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ENZYMOLOGICAL PROPERTIES AND IMMUNOLOGICAL CHARACTERIZATION OF α-GALACTOSIDASE ISOENZYMES FROM NORMAL AND FABRY HUMAN LIVER

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Summary

1. A method is described for the rapid isolation of α-galactosidases A and B (α-D-galactoside galactohydrolase, EC 3.2.1.22) from normal human liver.

2. When the same method is applied to Fabry liver, most of the α-galactosidase activity is recovered in the fraction corresponding to normal α-galactosidase B. In agreement with Romeo, G., D'Urso, M., Pisacane, A., Blum, E., De Falco, A. and Ruffilli, A. (1975) Biochem. Genet. 13, 615—628) [18], a small amount of α-galactosidase activity is found in the fraction corresponding to normal α-galactosidase A.

3. The kinetic properties of the B-like activity from Fabry liver are similar to those of normal α-galactosidase B. In agreement with Romeo et al. [18], it was found that the kinetic properties of the A-like activity from Fabry liver are similar to those of normal α-galactosidase A.

4. Using antisera raised against normal α-galactosidase A and normal α-galactosidase B, it is shown that the normal α-galactosidase isoenzymes are immunologically distinct and that the B-like activity from Fabry liver is immunologically related to normal α-galactosidase B. Furthermore, the A-like activity from Fabry liver is immunologically related to normal α-galactosidase B and not to normal α-galactosidase A.

5. Normal α-galactosidase B is converted into an A-like form during storage.

6. It is concluded that the B-like α-galactosidase in Fabry tissues is identical

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Abbreviations: anti-α-gal A Ig, anti-α-galactosidase A immunoglobulin; anti-α-gal B Ig, anti-α-galactosidase B immunoglobulin; ceramide-3, galactosyl (α 1 → 4) galactosyl (β 1 → 4) glucosylceramide; control Ig, control immunoglobulin.

to normal α-galactosidase B, and that the small amount of A-like activity found in Fabry material is due to a modified form of α-galactosidase B.

Introduction

Fabry's disease is due to the deficiency of a lysosomal α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) that hydrolyses the terminal α-galactosidic linkage present in galactosyl (α 1 → 4) galactosyl (β 1 → 4) glucosylceramide (ceramide-3; also known as ceramidetrihexoside) and in a few other glycosphingolipids [1]. Beutler and Kuhl [2] showed that there are two α-galactosidase isoenzymes (designated as α-galactosidase A and B) in human placenta, and that the A isoenzyme is deficient in Fabry tissues [3]. These findings have been confirmed by many other investigators studying various tissues and body fluids [4—9]. The deficiency leads to accumulation of ceramide-3 and other glycosphingolipids in the lysosomes [1,10].

The α-galactosidase isoenzymes have been purified from normal human placenta [2,11], liver [12,13] and urine [14]. The relationship between normal α-galactosidase B and the B-like residual α-galactosidase activity in Fabry tissues has been the subject of an immunological study by Beutler and Kuhl [2,15,16] and of an enzymological study by Rietra et al. [17]. Both groups concluded that the residual α-galactosidase activity in Fabry is identical to normal α-galactosidase B [2,15—17]. Furthermore, both groups have concluded [2,14—16] that the deficiency of α-galactosidase A in Fabry tissues is due to a complete absence of enzyme protein; no cross-reactivity could be detected between anti-α-galactosidase A antiserum and the residual α-galactosidase activity in Fabry fibroblasts [2,15,16], kidney [14] and urine [14]. In contrast, Romeo et al. [18] have reported the isolation of an A-like α-galactosidase activity from Fabry fibroblasts, that was enzymologically and immunologically related to normal α-galactosidase A. This implies that a physically altered α-galactosidase A is present in Fabry material. In addition, Romeo et al. [18] reported that there was considerable cross-activity between their anti-α-galactosidase A and B antisera and the purified preparations used to raise the antisera (contrast refs. 2,15—17).

In order to elucidate the reason for these discrepancies, we have extended our studies on the relationship between α-galactosidase isoenzymes from normal and Fabry material. For this purpose, we have developed a rapid method for isolating the isoenzymes from liver, and have prepared a specific antiserum against purified α-galactosidase B. We have been able to isolate an A-like activity not only from Fabry liver, but from normal liver as well. We conclude that α-galactosidase A and B are distinct proteins, and that the 'A-like' activity is formed from α-galactosidase B during aging of the preparations.

