Demonstration of Cross-Reacting Material in Tay–Sachs Disease

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(Received 5 October 1978)

Antibodies against placental hexosaminidase A and kidney α -subunits were raised in rabbits after cross-linking the antigens with glutaraldehyde. Anti-(α_n -subunit) antiserum (anti- α_n) precipitated hexosaminidase A but not hexosaminidase B, whereas anti-(hexosaminidase A) antiserum precipitated both hexosaminidases A and B. Specific anti-(hexosaminidase A) antiserum was prepared by absorbing antiserum with hexosaminidase B. Both anti- α_n and anti-(hexosaminidase A) antisera precipitated the CR (cross-reacting) material from eight unrelated patients with Tay–Sachs disease. Immunotitration, immunoelectrophoresis, double-immunodiffusion and radial-immunodiffusion techniques were used to demonstrate the presence of CR material. The CR-material–antibody complex was enzymically inactive. Antiserum raised against kidney or placental hexosaminidase A, without cross-linking with glutaraldehyde, failed to precipitate the CR material, implying that treatment of the protein with glutaraldehyde exposes antigenic determinants that are hidden in the native protein. Since anti-(hexosaminidase B) antiserum did not precipitate the CR material during the immunoelectrophoresis of Tay–Sachs liver extracts, it is suggested that altered α -subunits do not combine with β -subunits. By using immunotitration we have demonstrated the competition between the hexosaminidase B-free Tay–Sachs liver extract and hexosaminidase A for the common binding sites on monospecific anti-(cross-linked hexosaminidase A) antiserum. The amount of CR material in the liver samples from seven cases of Tay–Sachs disease was found to be in the same range as theoretically calculated α -subunits in normal liver samples. Similar results were obtained by the radial-immunodiffusion studies. The present studies therefore suggest that Tay–Sachs disease is caused by a structural-gene mutation.

The biochemical relationship between Tay–Sachs and Sandhoff diseases has been extensively studied since it was reported that the former is due to a deficiency of hexosaminidase A resulting in the storage of GM₂ ganglioside (Kolodny *et al.*, 1969; Okada & O'Brien, 1969; Hultberg, 1969; Sandhoff, 1969), and the latter is due to a deficiency of both hexosaminidase A and hexosaminidase B leading to the storage of GM₂ ganglioside and GA₂ globoside (Sandhoff *et al.*, 1968).

The studies performed with homogeneous isoenzymes (Srivastava *et al.*, 1974*a,b*; Srivastava & Beutler, 1974), along with somatic-cell-hybridization data (Lalley *et al.*, 1974; Lalley & Shows, 1975; Thomas *et al.*, 1974), demonstrated a genetic relationship between the two diseases and between the two

hexosaminidase isoenzymes. It was proposed that hexosaminidase A is a heteropolymer of two distinct polypeptide chains, α and β , and that hexosaminidase B is a homopolymer of the polypeptide β -chain (Robinson & Carroll, 1972; Srivastava & Beutler, 1973, 1974). This hypothesis has been confirmed by a number of investigators (Beutler & Kuhl, 1975; Lee & Yoshida, 1976; Srivastava *et al.*, 1976; Hoeksema *et al.*, 1977; Marinkovic & Marinkovic, 1977). Implicit in this theory was the hypothesis that Tay–Sachs disease, or hexosaminidase A deficiency, is the result of a mutation in the gene coding for the unique polypeptide chain, α , and Sandhoff disease is due to a mutation in the structural gene coding for the common polypeptide chain (Srivastava & Beutler, 1973, 1974). Although in two Sandhoff liver samples CR material was shown to be present, in three unrelated cases of Tay–Sachs disease no CR material was detected (Srivastava & Beutler, 1974). Other investigators have also failed to detect CR material in Tay–Sachs liver, by using antiserum

Abbreviations used: CR material, cross-reacting material; TL extract, Tay–Sachs liver extract; anti- α_n , antiserum against kidney α_n -subunit; IgG, immunoglobulin G.

raised against native hexosaminidase A (Carroll & Robinson, 1973; Bartholomew & Rattazzi, 1974).

