Immunological Properties of *N*-Acetyl- β -D-glucosaminidase of Normal Human Liver and of GM₂-Gangliosidosis Liver

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Antisera were raised to a partially purified preparation of human liver hexosaminidase and to highly purified preparations of hexosaminidase isoenzymes A and B. All the antisera precipitated the enzyme in an enzymically active form, which could be located on immunodiffusion and immunoelectrophoretic gels by using a histochemical substrate. The antisera to the purified isoenzymes were shown to react with hexosaminidase from human liver, kidney, brain and spleen, but did not cross-react with human liver β -glucosidase, β -galactosidase, α -mannosidase, β -xylosidase, arylsulphatase or acid phosphatase. Hexosaminidases A and B were immunologically identical. The immunological properties of the hexosaminidases from livers of patients with three types of GM₂-gangliosidoses were closely similar. No evidence could be found for cross-reacting material in enzyme-deficient states.

Sandhoff has demonstrated the important role of hexosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) (*N*-acetyl- β -D-glucosaminidase) in the degradation of glycosphingolipids (Sandhoff *et al.*, 1971; Wassle & Sandhoff, 1971). In GM₂-gangliosidosis, storage of a ganglioside having a terminal β -linked *N*-acetyl-galactosamine residue is accompanied by a deficiency of the enzyme activity considered to be responsible for its degradation, hexosaminidase A (O'Brien *et al.*, 1971). Several variants of GM₂-gangliosidosis have been described, each associated with a characteristic pattern of hexosaminidase isoenzymes and lipid storage. In classical Tay-Sachs disease (variant B) hexosaminidase A activity is deficient and ganglioside GM₂ is stored; in Sandhoff's disease (variant O) both isoenzymes A and B are absent and ganglioside GM₂ and kidney globoside are stored; in an exceptional case (variant AB) the activities of both isoenzymes are slightly elevated when assayed with artificial substrates, but ganglioside GM₂ degradation is impaired (Sandhoff *et al.*, 1971; Young *et al.*, 1970).

There is some evidence for a close interrelationship of hexosaminidases A and B (Carroll & Robinson, 1972*b*; Wassle & Sandhoff, 1971). Both have molecular weights of about 130000, both have pH optima of about 4.4 and their kinetic properties are very similar. Price & Dance (1972) showed that controlled heating of serum hexosaminidase A gives rise to an enzyme resembling the B form and to two intermediate forms (I forms). A similar result is obtained after neuraminidase treatment of partially purified hexosaminidase A (Robinson & Stirling, 1968). It was considered that the two isoenzymes might be differentiated by their immunological

properties. To this end we have raised antisera to the purified enzymes from human liver. The antisera have been used to test the immunological properties of the hexosaminidases from various human organs and from the livers of patients with variant B, variant O and variant AB GM₂-gangliosidoses. A preliminary account of part of this work has been published (Carroll & Robinson, 1972*a*).

Experimental

Materials

Methylumbelliferyl and Naphthol ASBI glycoside substrates were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Fast Garnet GBC salt was from G. T. Gurr, London S.W.6, U.K. Partially hydrolysed starch was a product of Connaught Medical Research Laboratories, Toronto, Ont., Canada, and agarose was supplied by Miles-Seravac, Maidenhead, Berks., U.K. Ion-exchange celluloses were DEAE-cellulose (Whatman DE 23) and CM-cellulose (CM 23) from W. and R. Balston Ltd., Maidstone, Kent, U.K. Sephadex G-200 was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. Ampholines were bought from LKB-Produkter AB, Stockholm, Sweden. Freund's complete adjuvant was a product of Difco Laboratories, Detroit, Mich., U.S.A. All reagents were of analytical grade unless otherwise stated.

