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A Low-Molecular-Weight Protein Cross-Reacting with Human Liver *N*-Acetyl-β-D-glucosaminidase

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Antisera were raised to preparations of hexosaminidase isoenzymes A and B purified from human liver. Protein that cross-reacted with the liver hexosaminidase was detected by an antibody-consumption method. A cross-reacting protein with a low molecular weight (20000) was partially characterized and purified from control human liver. This protein is also present in the liver of patients with Tay–Sachs disease or with Sandhoff's disease. Hexosaminidases A and B gave an immunological reaction of partial identity with the low-molecular-weight protein. The possible identity of the low-molecular-weight cross-reacting protein as a subunit of hexosaminidase is discussed.

The *N*-acetyl-β-D-glucosaminidase (hexosaminidase, EC 3.2.1.30) activity of human tissues exists in two major forms, hexosaminidases A and B, which differ with respect to isoelectric point and heat stability (Robinson & Stirling, 1968; Okada & O'Brien, 1969; Harzer & Sandhoff, 1971). Evidence for a structural and genetic interrelation of the two forms has been presented (Robinson & Carroll, 1972; Carroll & Robinson, 1973) and this is supported by the observation that hexosaminidases A and B from human liver (Carroll & Robinson, 1972) and from human placenta (Srivastava & Beutler, 1972) show a reaction of complete immunological identity. On the basis of the physicochemical and immunochemical properties of the isoenzymes, Carroll & Robinson (1973) and Srivastava & Beutler (1973) have proposed a structure for the enzyme based on a subunit concept. No report of a subunit of hexosaminidase has appeared in the literature.

The role of hexosaminidase in the pathogenesis of Tay–Sachs disease (GM_2-gangliosidosis, type 1) and of Sandhoff's disease (GM_2-gangliosidosis, type 2) is at present under active investigation (Tallman *et al.*, 1972; Tallman & Brady, 1972). Hexosaminidase A is deficient in Tay–Sachs disease and differential assay of this activity in the serum is the basis for the clinical detection of affected individuals and heterozygotes (Okada & O'Brien, 1969). However, the common identity of hexosaminidase A, as assayed with artificial substrates, and that enzyme responsible for catalysing the hydrolysis of the terminal *N*-acetylgalactosamine moiety of ganglioside GM_2 has not been established (Tallman *et al.*, 1972; Sandhoff & Wassle, 1971). Hexosaminidase B may catalyse the hydrolysis of asialo-GM_2 and globoside (Sandhoff & Wassle, 1971; Wenger *et al.*, 1972). Both hexosaminidases A and B are deficient in Sandhoff's disease, a rare condition characterized by the visceral accumulation of globoside (Sandhoff *et al.*, 1968; Sandhoff, 1969).

Antisera raised to preparations of hexosaminidases A and B purified from human liver (Carroll & Robinson, 1973) or from human placenta (Srivastava & Beutler, 1973) have been used to try to detect enzymically inactive but immunologically reactive hexosaminidase in Tay–Sachs disease and in Sandhoff's disease. We now report the isolation and partial characterization of a low-molecular-weight protein which cross-reacts with the hexosaminidase of human liver and which may constitute one of the proposed subunits.

Experimental

Materials

All materials with the following exceptions were as described previously (Carroll & Robinson, 1973). Crystalline lysozyme (grade 1) from egg white and *Micrococcus lysodeikticus* dried cells were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Crystalline human lysozyme was a gift from Professor D. C. Phillips and Dr. C. C. F. Blake, Laboratory of Molecular Physics, Oxford, U.K. All reagents were of analytical grade unless otherwise stated.

Methods

The assay and purification of hexosaminidases A and B from human liver, and the raising and routine testing of the anti-hexosaminidase sera have been described (Carroll & Robinson, 1973).

