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Hydrolysis of GM_1-Ganglioside by Human Liver β-Galactosidase Isoenzymes

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1. GM_1-ganglioside, specifically tritiated in the terminal galactose, was hydrolysed by two forms of 'acid' methylumbelliferyl β-galactosidase isolated on gel filtration. 2. Identification of GM_1-ganglioside β-galactosidase activity with the 'acid' methylumbelliferyl β-galactosidases was based on the following: coincident elution profiles on gel filtration; simultaneous inactivation by heat and other treatments; stabilization of both activities by chloride ions; mutual inhibition of hydrolysis by the two substrates. 3. The two isoenzymes (I) and (II) showed general requirements for a mixture of anionic and nonionic detergents in the hydrolysis of the natural substrate. 4. Isoenzyme (I) differed from (II) in molecular size, pH–activity profile, relative resistance to dilution and in sensitivity to various inhibitors. 5. The most significant difference between the isoenzymes is in substrate saturation kinetics: (I) was hyperbolic whereas (II) was sigmoid. The apparent Michaelis constants were 28 μM for (I) and 77 μM for (II). Isoenzyme (I) was insensitive to GM_2-ganglioside whereas (II) was inhibited, consistent with the hypothesis that GM_1-ganglioside (and its analogue) acts as modifier in isoenzyme (II) but not in (I). 6. Isoenzyme (I) was membrane-bound whereas (II) was soluble; the former probably represents isoenzyme (II) bound to membrane components, thereby becoming activated. 7. Membranes may serve a dual role in enzyme catalysis involving lipids: as a medium where both enzyme and substrate are effectively concentrated, and as actual activator of enzymes through binding of the latter to specific membrane components.

Multiple forms of β-galactosidase (EC 3.2.1.23) were detected in mammalian tissues by using synthetic substrates (see, for example, Furth & Robinson, 1965; Jungawala & Robins, 1968; Ho & O'Brien, 1970, 1971). Enzymic cleavage of naturally occurring β-galactosidic linkages have been demonstrated in glycolipids (Gatt & Rapport, 1966; Gatt, 1967; Bowen & Radin, 1968; Okada & O'Brien, 1968; Dawson & Stein, 1970), glycoproteins and mucopolysaccharides (Mahadevan *et al.*, 1969; MacBrinn *et al.*, 1969). Differential specificity for lactose is well known among the isoenzymes in small intestine (Asp & Dahlqvist, 1968; Alpers, 1969; Gray & Santiago, 1969). As yet, no definitive studies on the specificity of the β-galactosidase isoenzymes for glycolipids have been performed (nor for glycoproteins or mucopolysaccharides).

Galactocerebrosidase could be isolated from the bulk of the β-galactosidase activity measured with artificial glycosides (Bowen & Radin, 1968). Circumstantial evidence suggests the presence of specific β-galactosidases in the hydrolysis of galactocerebroside (Galβ1-1ceramide*), ceramide lactoside (Galβ1-4Glcβ1-1ceramide) and GM_1-ganglioside respectively: defect in the catabolism of each glycolipid was associated with a specific hereditary disease (Suzuki & Suzuki, 1970; Dawson & Stein, 1970; Okada & O'Brien, 1968). In GM_1-gangliosidosis, the deficiency in hydrolytic activity for GM_1-ganglioside was not accompanied by a decrease in hydrolysis of ceramide lactoside or of galactocerebroside (Brady *et al.*, 1970).

In the present paper, the hydrolysis of GM_1-ganglioside by the isoenzymes of β-galactosidase in human liver is described. GM_1-ganglioside β-galactosidase activity was identified with two 'acid' β-galactosidases; the two isoenzymes were compared and the significance of differences is discussed.

* Abbreviations: Abbreviations for individual gangliosides are those proposed by Svennerholm (1963), based on the number of sialic acid residues present in the molecule and chromatographic properties. The monosialogangliosides are abbreviated to GM and the disialogangliosides to GD. The number that follows refers to its order of migration on t.l.c. NeuNAc, *N*-acetylneuraminic acid; GM_1, Galβ1-3GalNAcβ1-4(NeuNAcα2-3)Galβ1-4Glcβ1-1ceramide; GM_2, GalNAcβ1-4(NeuNAcα2-3)Galβ1-4Glcβ1-1ceramide; GD_{1a}, NeuNAcα2-3Galβ1-3GalNAcβ1-4(NeuNAcα2-3)Galβ1-4Glcβ1-1ceramide; GD_{1b}, Galβ1-3GalNAcβ1-4(NeuNAcα2-8NeuNAcα2-3)Galβ1-4Glcβ1-1ceramide.

Experimental

Materials

Human livers were obtained at autopsy and stored at −20°C before use. They were kindly supplied by Dr. A. D. Patrick.