Methods

Enzyme assays. The fluorimetric assay procedure of Leaback & Walker (1961) was employed to determine hexosaminidase activity, as described by Robinson *et al.* (1972). One unit of enzyme activity

liberates 1 μ mol of product/min under these specified conditions. Other hydrolase activities were assayed similarly with the appropriate methylumbelliferyl substrate as described by Van Hoof & Hers (1968). Protein was measured by the Folin method (Lowry *et al.*, 1951) by using a bovine serum albumin standard (Sigma Chemical Co., St. Louis, Mo., U.S.A.) or, for dilute solutions of purified enzyme, by determination of the E_{280} .

Preparation of A and B fractions of human liver hexosaminidase. All steps were carried out at 4°C. A frozen sample of normal human liver (10g) was homogenized in 50ml of water and centrifuged (3×10^5 g-min). The supernatant was applied to a column (1 cm $\times$ 20 cm) of DEAE-cellulose equilibrated with 0.01 M-sodium phosphate buffer, pH 6.0, and eluted with this buffer at a flow rate of 30 ml/h. The hexosaminidase B was unadsorbed; the adsorbed A and minor intermediate components were eluted with a linear NaCl gradient (0–0.2 M) in the same buffer. Fractions containing activity were pooled and concentrated by dialysis under vacuum against the 0.01 M-sodium phosphate buffer, pH 6.0. The corresponding fractions from variant B and variant O liver were prepared in an identical manner.

Purification of human hexosaminidases A and B. The starting material was human liver obtained at *post mortem* and deep-frozen at –20°C until required. All purification steps were carried out at 0–4°C unless otherwise stated.

Liver (400 g) was diced and homogenized in 800 ml of 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 0.1% (w/v) Triton X-100 (step 1). The homogenate was stirred for 1 h before centrifuging (1.5×10^5 g-min) in the 6 $\times$ 250 ml angle rotor of an MSE 17 centrifuge. The supernatant, adjusted to pH 4.3 with solid citric acid, was warmed to 25°C over a period of 30 min, during which time some inactive protein was precipitated (step 2). After centrifuging as before, the clear supernatant was adjusted to pH 7.0 with solid tris. Ammonium sulphate (GPR grade, recrystallized) was added to 65% saturation according to the nomogram of Dixon & Webb (1958) (step 3). After overnight stirring, the precipitate was recovered by centrifugation as described above, dissolved in the minimum volume (approx. 50 ml) of 0.01 M-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl and centrifuged (5×10^5 g-min).

The clear supernatant from step 3 was applied to a column (5 cm $\times$ 100 cm) of Sephadex G-200, which had been equilibrated with 0.01 M-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl. The same buffer was used for elution at a flow rate of 60 ml/h and fractions of 10 ml were collected. The peak fractions (30–60) were combined and dialysed extensively against 0.01 M-sodium phosphate buffer, pH 6.0 (step 4). The enzyme solution from step 4 was applied

to a column (2 cm $\times$ 80 cm) of DEAE-cellulose equilibrated with 0.01 M-sodium phosphate buffer, pH 6.0. The column was eluted with the same buffer at a flow rate of 30 ml/h. Hexosaminidase B was unadsorbed and was eluted in the void volume (fractions 17–23); hexosaminidase A was eluted with a linear NaCl gradient (0–0.2 M) in the same buffer (step 5). The fractions (61–75) containing enzyme A activity were combined and concentrated by dialysis under vacuum against 1% (w/v) glycine solution, pH 6.0. The solution from step 5 containing hexosaminidase B was dialysed against 0.01 M-sodium phosphate buffer, pH 5.0, and then applied to a column (2 cm $\times$ 80 cm) of CM-cellulose equilibrated with the same buffer. The enzyme was eluted with a linear NaCl gradient (0–0.5 M) in the same buffer at a flow rate of 30 ml/h (step 6). The peak fractions (52–69) were combined and concentrated by dialysis under vacuum against 1% (w/v) glycine solution, pH 6.0. The solutions of hexosaminidase A and B were further purified separately by isoelectric focusing in a 110 ml LKB column (step 7), as described by Winchester (1971). Active fractions were pooled and concentrated by dialysis under vacuum against 0.01 M-sodium phosphate buffer, pH 6.0. The procedure is summarized in Table 1.