Recently we have purified the α - and β -polymers of hexosaminidase A after dissociation with *p*-hydroxy[^{203}Hg]mercuribenzoate (Srivastava *et al.*, 1976), and have raised antibodies in rabbits against glutaraldehyde cross-linked polymers of homogeneous subunits and hexosaminidase A. By using these antibodies, CR material was shown to be present in Tay-Sachs liver (Srivastava & Ansari, 1978) and it has now been quantified in the liver samples from seven unrelated patients with Tay-Sachs disease. The present studies indicate that, like Sandhoff disease, Tay-Sachs disease is also due to mutation in the structural gene.

Materials and Methods

4-Methylumbelliferyl β -D-N-acetylglucosaminide and the galactosaminide analogues were purchased from Koch-Light Co., Colnbrook, Bucks., U.K. Sephadex G-100 and G-200 were purchased from Pharmacia, Uppsala, Sweden, and other reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ouchterlony plates for double-immunodiffusion studies were obtained from Hyland Laboratories, Costa Mesa, CA, U.S.A. *p*-Hydroxy[^{203}Hg]mercuribenzoate was purchased from International Chemical and Nuclear Corporation, Irvine, CA, U.S.A., and Ampholine carrier ampholytes were obtained from LKB-Produkter, Stockholm, Sweden. Protein was determined by the method of Lowry *et al.* (1951), and hexosaminidase A and B activities were determined by using 4-methylumbelliferyl β -D-N-acetylglucosaminide as the substrate (Srivastava *et al.*, 1974a).

Homogeneous preparations of kidney and placental hexosaminidase A and B were prepared as described previously (Wiktorowicz *et al.*, 1977), and the homogeneity in each of the cases was confirmed by polyacrylamide-gel disc electrophoresis and by ultracentrifugation studies. The homogeneous preparation of human kidney hexosaminidase A was dissociated into subunits with *p*-hydroxy[^{203}Hg]mercuribenzoate, and the subunits (α_n and β_n) were purified as described previously (Srivastava *et al.*, 1976). Cross-linking was performed by incubation of 150 μg of protein with glutaraldehyde (final concn. 5% in a total volume of 0.5 ml) at 37°C for 1½ h. The turbid solution was dialysed for about 4 h against 1 litre of 0.145 M-NaCl. The dialysed mixture was diluted to 1 ml, mixed with 1 ml of Freund's adjuvant (complete) and divided into two equal portions. One portion was injected intradermally at ten sites into rabbits on day 0 and the second portion was injected on day 15 (Srivastava *et al.*, 1976). Antibodies against the native placental and kidney hexosaminidase A and hexosaminidase B were raised in rabbits

as described previously (Srivastava & Beutler, 1974). Immunodiffusion, immunoelectrophoresis and the staining of the gels for enzyme activities were performed as described previously (Srivastava & Beutler, 1974; Srivastava *et al.*, 1976).

For immunoelectrophoretic studies 1 g from each of the eight liver samples obtained from unrelated patients with Tay-Sachs disease and the two liver samples from patients who died of some other cause (normal) were homogenized in 9 ml of 10 mM-potassium phosphate buffer, pH 6.0, with a Potter-Elvehjem glass homogenizer. The homogenates were freeze-thawed three times in solid CO_2 /acetone, centrifuged at 10000g for 1 h, and the supernatants concentrated to about 0.2 ml by ultrafiltration in collodion bags. Samples (10 μl) were used for the immunoelectrophoretic studies as described previously (Srivastava *et al.*, 1976).

Preparation of absorbed (specific) anti-(cross-linked hexosaminidase A) antiserum

Specific anti-(cross-linked hexosaminidase A) antiserum was obtained by the addition of homogeneous placental hexosaminidase B to anti-(cross-linked hexosaminidase A) antiserum, centrifugation at 10000g after 24 h at 4°C, then the enzyme activity in the supernatant was determined. This was repeated until no more enzyme activity could be precipitated. This has been designated 'specific anti-(cross-linked hexosaminidase A)'.

Complete removal of hexosaminidase B from Tay-Sachs and normal liver extracts

About 2 g of Tay-Sachs liver was homogenized in 10 mM-phosphate buffer, pH 7.0, to make a 10% (w/v) homogenate. The homogenate was freeze-thawed three times and centrifuged at 10000g for 15 min. The supernatant was dialysed against 10 mM-potassium phosphate buffer, pH 5.0, and passed through a column (0.5 cm $\times$ 10 cm) of CM-cellulose (CM-52) equilibrated with the dialysis buffer. All of the enzyme activity (hexosaminidase B) was adsorbed. The complete removal of hexosaminidase B from all the fractions was confirmed by the determination of hexosaminidase activity with 4-methylumbelliferyl β -D-N-acetylglucosaminide, polyacrylamide-gel electrophoresis and by immunoelectrophoresis studies. The unadsorbed fraction was divided into four equal portions. One portion was designated 'hexosaminidase B-free TL extract'. The other three portions were passed through immunoaffinity (beaded agarose) columns of anti-(native hexosaminidase A), anti-(hexosaminidase B) or anti-(cross-linked hexosaminidase A). The unabsorbed fractions were concentrated by ultrafiltration by using collodion bags.