Ceramide and glucocerebroside (Glcβ1-1ceramide) were gifts from Dr. J. S. O'Brien and were purified by preparative t.l.c.

GM_2-ganglioside was prepared from a chloroform–methanol (2:1, v/v) extract of brain (obtained at autopsy) from a patient with Tay–Sachs disease. The extract was dried on a rotary evaporator several times from methanol. The denatured protein was filtered off by passing the total lipid mixture in chloroform–methanol (2:1, v/v) through glass wool. To the resultant clear solution was added 0.2vol. of water. After mixing, the phases were clarified by centrifugation (100*g*, 2min). The upper phase was aspirated off, washed once with a theoretical lower phase (Folch *et al.*, 1957) and evaporated to dryness. Pure GM_2-ganglioside (which comprised 90% of the mixture) was obtained by preparative t.l.c.

The following materials were from commercial sources as indicated: purified bovine brain gangliosides (type III) and neuraminidase (type VI from *Clostridium perfringens*) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; sodium taurocholate (A grade) and Triton X-100 (B grade) from Calbiochem, Los Angeles, Calif., U.S.A.; stearoyl-dihydrosphingosine from Miles Laboratories, Elkhart, Ind. U.S.A.; 4-methylumbelliferyl β-D-galactopyranoside 4-methylumbelliferyl β-D-glucopyranoside, galactocerebroside, 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 1,4-dioxan and naphthalene from BDH Chemicals Ltd., Poole, Dorset, U.K.: NaB^3H_4 (100mCi/mmol) from New England Nuclear, Boston, Mass., U.S.A.; silica gel H and silica gel pre-coated plates from E. Merck, Darmstadt, Germany; Sephadex G-150 from Pharmacia, Uppsala, Sweden.

Methods

Preparation of GM_1-ganglioside. GM_1-ganglioside was prepared by the method of Wenger & Wardell (1972). The mixture of bovine brain gangliosides (500mg) was dissolved in 100ml of 10mM-sodium acetate buffer, pH5.0, and incubated at 37°C for 24h with three additions (1unit each) of neuraminidase. The final product consisted of approx. 80% GM_1-ganglioside and 20% of the disialoganglioside GD_{1a} or GD_{1b}. This crude product was used in the preparation of radioactive GM_1-ganglioside without further purification.

Preparation of radioactive GM_1-ganglioside. GM_1-ganglioside, specifically labelled in the terminal galactose, was prepared by the galactose oxidase method (Radin *et al.*, 1969) as modified by Ho (1973). The radioactive product was dialysed exhaustively against distilled water to remove excess of NaB^3H_4 and purified by preparative t.l.c. on 1mm-thick silica gel H plates. Silica gel was removed from the purified product by dialysis against water.

Characterization of radioactive GM_1-ganglioside. The product co-chromatographed on silica gel t.l.c. with GM_1-ganglioside in the following solvent systems: chloroform–methanol–aq. NH_3 (2.5M) (60:40:9, by vol.); chloroform–methanol–water (35:15:2, by vol.); propan-1-ol–water (7:3, v/v). All radioactivity was associated with GM_1 ganglioside. The specific radioactivity was 5000c.p.m./nmol. This was used without further dilution with non-radioactive GM_1-ganglioside.

Identification of the products of hydrolysis. Radioactive GM_1-ganglioside was incubated with 2 units of partially purified 'acid' β-galactosidase (peak II, see the Results section) for 1h as in the assay procedure. The reaction mixture was plated and developed in the appropriate solvent system, and then scanned for radioactivity by sectioning the lanes and counting each section in the scintillation counter as in the assay procedure described below. Two peaks of radioactivity corresponding to galactose and GM_1-ganglioside were demonstrated. In a mixture containing boiled enzyme, no radioactive galactose was released. Orcinol staining for carbohydrates (0.2%, w/v, orcinol in 50%, v/v, H_2SO_4) and resorcinol staining for sialic acid (Svennerholm, 1957) revealed only the primary products GM_2-ganglioside and galactose in the mixture with active enzyme.

Enzyme assays. Radioactive GM_1-ganglioside was transferred in 5μg portions in chloroform–methanol (2:1, v/v) to small test tubes, evaporated to dryness by exposure to air, and redissolved in 15μl of 0.05M-sodium acetate buffer, pH4.0, containing 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100. Enzyme was added and the final volume of incubation adjusted to 30μl. Incubation was carried out for 15min at 37°C. The reaction was stopped by adding 0.1ml of chloroform–methanol (2:1, v/v). To check for possible formation of asialoganglioside GA_1 (structure as in GM_1-ganglioside with the omission of NeuNAc), some reactions were stopped by adding 0.2ml of chloroform–methanol (2:1, v/v). The lower phase, clarified by centrifugation, was aspirated off into a separate test tube. The ordinary reaction mixtures as well as the separated phases were dried in a desiccator connected to a vacuum pump. The solids were extracted with chloroform–methanol (2:1, v/v), and applied in 10mm streaks to silica gel pre-coated plates. The plates were developed in chloroform–methanol–aq. NH_3 (2.5M) (60:40:9, by vol.). Galactose (10μg)

