Biochem. J. (1973) **133**, 1–10
Printed in Great Britain

Hydrolysis of Ceramide Trihexoside by a Specific α-Galactosidase from Human Liver

By MAE WAN HO
Department of Biochemistry, Queen Elizabeth College, University of London, Campden Hill, London W8 7AH, U.K.

(*Received* 30 *October* 1972)

1. Partially purified ceramide trihexoside α-galactosidase from human liver was studied by using ceramide trihexoside specifically tritiated in the terminal galactose. 2. The hydrolysis of ceramide trihexoside was absolutely dependent on a mixture of sodium taurocholate and Triton X-100 and was markedly inhibited by human serum albumin and by NaCl. 3. The Lineweaver–Burk plot for ceramide trihexoside hydrolysis was upward curving. Ceramide lactoside inhibited hydrolysis of all concentrations of ceramide trihexoside. Ceramide digalactoside stimulated hydrolysis of low concentrations of ceramide trihexoside, but inhibited hydrolysis of high concentrations of the lipid. 4. α-Galactosidase activity assayed with the synthetic substrate 4-methylumbelliferyl α-D-galactopyranoside fractionated together with activity assayed with the natural substrate ceramide trihexoside. Both activities had identical heat-inactivation kinetics. 5. Characteristics of the hydrolysis of the synthetic substrate differed considerably from those of the natural substrate, including pH optimum, shape of the Lineweaver–Burk plot, and differential effects of inhibitors and activators. Mutual inhibition of hydrolysis between the synthetic and natural substrates was predominantly non-competitive. 6. These results are discussed in the light of special problems involved in the hydrolysis of lipids in an aqueous milieu.

The enzymic hydrolysis of lipids poses special problems some of which were considered by Gatt *et al.* (1972). In the present work, the hydrolysis of ceramide trihexoside (Galα1–4Galβ1–4Glc–ceramide) (Hakomori *et al.*, 1971), by a specific human α-galactosidase (EC 3.2.1.22) was investigated in the hope of gaining some insight into these problems.

Enzymic hydrolysis of biological importance occurs in an aqueous milieu. In the case of ceramide trihexoside, for instance, the limiting factor is likely to be the low solubility of the glycolipid (of the order of 10^{-5} M, experimentally determined) which compares unfavourably with the K_m of the enzyme for various synthetic glycosides which is of the order of 10^{-3} M (Ho *et al.*, 1972). Ceramide trihexoside is a soluble amphipath (Chapman, 1969) that forms micelles with the polar oligosaccharide chain projected into the aqueous phase. Micelle formation must decrease the chance of fruitful enzyme–substrate interactions by several orders of magnitude; partly because of a decrease in effective substrate concentration and partly by steric hindrance of enzyme recognition of the glycosidic bond in the micellar state. Basically, all the problems may be reduced to one of enzyme–substrate approach: how to bridge the gap between the enzyme in the bulk aqueous phase and the lipid in the micellar aggregate?

'Ceramide trihexosidase' as studied by Brady *et al.* (1967) was a measure of the release of total hexoses from a uniformly tritiated ceramide trihexoside. With the galactose oxidase method described by Radin *et al.* (1969), it was possible to tritiate specifically the terminal galactose and to follow the kinetics of its hydrolysis more precisely.

Experimental

Materials

Human livers were obtained at autopsy and stored at −20°C for 2 weeks before use. They were kindly supplied by Dr. A. D. Patrick.

Ceramide lactoside was purchased from Miles Laboratories, Elkhart, Ind., U.S.A. Ceramide digalactoside, ceramide glucoside, ceramide galactoside, ceramide, and free fatty acids were gifts from Dr. J. S. O'Brien, and were further purified by preparative t.l.c. Heart muscle (from a patient with Fabry's disease) which was stored in formalin, was chopped into small pieces and extracted with ethanol. Ceramide trihexoside was purified from the extract by column chromatography (Radin *et al.*, 1969) and preparative t.l.c.

Plates precoated with silica gel H and silica gel

were obtained from E. Merck, A.-G. Darmstadt, Germany. Galactose oxidase from *Dactylium dendroides* was a product of Worthington Biochem. Corp., Freehold, N.J., U.S.A. NaB^3H_4 (100mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Sources of other chemicals were as follows; 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 4-methylumbelliferyl α-D-galactopyranoside from Koch-Light Laboratories, Ltd., Colnbrook, Bucks., U.K.; 1,4-dioxan, naphthalene, and Triton X-100 from British Drug Houses Ltd., Poole, Dorset, U.K.; sodium taurocholate (A grade) from Calbiochem, Los Angeles, Calif., U.S.A.; orcinol from Sigma, St. Louis, Mo., U.S.A.; DE-23 cellulose from Whatman, Maidstone, Kent, U.K.; Sephadex G-150 from Pharmacia, Uppsala, Sweden.

Methods

Preparation of ceramide tri[^3H]hexoside. To obtain ceramide trihexoside specifically tritiated in the terminal galactose, the galactose oxidase method described by Radin *et al.* (1969) was used with slight modifications. The glycolipid (60mg) was reacted with three additions (125 units each) of galactose oxidase over a period of 24h of incubation at 24°C. T.l.c. analysis indicated that about 50% of the ceramide trihexoside was oxidized to the aldehyde. Reduction of the oxidized glycolipid was accomplished with 18mCi (3.4mg) of NaB^3H_4. After the radioactive ceramide trihexoside was isolated from the incubation mixture, it was purified by preparative t.l.c. The specific radioactivity of the product was 30000c.p.m./nmol (mol. wt. of ceramide trihexoside taken to be 1052).

Characterization of ceramide tri[^3H]hexoside and identification of products of reaction. The purified radioactive ceramide trihexoside co-chromatographed with ceramide trihexoside in the following solvent systems: chloroform–methanol–water (35:15:2, by vol.); chloroform–methanol–2.5M-NH_3 (60:40:9, by vol.); chloroform–acetone–methanol–acetic acid–water (10:4:2:2:1, by vol.) (Parsons & Patton, 1967). Scanning of the t.l.c. plate showed that essentially all of the radioactivity in the preparation was associated with ceramide trihexoside.