Materials and Methods

Purification of α-galactosidase isoenzymes from human liver

Livers were obtained at autopsy from a man with Fabry's disease and from a normal control. The tissue was removed within 6 h after death, frozen immediately at −20°C, and stored at this temperature until use. The control liver

showed no important pathological changes upon microscopic and macroscopic examination.

A portion of normal liver weighing 50 g was thoroughly homogenized in ice-cold distilled water in an Ultraturrax homogenizer, the temperature being kept at 0—3°C. The homogenate (concentration about 3% (w/v)) was centrifuged for 30 min at 50 000 × *g* and 0°C. The pH of the supernatant was adjusted to pH 7.0 by adding 1 M potassium phosphate buffer (pH 7.0) to a final concentration of 50 mM, and a 4 M NaCl solution was added to a final concentration of 500 mM. The next step involved adsorption of glycoproteins by concanavalin A and selective elution with methylglucose (see ref. 19). 10 ml packed concanavalin A-Sepharose 4B (Pharmacia), which had been exhaustively washed with distilled water, was added to the supernatant. After incubation with stirring at 0°C for 30 min, the mixture was poured into a column (inner diameter 3.3 cm and height 25 cm). The concanavalin A-Sepharose 4B was washed with 200 ml of a solution containing 50 mM potassium phosphate buffer (pH 7.0) and 500 mM NaCl. Glycoproteins were eluted from the column with 100 ml washing buffer to which methylglucose (Koch-Light) had been added to a final concentration of 1 M (see ref. 19). The eluate, which contained about 0.7% of the protein and about 75% of the α-galactosidase activity initially present in the homogenate, was concentrated by ultrafiltration (Amicon PM 30 filter) to a volume of about 8 ml and dialysed for 16 h against 2000 ml 10 mM potassium phosphate buffer (pH 6.5). The pH of the dialysed solution was adjusted to pH 4.0 by adding 0.4 ml 1 M acetate buffer (pH 4.0). The solution was layered onto a column (inner diameter 1.4 cm and height 20 cm) containing CM-cellulose (Whatman CM 23). The column had previously been equilibrated with 50 mM NaCl/50 mM acetate buffer (pH 4.0). Non-adsorbed material was washed from the column using the equilibration buffer. The α-galactosidase activity in this fraction was concentrated by ultrafiltration (Amicon PM 30 filter) to a volume of about 5 ml and called α-galactosidase B (see refs. 8 and 17). After the absorbance at 280 nm in the eluate had declined to zero, the eluant was changed to 100 mM sodium acetate. The α-galactosidase activity eluted after the buffer change was called α-galactosidase A (see refs. 8 and 17). The fractions containing this activity were pooled, dialysed against 10 mM potassium phosphate buffer (pH 6.5), and concentrated by ultrafiltration (Amicon PM 30 filter).

The procedure for isolating the α-galactosidase activity from Fabry liver was essentially the same as that described for normal liver, except that about 95 g of tissue was used. The fraction obtained after washing the CM-cellulose column with equilibration buffer contained most of the activity finally recovered and was called B-like α-galactosidase (see refs. 14 and 17). The fractions eluted with 100 mM sodium acetate contained very little α-galactosidase activity; this activity was called A-like α-galactosidase. The fractions were pooled, dialysed against 10 mM potassium phosphate buffer (pH 6.5) and concentrated by ultrafiltration (Amicon PM 30 filter).

Protein was measured according to Lowry et al., as modified by Eggstein and Kreutz [20], using crystallized egg albumin as standard.

Enzyme assay conditions

To determine *p*-nitrophenyl-α-galactosidase activity, the reaction mixture

contained 100 mM sodium acetate buffer (pH 4.6), 12 mM *p*-nitrophenyl-α-D-galactoside (Koch-Light), enzyme preparation (0.025—0.25 ml) and water to 0.5 ml. In control incubations, either enzyme or substrate was omitted. After incubation at 37°C for 10—60 min, the reaction was stopped by adding 1 ml 0.3 M glycine/NaOH buffer (pH 10.6). The liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm, using a molar extinction coefficient of $18.5 \cdot 10^6$ cm^2/mol [21]. One unit of enzyme activity is defined as 1 μmol *p*-nitrophenyl-α-D-galactopyranoside hydrolyzed/min at 37°C.