was applied as marker in some lanes. The developed plates were air-dried, and galactose and glycolipids made visible in an iodine chamber (5–10min). The section corresponding to galactose was scraped off into a scintillation vial; 0.4ml of water was added, followed by 10ml of 1,4-dioxan [containing 100g of naphthalene, 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 7g of 2,5-diphenyloxazole per litre]. Radioactivity was measured in c.p.m. in a Packard Tri-Carb Scintillation Counter. No asialoganglioside GA_1 was generated during the incubation, as evidenced by t.l.c. analysis of the lower phase on partitioning after enzyme reaction.

Methylumbelliferyl β-galactosidase was assayed in the same buffer system as used for GM_1-ganglioside hydrolysis (for 'acid' β-galactosidase) or in 0.05M-sodium acetate buffer, pH6.0 (for 'neutral' β-galactosidase activity). This substrate (1.0mM) was dissolved in the buffer; 15μl of enzyme was added to 15μl of the buffer–substrate solution and incubated for 15min at 37°C. The reaction was stopped by the addition of 1ml of 0.1M-glycine–NaOH buffer, pH10. 4-Methylumbelliferone released was measured fluorimetrically (Ho & O'Brien, 1971).

Gel filtration. Human liver was homogenized in a ground-glass hand homogenizer in 10vol. of 5mM-sodium phosphate buffer, pH7.0 (diluted from a stock mixture of 0.2M-Na_2HPO_4 and 0.2M-NaH_2PO_4), containing 5mM-NaCl, and centrifuged at 100000*g* for 45min to obtain a clear supernatant. This was applied to a column (100cm×2.5cm) of Sephadex G-150 equilibrated in the same buffer. Fractions (3ml) were collected at a flow rate of 30ml/h and monitored for both GM_1-ganglioside β-galactosidase and methylumbelliferyl β-galactosidase.

Preparation of GM_1-ganglioside β-galactosidases. The two forms of GM_1-ganglioside β-galactosidases (I and II, see Fig. 1) were prepared separately from each other as follows: peak I enzyme was obtained by gel filtration in 10mM-sodium phosphate buffer, pH7.0 (without chloride). Peak II enzyme was obtained by gel filtration of the 100000*g* supernatant after acid precipitation. Sodium acetate buffer (0.2M, pH4.0) was added to the supernatant to give pH5.0. This was left at 4°C for 1h and then centrifuged (1000*g*, 10min) to remove the precipitated protein.

Heat inactivation. Portions (50μl) of enzyme solution in 10mM-sodium phosphate buffer, pH7.0, were incubated at 37°C for various times in small test tubes sealed with Parafilm. At the end of the incubation the sample was plunged into a mixture of ice and water; 15μl portions were withdrawn for assay of methylumbelliferyl β-galactosidase and GM_1-ganglioside β-galactosidase.

Determination of kinetic constants. Kinetic constants ±S.E. were obtained for FORTRAN computer curve-fitting of data obtained from at least two independent experiments to equations (1) and (2).

$$v = \frac{V}{1 + K_m/s} \quad (1)$$

$$v = \frac{V}{1 + K_2/s + K_1 K_2/s^2} \quad (2)$$

K_m represents Michaelis constant for a single substrate reaction with a single binding site or non-interacting sites on the enzyme. K_1 and K_2 represent dissociation constants of the reactions:

$$E + S \rightleftharpoons SE$$
$$SE + S \rightleftharpoons SES$$

where the substrate acts as its own modifier (Dixon & Webb, 1964; Frieden, 1964). The assumption is made that substrate activation is essential (Frieden, 1964), i.e. the catalytic site of the non-activated enzyme has no affinity for the substrate.

Preliminary estimates of the constants were obtained graphically by Lineweaver–Burk plots. The reciprocal of eqn. (2):

$$1/v = 1/V + K_2/V(1/s) + K_1 K_2/V(1/s^2)$$

was used to obtain solutions for the general parabolic function

$$y = a + bx \times cx^2$$

Solutions for a, b and c give estimates of $1/V$, K_2/V and $K_1 K_2/V$ respectively.

Curve-fitting to eqn. (1) was done by the computer program of Cleland (1963). Curve-fitting to eqn. (2) was performed by the computer program based on the method of Rosenbrock (1960). A substrate concentration range of 1.1–111μM was examined.