To identify the products of reaction, hydrolysis of the radioactive substrate was allowed to proceed for 4h with purified enzyme (1.0 unit) as outlined in the assay procedure. A blank was set up with enzyme inactivated by boiling for 4min. The reaction was stopped with 1ml of chloroform–methanol (2:1, v/v) then it was evaporated to dryness in a stream of N_2. The solids were extracted with chloroform–methanol (2:1, v/v) and spotted on thin-layer plates precoated with silica gel. Standards of ceramide lactoside, ceramide trihexoside and galactose were also applied. After being developed in chloroform–methanol–water (35:15:2, by vol.), the lanes with reaction mixtures were scanned for radioactivity. The plate was then sprayed with 0.2% (w/v) orcinol in 50% (v/v) H_2SO_4 and heated for 5min at 110°C to locate glycolipids and hexoses. Two peaks of radioactivity in the positions of ceramide trihexoside and galactose were observed on scanning the incubation mixture containing active enzyme. In the mixture containing boiled enzyme, a single peak of radioactivity was present in the position of ceramide trihexoside. Orcinol staining revealed besides unchanged ceramide trihexoside, the primary products, ceramide lactoside and galactose in the active enzyme mixture. In the incubation mixture of the blank, no orcinol positive bands were detectable except ceramide trihexoside.

Assay of ceramide trihexoside α-galactosidase. Radioactive ceramide trihexoside was diluted with the non-radioactive glycolipid to a specific radioactivity of 3000–4000c.p.m./nmol. A portion (7μg) of ceramide trihexoside in chloroform–methanol (2:1, v/v) was transferred to a small test-tube and evaporated to dryness in a stream of N_2. This was redissolved with the aid of a vortex mixer in 25μl of a 0.1M-sodium acetate buffer, pH4.0, containing 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100. Enzyme solution was added, and the final volume of incubation mixture was adjusted to 50μl. The test tube was sealed with Parafilm and incubation was carried out for 15 or 30 min at 37°C. The reaction was stopped by the addition of 150μl water containing 10μg of galactose (Radin *et al.*, 1969) followed by 1ml of chloroform–methanol (2:1, v/v). The phases were mixed and then separated by centrifugation (120*g* for 2min). The upper phase was aspirated and washed with 0.8ml of a theoretical lower phase (Folch *et al.*, 1957). The phases were centrifuged to clarify these and the upper phase was aspirated and transferred to a scintillation vial, mixed with 10ml of 1,4-dioxan [containing 7g of 2,5-diphenyloxazole, 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 100g of naphthalene/l] and counted for radioactivity in a Packard Tri-Carb scintillation counter. The radioactive counting efficiency was 45–50%. One unit of ceramide trihexoside α-galactosidase activity represents hydrolysis of 1nmol/h.

Assay of methylumbelliferyl α-galactosidase. This was done as described previously (Ho *et al.*, 1972), or in the same buffer system as that used for ceramide trihexoside α-galactosidase. Kinetic parameters were similar in the two buffer systems.

Heat inactivation. Portions (50μl) of enzyme in 5mM-sodium phosphate buffer, pH7.0, were incubated for various times at 50°C, in small test tubes sealed with Parafilm. At the end of the incubation,

each sample was plunged into a mixture of ice and water at 0°C; 25μl was withdrawn for assay of ceramide trihexoside α-galactosidase and 10μl for assay of methylumbelliferyl α-galactosidase.

Purification of ceramide trihexoside α-galactosidase. Step 1. Human liver (20g) was homogenized in a Waring Blendor in 100ml of 10mM-sodium phosphate buffer, pH7.0, and centrifuged at 100000*g* for 1h to obtain a clear supernatant.

Step 2. Solid ammonium sulphate was added gradually with continuous stirring at 0°C to 30% saturation. The precipitate was spun down at 3000*g* for 15min, and the supernatant was adjusted to 60% saturation with ammonium sulphate as before. The precipitate was spun down, redissolved in water and dialysed for 24h against two changes of water. The protein precipitated during dialysis was spun down and removed.

Step 3. The clear solution was dialysed for 8h against 5mM-sodium phosphate buffer, pH7.0, containing 5mM-NaCl. This was applied to a Sephadex G-150 column (2.6cm×50cm) equilibrated in the same buffer. Fractions (2.15ml) were collected and active fractions containing ceramide trihexoside α-galactosidase were pooled.

Step 4. The pooled fractions were again adjusted to 60% saturation with ammonium sulphate. The precipitate was dialysed against two changes of water for 16h, then against 10mM-sodium phosphate buffer, pH6.0, for 8h.

Step 5. Whatman DE-23 cellulose was prewashed and equilibrated with 10mM-sodium phosphate buffer pH6.0, then packed under gravity in a 1cm×15cm column. The clear enzyme solution from Step 4 was applied in a total volume of 10ml. The column was washed with 50ml of the same buffer, then eluted with a linear NaCl gradient (0–0.3M) in 400ml of buffer.

Highly purified α-galactosidase. A 1600-fold purified α-galactosidase A from human placenta (Beutler & Kuhl, 1972*b*) was kindly supplied by Dr. E. Beutler.

Results

Purification of ceramide trihexoside α-galactosidase

The purification of ceramide trihexoside α-galactosidase is summarized in Table 1. Both ceramide trihexoside α-galactosidase and methylumbelliferyl α-galactosidase activities were monitored at every step. Both activities fractionated more-or-less together. The final purification was 57-fold and the recovery was 28%. This preparation was devoid of β-galactosidase activity, and was free from glycolipid.

Sephadex gel-filtration gave a non-symmetrical peak (Fig. 1*a*) containing both ceramide trihexoside α-galactosidase and methylumbelliferyl α-galactosidase activities. DEAE-cellulose chromatography separated a major component (with 98% total methylumbelliferyl α-galactosidase activity) from a minor component (Fig. 1*b*). Because of interference by salt in the ceramide trihexoside α-galactosidase assay, the latter activity was not monitored in the column effluent. After vacuum dialysis to concentrate and desalt the pooled peaks from DEAE-cellulose chromatography, peak 1 (α-galactosidase A; Beutler & Kuhl, 1972*b*) was found to contain all of the ceramide trihexoside α-galactosidase activity whereas peak 2 (α-galactosidase B; Beutler & Kuhl, 1972*b*) was inactive.

