Isolation and Relationship of Human Hexosaminidases*

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SUMMARY

A method for the preparation of highly purified $\beta$-hexosaminidase isozymes A and B from human placenta is presented. The purified enzymes possess identical molecular weight and exhibit similar kinetic parameters with artificial fluorogenic substrates. Both enzymes catalyze the hydrolysis of Tay-Sachs ganglioside (Cer Glc Gal(NeuAc) GalNAc) and the corresponding asialo-derivative (Cer-Glc-Gal-GalNAc). Both enzymes are composed of four subunits with a mass of 33,000 daltons each. The enzymes seem to differ only in the interactions of these subunits to form the fully associated enzyme by hydrophobic association and the formation of disulfide bonds. It is possible to convert hexosaminidase A into hexosaminidase B by heating under carefully controlled conditions. The newly formed B has the ion exchange properties and subunit interactions characteristic of highly purified hexosaminidase B isolated from fresh tissue. This conversion does not depend on the removal of N-acetylgalactosaminic acid. Thus, the two hexosaminidases seem to exist as conformers of each other. A model for the relationship of hexosaminidases in normal tissues and in the various clinical conditions which are characterized by the absence of one or more of these enzymes (Tay-Sachs disease and its variant forms) is presented. A benign form of hexosaminidase A deficiency is thought to be able to catalyze the breakdown of Tay-Sachs ganglioside, $\text{GM}_1$. $\beta$-Hexosaminidase A appears to be able to cleave this ganglioside while hexosaminidase B was previously considered to be inactive in this regard (3). It is now a well documented finding that patients with the classic form of Tay-Sachs disease are characterized by a missing hexosaminidase A (5-7) and the inability to catalyze the removal of the terminal N-acetylgalactosamine from Tay-Sachs ganglioside, $\text{GM}_1$ (8, 9).

We have previously shown that $\text{GM}_1$-neuraminidase and $\text{GM}_2$-hexosaminidase exist in preparations of brain lysosomes which will effectively catalyze the breakdown of $\text{GM}_1$ to ceramidetrihexoside and N-acetylgalactosaminic acid or $\text{GM}_2$ and N-acetylgalactosamine, respectively (10). The deficiency of $\text{GM}_1$-hexosaminidase in patients with classic Tay-Sachs disease (B variant, hexosaminidase A deficiency) and the O variant form (total hexosaminidase deficiency) leads to the accumulation of $\text{GM}_2$ and the related compound $\text{GA}_2$ in the brain of these patients (9).

To probe further the relationship of the hexosaminidases in normal and pathological tissue, we have undertaken the purification of both forms of this enzyme. Some of the properties of partly purified enzymes have been described (3) but the activity of these enzymes with the natural substrate $\text{GM}_2$ and the interrelationship of the enzymes have not been studied in a systematic fashion with highly purified human enzymes. In this communication we describe procedures for the purification of these enzymes to this degree and studies of their fine structure and substrate specificities. Through these investigations, we hope to provide a basis for understanding the relationship of hexosaminidases A and B in normal humans and their alteration in patients with the classic or variant forms of Tay-Sachs disease.

EXPERIMENTAL PROCEDURES

Materials

UDP-$\text{N}$-acetyl$[1-14C]$galactosamine (specific activity, $>43$ mCi per mmole) was purchased from New England Nuclear Company. Sodium $\text{[4]}$-borohydride (specific activity, $5$ mCi per mg) was also obtained from this company. $4\text{-Methylumbelliferyl-}\beta\text{-N-acetyl-}\alpha\text{-glucosaminide}$ and $4\text{-methylumbelliferyl-}\beta\text{-N-acetyl-}\alpha\text{-galacto-}$

* This work was presented in part at the Ninth International Congress of Biochemistry, Stockholm, July 1 to 7, 1973.