Results

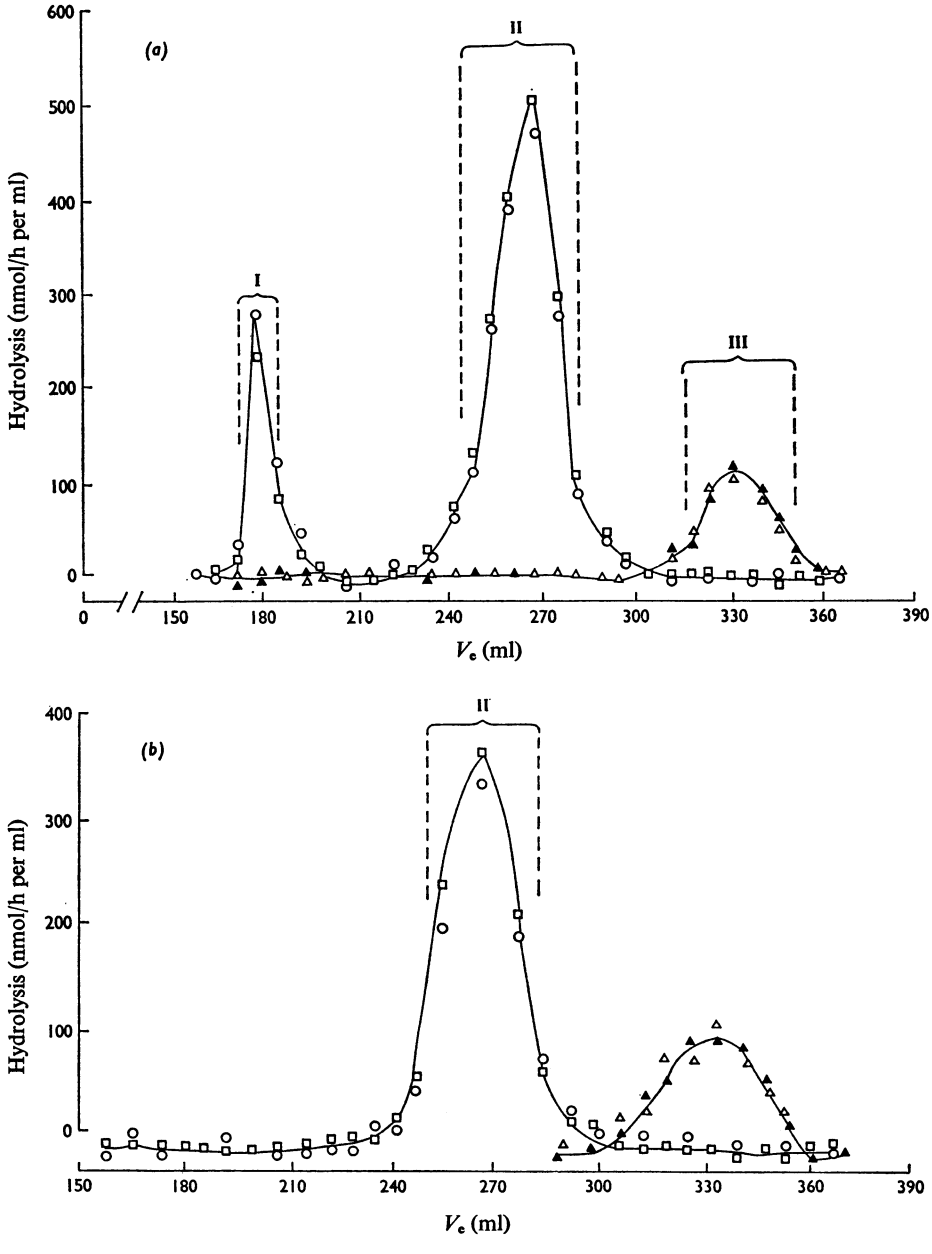
Identification of GM_1-ganglioside β-galactosidase with 'acid' β-galactosidase

Gel filtration of a high-speed supernatant of human liver gave three peaks of 4-methylumbelliferyl-β-galactosidase activity (Fig. 1*a*). In confirmation of previous findings (Ho & O'Brien, 1971), peaks I and II were 'acid' isoenzymes with optimum activity between pH4 and 5 (Fig. 3*a*, *b*) whereas peak III was a 'neutral' isoenzyme eluted coincident with 'neutral' β-glucosidase, and evidence described elsewhere (Ho & O'Brien, 1971) suggests this represents an enzyme hydrolysing both the β-galactoside and β-glucoside.

GM_1-ganglioside β-galactosidase eluted coincidently with both peaks of 'acid' β-galactosidase activity (Fig. 1*a*). Under the conditions used for assay, there was a 1:1 equivalence in activity for the synthetic and natural substances.