Assay of cross-reacting material. The anti-(hexosaminidase A) serum and a crude preparation of hexosaminidase A were used. The hexosaminidase A fraction prepared by batchwise DEAE-cellulose chromatography at pH7.0 (0.1–0.3M-NaCl eluate fraction) was further purified by gel filtration on a column (2.6cm×45cm) of Sephadex G-150. Antiserum (50μl) was incubated with either hexosaminidase A preparation or cross-reacting material (0–100μl) and the total incubation volume was made up to 150μl with phosphate-buffered saline (0.15M-NaCl in 0.01M-sodium phosphate buffer, pH7.0) containing 0.1% (w/v) bovine serum albumin. Controls were included in which enzyme solution was replaced by phosphate-buffered saline. After incubation for 1h at 37°C and 6h at 4°C solutions were centrifuged in a bench centrifuge (3000rev./min, 10min). Supernatants (100μl) were incubated with hexosaminidase A preparation (20μl) and phosphate-buffered saline plus albumin (30μl) for 16h at 4°C. After centrifugation as above supernatants were assayed for hexosaminidase activity. The consumption of antibody by hexosaminidase or by cross-reacting material in the test solution resulted in an increase in the hexosaminidase activity of the final superantant. A standard curve relating hexosaminidase activity to antibody consumption was constructed by using the crude preparation of hexosaminidase A. Units of cross-reacting material are expressed as that activity (in μmol of substrate hydrolysed/h) of the crude hexosaminidase A preparation which would consume the same amount of antibody.

Detection of low-molecular-weight cross-reacting protein. A 50% (w/v) homogenate of human liver prepared in sodium citrate buffer, pH4.4 (0.01M with respect to citrate), containing 0.1M-NaCl was centrifuged (5×10^5g-min) at 4°C. The clear supernatant (5ml) was applied to a column (2.6cm×45cm) of Sephadex G-150 equilibrated with the same buffer at 4°C and eluted with that buffer at a flow rate of 21ml/h. Fractions (2.4ml) were assayed for hexosaminidase activity and for cross-reacting material. The molecular weights of hexosaminidase and of low-molecular-weight cross-reacting protein were estimated by calibration of the column with rabbit γ globulin (mol.wt. 160000), bovine serum albumin (mol.wt. 69000), horseradish peroxidase (mol.wt. 49000) or albumin (mol.wt. 40000) and cytochrome *c* (mol.wt. 12000).

Purification of low-molecular-weight cross-reacting protein. All steps were carried out at 0–4°C. Frozen human liver (400g) obtained at post mortem was diced and homogenized in 800ml of sodium phosphate buffer, pH7.0 (0.01M with respect to citrate) (step 1). The clear supernatant (800ml) obtained after centrifugation (30min at 5×10^3g) was applied to DEAE-cellulose equilibrated with the same sodium phosphate buffer, pH7.0, and packed into a Buchner funnel (15cm×5cm) (step 2). The unadsorbed fraction (900ml) eluted with the phosphate buffer was dialysed for 16h against sodium citrate buffer, pH4.4 (0.01M with respect to citrate), before being applied to a similar bed of CM-cellulose equilibrated with the citrate buffer. Hexosaminidase B and low-molecular-weight cross-reacting protein were eluted with the citrate buffer containing 0.5M-NaCl (step 3). This fraction (130ml) was applied to a column (5cm×100cm) of Sephadex G-75 equilibrated with the above sodium citrate buffer, pH4.4, containing no NaCl and eluted with this buffer at a flow rate of 60ml/h, 15ml fractions being collected (step 4). Those fractions (61–80) containing low-molecular-weight cross-reacting protein were pooled and applied to a column (1cm×5cm) of CM-cellulose equilibrated with the citrate buffer. Adsorbed protein was eluted with a linear NaCl gradient (0–0.2M over 500ml) in the citrate buffer at a flow rate of 60ml/h, 6ml fractions being collected (step 5). Fractions 50–80 containing cross-reacting material were pooled and adjusted to 95% saturation with $(NH_4)_2SO_4$ (Dixon & Webb, 1958) (step 6). The precipitate recovered by centrifugation (30min at 15×10^3g) was dissolved in a small volume of sodium citrate buffer, pH4.4 (0.01M with respect to citrate) and this solution (1.2ml) applied to a column (2.6cm×45cm) of Sephadex G-150 equilibrated with 0.1M-NaCl in the citrate buffer. The column was eluted at a flow rate of 21ml/h and 2.4ml fractions were collected (step 7). Fractions 55–70, containing low-molecular-weight cross-reacting protein, were pooled and concentrated about 10-fold by dialysis under vacuum against 0.1M-NaCl in the above sodium citrate buffer, pH4.4 (step 8).