Properties of purified ceramide trihexoside α-galactosidase

The purified preparations from human liver and human placenta had identical properties where tested. These include ratio of methylumbelliferyl α-galactosidase/ceramide trihexoside α-galactosidase activities; effect of pH on activity; effect of analogues of ceramide trihexoside on ceramide trihexoside hydrolysis; heat inactivation kinetics of both methylumbelliferyl α-galactosidase and ceramide trihexoside α-galactosidase activities; and mutual inhibition

Table 1. *Purification of β-galactosidase from human liver*

For details see the text. Specific activity is expressed as nmol substrate cleaved/h per mg of protein.

	Ceramide trihexoside α-galactosidase (A)			Methylumbelliferyl α-galactosidase (B)			
Step	Total activity	Sp. act.	Recovery (%)	Total activity	Sp. act.	Recovery (%)	B/A
1 Homogenate	17626	5.1	100	120000	34.7	100	6.8
Supernatant (100000*g*)	17979	10.5	102	112200	67.8	93.5	6.5
2 $(NH_4)_2SO_4$ 60% ppt.	14894	56.6	84.5	98160	374.1	81.8	6.6
3 Sephadex G-150	8355	70.0	47.4	50400	418.2	42.0	6.0
4 $(NH_4)_2SO_4$ 60% ppt.	8354	86.5	47.4	49200	543.2	42.0	6.3
5 DE-23 peak I	5023	289.7	28.5	33600	1767.2	28.0	6.1

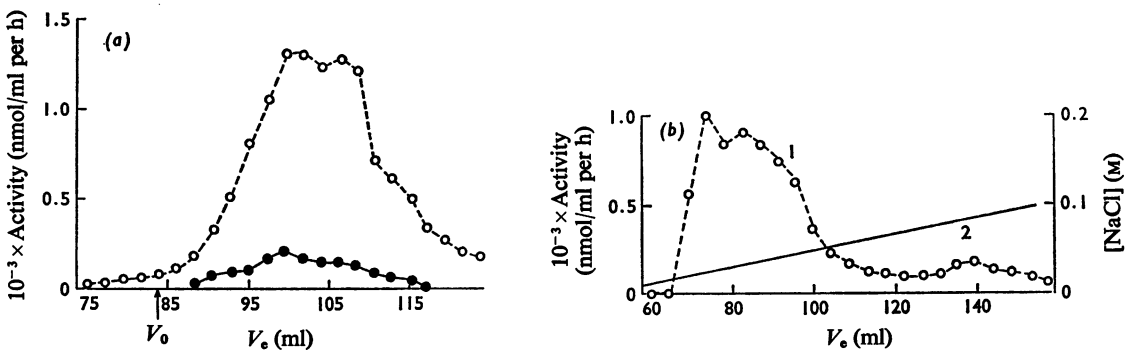

Fig. 1. *Fractionation of α-galactosidase from human liver*

(*a*) Sephadex G-150 gel-filtration of ceramide trihexoside α-galactosidase and methylumbelliferyl α-galactosidase. Conditions are as described in the Experimental section. Samples of each 2.15ml fraction were diluted 1/6 with water before the assay to minimize the effects of excess salt and protein. Gel filtration was performed in three batches on the same column. Each gave an identical elution pattern. ●, Ceramide trihexoside α-galactosidase; ○, methylumbelliferyl α-galactosidase. Fractions from 92.5–116ml were pooled. (*b*) DEAE-cellulose chromatography of methylumbelliferyl α-galactosidase (○). Conditions are as described in the Experimental section. A sample (10μl) of each 2.15ml fraction was assayed directly for methylumbelliferyl α-galactosidase. Fractions were pooled as follows; peak 1, 69–99ml; peak 2, 133–151ml. A linear sodium chloride gradient is shown (——).

between the synthetic and the natural substrates. Only the results obtained in the human liver preparation are given below.

Effect of pH on activity. Hydrolysis of methylumbelliferyl α-galactoside showed a broad optimum around pH4.5, where hydrolysis of ceramide trihexoside reached a sharp peak at pH3.5 (Fig. 2). Ceramide trihexoside α-galactoside as a routine was assayed at pH4.0, however, to minimize non-enzymic hydrolysis.

Time-course and protein concentration. Ceramide trihexoside hydrolysis was linear with time up to 60min of incubation at 37°C with less than 1nmol (14%) of the total substrate hydrolysed.

Ceramide trihexoside hydrolysis was linear with enzyme protein up to 20μg per assay volume of 50μl. A further increase in the amount of protein resulted in marked inhibition. That inhibition was caused by extraneous protein is suggested by strong inhibition with added human serum albumin (Fig. 3). Between 0.1–0.2% (w/v), albumin inhibited ceramide trihexoside α-galactosidase by about 80%. Inhibition by albumin was independent of substrate concentration and could not be relieved by increasing the substrate concentration by 2- to 3-fold. This indicated that substrate binding was not a significant factor in inhibition by albumin.

Effect of detergents. Ceramide trihexoside α-galactosidase activity was totally dependent on a mixture of Triton X-100 and sodium taurocholate. Either detergent alone gave no activity. The effect of other bile salts, e.g. sodium cholate and sodium deoxycholate, could not be tested as they were insoluble at the pH value of assay. The optimum concentration of taurocholate was 0.1–0.2% (Fig. 4*a*); of Triton X-100, 0.05% (Fig. 4*b*). Increasing the concentration of both detergents led to inhibition of enzyme activity (Fig. 4*c*). At the optimum concentration of Triton X-100, increase in enzyme activity with taurocholate was sigmoid, whereas that with Triton X-100 was hyperbolic.