Peak I β-galactosidase was precipitated by adjusting the pH of the 100000*g* supernatant to 5.0 and removed by centrifugation (see the Methods section). On gel filtration of the acid-treated supernatant, peak I methylumbelliferyl β-galactosidase had disappeared and, with it, the coincident GM_1-ganglioside β-galactosidase (Fig. 1*b*).

Peak II β-galactosidase was over 95% inactivated by gel filtration in the absence of chloride. This was accompanied by the simultaneous disappearance of coincident GM_1-ganglioside β-galactosidase activity (Fig. 1*c*). 'Neutral' β-galactosidase was unaffected under all conditions tested. Table 1 summarizes the parallel distribution of GM_1-ganglioside β-galactosidase and 'acid' methylumbelliferyl β-galactosidase activities.



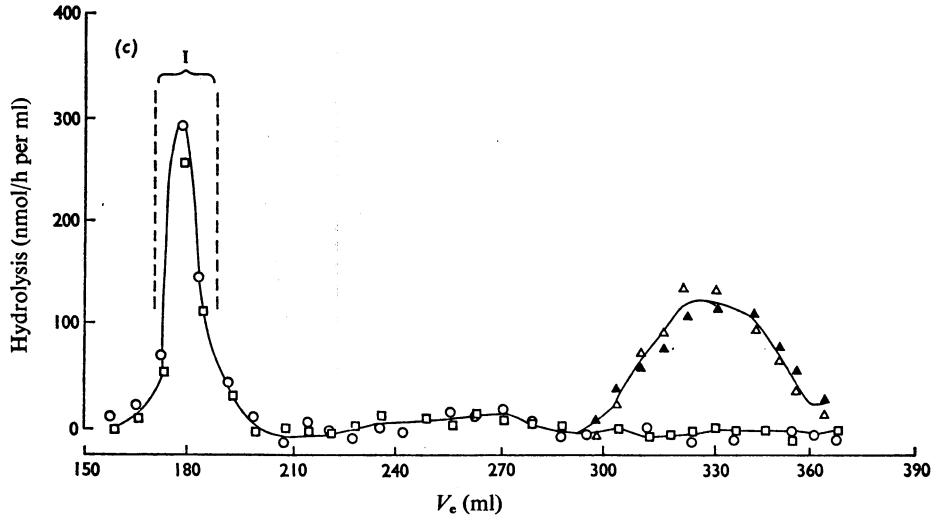


Fig. 1. *Gel filtration of human liver β-galactosidase*

Conditions are detailed under 'Methods'; 15 μl of each 3 ml fraction was assayed directly for methylumbelliferyl β-galactosidase, GM_1-ganglioside β-galactosidase and methylumbelliferyl β-glucosidase. ○, GM_1-ganglioside β-galactosidase; □, 'acid' methylumbelliferyl β-galactosidase; △, 'neutral' methylumbelliferyl β-galactosidase; ▲, methylumbelliferyl β-glucosidase assayed in 0.05 M-sodium acetate buffer, pH 6.0, with 2 mM-4-methylumbelliferyl β-D-glucopyranoside. (*a*) Gel filtration in 5 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-NaCl. (*b*) Gel filtration of acid-precipitated liver supernatant (see under 'Methods') in 5 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-NaCl. (*c*) Gel filtration in 10 mM-sodium phosphate buffer, pH 7.0.

Table 1. *Distribution of GM_1-ganglioside β-galactosidase and 'acid' methylumbelliferyl β-galactosidase activities*

Activities for both GM_1-ganglioside and 4-methylumbelliferyl β-galactoside were assayed in 0.05 M-sodium acetate buffer, pH 4.0, containing 0.1% (w/v) sodium taurocholate and 0.05% (v/v) Triton X-100.

	GM_1-ganglioside β-galactosidase (*A*)			Methylumbelliferyl β-galactosidase (*B*)			
Fraction	Total activity	Sp. activity	%	Total activity	Sp. activity	%	*A*/*B*
Homogenate 100000*g*	17000	220	100	17770	230	100	0.95
Supernatant 100000*g*	17300	427	102	16820	421	95	1.03
Residue	220	5	1.3	270	6	1.5	0.85
(*a*)* Peak I	2300	701	13.5	2100	636	12	1.10
(*a*) Peak II	13800	1020	81.0	13890	1021	78	0.92
(*a*) Peak III	0	0	0	0	0	0	0
(*c*)* Peak I	2335	778	13.7	2120	707	12	1.10
(*b*)* Peak II	10728	1506	63.0	11102	1563	62	0.96

* Peaks pooled as shown in Fig. 1(*a*), 1(*b*) and 1(*c*).

Properties of the GM_1-ganglioside β-galactosidases

Peak I was obtained from gel filtration under conditions described in Fig. 1(*c*). Peak II was obtained under conditions described in Fig. 1(*b*).