The abbreviations used are: Cer, ceramide ($N$-acylsphingosine); NeuAc, $\text{N}$-acetylneuraminic acid; $\text{GM}_2$, Cer-Glc-Gal(NeuAc); $\text{GM}_3$, Cer-Glc-Gal(NeuAc)-GalNAc; $\text{GA}_2$, Cer-Glc-Gal-GalNAc; $\text{MU}$, $4\text{-Methylumbelliferone}$.
from the brain tissue of patients with Tay-Sachs disease by the method of Gatt and Berman (11). Gm was obtained by mild acid hydrolysis of Tay-Sachs ganglioside in the following manner. Tay-Sachs ganglioside was passed over a small column of Dowex 50, H+. The acidified GM was refluxed in distilled water for 1 hour and dialysed. The retentate was lyophilized and asialoglycolipid was separated from GM by thin layer chromatography on silica gel G plates in a solvent system of chloroform-methanol-water, 65:25:4 (v/v). The plates were scraped and the GM was recovered from the plates by eluting with chloroform-methanol-water, 60:25:4. It was further purified by recrystallization from methanol. The yield of GM from GM was about 70%. Other methods for GM hydrolysis using 0.1 N HCl result in a number of degradation products such as glucocerebroside and lacto-syleceramide of which only a portion is the desired GM.

Preparation of Radioactive Glycolipids

[14C]GalNAc-GM, labeled specifically in the N-acetylgalactosaminyl portion of the molecule, was prepared from UDP-N-acetyl-[1-14C]galactosamine and GM as previously described (12). The final specific activity of this compound after dilution with cold GM was 1.0 X 107 cpm per milliliter. [3H]GalNAc-GM was prepared by a galactose oxidase method (13). Thirty milligrams of GM were dissolved in 7.5 ml of freshly distilled tetrahydrofuran. To this solution, 250 units of galactose oxidase (Kabi, Stockholm, Sweden) in 7.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) were added and the solution was incubated for 24 hours at room temperature. At this time, an additional 250 units of galactose oxidase were added and the reaction was allowed to proceed for an additional 12 hours. Chloroform and methanol were added and the incubations subjected to partitioning according to the method of Folch et al. (14). Lower phases were dried and the GM, oxidized at C4 of the terminal N-acetylgalactosamine, was redissolved in 6 ml of tetrahydrofuran; 6 mg of tritiated NaNH4 dissolved in 350 ml of 0.001 N NaOH were added to this solution and the reaction was continued overnight. An additional 20 mg of cold NaNH4 were added and after an additional 12 hours, 20 ml of chloroform and 1 N acetic acid (19:1) were added and this solution was washed with methanol-water, 1:1. Lower phases were dried and the [3H]GalNAc-GM was applied on a preparative thin layer chromatogram and developed with a solution of chloroform-methanol-water, 65:25:4 (v/v/v). The radioactive glycolipid was localized by scanning with a Varian Aerograph chromatogram scanner and extracted with chloroform-methanol-water, 10:1:0.5 (v/v/v). The final yield was 26 mg with a specific activity of 14 mCi per milliliter. Over 90% of the label was localized in the N-acetylgalactosaminyl portion of the molecule. This compound was used without further dilution.

Assay of Enzymes

Fluorescent Enzymatic Assay

Incubation mixtures contain 10 μl of enzyme extract, 150 μl of citrate-phosphate buffer (150 mM, pH 4.4), and 50 μl of substrate solution (5 mM 4-MU-hexosamine) in a total volume of 0.21 ml. The blank, containing the same amounts of buffer and substrate, but without the enzyme extract, is treated identically. An enzyme blank is usually unnecessary in the fluorometric assay with purified enzyme. After incubation at 37° for up to 1/2 hour the reactions are stopped by adding 0.8 ml of glycine-sodium hydroxide buffer at pH 10. Ten-fold dilution is performed, if necessary, by mixing 0.1 ml of samples and blanks with 0.9 ml of the pH 10 buffer. Further dilution was not attempted. Very active enzyme preparations were dialyzed with the citrate-phosphate buffer before addition to the incubation mixture. The fluorescence was measured and the result was compared with the fluorescence of known amounts of 4-MU.