Albumin cancels out the sharp increase in enzyme activity with either detergent (Figs. 4*a*, 4*b* and 4*c*). Increasing the concentration of taurocholate (from 0.1 to 0.2%) offset albumin inhibition to some extent, so that a new maximum was reached that was about 65% of that in the absence of albumin (Fig. 4*a*). Increasing Triton X-100 concentration (from 0.05–0.1%) was inhibitory and did not offset albumin inhibition appreciably (Fig. 4*b*).

Effect of salt. Low concentrations of strong salts, e.g. NaCl (0.2M) completely abolished ceramide trihexoside α-galactosidase activity. Ammonium sulphate had a similar effect.

Effect of substrate concentration. The substrate saturation curve was non-hyperbolic: the Lineweaver–Burk plot showed an upward curvature at low substrate concentrations. At high substrate concentrations (up to 0.13mM) a near-linear portion of the curve could be extrapolated to give an apparent K_m of about 5×10^{-5}M (Fig. 5). Ceramide trihexoside concentrations >0.13mM did not further increase enzyme activity, or were slightly inhibitory. Lineweaver–Burk plots of this type have never been

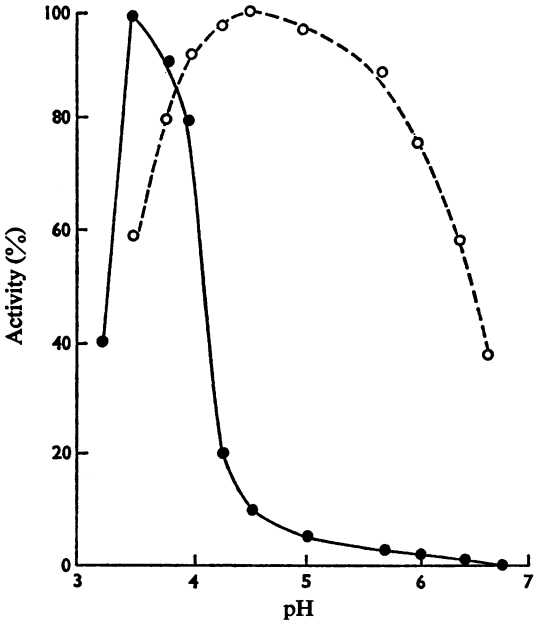

Fig. 2. *pH–activity curve of ceramide trihexoside α-galactosidase and methylumbelliferyl α-galactosidase*

Acetic acid (0.1 M) and sodium acetate (0.1 M), 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 pH 3.3–6.8. This was used directly for ceramide trihexoside α-galactosidase assay, and diluted 1/2 in water for methylumbelliferyl α-galactosidase assays. ●, Ceramide trihexoside α-galactosidase; ○, methylumbelliferyl α-galactosidase. The value of 100% indicates the maximal activity attained in each case.

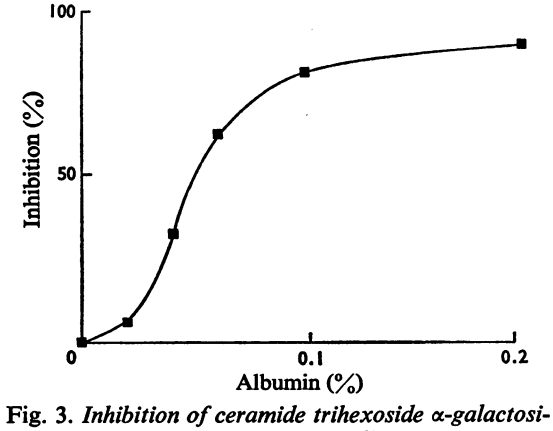

Fig. 3. *Inhibition of ceramide trihexoside α-galactosidase by human serum albumin*

The final concentration of added albumin is expressed as % (w/v). For further details see the text.

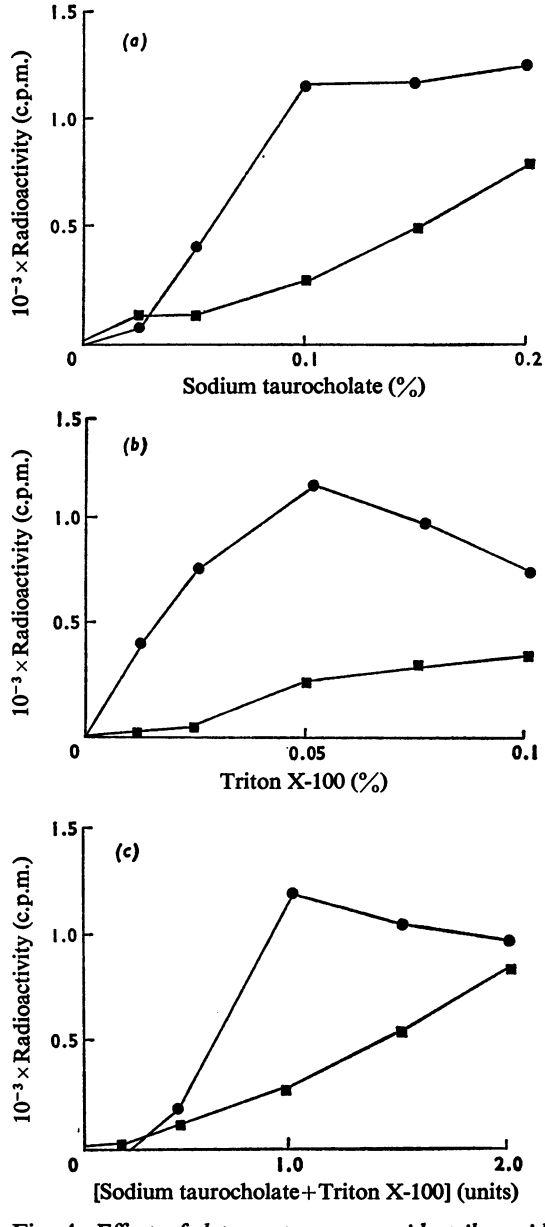

Fig. 4. *Effect of detergents on ceramide trihexoside α-galactosidase*

(*a*) Effect of various concentrations of sodium taurocholate in the presence of 0.05% Triton X-100. (*b*) Effect of various concentrations of Triton X-100 in the presence of 0.1% sodium taurocholate. (*c*) Effect of various concentrations of sodium taurocholate plus Triton X-100. One unit represents a concentration of 0.1% taurocholate and 0.05% Triton X-100. ●, In the absence of human serum albumin; ■, in the presence of human serum albumin (0.1%).