Gel electrophoresis. Electrophoresis in 5% polyacrylamide gels was carried out in 0.1 M-sodium phosphate buffer, pH 7.4, at 8 mA/gel for 3 h at 4°C, by the procedure of Coston & Loomis (1969). Protein bands were stained with Amido Black, and glycoprotein with the periodic acid–Schiff reagent. Enzyme activity was detected either by incubating the intact gel with the appropriate methylumbelliferyl glycoside substrate at pH 4.5 at 37°C and subsequently viewing the fluorescent band of liberated product under a u.v. lamp, or by cutting the gel into 2 mm slices and incubating the homogenized slices with substrate and assaying as described above.

Raising and testing antisera. All rabbits were bled before immunization to provide control sera. Control sera and antisera were stored at –20°C in the presence of 0.2% (w/v) NaN₃ as preservative. Soluble enzyme preparations were emulsified with complete Freund's adjuvant (1:1, v/v). Three injections were given at 7-day intervals. Rabbits 1 and 2 both received 2 ml of a partially purified preparation of human liver hexosaminidase containing both isoenzymes (enzyme activity 17 μ mol/min, 16.5 mg of protein); rabbit 3 received 2 ml of a purified hexosaminidase B preparation (enzyme activity 1.0 μ mol/min, 0.55 mg of protein); rabbit 4 received 0.75 ml of a purified hexosaminidase A preparation (enzyme activity 1.2 μ mol/min, 0.9 mg of protein). The animals were bled on day 21 and subsequently at 1-week intervals. Booster injections of non-emulsified enzyme preparation were given when the antibody titre began to fall.

Table 1. *Purification of human liver N-acetyl- β -hexosaminidases*

Details of the purification steps are described in the Experimental section.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1. Supernatant	21 700	5000	0.23	100
2. Heated supernatant (25°C)	9 700	4700	0.48	94
3. Ammonium sulphate precipitate (65% saturation)	3 400	4500	1.3	90
4. Sephadex G-200	730	1500	2.1	30
5. DEAE-cellulose column				
(a) Hexosaminidase A	28	160	5.8	3.2
(b) Hexosaminidase B	167	700	4.2	14
6. CM-cellulose (hexosaminidase B)	33	290	8.7	5.8
7. Isoelectric focusing				
(a) Hexosaminidase A	2.5	50	20.0	1.0
(b) Hexosaminidase B	3.9	54	14.0	1.1

Antisera were tested by immunoelectrophoresis, by immunodiffusion and by precipitation in solution. Immunoelectrophoresis was carried out on a micro-scale (Scheidegger, 1955) by using 3% (w/v) starch-agarose gel (Connaught hydrolysed starch-agarose, 3:1, w/w) in the phosphate buffer system described by Robinson *et al.* (1967). A potential of 25 V/cm, 2.5 mA/gel was applied for 45 min at room temperature. Antiserum was suitably diluted with phosphate-buffered saline (0.15 M-NaCl in 10 mM-sodium phosphate buffer, pH 7.0) before use. After overnight development in a moist atmosphere at room temperature, the gels were washed with three changes of phosphate-buffered saline over 2 days. Proteins were stained with Amido Black (0.5%, w/v, in 10%, v/v, acetic acid) and enzymically active precipitin arcs were detected by staining with the Naphthol ASBI substrate and Fast Garnet GBC salt as described by Raunio (1968). Double-diffusion analysis was carried out in 3% starch-agarose gels in phosphate-buffered saline and subsequent treatment was as described above.