Radioactive Assays

[14C]GalNAc-GM—Incubations contained in 200 μl, 20 μmoles of citrate-phosphate buffer (pH 4.2), 200 to 240 μg of crude sodium taurocholate (the gift of Dr. K. Sandhoff), hexosaminidase A or B, and 20 nmoles of [14C]GalNAc-GM. Incubations were carried out for 3 to 6 hours and the extent of the reaction was determined as previously described (10). Both boiled enzyme and "zero time" blanks were run. Blank values were about 1/3 of the values of the incubation.

[3H]GalNAc-GM—Incubations contained in 200 μl, 20 μmoles of glycerine-HCl (pH 3.8), 240 μg of sodium taurocholate, hexosaminidase A or B, and 12 nmoles of [3H]GalNAc-GM. Incubations were carried out for 1 to 3 hours at 37° and worked up using our standard method (10). Boiled enzyme and "zero-time" controls were carried out; these values, although higher than the [3H] blanks (above), were in all cases less than 1/2 the absolute counts obtained in the "active" incubation.

RESULTS

Purification of β-Hexosaminidases

Fresh human placenta were placed on ice immediately after delivery and processed within 12 hours. All subsequent procedures were carried out at cold room temperature (3°). Placentas were perfused with 0.9% NaCl saline solution to remove clots and dissected free from fibrous tissue. Portions of 2500 g were homogenized in a Waring Blender (two 1-min runs) in 5 volumes of 25 mm sodium phosphate buffer, pH 6. The homogenate was centrifuged at 10,000 x g for 20 min and the supernatant was retained.

Ammonium Sulfate Precipitation

Ammonium sulfate fractionation was performed (using 70.5 g of (NH4)2SO4 per 100 ml of solution as 100% saturation) and the fraction which precipitated between 24 and 59% was collected by centrifugation at 10,000 x g for 30 min and resuspended in 25 mm sodium phosphate buffer, pH 6. The homogenate was centrifuged at 10,000 x g for 20 min and the supernatant was retained.

Sephadex G-200

The retentate was centrifuged at 50,000 x g for 1 hour and half of the supernate was applied to an upward flowing column of Sephadex G-200 (Pharmacia column, 10 x 100 cm) equilibrated with the same phosphate buffer. Fractions (25 ml) were collected and analyzed for β-hexosaminidase. A single peak of β-hexosaminidase activity was found; fractions containing 80% of the total assayed activity were pooled and retained. Pooled fractions from two such runs were combined and concentrated to a volume of 300 ml. (Amicon system, PM-10 membranes).

DEAE-Sephadex

Sodium phosphate buffer (25 mm) was titrated to pH 6.0 at 25°. DEAE-Sephadex A-25 was swollen in this buffer and titrated to pH 6.0 (25°). The gel suspension was washed twice with fresh buffer and retitrated each time. A column (5 x 50 cm) was packed with this gel and washed with 2 liters of the buffer. Combined fractions (300 ml) from two runs of the previous step were titrated to pH 6.0 (25°) and applied slowly (downward flow) to the column; the column was washed with 300 ml of the phosphate buffer and 2 liters of a linear NaCl gradient from 0 to 600 mm in the same phosphate buffer were applied. Fractions (25 ml) were collected and a sharp peak of β-hexosaminidase activity was obtained. The enzyme which was adsorbed to the column and eluted with the salt gradient is β-hexosaminidase A. Fractions containing 80% of the β-hexos-
aminidase A activity were pooled, concentrated to 30 to 50 ml (Amicon system, PM-10 membranes), and dialyzed overnight against the sodium phosphate buffer. Fractions containing hexosaminidase A activity were pooled, concentrated to 30 to 50 ml and dialyzed against the sodium phosphate buffer. Fractions containing hexosaminidase B which appeared in the void volume were treated identically.