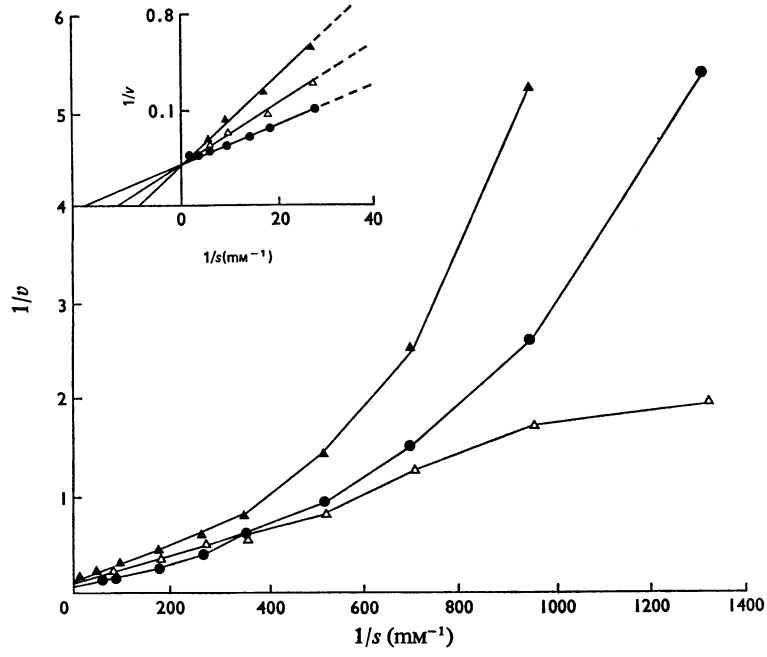

Fig. 5. *Effect of ceramide trihexoside concentration on rate of ceramide trihexoside hydrolysis*

Lineweaver–Burk plots were obtained in the absence (●) and presence of inhibitors; ▲, 0.1 mM-ceramide lactoside; △, 0.1 mM-ceramide digalactoside. $1/v$ is expressed as 10^2/c.p.m. Inhibitors were added simultaneously with substrate. Similar results were obtained in four other experiments at different enzyme concentrations.

shown for any lipid glycosidases; one reason may be that a wide enough range of substrate concentration was not tested. In the present case, a satisfactory linear Lineweaver–Burk plot can be demonstrated within a 3- to 4-fold difference in substrate concentrations (where most Lineweaver–Burk plots in the literature have been obtained). Gatt *et al.* (1972) predicted that enzymes which utilized only substrates in the micellar state would exhibit sigmoid kinetics. This seems unlikely in the present case, since the concentrations of detergents (much in excess of the substrate) were kept constant over the entire range of substrate concentrations, and mixed micelles of detergents and lipid would be expected at all concentrations of ceramide trihexoside tested. Non-linearity was much accentuated when the ratio of total detergents to lipid was maintained (i.e., concentrations of both detergents and lipid were varied in parallel). Enzyme activity decreased so rapidly that only two values, corresponding to the highest substrate and detergent concentrations, could be determined. Varying the concentration of one detergent with the substrate while maintaining that of the other detergent gave a similar deleterious effect. An upward curving Lineweaver–Burk plot indicates a decrease in K_m with substrate concentration and would be expected if the substrate itself were to function as a modifier. The existence of modifiers that are structural analogues of the substrate is compatible with this suggestion.

Effect of ceramide trihexoside analogues. Two analogues of ceramide trihexoside were tested for inhibition of ceramide trihexoside hydrolysis; ceramide lactoside (Galβ1–4Glc–Ceramide) and ceramide digalactoside (Galα1–4Gal–Ceramide) (Li *et al.*, 1972). The first is the product of the hydrolytic reaction; the second has the correct anomeric configuration in its terminal galactose to act as alternative substrate to ceramide trihexoside.

Ceramide lactoside inhibited at all ceramide trihexoside concentrations, causing an increased upward curvature in the reciprocal plot (Fig. 5). Ceramide digalactoside stimulated hydrolysis of low concentration of ceramide trihexoside, but inhibited hydrolysis of high concentrations of ceramide trihexoside (Fig. 5). Examination of the linear portion of the curve at high substrate concentrations revealed an apparently competitive type of inhibition (Fig. 5, insert).

Effect of other related lipids. To test further the

specificity of the inhibition, ceramide trihexoside hydrolysis was allowed to proceed in the presence of 0.1 mM of other lipids. At the optimum ceramide trihexoside concentration, ceramide lactoside inhibited by 37%, ceramide glucoside by 30%, and ceramide digalactoside by 25%. Ceramide galactoside, ceramide and free fatty acids had no effect. The kinetics of ceramide glucoside inhibition was identical with that of ceramide lactoside.

Unusual specificity is seen in the differential effects, first of ceramide lactoside and ceramide digalactoside, and secondly of ceramide glucoside and ceramide galactoside. The two analogues that inhibited hydrolysis at all ceramide trihexoside concentrations, ceramide lactoside and ceramide glucoside, are both stereospecifically unsuitable for binding to the catalytic site. This suggests the presence of a second site on the enzyme (other than the catalytic site) where recognition of the glycolipids takes place, and which can then exert a modifier influence on ceramide trihexoside hydrolysis. The modifier effect is 'negative' in the case of ceramide lactoside and ceramide glucoside, and 'positive' in the case of ceramide trihexoside. Ceramide digalactoside, an alternative substrate, may mimic the activating effect of ceramide trihexoside at low concentrations of the latter; at high ceramide trihexoside concentrations, inhibition is expected because its terminal galactosidic linkage is anomerically favourable, and can compete for the activated catalytic site. The more straightforward competitive nature of ceramide lactoside inhibition suggests that binding to the modifier site is an essential activation of the enzyme for binding to the catalytic site (Dixon & Webb, 1964; Frieden, 1964). Further experiments lend support to this idea.