To determine the α-galactosidase activity with galactosyl (α 1 → 4) galactosyl (β 1 → 4) glucosylceramide (ceramide-3) as substrate, the reaction mixture contained 100 mM sodium acetate (pH 4.6), 0.4% (w/v) sodium taurocholate (Mann Research Laboratories, New York, U.S.A.), [^{3}H]ceramide-3 (spec. act. 22 400 dpm/nmol; see below), enzyme preparation (0.01—0.03 ml), and water to 100 μl. In determining the V and K_m values for the different isoenzymes, the substrate concentration was varied between 10 and 280 μM. In control incubations, enzyme preparation was omitted and water was added to 100 μl. After incubation at 37°C for 5—20 min, the reaction was stopped by adding 2 ml chloroform/methanol (2 : 1, v/v) and 0.4 ml 0.4 mM galactose in 0.2 M acetic acid (see ref. 22). After mixing and centrifugation (1000 × *g* for 2 min), 250 μl of the upper phase was transferred to a scintillation vial and mixed with 10 ml counting solution containing toluene/ethanol (3 : 1, v/v), 2,5-diphenyloxazole (2 g/l) and 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)-benzene (25 mg/ml). Radioactivity was measured with a Nuclear Chicago scintillation counter (Isocap 300). The counting efficiency was 25—35%.

Ceramide-3 was purified from human erythrocytes by the method of Vance and Sweeley [23] and characterized gas chromatographically. It was specifically labelled with ^{3}H in the terminal galactose residue essentially as described by Radin et al. [24] for galactosylceramide. The specific activity was $11 \cdot 10^6$ dpm/nmol.

Preparation of antisera

Anti-α-galactosidase A antibodies. Anti-α-galactosidase A antibodies (anti-α-gal A Ig) were prepared as described in ref. 17, using α-galactosidase A purified from normal urine as the antigen.

Anti-α-galactosidase B antibodies. α-Galactosidase B was purified from normal human liver as described above. A mixture of 0.35 ml purified enzyme (200 munits/ml; 400 μg protein/ml), 0.5 ml phosphate-buffered saline and 1 ml complete Freund's adjuvant was injected intramuscularly into a rabbit in four different places. Three booster injections of 0.25 ml of the same enzyme preparation with incomplete Freund's adjuvant were given 3, 5 and 7 weeks later. 2 weeks later the rabbits were plasmaphoresed and complement was inactivated by incubation of the antiserum at 56°C for 30 min. A crude immunoglobulin fraction was prepared by 50% $(NH_4)_2SO_4$ precipitation followed by dialysis against phosphate-buffered saline (pH 7.4), and was used as a source of anti-α-galactosidase B antibodies (anti-α-gal B Ig).

A control immunoglobulin preparation was obtained in a similar way from rabbits injected with complete Freund's adjuvant only (control Ig).

Incubation of antisera with α-galactosidase preparations

All incubations of antisera with α-galactosidase preparations were carried out first for 30 min at 37°C and then for 2 h at 0°C. 20 mM potassium phosphate (pH 7.0) was present. The incubation mixture was subsequently centrifuged at 10 000 × *g* for 4 min at room temperature. The resultant supernatant was assayed for *p*-nitrophenyl-α-galactosidase activity according to Rietra et al. [8].

In order to test the immunological relationship between the α-galactosidase isoenzymes from normal and Fabry liver, the following experiments were carried out. 100 μl of an α-galactosidase A or α-galactosidase B preparation from normal liver were incubated with 100 μl anti-α-gal A Ig, anti-α-gal B Ig or control Ig in a series of 2-fold dilutions from 1 : 4 to 1 : 512. Dilutions were made in phosphate-buffered saline (pH 7.4). After incubation and centrifugation, 50 μl samples of the supernatant were taken and assayed for α-galactosidase.

The B-like α-galactosidase from Fabry liver was tested as described above. Furthermore, to protect the enzyme against inactivation in dilute protein-containing solutions, all serum dilutions were made in phosphate-buffered saline containing bovine serum albumin (final concentration 0.1%), and the enzyme assay mixture also contained 0.1% bovine serum albumin.