The precipitation of hexosaminidase from solution by antibody was studied at constant concentration of enzyme activity and various antiserum concentrations. Enzyme samples and antiserum were dialysed against phosphate buffered saline before use. To enzyme solution (50 μ l) was added a sample of the antiserum (0–100 μ l) and phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin was added to make the total volume 300 μ l. Duplicate mixtures were incubated overnight at 4°C, the precipitates recovered by centrifugation and suspended in 300 μ l of phosphate-buffered saline, and supernatants and suspended precipitates assayed for enzymic activity. In those experiments designed to detect enzymically inactive cross-reacting material, 100 μ l of phosphate-buffered saline was replaced by an equal volume of the solution of suspected cross-

reacting material. A single antiserum concentration, calculated to be just sufficient to precipitate all the enzyme activity in the absence of cross-reacting material, was used.

Results

After step 7 of the procedure described above hexosaminidases A and B from human liver had been purified about 80 times and 60 times respectively (Table 1). Both isoenzymes were recovered in low yield (about 1% of the starting activity) and were still heterogeneous on polyacrylamide-gel electrophoresis (Fig. 1). The preparation of enzyme A contained one major enzymically active component and three minor proteins; enzyme preparation B contained two major enzyme components and one minor protein. All the proteins were glycoproteins, as shown by periodate-Schiff staining. The ratio of *N*-acetyl- β -D-glucosaminidase to *N*-acetyl- β -D-galactosaminidase activity remained at about 8:1 throughout the purification. The purified isoenzymes and a sample containing both isoenzymes taken after step 4 of the purification were used to raise antibodies as described above.

All these enzyme samples evoked a good immune response. The antisera precipitated the enzyme from solution and in gel media in an enzymically active form, so that precipitin lines could be stained with a histochemical substrate (Hayashi, 1965; Raunio, 1968). Neither the anti-(enzyme A) nor anti-(enzyme B) serum was completely specific, since in immunodiffusion gels two precipitin lines were present, of which only one possessed hexosaminidase activity. The contaminating antigen was not identified.

Hexosaminidases A and B were immunologically identical. The anti-(enzyme A) serum precipitated both forms of the enzyme, the B form being more readily precipitated than the A form (Fig. 2). The

Fig. 1. Protein components of (a) hexosaminidase A and (b) hexosaminidase B preparations from step 7 of the purification scheme separated in 5% polyacrylamide gels at pH7.4

The location of enzymically active bands is indicated by E. The origin is at the top and migration is towards the anode. Details of electrophoresis and staining methods are described in the text.

anti-(enzyme B) serum behaved identically. Srivastava & Beutler (1972) have reported that an antiserum to human placental hexosaminidase B also cross-reacts with enzyme A from the same tissue. In immunodiffusion experiments the arcs of hexosaminidases A and B fused with no trace of a spur, even after incubation for several days (Plate 1a). In immunoelectrophoresis experiments the arcs of precipitated enzyme could rarely be seen until stained for enzyme activity. An intermediate component of hexosaminidase (form I) isolated from human liver by DEAE-cellulose chromatography as described below showed a reaction of complete immunological identity with the A and B enzymes.

All the antisera could precipitate the total hexosaminidase activity of human liver, spleen, kidney and brain. The enzymes from these tissues were immunologically identical, as judged by immunodiffusion and immunoelectrophoresis. The *N*-acetyl- β -D-glucosaminidase and *N*-acetyl- β -D-galactosaminidase activities were always precipitated in parallel by the antibody. The antisera raised to the purified isoenzymes did not cross-react with β -glucosidase, β -

Fig. 2. Precipitation of purified human liver hexosaminidases A and B by anti-(hexosaminidase A) serum compared with control serum taken before immunization

Details of immunization and assay procedures are described in the text. Assays were made on supernatants (filled symbols) and precipitates (open symbols) after centrifugation of suspensions containing various volumes of antiserum and a fixed volume of enzyme preparation (after step 7 of the purification procedure) at pH7.0. $\circ$, $\bullet$, Hexosaminidase A; $\triangle$, $\blacktriangle$, hexosaminidase B; $\blacksquare$, control.

galactosidase, α -mannosidase, β -xylosidase, arylsulphatase or acid phosphatase activities of human liver, since these activities remained in solution after all the hexosaminidase had been precipitated. However, the antiserum raised to the partially purified preparation of hexosaminidase also precipitated α -mannosidase, and subsequent analysis of this preparation showed it to contain two electrophoretically distinct components of α -mannosidase activity.