Further Purification of Hexosaminidase A

CM-Sephadex—CM-Sephadex C-25 was swollen in 0.02 M sodium acetate buffer (pH 4.80) titrated to pH 4.80 ± 0.02 (25°C), washed twice with fresh buffer, retitrated if necessary, and packed into a column (0.9 × 20 cm). The column was washed with 50 ml of the same buffer. The protein sample (~30 ml) of β-hexosaminidase from DEAE-Sephadex was titrated to pH 4.80 (25°C) and applied to the column. The column was washed with 50 ml of the acetate buffer and eluted with a 400-ml linear NaCl gradient (0 to 500 mM). At this pH, β-hexosaminidase A was bound to the column and eluted by the salt gradient in a single peak. These fractions were pooled, titrated to pH 6.0, and dialyzed against sodium phosphate buffer (pH 6.0, 25 mM). As a final step, hexosaminidase A was recycled through a DEAE-Sephadex column at pH 6.0. A summary of this purification is given in Table I.

Further Purification of Hexosaminidase B

CM-Sephadex—CM-Sephadex C-25 was swollen in 0.02 M sodium acetate buffer (pH 5.00), titrated to pH 5.00 ± 0.02 (25°C), washed twice with fresh buffer, retitrated if necessary, and packed into a column (0.9 × 20 cm). The column was washed with 50 ml of the same buffer. The protein sample (~30 ml) of β-hexosaminidase B from DEAE-Sephadex was titrated to pH 5.00 at 25°C and applied to the column. The column was washed with 50 ml of the acetate buffer and eluted with a 400-ml linear NaCl gradient (0 to 500 mM). At this pH, β-hexosaminidase B was bound to the column and eluted by the salt gradient in a single peak. These fractions were pooled, titrated to pH 6.0, and dialyzed against sodium phosphate buffer at pH 6.0 and 25 mm.

DEAE-Sephadex—DEAE-Sephadex A-25 (100 g) was swollen in 25 mM sodium phosphate buffer and the pH was adjusted to 7.6. Combined fractions from the previous step were titrated to pH 7.6 and applied slowly to the column. The column was washed with 200 ml of the phosphate buffer (pH 7.6) and a linear NaCl gradient, 0 to 500 mM in the phosphate buffer, was applied. Fractions containing activity were pooled and dialyzed against phosphate buffer at pH 6.0. This enzyme represents purified β-hexosaminidase B. A summary of the purification is given (Table II).

Purity of Preparations

Polyacrylamide Electrophoresis in Discontinuous Gel System

Further evidence of purity was obtained through polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (vide infra).

Molecular Weight Determinations

Sephadex G-200—The molecular weight of each enzyme was determined on a calibrated column of Sephadex G-200. The Sephadex G-200 column conditions were identical with those utilized in the enzyme purification except that purified enzymes and smaller columns were used. The column was calibrated using standard proteins with known molecular weights. The molecular weight of each enzyme was determined in the presence of sodium dodecyl sulfate (vide infra).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ammonium sulfate (25-55%)</td>
<td>79.4</td>
<td>3.2 x 10^5</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>4.0</td>
<td>4.6 x 10^4</td>
<td>7,600</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 6.0)</td>
<td>0.24</td>
<td>7.0 x 10^4</td>
<td>73</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>CM-Sephadex (pH 4.8)</td>
<td>1.5 x 10^-4</td>
<td>30,600</td>
<td>3,437</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 6.0)</td>
<td>4.0 x 10^-4</td>
<td>5.5 x 10^4</td>
<td>4,100</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.

Polyacrylamide Electrophoresis in Discontinuous Gel System

Further evidence of purity was obtained through polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (vide infra).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sephadex (pH 6.0)</td>
<td>5.9 x 10^-3</td>
<td>28,100</td>
<td>7,040</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>CM-Sephadex (pH 5.0)</td>
<td>1.1 x 10^-3</td>
<td>5.8 x 10^4</td>
<td>4,800</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 7.6)</td>
<td>1.4 x 10^-5</td>
<td>5.5 x 10^4</td>
<td>4,100</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

* Hexosaminidase B represents ~45% of the total activity present before this step. 4-Methylumbelliferyl-N-acetyl-D-glucosaminide was used to monitor enzymatic activity at each step.
FIG. 1. Anionic gel with both purified hexosaminidase A (lower band) and hexosaminidase B (upper band). Gels were run with between 25 and 50 μg of protein per gel as described in Ref. 15 and the text.