Effect of detergents, albumin and strong salts. The requirements for the two peaks of GM_1-ganglioside β-galactosidase activity were identical with those tested in detail for ceramide trihexoside α-galactosidase (Ho, 1973). Both sodium taurocholate and Triton X-100 were required for maximal activity. Protein (0.1% albumin) and strong salts (0.2 M-NaCl) both inhibited by more than 80%.

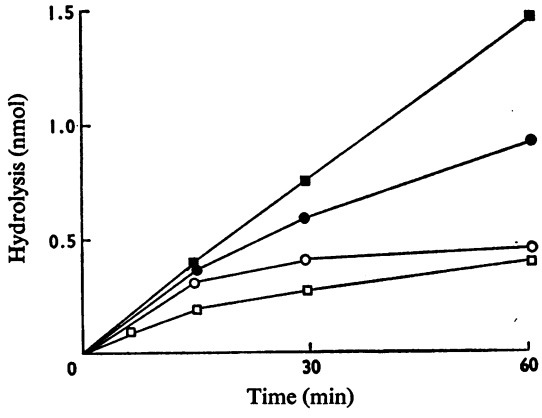


Fig. 2. *Time-course of peak II β-galactosidase activities in the presence and absence of chloride ions*

○, ●, GM_1-ganglioside β-galactosidase; □, ■, methylumbelliferyl β-galactosidase. Open symbols, without chloride; filled-in symbols, with 5mM-NaCl.

Time-course and effects of dilution with and without low concentrations of chloride. 'Acid' β-galactosidase activity was stimulated and stabilized by chloride ions (Ho & O'Brien, 1970, 1971). The effect of low concentrations of chloride (5mM) was tested since higher concentrations interfered with interactions between the lipid substrate and enzyme in a non-specific way (Ho, 1973).

A non-linear time-course was exhibited by peak I and peak II enzyme activities for both the natural and the synthetic substrates; 5mM-NaCl in the assay mixture stabilized both activities. The stabilization was particularly marked in peak II and is illustrated in Fig. 2. Enzyme activities were linear during the first 15min of incubation. All kinetic studies were therefore carried out with an incubation period of 15min.

Dilution resulted in rapid deterioration of methylumbelliferyl β-galactosidase activity in peak II but not in peak I. GM_1-ganglioside β-galactosidase activity in both peaks was far more resistant to dilution than the corresponding activity for the synthetic substrate. This suggested that the natural substrate protects against inactivation caused perhaps by dissociation of enzyme subunits on dilution. The effect of chloride was to stimulate peak I methylumbelliferyl β-galactosidase activity at all protein concentrations tested. Peak II methylumbelliferyl β-galactosidase was both stimulated and completely protected against inactivation on dilution. Little or no stimulation of GM_1-ganglioside β-galactosidase activity was evident in either peak I or peak II, suggesting that the natural substrate itself may function in some way similar to chloride in stimulating hydrolysis, e.g. in the maintenance of an active enzyme conformation.

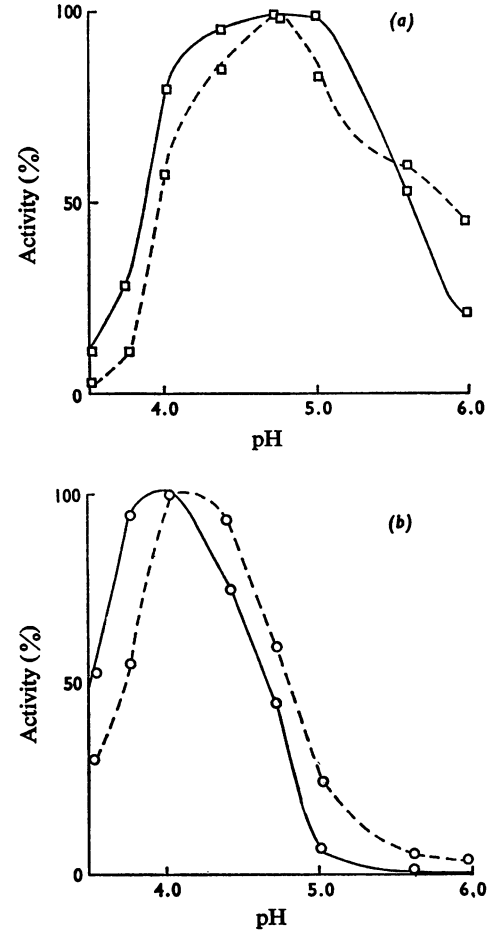


Fig. 3. *pH–activity of β-galactosidases*

Sodium acetate (0.1M) and acetic acid (0.1M), each containing 0.1% (v/v) Triton X-100 and 0.2% (w/v) sodium taurocholate, were mixed in various proportions for a range of pH3.5–6.0 and used directly for assay of (*a*) methylumbelliferyl β-galactosidase and (*b*) GM_1-ganglioside β-galactosidase. Continuous line, peak I; broken line, peak II. All assays were performed with a 15min incubation.

pH–activity profile. There was a small but consistent difference in the pH–activity profile between peaks I and II, in agreement with previous results (Ho & O'Brien, 1971). Under the present conditions of assay, methylumbelliferyl β-galactosidase activity

exhibited a broad optimum between pH4 and 5 for peak I, and at around pH4.7 for peak II (Fig. 3*a*). GM_1-ganglioside hydrolysis exhibited optimum activity between pH3.8 and 4.2 for peak I, and between pH4.0 and 4.3 for peak II (Fig. 3*b*).