On storage of the A enzyme at 4°C, a form resembling B in its physical and heat-stability properties appeared (Wassle & Sandhoff, 1971). No difference could be detected between the immunoelectrophoretic behaviour of this B-like enzyme and that of the normal B enzyme.

Hexosaminidase A, B and I fractions were isolated from normal human liver and from livers of patients with classical Tay-Sachs disease or the variant O form by DEAE-cellulose chromatography (Price & Dance, 1972; Young *et al.*, 1970). The enzymes had the expected characteristic mobilities on starch-gel electrophoresis at pH7.0. The I fraction from normal liver contained two enzymic components with isoelectric points of 5.8 and 6.0 as determined by isoelectric focusing. The hexosaminidase A activity of the liver in Tay-Sachs disease was only 3% of that found in a normal sample, and the total residual

EXPLANATION OF PLATE I

Immunodiffusion plates

(a) Complete identity of hexosaminidases A, I and B from human liver shown by using anti-(human hexosaminidase A) serum (As). (b) Anti-(human hexosaminidase A) serum reacting with hexosaminidase B from liver of normal (N) and Tay-Sachs (TS) subjects and the corresponding fraction from variant O liver (O). (c) Anti-(human hexosaminidase A) serum reacting with hexosaminidase A from liver of normal (N) and Tay-Sachs (TS) subjects and the corresponding fraction from variant O liver (O). Precipitin arcs were stained with Naphthol ASBI hexosaminide (Raunio, 1968).

hexosaminidase activity of variant O liver amounted to only 0.1% of normal. A concentrated sample of an extract of liver from a variant AB patient was kindly made available by Dr. Konrad Sandhoff of the Max-Planck Institute, Munich, Germany. The total hexosaminidase of the variant AB extract was precipitated by the antibody in a manner identical with a mixture of normal hexosaminidases A and B in proportions similar to those of the variant AB extract (30% A, 70% B as determined by isoelectric focusing; K. Sandhoff, personal communication). The A and B isoenzymes of this variant AB extract, prepared by isoelectric focusing, behaved identically with the normal isoenzymes in immunoelectrophoresis and immunoprecipitation experiments.

The B enzyme of liver from patients with Tay-Sachs disease was identical with normal B enzyme in its precipitation by the antibody, and the two enzymes were immunologically identical as judged by the fusion of their precipitin arcs in immunodiffusion gels (Plate 1b). Similarly the residual A enzyme from liver of a Tay-Sachs patient formed a precipitin line that fused with that of normal A enzyme. This was more readily seen when the precipitin lines were stained for enzymic activity (Plate 1c). However, only partial precipitation from solution of residual A enzyme from a Tay-Sachs patient was achieved even with high concentrations of antiserum. Immunoelectrophoresis revealed heterogeneity of the B enzyme in the Tay-Sachs and variant AB liver extracts (cf. Robinson *et al.*, 1972). The arcs of enzyme activity were less discrete than those of normal B enzyme and in addition they extended to a slightly anodic position. The arc of residual A enzyme of liver of a Tay-Sachs patient had a slower anodic mobility at pH 7.0 than the corresponding normal enzyme.

Neither of those fractions of variant O liver that correspond to enzymes A and B gave a precipitin line in immunodiffusion experiments. In addition, the Tay-Sachs patient 'A' fraction and the variant O 'A' and 'B' fractions did not interfere in the precipitation of normal hexosaminidase A by the antibody, indicating an absence of a significant amount of enzymically inactive cross-reacting material in these fractions.

