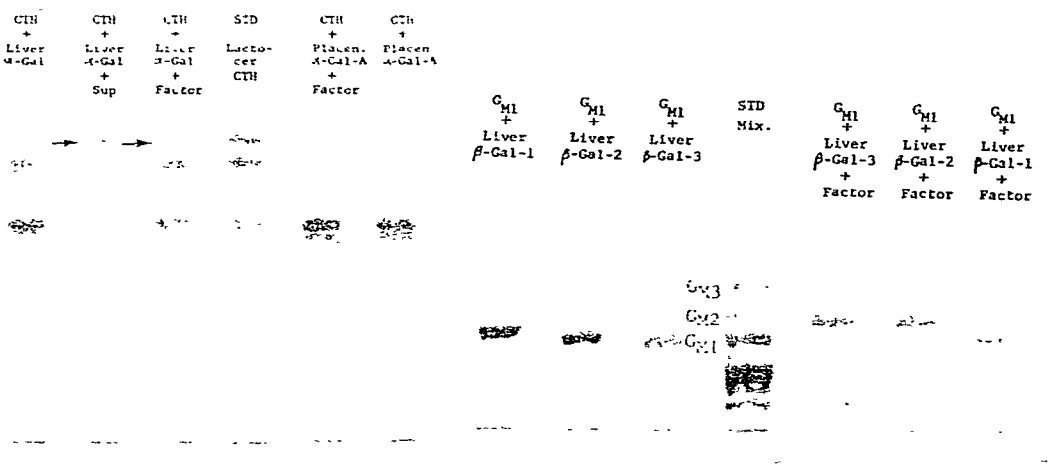

Fig. 2. Thin-layer chromatograms[4] of the effect of activator on α-D- and β-D-galactosidase. From left, six runs: Effect of the factor (activator) on the hydrolysis of CTH by liver and placenta α-D-galactosidase. α-Gal, α-D-galactosidase; Sup, the heat-stable preparation[1]; STD, standard. Liver α-D-galactosidase (0.02 unit) and placenta α-D-galactosidase-A (0.03 unit) were separately incubated with CTH for 16 h at 37° in the presence or absence of the heat-stable preparation (2 mg) or of the partially purified factor (100 μg), according to the procedure previously described[1]. Arrow indicates the lactosylceramide produced by enzyme hydrolysis. The band below lactosylceramide is an artifact from the enzyme. Standard CTH plus lactosylceramide shows two bands due to the heterogeneity in fatty acid composition. The plate was sprayed with 1% orcinol in 3% sulfuric acid[4].
From right, seven runs: Effect of the factor (activator) on the hydrolysis of G_{M1} by β-D-galactosidases of human liver. STD Mix, standard gangliosides mixture. β-D-Galactosidase-1 (0.03 unit), β-D-galactosidase-2 (0.04 unit), and β-D-galactosidase-3 (0.04 unit) were separately incubated with G_{M1} for 16 h at 37° in the presence or absence of the factor (100 μg), according to the procedure previously described[1]. The plate was sprayed with the resorcinol reagent[7].

glycosidases could be detected. As shown in Fig. 1, three *N*-acetyl-β-D-hexosaminidase peaks, *N*-acetyl-β-D-hexosaminidase B, A_1, and A_2, were obtained by DEAE-cellulose chromatography. Both *N*-acetyl-β-D-hexosaminidase A_1 and A_2 were able to hydrolyze G_{M2} in the presence of the activator. Without the activator, these two isozymes hydrolyze G_{M2} only with great difficulty. *N*-Acetyl-β-D-hexosaminidase B, on the other hand, was not able to hydrolyze G_{M2} in the presence or absence of the activator. Hydrolysis of G_{M2} by *N*-acetyl-β-D-hexosaminidase A and B has been previously published[1]. All the β-D-galactosidases contained in the three peaks shown in Fig. 1 were unable to hydrolyze G_{M1}, except in the presence of the activator. Since β-D-galactosidases-2 and -3 were contaminated with *N*-acetyl-β-D-hexosaminidases A_1 and A_2, respectively (Fig. 1), incubation of these two enzymes with G_{M1} resulted in the production of a small amount of G_{M3}, in addition to G_{M2} (Fig. 2). From Table I, it appears that the activator stimulates the hydrolysis of G_{M1} better than that of G_{M2} or CTH. However, it should be pointed out that the unit of enzyme applied, which was based on the hydrolysis of synthetic substrates, does not reflect its ability to hydrolyze sphingoglycolipids. It is of interest to note that the activator also stimulates the hydrolysis of CTH by the purified α-D-galactosidase isolated from human placenta.

The requirement of a heat-stable activator for glycosidases to hydrolyze the complex carbohydrate chain in sphingoglycolipids suggests that the mechanism for the catabolism of sphingoglycolipids is far more complex than that previously postulated. Whether all of the glycosidases involved in the catabolism of sphingoglycolipids require an activator or not remains to be determined. Complete characterization of the activator isolated from human liver will be published elsewhere.

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