Relationship between methylumbelliferyl α-galactosidase and ceramide trihexoside α-galactosidase. The two enzyme activities co-purified to a large extent; the ratio of one to the other remained between 6 and 6.8 (Table 1). This same ratio was maintained in the highly purified placental enzyme. Heat inactivation kinetics were identical for both activities (Fig. 6). The two activities appeared closely associated, if not identical. This is supported by the mutual inhibition of hydrolysis between the synthetic and the natural substrates (see below).

Mutual inhibition between ceramide trihexoside and methylumbelliferyl α-galactoside. Methylumbelliferyl α-galactoside markedly inhibited ceramide trihexoside hydrolysis at all ceramide trihexoside concentrations, much accentuating the upward curvature of the Lineweaver–Burk plot. Examination of the near-linear portion of the latter demonstrated a 'mixed' type of inhibition (Fig. 7*a*). The hydrolysis of methylumbelliferyl α-galactoside was hyperbolic, the Lineweaver–Burk plot yielding a K_m of 2 mM (Fig. 7*b*), compatible with a single binding site (or non-interacting sites) for methylumbelliferyl α-galactoside.

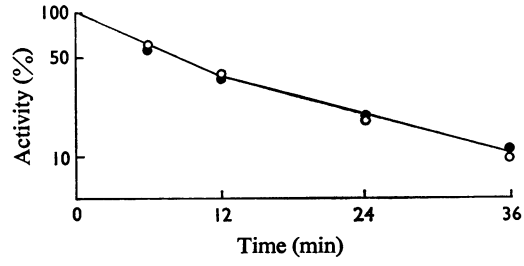

Fig. 6. *Heat inactivation of ceramide trihexoside α-galactosidase and methylumbelliferyl α-galactosidase*

Residual activity was measured after various time-periods at 50°C. ●, Ceramide trihexoside α-galactosidase; ○, methylumbelliferyl α-galactosidase.

Ceramide trihexoside gave a weak but definite inhibition of methylumbelliferyl α-galactoside hydrolysis that appeared to be entirely non-competitive (Fig. 7*b*). Mutual inhibition of hydrolysis suggests that the two activities are associated whereas the non-competitive nature of the inhibitions indicate that the two substrates do not always compete for the same sites on the enzyme. One simple explanation is that methylumbelliferyl α-galactoside has access to only one of two sites available to ceramide trihexoside; the catalytic site. That it has no access to the modifier site is not surprising, since it lacks the essential 'aglycone' requirements. Similarly, ceramide trihexoside may be imagined as having no access to the catalytic site except by first binding to the modifier site to bring about an essential conformational change.

Differential effects of inhibitors, activators. Ceramide lactoside, a potent inhibitor of ceramide trihexoside hydrolysis, had no effect on the hydrolysis of methylumbelliferyl α-galactoside. This is compatible with the following suppositions; (*a*) ceramide lactoside does not compete for the catalytic site, but (*b*) competes with ceramide trihexoside for the modifier site.

Galactose inhibited methylumbelliferyl α-galactoside hydrolysis competitively, as expected (Fig. 8*a*), but inhibition of ceramide trihexoside hydrolysis was again 'mixed' (Fig. 8*b*). The reason for this is that galactose competes for the catalytic site, but not for the modifier site. Unlike ceramide trihexoside hydrolysis, methylumbelliferyl α-galactoside hydrolysis was not inhibited by albumin, and did not require detergents.

Discussion

Two α-galactosidases with activity towards the synthetic substrate methylumbelliferyl α-galactoside were first separated in human placenta by Beutler &

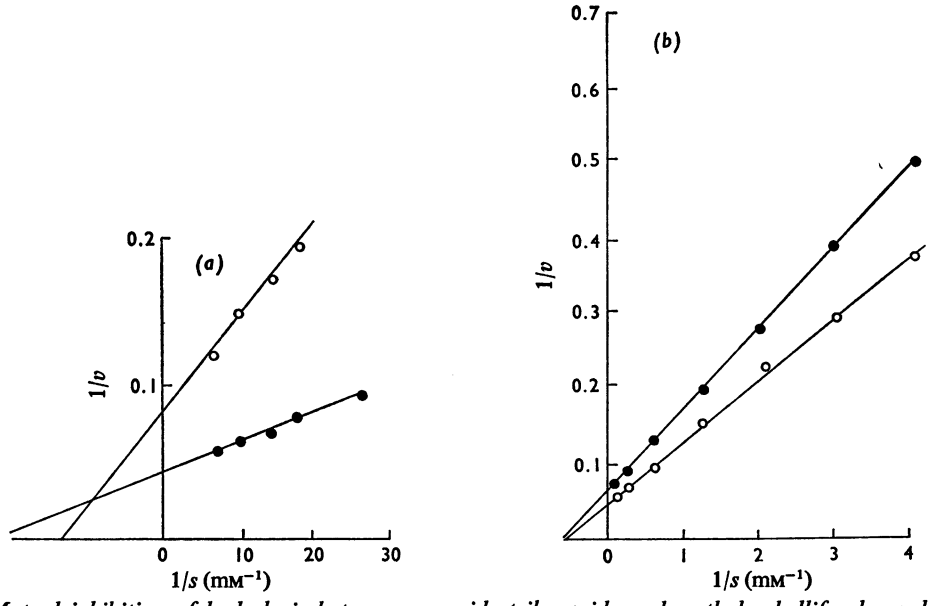

Fig. 7. *Mutual inhibition of hydrolysis between ceramide trihexoside and methylumbelliferyl α-galactoside*

(a) Inhibition of ceramide trihexoside hydrolysis by methylumbelliferyl α-galactoside (5mM). ●, No inhibitor; ○, with inhibitor. (*b*) Inhibition of methylumbelliferyl α-galactoside hydrolysis by ceramide trihexoside (0.13mM). ○, No inhibitor; ●, with inhibitor. $1/v$ is expressed as 10^2/c.p.m., or 1/fluorescence units. Inhibitor was added simultaneously with substrate. Similar results were obtained in two other experiments using different enzyme concentrations.