In order to achieve a complete removal of hexos-

aminidase A and B from the normal liver homogenate, the homogenization of the liver and adsorption by CM-cellulose (CM-52) was performed the same way as in the case of TL extract. The unadsorbed fraction was further passed through a column of anti-(native hexosaminidase A) insolubilized on beaded Sepharose. The non-absorbed fraction was found to be free of hexosaminidase activity and hexosaminidase A and B antigens.

Radial immunodiffusion

Radial immunodiffusion was performed as described by Mancini *et al.* (1965). Briefly, 3.0% (w/v) agarose in 10mM-potassium phosphate buffer, pH 6.0, was heated with constant stirring. It was cooled to about 60°C, and mixed with an equal volume of various dilutions of anti-(cross-linked hexosaminidase A) serum heated to about 55°C. A portion (10ml) of the mixture was poured on to glass slides (2.5cm×25.0cm). Holes 3.0mm in diameter were punched into each agar plate, approx. 3.0cm apart. In each plate 10μl of different dilutions of hexosaminidase A (5.6units/ml) made in 10mM-potassium phosphate buffer, pH 6.0, were added to wells. Different dilutions of hexosaminidase B-free TL extract were added to some wells, with a constant amount of hexosaminidase A (0.5munit). The immunodiffusion plates were incubated in a humidified chamber at room temperature for 48h. Non-precipitated proteins were extracted by incubation of the slides with a large excess of 0.145M-NaCl and mild agitation on a Dubnoff shaker at room temperature.

After extraction the agar gels were covered with a filter paper (Whatman no. 3 MM) and dried at room temperature. Staining for hexosaminidase activity was performed by using the chromogenic substrate Naphthol-AS-BI *N*-acetyl-β-D-glucosaminide and Fast Garnet GBC Salt as described by Raunio (1968) or by using 4-methylumbelliferyl β-D-*N*-acetylglucosaminide (Srivastava *et al.*, 1976). Photographs of the stained gels were taken and slides were made for projection on to a screen. The diameters of the projected images were measured. The area of the well was subtracted from the area of the enzyme-activity circle, and the latter was plotted as a function of hexosaminidase A activity.

Results

Immunological studies performed with kidney anti-(cross-linked-α_n) antiserum

The antibodies raised in rabbits against α_n-subunit cross-linked with glutaraldehyde precipitated kidney hexosaminidase A but not hexosaminidase B (Srivastava *et al.*, 1976). The hexosaminidase A-anti-α_n complex did not sediment at 12000g after 1h, consequently the reaction of hexosaminidase A

with anti-α_n antiserum was determined by precipitation of the hexosaminidase A-anti-α_n complex with goat anti-(rabbit IgG) antiserum (anti-globulin co-precipitation). The addition of goat anti-(rabbit IgG) antiserum precipitated almost all of the hexosaminidase A-anti-α_n antiserum complex as determined by the absence of enzyme activity in the supernatant.

The identity of the antigenic determinant groups of hexosaminidase A and α_n-subunit was demonstrated by measuring the inhibition of precipitation of hexosaminidase A-anti-(hexosaminidase A) complex by α_n-subunit (Fig. 1). Addition of α_n-subunit to anti-(hexosaminidase A) antiserum decreased the amount of anti-(hexosaminidase A) antibody available for the binding of hexosaminidase A (Fig. 1).

The precipitin arc obtained with hexosaminidase A and anti-α_n antiserum on immunodiffusion was enzymically inactive.