Polyacrylamide-gel electrophoresis. Disc electrophoresis was carried out in 7.5% (w/v) polyacrylamide gels at pH4.3 with the β-alanine–acetate buffer system described by Reisfeld *et al.* (1962). A potential of 50V (7mA/gel) was applied for 1h at 4°C. Protein bands were stained with Amido Black [0.5% (w/v) in 10% (v/v) acetic acid] and the gels destained in 7% (v/v) acetic acid.

Assay of lysozyme activity. Lysozyme activity was measured as described by Harrison *et al.* (1968). A standard curve over the range 0–20μg/ml was constructed by using crystalline lysozyme from egg white.

Immunoprecipitation of lysozyme. A solution (20μg/ml) of human lysozyme in phosphate-buffered saline (50μl) was incubated with either anti-(hexosaminidase A) serum (0–250μl), anti-(hexosaminidase B) serum (0–250μl) or normal rabbit serum (0–250μl) and the total incubation volume made up to 300μl with phosphate-buffered saline plus albumin. After 16h at 4°C the solutions were centrifuged in a bench centrifuge (3000rev./min, 10min) and the supernatants were assayed for lysozyme activity.

Raising and testing of antiserum to low-molecular-weight cross-reacting protein. Antibodies to the low-

molecular-weight cross-reacting protein were raised by injection of a rabbit with the preparation from step 8 of the purification scheme. The preparation was emulsified with complete Freund's adjuvant (1:1, v/v). The rabbit received a total of 2.2mg of protein in three injections given at 7 day intervals. The reaction of the antiserum with the homologous antigen and with hexosaminidases A and B purified from human liver (Carroll & Robinson, 1972) was tested by immunodiffusion in starch–agarose gel and by precipitation in solution as described by Carroll & Robinson (1973).

Results and Discussion

Protein that cross-reacted with an antiserum raised to hexosaminidase A purified from human liver was detected by an antibody-consumption method. This cross-reacting material was quantified by comparison with a standard curve constructed by using a crude preparation of hexosaminidase A of known specific activity prepared so as to exclude low-molecular-weight material. Such a method has been used to detect the presence of immunologically reactive but enzymically inactive enzyme protein in several inborn errors of metabolism (see, e.g., Dreyfus, 1972). The method has several advantages: it is quantitative and does not rely on either the complete purification of the enzyme concerned or the presence of precipitating antibodies.

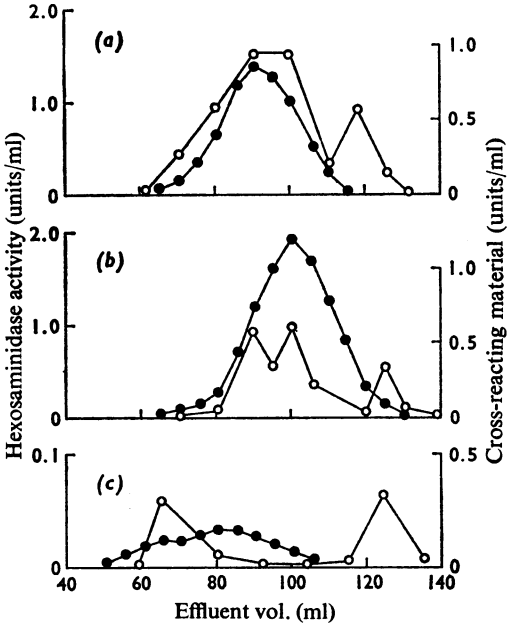

Fig. 1. *Gel filtration on Sephadex G-150 of hexosaminidase activity and of cross-reacting material from liver of (a) normal individual, (b) patient with Tay–Sachs disease and (c) patient with Sandhoff's disease*

For details see the text. ●, Enzyme; ○, cross-reaction to anti-hexosaminidase serum. One unit of either activity is equivalent to the hydrolysis of 1 μmol of substrate/h under the conditions of assay. The peak elution volumes of the molecular-weight markers were: Blue Dextran, 59ml; fibrinogen, 72ml; immunoglobulin G, 81ml; phosphatase, 98ml; peroxidase, 106ml; ovalbumin, 108ml; cytochrome *c*, 128ml; sucrose, 190ml.