Romeo's A-like α-galactosidase from Fabry liver was incubated as described for the B-like activity, using the following dilutions of antisera: anti-α-gal A Ig in 4-fold dilutions from 1 : 8 to 1 : 2048, anti-α-gal B Ig in 4-fold dilutions from 1 : 4 to 1 : 1024 and control Ig in 4-fold dilutions from 1 : 4 to 1 : 512. 250 μl enzyme preparation was incubated with 250 μl antiserum, and a sample of 150 μl of the supernatant after centrifugation was assayed for α-galactosidase.

In order to test for the presence of Romeo's 'A-like' activity in an α-galactosidase A preparation from normal livers the following experiment was carried out. An α-galactosidase A preparation was first incubated with the optimal amount of anti-α-gal A Ig required to cause maximum precipitation of enzyme activity. After incubation and centrifugation, a small sample was assayed for residual activity, and the remaining portion of the supernatant was incubated with a second batch of anti-α-gal A Ig, calculated to be the optimal amount to precipitate the remaining activity. After the second incubation and centrifugation, a small sample of the supernatant was assayed for α-galactosidase. The remainder of the supernatant was incubated with an optimal amount of anti-α-gal B Ig required to precipitate the remaining α-galactosidase activity. After the third incubation and centrifugation, a sample of the supernatant was assayed for α-galactosidase activity. The optimal amounts of antiserum to be used in this experiment were read from the curves in Figs. 2A and 2B for anti-α-gal A Ig and anti-α-gal B Ig, respectively.

All enzyme assays were carried out in duplicate and corrected for blank determinations in which substrate was added after termination of the reaction with 1 ml 0.3 M glycine/NaOH buffer (pH 10.6).

Results and Discussion

Isolation of α-galactosidase isoenzymes from normal human liver

Table I shows the results of a typical experiment in which α-galactosidase A

TABLE I

PURIFICATION OF α-GALACTOSIDASE ISOENZYMES FROM NORMAL HUMAN LIVER

The initial homogenate was prepared from 50 g liver. For experimental details, see text.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
Homogenate	3.60	—	—	100
Concanavalin A-Sepharose 4B	2.73 [a]	35	0.078	75
CM-cellulose				
A fraction	1.66 [b]	20	0.083	46
B fraction	0.42 [c]	0.9	0.47	12

[a] Range 2.53—2.96 (n = 5).
[b] Range 1.37—1.66 (n = 3).
[c] Range 0.33—0.73 (n = 3).

and B were isolated from normal human liver by the procedure described in Materials and Methods. The first step is based on the procedures described for some lysosomal enzymes [10], including α-mannosidase [25] and β-galactosidase [26]. These methods make use of the fact that most lysosomal enzymes are glycoproteins. The homogenate is treated with concanavalin A coupled to Sepharose 4B in order to bind glycoproteins, the mixture is poured into a column, and the column is washed and eluted. No flow problems are encountered in the column, and the concanavalin A-Sepharose 4B can be regenerated and re-used several times. About 75% of the α-galactosidase activity present in the homogenate was recovered after this step, with a high degree of reproducibility (2.53—2.96 units/50 g liver in five experiments). After the CM-cellulose step, 46% of the original α-galactosidase activity in the homogenate was recovered in the α-galactosidase A fraction (specific activity 0.08 unit/mg protein) and 12% in the B fraction (specific activity, 0.47 unit/mg protein). The total activity in the α-galactosidase A fraction ranged from 1.37 to 1.66 units (n = 3) and in α-galactosidase B fraction, from 0.33 to 0.73 unit in fresh preparations (n = 3). The α-galactosidase A and B fractions are characterized below (see refs. 14 and 17).

Although the final fractions have a lower specific activity than the more highly purified preparations described in the literature [2,11—14], our procedure has the advantage of rapidity (the entire procedure takes 20 h), of providing complete separation of α-galactosidase A and B (see below), and of high yield.