Immunoelectrophoretic studies. The results are presented in Fig. 2. Tay-Sachs liver samples exhibited only hexosaminidase B activity (Fig. 2a). In the immunoelectrophoretic studies using anti-α_n antiserum, precipitin lines were obtained with hexosaminidase A and not with hexosaminidase B (Fig. 2b). Anti-α_n antiserum gave precipitin lines with all of the eight Tay-Sachs and the normal liver samples at positions identical with that of hexosaminidase A (Fig. 2b), whereas anti-(hexosaminidase A) serum prepared against native hexosaminidase A (without cross-linking) did not give precipitin lines against

Fig. 1. Inhibition of precipitation of kidney hexosaminidase A with anti-(hexosaminidase A) antiserum in the presence of α_n

At a relative concentration of hexosaminidase A/anti-(hexosaminidase A), such that 50% of hexosaminidase A was precipitated, varying amounts of α_n-subunit were added to anti-(hexosaminidase A) antiserum. The incubation mixture (220μl) contained 100μl of hexosaminidase A (140munits), 20μl of varying concentrations of α_n-subunit (containing 0–0.4μg of protein) and 100μl of a 1:6 dilution of anti-(hexosaminidase A) antiserum. The samples were incubated overnight at 4°C, centrifuged at 12000g for 1h and supernatants were assayed for the enzyme activity (Srivastava *et al.*, 1974a).

Fig. 2. Immuno-electrophoresis studies

These were performed as described in the text. Wells contained as follows: slide 1: left, hexosaminidase A; right, hexosaminidase B; slide 2 (left and right), Tay-Sachs liver extracts. (a) Enzyme stain; (b) anti-(cross-linked α_n) in centre slots; (c) anti-(native hexosaminidase A) in centre slots. Abbreviation used: Hex, hexosaminidase.

Tay-Sachs liver extracts corresponding to hexosaminidase A (Fig. 2c). The results of one typical Tay-Sachs liver are presented in Fig. 2.

Immunological studies performed by using anti-(cross-linked placental hexosaminidase A) antiserum

Antiserum produced in rabbits against a homogeneous preparation of placental hexosaminidase A cross-linked with glutaraldehyde was used in all of the studies described in this section. The results of these studies are presented in Figs. 3, 4 and 5.

Immuno-electrophoresis. When electrophoresis was performed with Tay-Sachs liver extracts (Srivastava *et al.*, 1976), enzyme activity was observed only in the position corresponding to the control hexosaminidase B, whereas in normal liver both hexosaminidase A and B activities were present. On immunodiffusion against anti-(cross-linked hexosaminidase A) antiserum, precipitin arcs in both

normal and Tay-Sachs livers were observed corresponding to homogeneous hexosaminidase A and B (Fig. 3a). After the extraction of the non-precipitated proteins with 0.145M-NaCl, all the precipitin arcs were enzymically active, with the exception of the arcs obtained against Tay-Sachs liver extract, which corresponded to the hexosaminidase A position (Fig. 3b).

Immunotitration. Hexosaminidase A was precipitated with specific anti-(cross-linked hexosaminidase A) serum as described in the legend to Fig. 4. The addition of a fixed amount of hexosaminidase B-free Tay-Sachs liver extract (see the Materials and Methods section) shifted the immunotitration curve to the right. This indicated competition between the hexosaminidase B-free Tay-Sachs liver extract and hexosaminidase A for common binding sites on

Fig. 3. Immuno-electrophoresis of normal and Tay-Sachs liver extracts by using anti-(cross-linked hexosaminidase A) antiserum

Immunoelectrophoresis was performed as described in the text. (a) Immunodiffusion; (b) enzyme stain after immunodiffusion and extraction of non-precipitated proteins with 0.145M-NaCl. Slide 1: left, hexosaminidase A; right, hexosaminidase B. Slide 2: left, Tay-Sachs liver extract; right, normal liver extract. Abbreviation used: Hex, hexosaminidase.

the specific anti-(hexosaminidase A) antibody molecules.

The amount of specific anti-(hexosaminidase A) antiserum that precipitated about 60% of hexosaminidase A, as marked by an arrow in Fig. 4, was used in subsequent titration studies presented in Fig. 5. When varying amounts of hexosaminidase B-free TL extract were added to the fixed amounts of hexosaminidase A and specific anti-(cross-linked hexosaminidase A) antiserum, a decrease in the amount of hexosaminidase A precipitated was observed (Fig. 5). Similar results were obtained when hexosaminidase B-free TL extract adsorbed on insolubilized anti-(hexosaminidase B) or anti-(native hexosaminidase A) columns was added. This also indicated competition between hexosaminidase B-free TL extract and hexosaminidase A for common binding sites on the specific anti-(cross-linked hexosaminidase A) antibody molecules. On the other hand, the addition of varying amounts of (1) normal liver extract adsorbed on CM-cellulose (CM-52) and adsorbed on anti-(native hexosaminidase A) column, or (2) hexosaminidase B-free TL extract adsorbed on anti-(cross-linked hexosaminidase A) column did

not interfere in the precipitation of hexosaminidase A by monospecific anti-(cross-linked hexosaminidase A) serum.