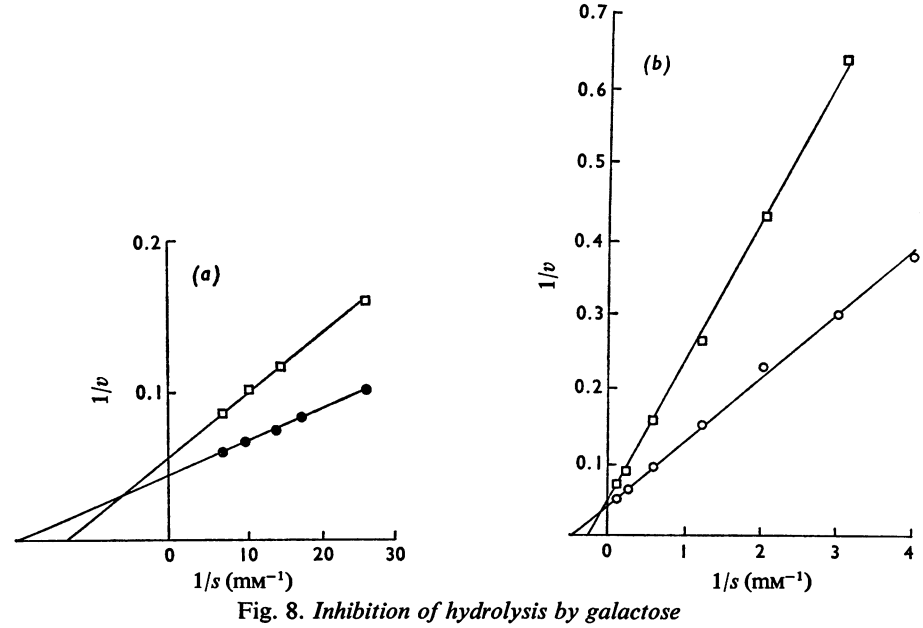

Fig. 8. *Inhibition of hydrolysis by galactose*

(*a*) Inhibition of ceramide trihexoside hydrolysis by galactose (5mM). ●, No inhibitor; □, with inhibitor. (*b*) Inhibition of methylumbelliferyl α-galactoside hydrolysis by galactose (10mM). ○, No inhibitor; □, with inhibitor. $1/v$ is expressed as in Fig. 7. Inhibitor was added simultaneously with substrate. Similar results were obtained in one other experiment at a different enzyme concentration.

Kuhl (1972*b*). The proportion of α-galactosidase B, according to these workers was 20% of the total. In the present case, α-galactosidase B was merely 2% of the total activity. This discrepancy may arise out of intrinsic differences between different tissues; or between the same tissues from different individuals. Whether the different proportions of A and B in the same sample could arise from extrinsic factors such as methods of fractionation remains to be investigated.

The characteristics of ceramide trihexoside α-galactosidase studied with specifically labelled ceramide trihexoside differ radically from those obtained with uniformly tritiated ceramide trihexoside (Brady *et al.*, 1967) or non-radioactive substrate (Mapes *et al.*, 1970). The non-specific nature of these previous assays, which involved a measure of total hexoses released (3mol/mol of ceramide trihexoside), or total galactose released (2mol/mol) renders comparisons difficult. Considerable caution must be exercised in interpreting kinetic information obtained under those conditions.

Bensaude *et al.* (1971), by using specifically labelled ceramide trihexoside, demonstrated very low activities for the natural substrate; about 1/100 of that for the synthetic substrate. This compares unfavourably with the activity attained with the present optimum assay conditions.

Rigorous interpretation of the enzyme kinetic results is difficult. The major obstacle is in determining the actual or effective concentration of a substrate that forms micelles in aqueous solution. Qualitative observations are however, valid, since they do not depend on the exact magnitude of the substrate concentration scale. To obviate uncertainties about the cause of the non-linearity of the Lineweaver–Burk plot (such as the physical state of the substrate), interpretation was mostly based on extrapolations from the linear or near-linear portions. The results suggest interesting possibilities in the mechanisms of enzyme–substrate approach and recognition already alluded to.

If micelle formation of the substrate does not involve the enzyme protein, the true substrate concentration should be decreased by several orders of magnitude (Gatt *et al.*, 1972). If the enzyme complexes with the micelles, however (Elworthy *et al.*, 1968), the result would be a very high local concentration of both enzyme and substrate. The association of numerous lipid-metabolizing enzymes with membranes (Morell & Braun, 1972) where lipids are concentrated may not be fortuitous. Such membrane associations differ from the somewhat unphysiological association of enzyme with lipid–detergent micelles in solution. The end result, however, is similar; the probability of fruitful enzyme–substrate interactions is greatly increased.

Solubilization of the lipid substrate by itself is insufficient for enzyme activity. Either Triton X-100 or sodium taurocholate alone will give optically clear solutions, but both are required for enzyme activity. A ratio of the mixture of detergents to substrate adequate for solubilization is not sufficient for enzyme activity; the same absolute concentrations of both detergents must be present at all substrate concentrations to give full enzyme activity. It is possible that the mixture of detergents at their optimum concentrations provide a favourable environment (by virtue of their physical characteristics) in which enzyme–substrate interactions can occur in protein–lipid–detergent micelles.

Initial interactions between protein and lipid–detergent micelles are electrostatic (Sweet & Zull, 1970; Cherry *et al.*, 1971; see also Razin, 1972, for references), hence the need for the anionic detergent taurocholate. NaCl totally inhibits enzyme activity presumably by the neutralization of charge. Anionic detergents are needed for many lipid glycosidases (Gatt, 1966; Bowen & Radin, 1968; Wenger & Wardell, 1972).

The requirement for Triton X-100 suggests that secondary hydrophobic protein–lipid interactions (Sweet & Zull, 1970) are essential for biological activity.