Fig. 1, sodium dodecyl sulfate gels of hexosaminidase A (left) and hexosaminidase B (right) after preincubation in the presence of 0.1% sodium dodecyl sulfate at pH 7.2 for 1 hour. Conditions of the electrophoresis are described in the text. a, sodium dodecyl sulfate gels of hexosaminidase A (left) and hexosaminidase B (right) after preincubation in the presence of 0.1% sodium dodecyl sulfate at pH 7.2 for 1 hour. Conditions of the electrophoresis are described in the text.

Fig. 2. Molecular weight determination of hexosaminidases A and B on a calibrated Sephadex G-200 column. Conditions and standard proteins are described in the text.
degree of purity judged by disc electrophoresis at pH 4.3 and 8.9, showed a heterogeneous population (with an almost statistical distribution) of proteins of molecular weights between 120,000 and 140,000 (Fig. 3a). There was a very small amount of protein with molecular weight similar to hexosaminidase A. With the same preparation, when the enzymes were pre-incubated in 3 M urea, 0.5% dithiothreitol, and 10% mercaptoethanol in the presence of 0.1% sodium dodecyl sulfate at pH 7.2 and electrophoresis was carried out in 10% gels, two bands occurred with molecular weights of approximately 33,000 and 65,000 (Fig. 3b). Hexosaminidase B showed a very small band of approximately 90,000 to 100,000. No bands were detected of weight smaller than 33,000 or in the region of the high molecular weight material which was seen without reduction. If pre-incubation is carried out as above in the presence of reducing agent, urea, and 0.1% sodium dodecyl sulfate at pH 10.5, one band of molecular weight 33,000 can be detected for either hexosaminidase A or B.

**Kinetic Properties of Purified Enzymes**

**Fluorogenic Substrates**

Both enzymes exhibited linearity with time up to 2 hours at 37°C in dilute enzyme solutions. When short time incubations were employed, linearity with protein was obtained up to 10 μg per incubation when either 4-MU-N-acetyl-D-glucosaminide or 4-MU-N-acetyl-D-galactosaminide were used as substrates. The optimal pH for each enzyme was 4.4 in a 150 mM phosphate-citrate buffer for each substrate. The observed $K_m$ and $V_{max}$ for each enzyme and substrate are reported (Table III).

**Sphingolipid Substrates**

$G_{M2}$—Neither enzyme exhibited significant activity toward [3H]GalNAc-GM$_2$ in the absence of sodium taurocholate. The response of the enzymes to increasing concentrations of sodium taurocholate with a constant substrate concentration was complex (Fig. 4) and involved both a stimulatory and an inhibitory effect. The optimal concentration of sodium taurocholate was 1.2 mg per ml. Under optimal detergent concentrations, both enzymes exhibited linearity with time at least up to 4 hours and with protein up to at least 50 μg per incubation. The optimal pH was 3.8 in a 150 mM citrate-phosphate buffer. Using a constant optimal detergent concentration, the effect of increasing substrate concentration is to increase enzymatic activity up to a substrate concentration of about 60 μM; further increases of substrate concentration are inhibitory (Fig. 5). The velocity at this substrate concentration was approximately 100 μmoles per mg of protein per hour for either enzyme. If both substrate and detergent were varied with a constant optimal ratio (83.3 μmoles of G$_{M2}$ per mg of sodium taurocholate), the activity was rapidly optimized and decreased immediately. A square hyperbola, characteristic of classical Michaelis-Menten kinetics, was not obtained.