The reactivity of various antisera with hexosaminidases and CR material from the tissues of normal and Tay-Sachs patients has been summarized in Table 1.

Quantification of CR material in Tay-Sachs liver by immunotitration. For the quantification of CR material, various amounts of hexosaminidase B-free TL extract prepared from the liver samples of seven unrelated patients with Tay-Sachs disease were added to a fixed amount of hexosaminidase A and specific anti-(cross-linked hexosaminidase A) serum as presented in Fig. 5. The amount of hexosaminidase A not precipitated due to the addition of a known amount of hexosaminidase B-free TL extract was calculated. The hexosaminidase A units were converted into mg of protein by dividing by 200, because the specific activity of homogeneous

Fig. 4. Inhibition of hexosaminidase A: specific anti-(cross-linked hexosaminidase A) precipitation by hexosaminidase B-free Tay-Sachs liver extract

The first set of incubation mixtures (without Tay-Sachs liver extract) (○), in a total volume of 300 μl, contained 50 μl of placental hexosaminidase A (150 munits/ml) with various amounts of specific anti-(cross-linked hexosaminidase A) antiserum (diluted 1:8) and 10 mM-phosphate buffer, pH 7.0, containing 4% (w/v) bovine serum albumin. The second set of incubation mixtures (□) contained, in addition, a fixed amount of hexosaminidase B-free Tay-Sachs liver extract (2 μg of liver in 20 μl). The samples were incubated overnight at 4°C, centrifuged at 10000g for 1 h and supernatants were assayed for enzyme activity (Srivastava *et al.*, 1974a). The first set was repeated four times, and the range of values is shown by the horizontal bars. The second set of experiments was repeated once; the average values are plotted.

Fig. 5. Immunotitration to demonstrate and quantify the cross-reacting material in Tay-Sachs liver extracts

A fixed amount of hexosaminidase A and anti-(cross-linked hexosaminidase A) antiserum that precipitated about 60% of hexosaminidase A (marked by an arrow in Fig 4) was used in all of the incubations. The incubation mixture (250 μl) contained 50 μl of hexosaminidase A (150 munits/ml), varying amounts of various hexosaminidase B-free liver extracts (as described in the text), and 10 mM-phosphate buffer, pH 7.0, containing 4% (w/v) bovine serum albumin. The samples were incubated overnight at 4°C, centrifuged at 10000g for 1 h, and the supernatants assayed for enzyme activity. Δ, Hexosaminidase B-free Tay-Sachs liver extract (TL extract); ○, TL extract passed through anti-(hexosaminidase B) column; ●, TL extract passed through anti-(native hexosaminidase A) column; ■, TL extract passed through anti-(cross-linked hexosaminidase A) column; □, hexosaminidase B-free normal liver extract passed through anti-(native hexosaminidase A) column. These are new data. A similar graph has already been published by Srivastava & Ansari (1978).

Table 1. Reactivity of various antisera with hexosaminidases and cross-reacting material from the tissues of normal and Tay-Sachs patients

The value of n is 8; the value of n' has not been determined so far. α' represents altered α -subunits. Abbreviations used: Hex, hexosaminidase; CRM, cross-reacting material.

Antiserum	Reactivity against respective antisera			
	Proteins obtained from normal human liver		Proteins obtained from Tay-Sachs liver	
	Hex A ($\alpha\beta$) ₃	Hex B ($\beta\beta$) ₃	Hex B ($\beta\beta$) ₃	CRM α'_n
1. Anti-(Hex A)	+	+	+	—
2. Anti-(Hex B)	+	+	+	—
3. Anti-(Hex A) absorbed with Hex B	+	—	—	—
4. Anti-(cross-linked Hex A)	+	+	+	+
5. Anti-(cross-linked Hex A) absorbed with Hex B [specific anti-(Hex A)]	+	—	—	+
6. Anti-(cross-linked α_n)	+	—	—	+

Table 2. Quantification of cross-reacting material in the liver samples from Tay-Sachs patients

The results are means $\pm$ S.E.M. The amounts of α -subunits were calculated from the titration graph (Fig. 5) assuming the following: (1) that specific activity of hexosaminidase A is 200 units/mg of protein; (2) that hexosaminidase A is composed of equal number of α - and β -subunits; and (3) that both the subunits have the same molecular weight. Abbreviations used: T, Tay-Sachs liver; N, normal liver; CRM, cross-reacting material.