Serum albumin interferes with enzyme–detergent interactions in a non-specific way. At the pH value of assay, both albumin and α-galactosidase carry a small net positive charge, and a competition for taurocholate is expected. Thus, inhibition of enzyme activity by albumin was greatly decreased by increased amounts of taurocholate, and not by increased concentrations of Triton X-100 or ceramide trihexoside.

Lipid–protein interactions are thus a combination of relatively non-specific electrostatic and hydrophobic forces. To promote enzyme–substrate recognition, extra specificity is desirable.

The present kinetic results suggest an extended 'aglycone' specificity mediated via a modifier site (Gerhart & Pardee, 1962; Dixon & Webb, 1964; Frieden, 1964). The potentials of such 'aglycone' specificity in the regulation of neutral glycolipid and ganglioside metabolism are indicated by both product (ceramide lactoside) and end-product (ceramide glucoside) inhibition of enzyme activity. 'Two-sited' kinetics has been observed for cerebroside β-galactosidase on the basis of inhibition by various synthetic inhibitors (Arora & Radin, 1972); although the interpretation offered differs somewhat.

Ho (1972*a*,*b*) suggested that in Fabry's disease the enzyme activity which hydrolysed methylumbelliferyl α-galactoside, but not ceramide trihexoside, represents an enzyme with a defective modifier site. Subsequent studies (Beutler & Kuhl, 1972*b*; A. G. Norden & J. S. O'Brien, personal communication) indicate the residual enzyme activity in Fabry's

disease may be identical with α-galactosidase B, which was not detected by Ho *et al.* (1972). The origin and function of α-galactosidase B is at present unknown. Its true activity *in vivo* is uncertain (see the Results section). α-Galactosidase B may represent a homopolymer of a hypothetical catalytic subunit. In a preliminary report Beutler & Kuhl (1972*a*) indicated immunological non-identity between α-galactosidases A and B since each anti-serum failed to precipitate a considerable percentage of the activity of the other enzyme from solution. This may not be incompatible with the foregoing hypothesis. If the combination of a hypothetical modifier subunit with the catalytic subunit is involved in the formation of α-galactosidase A, a masking of the antigenic site of the catalytic subunit may be envisaged, so that a new antigenic site characteristic of the modifier subunit becomes reactive. Under such conditions, enzymes A and B would contain a common catalytic subunit and yet exhibit little cross-reactivity.

This work was done in the laboratories of Dr. J. S. O'Brien and Dr. D. Robinson. Their advice and interest are gratefully acknowledged. Special thanks are due to Dr. D. Chapman, Dr. J. Everse and Dr. N. O. Kaplan, who gave critical comments and helpful suggestions, and to Dr. E. Beutler and Dr. W. Kuhl for preprints of their work. Valuable materials were made available for study by Dr. J. S. O'Brien, Dr. E. Beutler and Dr. A. D. Patrick as detailed in the text. Excellent technical assistance was given by Mr. D. Venezuela. The author was supported in part by a fellowship from the National Genetics Foundation.

References

Arora, R. C. & Radin, N. S. (1972) *J. Lipid Res.* **13**, 86–91
Bensaude, I., Callahan, J. & Philippart, M. (1971) *Biochem. Biophys. Res. Commun.* **43**, 913–918
Beutler, E. & Kuhl, W. (1972*a*) *Nature (London) New Biol.* **239**, 207–208
Beutler, E. & Kuhl, W. (1972*b*) *J. Biol. Chem.* in the press
Bowen, D. & Radin, N. S. (1968) *Biochim. Biophys. Acta* **152**, 587–610
Brady, R. O., Gal, A. E., Bradley, R. M. & Martensson, E. (1967) *J. Biol. Chem.* **242**, 1021–1026
Chapman, D. (1969) *Introduction to Lipids*, p. 14, McGraw-Hill, London
Cherry, R. J., Berger, K. U. & Chapman, D. (1971) *Biochem. Biophys. Res. Commun.* **44**, 644–652
Dixon, M. & Webb, E. C. (1964) *Enzymes*, pp. 82–84, Academic Press, New York
Elworthy, P. H., Florence, A. T. & Macfarlane, C. B. (1968) *Solubilization by Surface Active Agents*, pp. 248–285, Chapman and Hall, London
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
Frieden, C. (1964) *J. Biol. Chem.* **239**, 3522–3531
Gatt, S. (1966) *Biochem. J.* **101**, 687–691
Gatt, S., Barenholz, Y., Borkovski-Kubiler, I. & Leibovitz-Ben Gershon, Z. (1972) In *Sphingolipids, Sphingolipidoses and Allied Disorders* (Volk, B. W. & Aronson, S. M., eds.), pp. 237–256, Plenum Press, New York
Gerhart, J. C. & Pardee, A. B. (1962) *J. Biol. Chem.* **237**, 891–896
Hakomori, S. I., Siddiqui, B., Li, Y.-T., Li, S.-C. & Hellerqvist, C. G. (1971) *J. Biol. Chem.* **246**, 2271–2277
Ho, M. W. (1972*a*) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 437 (Abstr.)
Ho, M. W. (1972*b*) *Abstr. FEBS Meet. 8th* no. 920
Ho, M. W., Beutler, S., Tennant, L. & O'Brien, J. S. (1972) *Amer. J. Hum. Genet.* **24**, 256–266
Li, Y.-T., Li, S.-C. & Dawson, G. (1972) *Biochim. Biophys. Acta* **260**, 88–92
Mapes, C. A., Anderson, R. L. & Sweeley, C. C. (1970) *FEBS Lett.* **7**, 180–182
Morell, P. & Braun, P. (1972) *J. Lipid Res.* **13**, 293–310
Parsons, J. G. & Patton, S. (1967) *J. Lipid Res.* **5**, 696–702
Radin, N. S., Hof, L., Bradley, R. M. & Brady, R. O. (1969) *Brain Res.* **14**, 497–505
Razin, S. (1972) *Biochim. Biophys. Acta* **265**, 241–296
Sweet, C. & Zull, J. E. (1970) *Biochim. Biophys. Acta* **219**, 253–263
Wenger, D. A. & Wardell, S. (1972) *Physiol. Chem. Phys.* **4**, 224–230