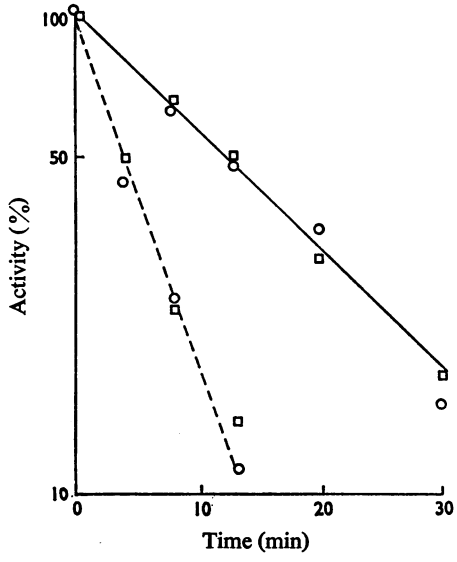


Fig. 4. *Heat inactivation of β-galactosidases*

Conditions are outlined under 'Methods'. Residual activities were determined in the presence of 5mM-NaCl. ○, GM_1-ganglioside β-galactosidase; □, methylumbelliferyl β-galactosidase; continuous line, peak I; broken line, peak II.

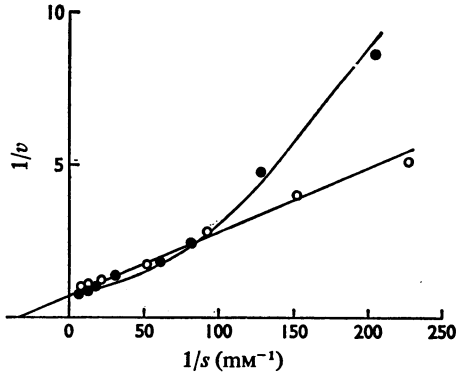


Fig. 5. *Lineweaver–Burk plots for GM_1-ganglioside hydrolysis*

Each point represents the average of two determinations $1/v$ is expressed as 10^3/c.p.m. ○, Peak I; ●, peak II.

Heat inactivation. Peaks I and II were further distinguished by heat inactivation (Fig. 4). For both isoenzymes, activities for the natural and the synthetic substrates decreased at the same rate. Peak I appeared to be the more stable of the two consistent with its resistance to dilution.

Substrate saturation. By far the most impressive difference between peaks I and II was in the substrate saturation kinetics. Peak II behaved like ceramide trihexoside α-galactosidase (Ho, 1973) in that an upward-curving Lineweaver–Burk plot was obtained, suggesting that GM_1-ganglioside acts like ceramide trihexoside as a modifier for its own hydrolysis (Fig. 5). K_1, the dissociation constant of the modifier site, was estimated to be $2.1 \pm 0.17\,\mu$M; K_2, the dissociation constant of the activated enzyme–substrate complex, was $77 \pm 0.24\,\mu$M. In contrast, peak I gave a linear Lineweaver–Burk plot in the same range of substrate concentrations. The Michaelis constant in this case was $28 \pm 3\,\mu$M, a difference of two- to three-fold compared with K_2 of peak II.

Effect of inhibitors. Two groups of inhibitors were tested: water-soluble sugars and glycosides, and lipid analogues of GM_1-ganglioside. The results are summarized in Table 2.

Inhibition by the first group of inhibitors was

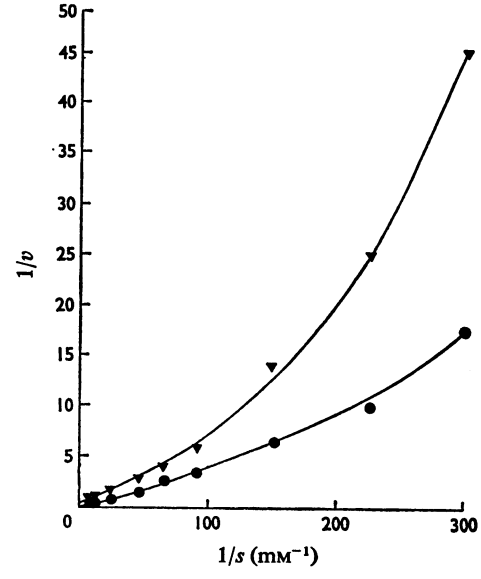


Fig. 6. *Effect of GM_2-ganglioside on GM_1-ganglioside hydrolysis in peak II*

Lineweaver–Burk plots were obtained in the absence (●) and presence (▲) of 0.1mM-GM_2-ganglioside. $1/v$ is expressed as 10^3/c.p.m. Inhibitor was added simultaneously with substrate. Each point represents the average of two determinations.