Gel filtration of an extract of control human liver on Sephadex G-150 resolved the cross-reacting material into two components, the larger of which was co-eluted with the hexosaminidase activity (Fig. 1). The molecular weights of the two components were 130000 and 20000. Similar elution profiles of cross-reacting material were obtained after gel filtration of extracts of liver from patients with Tay–Sachs disease or Sandhoff's disease.

In liver from patients with Tay–Sachs disease, some of the high-molecular-weight cross-reacting material must be due to the hexosaminidase B that is present. In liver from patients with Sandhoff's disease the peak of hexosaminidase activity was, as expected, greatly decreased (Sandhoff *et al.*, 1968) and the high-molecular-weight cross-reacting material present cannot wholly be attributed to active enzyme species. Further, in our previous paper (Carroll & Robinson, 1973) it was found that those components of liver from patients with Sandhoff's disease that had the charge characteristics of hexosaminidases A and B did not give appreciable cross-reaction.

The high-molecular-weight cross-reacting material described here may therefore represent a protein species that has different physical characteristics from native hexosaminidases. It is noteworthy that in liver from control and particularly from pathological patients the ratio of cross-reacting material to hexosaminidase was greater in the leading edge than in the peak tubes after Sephadex filtration. This suggests that some of the cross-reacting material may have an apparent molecular weight greater than that of hexosaminidase. Gel filtration of the DEAE-absorbed fraction of control liver partially resolved two components, the smaller one (mol.wt. 130000) having associated hexosaminidase activity. The larger component had a molecular weight greater than 200000 and was not identified. The purification of low-molecular-weight cross-reacting protein from liver of control subjects provided a preparation free of contaminating hexosaminidase activity. This activity was removed by gel filtration, by batchwise DEAE-cellulose chromatography and by chromatography on CM-cellulose, which bound the low-molecular-weight protein more strongly than hexosaminidase B at pH4 (Fig. 2). The final preparation contained 3.9mg of protein and had specific activity eight times that of the liver homogenate from which it was derived.

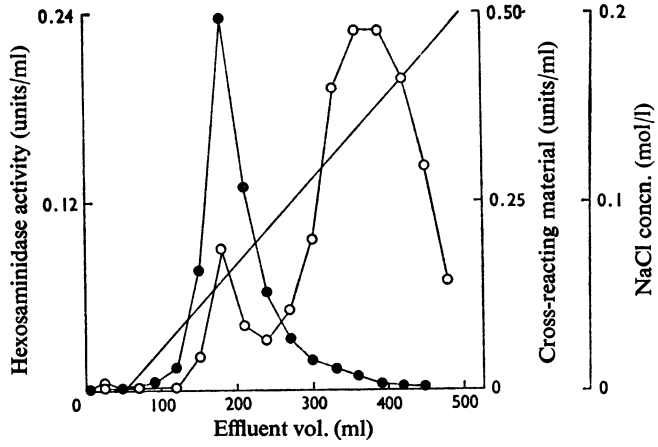

Fig. 2. *Separation of low-molecular-weight cross-reacting material from hexosaminidase activity by chromatography on CM-cellulose*

The original sample was fractions 61–80 from step 4 of the purification scheme described in the text. Details of assay conditions for hexosaminidase activity (●) and for cross-reacting material (○) are given in the text. The NaCl gradient, as determined conductimetrically, is indicated as a solid line without points.

The purified low-molecular-weight cross-reacting protein was still heterogeneous, since electrophoresis in polyacrylamide gel at pH4.3 resolved two major protein components and one minor component. The purified material was stable only under acidic conditions; dialysis against 20-fold-diluted McIlvaine sodium phosphate–citric acid buffer, pH7.0 (McIlvaine, 1921), for 16h at 4°C resulted in a 30% loss of cross-reacting activity, whereas dialysis against a similar sodium phosphate–citric acid buffer, pH4.4, resulted in no such loss. The preparation had no hydrolytic activity towards the disaccharide *NN'*-diacetylchitobiose, prepared by acid hydrolysis of chitin (J. L. Stirling, personal communication), but contained significant lysozyme activity with a specific activity 1% of that of crystalline egg-white lysozyme.