Tay-Sachs liver		Normal liver	
Sample	CRM [altered (α') subunits] ($\mu\text{g/g}$)	Sample	α -Subunits ($\mu\text{g/g}$)
T ₁	4.00	N ₁	3.00
T ₂	6.24	N ₂	3.55
T ₃	4.20	N ₃	3.80
T ₄	4.75	N ₄	3.12
T ₅	4.75	N ₅	3.12
T ₆	4.50	N ₆	3.80
T ₇	3.75	N ₇	3.12
Mean $\pm$ S.E.M.	4.6 $\pm$ 0.24		3.36 $\pm$ 0.13

Fig. 6. Quantification of CR material in Tay-Sachs liver by radial immunodiffusion

The experimental conditions are described in the text. The six horizontal enzyme-activity circles of radial immunodiffusion represent from left to right a decreasing amount of hexosaminidase applied: 1.75, 1.5, 1.0, 0.5, 0.2 and 0.1 munits respectively. The final concentration of antiserum in the agar was 1:300. The two vertical circles represent two dilutions of hexosaminidase B-free Tay-Sachs liver extract added to the corresponding amount of hexosaminidase A (0.5 munit). The projected area of the enzyme-activity circles is marked by the arrows. The gels were stained for the enzyme activity 48 h incubation by using 4-methylumbelliferyl β -D-N-acetylglucosaminide. The amount of altered α -subunits was calculated by assuming that the specific activity of hexosaminidase A is 200 units/mg of protein, and that anti-(cross-linked hexosaminidase A) antiserum has equal amounts of anti- α and anti- β antibodies.

hexosaminidase A preparations is about 200 units/mg of protein (Srivastava *et al.*, 1976). To calculate the amount of α -subunits, this value was divided by 2, assuming that hexosaminidase A has equal numbers of α - and β -subunits and that both the subunits have similar molecular weights. By using this method, we have found that the CR material in the livers of seven unrelated cases of Tay-Sachs disease was 4.6 ± 0.24 (S.E.M.) $\mu\text{g/g}$ of tissue (Table 2).

Quantification of CR material in Tay-Sachs liver by radial immunodiffusion. Radial immunodiffusion, which is a function of the antigen concentration at a fixed antiserum concentration, was used to obtain a standard graph (Fig. 6). The increase in the area of the precipitin circle, made evident by a positive or fluorescent stain for the enzyme activity, was observed when either increased amounts of hexosaminidase A or hexosaminidase B-free TL extract were tested. The increase in the area was found to be proportional to the amount of hexosaminidase A or hexosaminidase B-free TL extract. By taking into consideration the same assumption made for the quantification of CR material with the titration method, $3.3 \mu\text{g}$ of CR material/g of liver was found in Tay-Sachs samples. Representative immunodiffusion patterns are presented in Fig. 6.

Discussion

Although it was proposed that Tay-Sachs disease is the result of a mutation in the α structural gene (Srivastava & Beutler, 1973, 1974), the presence of CR material by using antisera against native hexosaminidase A was not demonstrated (Srivastava & Beutler, 1974; Bartholomew & Rattazzi, 1974; Carroll & Robinson, 1973). We have now obtained antisera against four antigen preparations that precipitate hexosaminidase A. The preparations include: (1) native hexosaminidase A, (2) α_n -subunits cross-linked with glutaraldehyde, (3) hexosaminidase A cross-linked with glutaraldehyde, and (4) native hexosaminidase B. As found previously (Srivastava & Beutler, 1974; Bartholomew & Rattazzi, 1974; Carroll & Robinson, 1973), anti-(hexosaminidase A) antiserum obtained against native hexosaminidase A precipitated both hexosaminidase A and hexosaminidase B but not CR material in TL extracts (Fig. 2c). Anti- α_n antiserum precipitated only hexosaminidase A, but not hexosaminidase B, and also precipitated hexosaminidase A-type CR material from TL extracts as observed in immunoelectrophoretic studies. Anti-(hexosaminidase B) antiserum precipitated hexosaminidase A and hexosaminidase B, but not CR material from Tay-Sachs livers. The inability of anti-(native hexosaminidase A) or anti-(hexosaminidase B) antisera to detect CR material suggests that β -subunits are not associated with the altered α -subunits. On immuno-

electrophoresis, the presence of precipitin arcs corresponding to hexosaminidase A in TL extracts against anti-(cross-linked hexosaminidase A) and α_n -antiserum demonstrated the presence of CR material in this disease.