$G_{M3}$—In contrast to our experiments with crude brain preparations (10), the purified placental hexosaminidases required sodium taurocholate for activity with [3H]GalNAc-GM$_3$. In experiments similar to the above, an optimal detergent concentration of 1.0 mg per ml was obtained. Both hexosaminidases A and B possessed activity toward [3H]GalNAc-GM$_3$ and both possessed a similar ratio of natural G$_{M3}$ to artificial (4-MU-N-acetyl-D-galactosaminide) activity (Table IV) when each was assayed under optimal conditions. When the artificial substrates were used in the presence of sodium taurocholate to assay enzyme activity, both enzymes were moderately (~20%) inhibited. Each enzyme showed an optimal pH of 4.2 using a 75 mM citrate-phosphate buffer (Fig. 6). The enzymes showed

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**Table III**

$K_m$ and $V_{max}$ of purified placental hexosaminidases A and B with artificial substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>4-MU-β-N-acetyl-D-glucosaminide</th>
<th>4-MU-β-N-acetyl-D-galactosaminide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (μM)</td>
</tr>
<tr>
<td>Hexosaminidase A</td>
<td>1.1</td>
<td>173</td>
</tr>
<tr>
<td>Hexosaminidase B</td>
<td>1.0</td>
<td>158</td>
</tr>
</tbody>
</table>

*a Millimoles of 4-MU released per mg of protein per hour.*

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**Fig. 4.** Effect of varying detergent concentration on G$_{M2}$ hydrolysis. Incubations were carried out as described under "Experimental Procedures" with the exception of varying detergent concentration.

**Fig. 5.** Effect of varying substrate concentration on G$_{M2}$ hydrolysis. Only the substrate concentration was varied; the amounts of hexosaminidases A and B present in these incubations were not identical (approximately 10 μg of hexosaminidase B and 6 μg of hexosaminidase A per incubation).
Ratio of GM₂-cleaving to artificial substrate activity of purified placental hexosaminidases

Incubations were carried out in duplicate for either natural or artificial (4-MU-N-acetyl-D-glucosaminide) cleaving activity with highly purified enzyme preparations as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Artificial activity</th>
<th>GM₂ activity</th>
<th>Ratio of natural to artificial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexosaminidase A</td>
<td>2.4 μmoles/hr</td>
<td>121 μmoles/hr</td>
<td>50.4</td>
</tr>
<tr>
<td>Hexosaminidase B</td>
<td>0.6 μmoles/hr</td>
<td>34.5 μmoles/hr</td>
<td>57.5</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of pH on GM₂ hydrolysis. Incubations were carried out as described except that pH was varied as indicated. Incubations contained 2.1 μmoles per hour of 4-MU-N-acetyl-D-glucosaminide activity for hexosaminidase A and 0.7 μmole per hour for hexosaminidase B.

Attempts to increase the GM₂-cleaving activity of our enzymes through the addition of Clostridium perfringens neuraminidase (Sigma type VI) which has been shown to stimulate the liver hexosaminidases (17) were largely unsuccessful, (Table V). At pH 3.8, where maximal stimulation of the liver hexosaminidases was noted, our GM₂-cleaving hexosaminidase A preparations were inhibited by the addition of this neuraminidase preparation. Heat denaturation of the neuraminidase before addition to the

FIG. 7. Linearity of GM₂ hydrolysis with time. Incubations were carried out for varying lengths of time as described in the text. The amount of enzyme was described in Fig. 6. In this preparation, the ratio of GM₂ (natural) to artificial substrate hydrolysis (in the sense of Table IV) was 101.2 for A and 78.9 for B.

FIG. 8. Effect of varying substrate concentration on GM₂ hydrolysis. Incubations were carried out as described in the text and the concentration of [14C]GalNAc-GM₂ was varied as indicated. Two micromoles per hour of artificial substrate activity was used for either hexosaminidase A or B.

Table V

Effect of clostridium perfringens neuraminidase (type VI) on hexosaminidase A activity on [14C]GalNAc-GM₂

Incubations were carried for 16 hours at 37° under the conditions described under “Experimental Procedures.” The pH was varied as indicated.