However, neither the anti-(hexosaminidase A) serum nor the anti-(hexosaminidase B) serum precipitated human lysozyme either from solution or in gel media. In one experiment with an extract of human kidney cortex, 85% of the hexosaminidase activity was precipitated at an antiserum concentration that failed to precipitate the lysozyme activity. It is concluded therefore that the low-molecular-weight cross-reacting protein and lysozyme are not identical. We attribute the presence of lysozyme activity in the purified preparation of the low-molecular-weight protein to the co-purification of these two proteins which have similar physicochemical properties (Jollès & Jollès, 1961).

The antiserum raised to the low-molecular-weight cross-reacting protein precipitated that protein from solution. The reaction between the antiserum and the homologous antigen in immunodiffusion gels gave a single precipitin line, even at antigen concentrations which were about five times that required for equivalence. The antiserum failed to precipitate either hexosaminidase A or B from solution. However, in immunodiffusion gels the reaction between the antiserum and highly purified hexosaminidase A or B gave a single enzymically inactive precipitin line which partially fused with the precipitin line formed between the antiserum and the low-molecular-weight cross-reacting protein (Fig. 3). The antigen in the hexosaminidase A and B preparations is probably not native enzyme, but may be either denatured enzyme or a free subunit in which antigenic determinants otherwise masked have become exposed (cf. pepsinogen and pepsin; Van Vunakis & Levine, 1963).

Our efforts to denature hexosaminidase preparations have invariably led to precipitation, thus preventing meaningful experiments on loss of antigenicity. On the other hand, the inactivation of hydrolytic activity by iodination was found not to destroy antigenicity.

The characteristics of the hypothetical subunits of human hexosaminidase have been predicted (Robinson *et al.*, 1973) and the low-molecular-weight cross-reacting protein described in the present paper has two such characteristics, namely a small molecular size and a structural relationship with hexosaminidase on the basis of its immunological cross-reaction. It has no detectable activity against either aryl *N*-acetyl-β-D-glucosaminide or *N*-acetyl-β-D-galactosaminide substrates or against the disaccharide *NN'*-diacetylchitobiose. The charge properties of the pro-

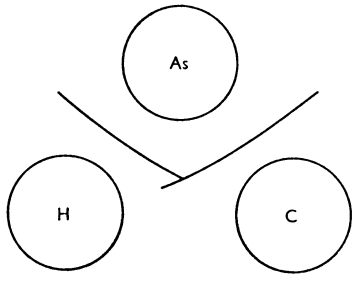

Fig. 3. *Immunodiffusion of anti-(hexosaminidase A) serum against purified hexosaminidase A and low-molecular-weight cross-reacting protein from step 8 of the purification scheme described in the text*

As, Antiserum well; H, hexosaminidase A well; C, cross-reacting protein well.

tein preclude its identity with the specifier subunit proposed by Carroll & Robinson (1973) or the α subunit proposed by Srivastava & Beutler (1973). However, it may be the active-site subunit (the β subunit) if one postulates that activity is manifested only in the oligomeric form (mol.wt. 130000) of the enzyme. In this respect, hexosaminidase would resemble lactate dehydrogenase (Markert & Appella, 1963). We have been unable to prepare a specific antiserum to hexosaminidase A by absorption with purified hexosaminidase B of those antibodies that react with both isoenzymes. Such an antiserum, as prepared by Srivastava & Beutler (1973), would possibly be specific for the specifier subunit and could be used to detect that subunit by methods analogous to those described in the present paper.

The presence of the low-molecular-weight cross-reacting protein in Tay–Sachs disease and Sandhoff's disease is compatible with the present theory of the biochemical and genetic relationships of the hexosaminidases in the GM_2-gangliosidoses (Carroll & Robinson, 1973; Srivastava & Beutler, 1973). The mutation in Tay–Sachs disease probably affects the synthesis of the specifier subunit, leaving free enzymic subunits to combine to produce elevated amounts of hexosaminidase B. In Sandhoff's disease it is postulated that a mutation at the active site destroys the activity of the enzymic subunit.

A further elaboration on this concept involving three different subunits has been briefly proposed by Srivastava *et al.* (1973).

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