Discussion

Although the enzyme preparations used here were inevitably a small fraction of the total starting activity, they exhibited all the physical characteristics of the major fractions from which they were obtained, and there is no reason to suppose that they are minor forms and not representative samples.

Hexosaminidase of human liver has proved to be a good immunogen in rabbits, since potent antisera were produced in response to the injection of small

amounts of enzyme. All of the antisera precipitated the enzyme without inhibition of its activity under the conditions used. This was of value in the immunoelectrophoresis and immunodiffusion experiments, since precipitin arcs of enzyme were seldom visible. However, incubation of the gels with the histochemical substrate Naphthol ASBI *N*-acetyl- β -D-glucosaminide revealed the precipitin arcs as deep red lines where the enzyme was active. A similar situation with rat epididymal β -galactosidase and α -mannosidase has been reported by Raunio (1968). This lack of immuno-inhibition may be related to the small size of the substrate used to assay the enzymic activity (Cinader, 1963).

The antisera used here were incompletely specific and even when raised to the highly purified hexosaminidase isoenzymes at least one other antigen-antibody system was present. The degree of specificity obtained, however, justified the experiments described here and the antigenic preparation of hexosaminidase A was free of contaminating enzyme B as judged by isoelectric focusing and starch-gel electrophoresis. The converse was also true.

In immunodiffusion and immunoelectrophoresis experiments, complete fusion of the precipitin arcs of hexosaminidases A and B was observed, with no trace of spur formation even after incubation for several days. This reaction of complete immunological identity extended to the minor intermediate (I) component of hexosaminidase. These results support the mounting evidence for a close structural, functional and genetic relationship between the major forms of the enzyme in human tissue (Wassle & Sandhoff, 1971). The fact that they are also glycoproteins has been suspected for some time (Robinson & Stirling, 1968) and we have discussed the possibility that the A and B enzymes may have a subunit structure, one subunit of which is shared by both isoenzymes and which has the antigenic sites responsible for the enzymes' common immunogenicity (Carroll & Robinson, 1972b). Although such a hypothesis could account for the observed immunological relationship of the two forms, the evidence on which it is based is far from substantial. The immunological identity of closely related forms of an enzyme has been previously reported (Dingle *et al.*, 1971) for the major isoenzymes of cathepsin D from human liver.

Although we are primarily concerned with the function of hexosaminidase in the degradation of gangliosides in the brain, this was not a practicable source of the enzyme and it was necessary to raise antisera to hexosaminidase prepared from liver. It was possible that the antisera raised against the liver enzyme might fail to react with that of brain, for several enzymes are known to occur in organ-specific forms that are immunologically distinct (Sussman *et al.*, 1968; Henion & Sutherland, 1957; Sherwin *et al.*, 1969). However, the hexosaminidases from

human brain, kidney and spleen were indistinguishable from the liver enzyme by immunodiffusion and immunoprecipitation.

The use of antibodies to detect enzymically inactive cross-reacting enzyme protein in tissues of patients with glycosphingolipid storage disease has been exploited so far only by Austin and co-workers in their study of arylsulphatase A in metachromatic leukodystrophy (Neuwelt *et al.*, 1971).

From the results described in the present paper, it appears that the deficiency of hexosaminidase A activity in Tay-Sachs disease is a result of a decreased amount of an active enzyme and not of a normal amount of an enzyme with decreased activity. However, the residual A enzyme is not completely normal as judged by the results of immunoelectrophoresis and immunoprecipitation. The almost complete absence of hexosaminidase activity in variant O liver can be related to the corresponding absence of any enzymically inactive cross-reacting protein. In this case it seems that there is a completely deficient synthesis of the enzyme or of an essential subunit that bears the antigenic site. The alternative possibility of synthesis in normal amounts of an enzyme protein lacking immunological reactivity seems less plausible.

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