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Neuraminidase</td>
</tr>
<tr>
<td>----</td>
<td>----------------</td>
</tr>
<tr>
<td>3.8</td>
<td>377</td>
</tr>
<tr>
<td></td>
<td>891²</td>
</tr>
<tr>
<td>4.2</td>
<td>614</td>
</tr>
<tr>
<td>5.0</td>
<td>600</td>
</tr>
</tbody>
</table>

* 100 μg per incubation.

² Heat-denatured neuraminidase.
N-acetyl-

-400

-300

-200

-100

0

100

200

300

400

500

600

700

800

900

1000

Fig. 9 (left and center). Heat stability of the hexosaminidas. Preincubation was carried out for varying times and temperatures as indicated. Enzymes were assayed as in the text with 4-MU-N-acetyl-

-400

-300

-200

-100

0

100

200

300

400

500

600

700

800

900

1000

hexosaminidase A and B. Preincubation was carried out for 4 hours at the temperature indicated and hexosaminidase A was separated from B as described in the text. Assays were carried out as in Fig. 9.

**FIG. 10 (right). Effect of temperature on the conversion of hexosaminidase A to B.**

Heat Dependence—We observed during the purification of hexosaminidase A that if partly purified enzyme was stored at 5°C, a small amount of this enzyme seemed to undergo transformation to hexosaminidase B. Such observations had been made earlier (2, 3) but were not systematically investigated. To quantify this transformation small (1 × 25 mm) columns of DEAE-cellulose which had been suspended in 25 mM phosphate (pH 6.0), pH adjusted to 6.0, and washed several times with this buffer were prepared in Pasteur pipettes. It was observed in the course of purifying the enzymes that hexosaminidase A is retarded on an ion exchange column under these conditions and this retardation was confirmed for the small columns. The formation of hexosaminidase B can therefore be determined by monitoring the activity which passes through the column. Small quantities (5 to 10 μg) of purified hexosaminidase A in 0.1 ml of sodium phosphate (25 mM, pH 6.0) buffer were incubated at various temperatures for 4 hours. At the end of this period, the samples were diluted to 1 ml with the buffer, an aliquot was removed for analysis of enzymatic activity, and the remainder was placed on the DEAE-cellulose column. The columns were eluted with an additional 2.0 ml of buffer and the effluent was analyzed for hexosaminidase activity (Fig. 10).

The conversion of hexosaminidase A to B increased as the temperature was elevated. Almost total conversion was obtained at 50°C along with a 10% loss in total activity. These data are consistent with the heat stability profile noted for the purified enzyme (Fig. 9b) where only total activity was monitored. N-Acetylgalactosamine is a competitive inhibitor of the hydrolysis of 4-MU-GalNAc. The conversion of hexosaminidase A to B was inhibited by this substance (Table VII). It should be noted that after the dilution following heating at 50°C, the concentration of N-acetylgalactosamine was no longer at a significantly inhibitory level. A similar protective effect was noted with G\textsubscript{Mg} at substrate level concentrations without sodium taurocholate (Table VII). This newly formed hexosaminidase B had a pattern on gel electrophoreses in the presence of 0.1%
TABLE VII

Effect of GM2 and N-acetyl-D-galactosamine on hexosaminidase A to B transformation

<table>
<thead>
<tr>
<th>Addition</th>
<th>B Formed</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine (2 mM)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine (2 mM)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>GM2 (200 μM)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>GM2 (200 μM)</td>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

*Concentration in preincubation is indicated in parentheses.
*Added after preincubation. Preincubation was carried out for 3/8 hour at 45° and samples were then treated as described in the text.

TABLE VIII

Neuraminidase treatment of hexosaminidase A

Incubations were carried out in duplicate at each temperature with 25 μl of highly purified neuraminidase at pH 5.5 in a 0.05 M potassium acetate buffer with 10 mM CaCl2. After incubation one sample was taken for NeuAc determination and the other for per cent hexosaminidase B as described in the text.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>NeuAc released</th>
<th>B Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmolcs</td>
<td>%</td>
</tr>
<tr>
<td>5°</td>
<td>10.7</td>
<td>3.5</td>
</tr>
<tr>
<td>39</td>
<td>7.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Theoretically 13.5 nmolcs of NeuAc present per incubation.
*Theoretically 4.8 nmolcs of NeuAC present per incubation.

sodium dodecyl sulfate similar to hexosaminidase B obtained from fresh placental tissue.