The immunotitration studies using specific anti-(cross-linked hexosaminidase A) serum (which precipitated hexosaminidase A but not hexosaminidase B) indicated that hexosaminidase B-free TL extract contains a protein that competes with native hexosaminidase A for binding sites on the antibody molecules, and that this protein is not absorbed by either anti-(hexosaminidase B) or anti-(native hexosaminidase A) antisera. This would, therefore, confirm the presence of the CR material in Tay-Sachs liver as reported previously (Srivastava & Ansari, 1978). The CR material is unique to Tay-Sachs liver because the addition of varying amounts of normal liver extract adsorbed on CM-cellulose (CM-52) and absorbed by anti-(native hexosaminidase A) antibody did not interfere in the precipitation of hexosaminidase A by specific anti-(cross-linked hexosaminidase A) serum (Srivastava & Ansari, 1978). The possibility of a non-specific interference by liver extracts was ruled out by the absence of competition between hexosaminidase A and hexosaminidase B-free TL extract, absorbed by anti-(cross-linked hexosaminidase A) serum, for the binding sites on the antibody molecules. In addition, this also rules out the possibility that the antigen (hexosaminidase A) used in raising the antibodies was contaminated with a small amount of protein that became immunogenic after cross-linking.

Although antisera derived from glutaraldehyde-cross-linked α -polymers and hexosaminidase A are able to detect CR material, the antisera obtained against native hexosaminidase A failed to detect CR material in Tay-Sachs disease. This difference may be due to an apparent conformational change that may occur when proteins are modified by glutaraldehyde. Thus cross-linking may expose new regions of the proteins, which become antigenic-determinant groups (heterotopes). The observation that modified proteins may direct antibody response against certain determinants not immunogenic on the native protein has ample precedence (Bonavida & Sercarz, 1971; Benjamini *et al.*, 1972). Heterotopes have been shown to be present in lysozyme (Bonavida & Sercarz, 1971; Thompson *et al.*, 1972) and flagellin (Benjamini *et al.*, 1972), as evidenced by carboxymethylation and acetoacetylation of lysosome and flagellin respectively.

It is therefore possible that the mutation in the α -subunit that leads to Tay-Sachs disease results in a drastic alteration of tertiary structure such that it can no longer bind β -subunits or cross-react with antibody raised against the native enzyme. Glutaraldehyde cross-linking of the purified antigens (α_n -

subunits and hexosaminidase A) may, however, mimic the possible conformational alteration observed in Tay-Sachs disease, such that it exposes heterotopic determinants. The antibodies raised against these determinants therefore recognize both native hexosaminidase A and Tay-Sachs CR material. The amount of altered α -subunits present in Tay-Sachs disease quantified by immunotitration or by radial immunodiffusion was either the same or slightly higher than the calculated α -subunits in normal liver. Since the amount of altered α -subunits was similar (same variation found in the normal liver hexosaminidase A) in all of the seven unrelated cases of Tay-Sachs disease and they had similar electrophoretic mobility, it may be inferred that in all the cases the mutation was in the same site. This kind of similarity would be expected in the mutations involving a certain population dominated by endogamous marriages.

The present studies demonstrate that, like Sandhoff disease, Tay-Sachs disease is due to a structural-gene mutation, as proposed previously (Srivastava & Beutler, 1974), and that in both diseases non-functional proteins are synthesized. Thus the demonstration of CR material in Tay-Sachs disease and the availability of purified α -subunits will allow the development of a radioimmunoassay for the quantification of CR material in heterozygotes and homozygotes of Tay-Sachs disease.