Isolation of α-galactosidase activity from Fabry liver

The α-galactosidase activity present in Fabry liver was isolated by the same procedure used for normal human liver. As shown in Table II, two fractions were obtained after the CM-cellulose step. That designated as the B-like fraction contained 55% of the total activity present in the original homogenate, with a specific activity of 0.1 unit/mg protein. A minor portion of the activity (1% of that present in the original homogenate) was recovered (like α-galactosidase A from normal liver) in the fractions eluted with 100 mM sodium

TABLE II

PURIFICATION OF α-GALACTOSIDASE FROM FABRY LIVER

The initial homogenate was prepared from 95 g liver. For experimental details, see text.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
Homogenate	1.00	—	—	100
Concanavalin A-Sepharose 4B	0.70	26.4	0.026	70
CM-cellulose				
A-like fraction	0.0085	7.0	0.0012	1
B-like fraction	0.55	5.5	0.10	55

acetate; it was called the A-like fraction (specific activity, 0.0012 unit/mg protein).

Enzymological properties of α-galactosidase isoenzymes from normal and Fabry human liver

The amount of *p*-nitrophenyl-α-galactoside hydrolysed by all four α-galactosidase preparation increased linearly with time for at least 90 min (not shown). There was a linear relationship between rate of substrate hydrolysis and amount of enzyme preparation added for α-galactosidase A from normal liver and the A-like activity from Fabry liver (not shown). Normal α-galactosidase B, however, showed a deviation from linearity, particularly at low concentrations of enzyme protein (Fig. 1), probably due to enzyme inactivation. Indeed, dilution of an α-galactosidase B preparation and subsequent concentration by ultrafiltration led to a 56% loss of activity. An almost linear relationship between rate of substrate hydrolysis and amount of α-galactosidase could be obtained by adding 1 mg bovine serum albumin to the reaction medium. In subsequent experiments with α-galactosidase B and Fabry B-like activity in which artificial substrate was used, 0.1% albumin was always present in the reaction mixture.

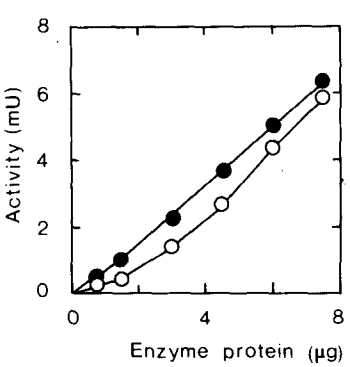

Fig. 1. Effect of concentration of enzyme protein in the reaction mixture on the *p*-nitrophenyl-α-galactosidase activity of an α-galactosidase B preparation from Fabry liver in the absence and presence of albumin. For conditions, see text. ○———○, control. ●———●, + 1 mg albumin.

The kinetic parameters of the α-galactosidase isoenzymes from normal and Fabry liver with *p*-nitrophenyl-α-galactoside (PNP-α-gal) as substrate are shown in Table III. The K_m for the artificial substrate of the B-like activity from Fabry liver is, like that of α-galactosidase B from normal liver, higher than that of normal α-galactosidase A. The activity of both the Fabry B-like isoenzyme and normal α-galactosidase B is slightly stimulated by myoinositol. These findings are in agreement with the results of earlier studies [2—4,17], in which it was concluded that the residual α-galactosidase activity in Fabry tissues is identical to normal α-galactosidase B.

α-Galactosidase A and B and the B-like activity from Fabry liver all hydrolyse ceramide-3, albeit at different rates. Calculation of the ratio $V_{PNP\text{-}\alpha\text{-}gal}/V_{ceramide\text{-}3}$ yields values of only 4 in the case of normal α-galactosidase A and of about 80 and 200 in the case of normal α-galactosidase B and the B-like activity, respectively (Table III). Rietra et al. [17] have shown that about 20% of the α-galactosidase activity in whole homogenate of liver with *p*-nitrophenyl-α-galactoside as substrate is heat stable, and thus due to α-galactosidase B (cf. ref. 2). From this fact, and from the $V_{PNP\text{-}\alpha\text{-}gal}/V_{ceramide\text{-}3}$ ratios in Table III, it can be concluded that at least 98% of the ceramide-3-hydrolysing activity of normal liver is due to α-galactosidase A and < 2% to α-galactosidase B. The K_m for ceramide-3 of α-galactosidase A was 460 ± 70 μM, and 430 and 260 μM for α-galactosidase B and the Fabry B-like activity, respectively (Table III). The low activity towards ceramide-3 of normal α-galactosidase B and the B-like α-galactosidase activity from Fabry liver provides further evidence that the two activities are identical.