Table 2. *Inhibition of β-galactosidase activities*

The inhibitor was added simultaneously with substrate at a concentration indicated in parentheses. Each value represents the average of two or three determinations.

	Inhibition (%)			
	Peak I		Peak II	
Inhibitor	Methylumbelliferyl-β-galactosidase	GM_1-ganglioside β-galactosidase	Methylumbelliferyl-β-galactosidase	GM_1-ganglioside β-galactosidase
Galactose (10mM)	69	0	60	0
Glucose (10mM)	0	0	0	0
Lactose (10mM)	30	59	43	50
N-Acetylneuraminic acid (5mM)	0	0	0	0
4-Methylumbelliferyl β-galactoside (0.5mM)	—	10	—	10
GM_2-ganglioside (0.1mM)	0	4	0	32
Ceramide lactoside (0.1mM)	0	34	0	30
Galactocerebroside (0.1mM)	0	60	0	30
Glucocerebroside (0.1mM)	0	60	0	0
Ceramide (0.1mM)	0	12	0	0
GM_1-ganglioside (0.1mM)	29	—	20	—

similar for peaks I and II. Galactose inhibited activity for the synthetic but not the natural substrate, whereas lactose inhibited activities for both substrates. None of the lipids tested inhibited activity for the synthetic substrate. Peak II GM_1-ganglioside β-galactosidase was inhibited to about equal extents by GM_2-ganglioside, the product of hydrolysis, as well as by ceramide lactoside and galactocerebroside. Glucocerebroside and ceramide were non-inhibitory. In contrast, peak I GM_1-ganglioside β-galactosidase was not significantly inhibited by GM_2-ganglioside though it was rather strongly inhibited by galactocerebroside and glucocerebroside.

GM_2-ganglioside inhibited hydrolysis of GM_1-ganglioside in peak II at all concentrations of GM_1-ganglioside tested (Fig. 6). The extrapolated maximal velocity at high substrate concentrations remained unchanged.

Discussion

Under the present assay conditions, a high activity for the natural substrate is attained. The 1:1 equivalence of enzyme activity for the synthetic and natural substrates compares well with other glycosidases recently studied (Ho *et al.*, 1973; Ho, 1973). In all cases, activity for the natural substrate is of the same order of magnitude as that for the synthetic substrate. This indicates that high activities are attainable for natural substrates and optimum conditions of assay should be rigorously sought after.

The detergent requirements for hydrolysis of GM_1-ganglioside are similar to those of ceramide trihexoside hydrolysis (Ho, 1973). The suggestion was made (Ho, 1973) that the mixture of detergents (in simulation of physiological membranes) provides for the concentration of both enzyme and substrate in a hydrophobic milieu where catalysis takes place. Albumin and chloride ions interfere with initial electrostatic interactions between enzyme and detergent in a non-specific way.

The two forms of GM_1-ganglioside β-galactosidase are of considerable interest. Demonstration of hyperbolic substrate saturation kinetics in isoenzyme (I) to a large extent eliminates the physical state of the lipid substrate as a contributing cause to sigmoid kinetics of isoenzyme (II). Assays for the two isoenzymes were performed under identical conditions for the same range of substrate concentrations. Any difference must be attributed to enzyme characteristics. The two isoenzymes differ in molecular size and are electrophoretically distinct (Ho & O'Brien, 1971). General properties are, however, quite similar: acid pH optimum, stimulation and stabilization by chloride ions, substrate specificity (both hydrolysing GM_1-ganglioside) and relative instability to heat. That they are the same enzyme or very closely related is indicated by their simultaneous absence in GM_1-gangliosidosis (Ho & O'Brien, 1971). Peak I may represent peak II enzyme bound to membrane components. More peak I material could be released from the particulate fraction of human

liver or spleen by treatment with 0.02% (v/v) Triton X-100 (M.W. Ho, unpublished work). It is conceivable that peak I is an activated peak II enzyme 'dissolved' in the lysosomal membrane. This is suggested by the two- to three-fold decrease in apparent K_m in peak I compared to that (K_2) of peak II. Thus membranes may serve a dual role in enzyme catalysis involving lipids: as a medium where both enzyme and substrate are effectively concentrated [see Ho (1973) for discussion] and as actual activator of enzymes through binding of the latter to specific membrane components.

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