While the present paper was being prepared, Carroll (1978) reported the presence of a protein in normal human liver that was immunologically identical with a protein having the charge and size characteristics of the A isoenzyme of hexosaminidase but had no enzyme activity towards 4-methylumbelliferyl β -D-N-acetylglucosaminide. However, he further states that this protein was serologically unrelated to hexosaminidase A. Since the conclusions drawn in that paper (Carroll, 1978) were based on double-immunodiffusion studies using relatively crude preparations of human liver, those results cannot be correlated with the present findings. Various controls included in our present titration studies eliminate the possibility of a contaminating antigen in the homogeneous preparations of hexosaminidase A and B, which might be responsible for the demonstration of CR material. Moreover, Carroll (1978) has mistakenly reported that he used the γ -globulin fraction from the same antisera we used in our previous study (Srivastava & Beutler, 1974). We, in fact, supplied him with freeze-dried whole antiserum raised against a different preparation of placental hexosaminidase A and B. By using this preparation of antiserum, we have not been able to duplicate his (Carroll, 1978) results.

This work was supported in part by U.S. National Institutes of Health grants nos. GM 21655, 1 T32, GM

07204 and EY 02260, and the National Foundation-March of Dimes. We are grateful to Dr. Abraham Saefer of Isaac Albert Research Institute, Brooklyn, New York, for kindly giving us Tay-Sachs liver samples.

References

- Bartholomew, W. R. & Rattazzi, M. C. (1974) *Int. Arch. Allergy Appl. Immunol.* **46**, 512-524
- Benjamini, E., Scibienski, R. J. & Thompson, K. (1972) *Contemp. Top. Immunochim.* **1**, 1-49
- Beutler, E. & Kuhl, W. (1975) *Nature (London)* **258**, 262-264
- Bonavida, B. & Sercarz, E. (1971) *Eur. J. Immunol.* **1**, 166-170
- Carroll, M. (1978) *Biochem. J.* **173**, 191-196
- Carroll, M. & Robinson, D. (1973) *Biochem. J.* **131**, 91-96
- Hoeksema, H. L., Reuser, A. J. J., Hoogeveen, A., Westerveld, A., Braidman, I. & Robinson, D. (1977) *Am. J. Hum. Genet.* **29**, 14-23
- Hultberg, B. (1969) *Lancet* **ii**, 1195
- Kolodny, E. H., Brady, R. O. & Volk, B. W. (1969) *Biochem. Biophys. Res. Commun.* **37**, 526-531
- Lalley, P. A. & Shows, T. B. (1975) *Am. J. Hum. Genet.* **27**, 57A
- Lalley, P. A., Rattazzi, M. C. & Shows, T. B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1569-1573
- Lee, J. E. S. & Yoshida, A. (1976) *Biochem. J.* **159**, 535-539
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235-254
- Marinkovic, D. V. & Marinkovic, J. N. (1977) *Biochem. J.* **163**, 133-140
- Okada, S. & O'Brien, J. S. (1969) *Science* **165**, 698-700
- Raunio, V. (1968) Ph.D. Thesis, University of Turku; in *Acta Pathol. Microbiol. Scand. Suppl.* **195**
- Robinson, D. & Carroll, M. (1972) *Lancet* **i**, 322-323
- Sandhoff, K. (1969) *FEBS Lett.* **4**, 351-354
- Sandhoff, K., Andrae, U. & Jatzkewitz, H. (1968) *Life Sci.* **7**, 283-288
- Srivastava, S. K. & Ansari, N. H. (1978) *Nature (London)* **273**, 245-246
- Srivastava, S. K. & Beutler, E. (1973) *Nature (London) New Biol.* **241**, 463-465
- Srivastava, S. K. & Beutler, E. (1974) *J. Biol. Chem.* **249**, 2054-2057
- Srivastava, S. K., Awasthi, Y. C., Yoshida, A. & Beutler, E. (1974a) *J. Biol. Chem.* **249**, 2043-2048
- Srivastava, S. K., Yoshida, A., Awasthi, Y. C. & Beutler, E. (1974b) *J. Biol. Chem.* **249**, 2049-2053
- Srivastava, S. K., Wiktorowicz, J. E. & Awasthi, Y. C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2833-2837
- Thomas, G. H., Taylor, H. A., Miller, C. S., Axelman, J. & Migeon, B. R. (1974) *Nature (London)* **250**, 580-582
- Thompson, K., Harris, M., Benjamini, E., Mitchell, G. & Nobel, M. (1972) *Nature (London) New Biol.* **238**, 20-21
- Wiktorowicz, J. E., Awasthi, Y. C., Kurosky, A. & Srivastava, S. K. (1977) *Biochem. J.* **165**, 49-53