In agreement with Romeo et al. [18], we have been able to isolate an A-like α-galactosidase fraction from Fabry liver with kinetic parameters similar to those of normal α-galactosidase A (low K_m for artificial substrate, inhibition by myoinositol: see Table III).

Ceramide-3-hydrolysing activity was not detected in the A-like preparation

TABLE III

KINETIC PROPERTIES OF THE α-GALACTOSIDASE-CONTAINING FRACTIONS PURIFIED FROM NORMAL AND FABRY HUMAN LIVER

To determine the K_m for *p*-nitrophenyl-α-galactoside, the substrate concentration was varied between 2.8 and 18 mM. The effect of myoinositol (400 mM) was tested at a *p*-nitrophenyl-α-galactoside concentration of 4 mM (see ref. 8). For other experimental details, see text.

Property	α-Galactosidase fraction from			
	Normal liver		Fabry liver	
	A	B	A-like	B-like
K_m for p-nitrophenyl-α-galactoside (mM)	13 [a]	24 [a]	13 [b]	30 [a]
Inhibition (—) or stimulation (+) by myoinositol (%)	—36	+6	—18	+20
K_m for ceramide-3 (μM)	460 ± 70 [c]	430	—	260
$V_{PNP\text{-}\alpha\text{-}gal}/V_{ceramide\text{-}3}$	4.0 ± 0.6 [c]	80	—	200

[a] cf. ref. 17.
[b] In a second experiment, a K_m of 9 mM was found.
[c] Means ± S.E.M.

from Fabry liver. It should be pointed out, however, that the specific activity of this preparation with *p*-nitrophenyl-α-galactoside as substrate was very low (Table II), so that the relatively high protein concentration in the reaction medium may have inhibited any ceramide-3-hydrolysing activity present. The assay with the glycolipid substrate is carried out in the presence of 0.4% taurocholate, and excess protein inhibits activity by binding the detergent (Schram, A.W., unpublished observations).

Immunological characterization of α-galactosidase isoenzymes from normal and Fabry human liver

Fig. 2A shows that anti-α-gal A Ig strongly reduces α-galactosidase A activity from normal liver, and that the magnitude of this reduction is dependent on

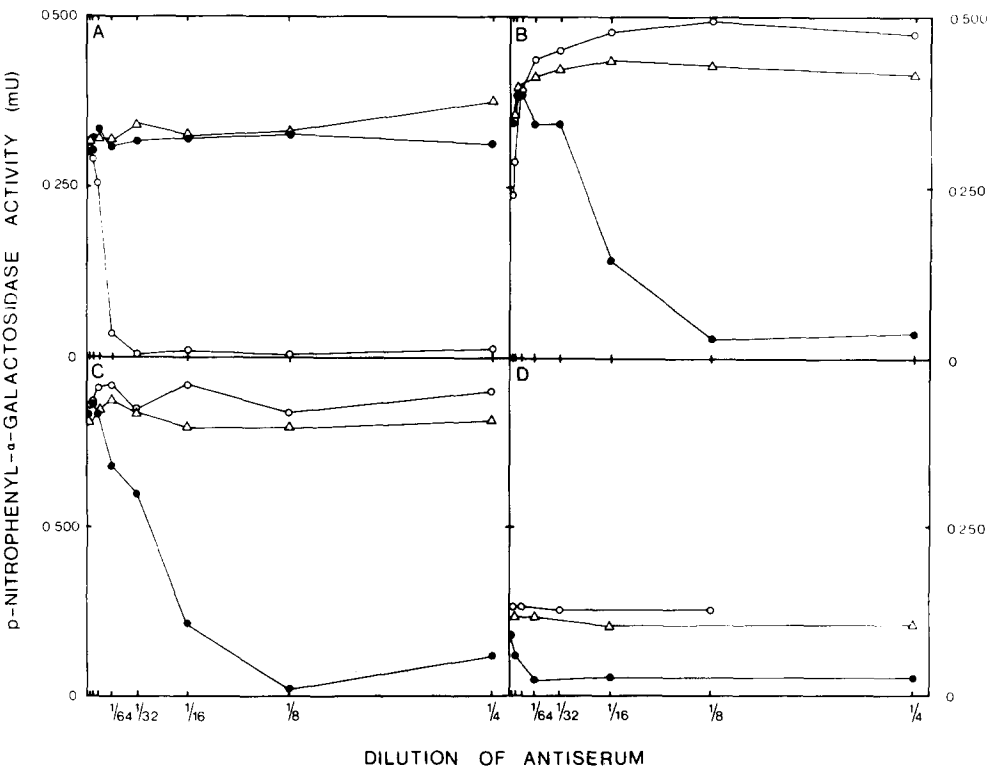

Fig. 2. Effect of preincubation with anti-α-gal A Ig (○———○), anti-α-gal B Ig (●———●) or control Ig (△———△) on the *p*-nitrophenyl-α-galactosidase activity of human α-galactosidase isoenzyme preparations. (A) α-Galactosidase A from normal liver. (B) α-Galactosidase B from normal liver. (C) α-Galactosidase B-like isoenzyme from Fabry liver. (D) α-Galactosidase A-like isoenzyme from Fabry liver. In A and B, the antisera were diluted in phosphate-buffered saline. In C and D, the antisera were diluted in phosphate-buffered saline containing 0.1% bovine serum albumin, and the enzyme assay reaction mixture contained 0.1% bovine serum albumin. In all cases, the *p*-nitrophenyl-α-galactosidase activity was measured in the supernatant after centrifugation of the isoenzyme/antiserum mixture. For other details, see Materials and Methods. In B, those dilutions of antiserum in which the amount of protein introduced into the enzyme assay reaction mixture was < 20 μg were 1/32 (○), 1/128 (●) or 1/128 (△). Note the difference in the scale on the abscissa in C.

the relative amount of antigen and antibody present in the system *. In contrast, there is no effect of anti-α-gal B Ig or control Ig on α-galactosidase A activity.

The effect of the antisera on α-galactosidase B activity from normal liver is shown in Fig. 2B. Anti-α-gal B Ig reduces the enzyme activity to an extent depending on the relative amount of antigen and antibody present in the system, while anti-α-gal A Ig or control Ig does not. If the antiserum is diluted beyond a certain value, a non-specific reduction of enzyme activity is observed with all antisera. This is due to inactivation of α-galactosidase B that occurs in dilute protein solutions.

Fig. 2C shows that anti-α-gal B Ig reduces the B-like activity from Fabry liver, whereas anti-α-gal A Ig or control Ig does not.

The results presented in Figs. 2A, 2B and 2C show that there is no cross-reactivity between anti-α-gal A Ig and α-galactosidase B from Fabry liver, and between anti-α-gal B Ig and normal α-galactosidase A. In agreement with Beutler and Kuhl [2,15,16], we conclude that there is no immunological relationship between α-galactosidase A and B. The considerable cross-reactivity (> 50%) reported by Romeo et al. [18] must be due to cross-contamination of the isoenzymes in the preparations used to raise the antisera.

The α-galactosidase B preparation used for the experiment of Fig. 2B had an activity of about 0.95 munit when measured directly after isolation but an activity of only 0.475 munit/ml when the immunological experiment was carried out 2 weeks later. Thus the real amount of antigen introduced into the system was composed of enzymically active protein, which is measured in the experiments, and enzymically inactive material, which is not. In the experiment of Fig. 2C, freshly prepared B-like α-galactosidase from Fabry liver was used, with an activity of 0.95 munit/ml. Thus the amount of antigen introduced into the system in Figs. 2B and 2C was probably very similar. In both cases, optimum precipitation of activity was obtained at a dilution of anti-α-gal B Ig of 1/8, suggesting strongly that the two α-galactosidase B preparations are immunologically identical.

The effect of the antisera on Romeo and coworkers' [18] A-like α-galactosidase activity is shown in Fig. 2D. Anti-α-gal B Ig reduces the A-like activity, depending on the relative amount of antigen and antibody present in the system, whereas there is no effect of anti-α-gal A Ig or control serum. It is clear that the A-like α-galactosidase activity from Fabry liver is immunologically related to α-galactosidase B and not, as claimed by Romeo et al. [18], to α-galactosidase A.

It can be calculated from Figs. 2C and 2D that the amount of Fabry α-galactosidase B precipitated at the optimum at a serum dilution of 1/8 is 0.8 munit/ml and the amount of A-like α-galactosidase precipitated at the optimum at a serum dilution of 1/64 is 0.112 munit/ml. Thus the ratio precipitated α-galactosidase B : precipitated A-like α-galactosidase is 7.3 : 1, whereas the ratio of optimum serum dilutions is 1 : 8. Thus the antigen : antibody ratios at the

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* Exactly similar results were obtained with an antiserum raised against α-galactosidase A purified from normal human liver.

TABLE IV

PRESENCE OF ROMEO'S A-LIKE α-GALACTOSIDASE IN A NORMAL LIVER α-GALACTOSIDASE A PREPARATION

An α-galactosidase A preparation was successively absorbed with antisera as indicated in column 2. After each absorption step the incubation mixture was centrifuged and α-galactosidase activity was determined in the supernatant. The substrate was 12 mM *p*-nitrophenyl-α-galactoside. For other experimental details, see Materials and Methods.

Step	Successive absorption with	α-Galactosidase activity in supernatant	
		munits/ml	%
1	—	52.5	100
2	Anti-α-galAIg	5.33	10
3	Anti-α-galAIg	6.03	11
4	Anti-α-galBIg	0.27	0.5

optima are almost identical, leading to the conclusion that the A-like α-galactosidase from Fabry liver is immunologically identical to α-galactosidase B.

A-like α-galactosidase from normal liver

Table IV shows the results of an experiment in which an α-galactosidase A preparation from normal liver was successively incubated with anti-α-gal A Ig and anti-α-gal B Ig. The first incubation with anti-α-gal A Ig resulted in a 90% reduction in enzyme activity. A second incubation with anti-α-gal A Ig had no further effect on the remaining activity. However, a third incubation with anti-α-gal B Ig removed almost all of this activity. It is clear that α-galactosidase A preparations from normal liver contain some A-like α-galactosidase, immunologically related to α-galactosidase B. In this preparation, 10% of the total activity was due to A-like α-galactosidase; in another preparation, 6% was found.

Conversion of α-galactosidase B to A-like α-galactosidase during aging of the preparation

When a freshly prepared α-galactosidase B preparation from normal liver was immediately subjected to a second CM-cellulose chromatography step, 52% of the input activity was recovered after washing the column with equilibration

TABLE V

CONVERSION OF α-GALACTOSIDASE B TO A-LIKE α-GALACTOSIDASE DURING STORAGE

α-Galactosidase B was prepared from normal human liver by concanavalin A-Sepharose 4B adsorption and CM-cellulose chromatography as described in Table I and in the text. A portion containing 300 munits was subjected to a second CM-cellulose chromatography step either immediately or after 7 days of storage. The input activity had declined to 276 munits after 7 days

Time after first CM-cellulose step (days)	Input α-galactosidase activity (munits)	Activity (munits) after second CM-cellulose step		
		B fraction	A-like fraction	Total
0	300	156	5	161
7	276	58	88	146

buffer (B fraction) and about 2% after elution with 100 mM sodium acetate (A-like fraction; Table V). When the second CM-cellulose chromatography step was carried out on an α-galactosidase B preparation that had been stored at about 4°C for 7 days, only 22% of the activity was recovered in the B fraction, whereas 32% was recovered in the A-like fraction. It is clear that α-galactosidase B is partly converted to an A-like form during storage.

Conclusions

The following conclusions may be drawn from the results presented in this paper and from earlier observations.

1. B-like α-galactosidase activity in Fabry material is enzymologically and immunologically identical to α-galactosidase B from normal human tissues (see also refs. 2, 15—17).

2. α-Galactosidase A and α-galactosidase B are enzymologically and immunologically distinct proteins (see also refs. 2, 15—17; contrast ref. 18).

3. Cross-reactive material immunologically related to α-galactosidase A is absent in Fabry material [2,14—17].

4. During storage, α-galactosidase B is converted to a form with some kinetic and physical-chemical properties similar to those of α-galactosidase A (see also ref. 18). This form remains immunologically identical to α-galactosidase B (contrast ref. 18).

The question remains of the identity and function of α-galactosidase B. Evidence is presented in the accompanying paper [27] that this activity is exhibited by a protein with *N*-acetyl-α-galactosaminidase activity and that α-galactosidase B should more properly be referred to as *N*-acetyl-α-galactosaminidase.

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