**Neuraminidase Treatment**

A transformation of hexosaminidase A to B has been reported by Robinson and Stirling in the presence of a crude preparation of neuraminidase (2). However, they did not examine the effects of temperature or other parameters such as possible contaminants in their neuraminidase preparation in detail. Highly purified neuraminidase was prepared from an extract of C. perfringens utilizing the affinity column method of Cuatrecasas and Illiano (18) (a gift from Dr. P. Fishman). Mixtures containing 25 μl of this enzyme were incubated with fetus or partly purified hexosaminidase (after Step 5). Total N-acetylneuraminic acid in each incubation was determined using the resorcinol method of Svennerholm (19). Duplicate incubations were carried out for 16 hours at 37° or 1 week at 5°. Control vessels which contained heat-denatured neuraminidase were incubated simultaneously. Aliquots were removed for the determination of free N-acetylneuraminic acid (20) and the amount of hexosaminidase B was determined as above after dilution with pH 6.0 buffer (Table VIII). More N-acetylneuraminic acid was released at the lower temperature after 1 week (perhaps due to the instability of the highly purified neuraminidase at 37°) but more significantly, only at 37° was an appreciable amount of hexosaminidase B formed (48%) indicating that removal of N-acetylneuraminic acid alone does not cause transformation of A to B.

**DISCUSSION**

We present a method for the preparation of highly purified hexosaminidases A and B from human placental tissue. The molecular weight is identical for both enzymes as determined by gel filtration on Sephadex G-200. Our value of 127,000 is in agreement with that reported earlier (3) for human liver and slightly lower than the reported weights of the beef spleen enzymes (4). The molecular weights of hexosaminidases A and B were determined using gel electrophoresis in the presence of urea, sodium dodecyl sulfate, and reducing agent at high pH, a uniform subunit with a mass of 33,000 daltons is obtained. Subunits of masses of 33,000 and 66,000 daltons were seen after preincubation in the presence of these agents near neutrality. These results are in the range of subunit weights obtained with the beef spleen enzyme (4), and the presence of only a single band on the high pH gels when hexosaminidases A and B are run simultaneously indicates that the subunits of each enzyme are of identical mass. In separate experiments, when both enzymes were added to the same gel, no differences could be detected. An interesting difference was noted when urea and reducing agents were not included in the preincubation. In this case, only trace amounts of the 33,000 molecular weight unit were seen.

Hexosaminidase A had a dark band at the 65,000 level and trace amounts of a higher 120,000 to 140,000 molecular weight protein. In contrast, hexosaminidase B was rather resistant to breakdown into its subunits and displayed a pattern of proteins of molecular weight ranging from 120,000 to 140,000. This pattern was qualitatively like the trace amounts of high molecular weight A. From these data it would seem that disulphide bonds play an important part in the association of the two 65,000 units in B to form the tetramer. In hexosaminidase A, this type of bond does not yet exist between the units or is labile under the conditions employed; indeed, it is likely that the units are hydrophobically associated but not covalently linked. The heterogeneity of both enzymes on sodium dodecyl sulfate gels at the high molecular weight range is interesting because it may represent the association of varying amounts of sodium dodecyl sulfate with holoenzyme. Presumably the enzymes are quite close to their native state and may contain varying amounts of carbohydrates (4). Carbohydrate side chains change the interaction of sodium dodecyl sulfate with proteins (21).

This finding would also explain the ability to convert A into B by the action of two enzymes in the presence of a crude preparation of neuraminidase, including heat-denatured neuraminidase and a reducing agent at high pH, a uniform subunit with a mass of 33,000 daltons is obtained. Subunits of masses of 33,000 and 66,000 daltons were seen after preincubation in the presence of these agents near neutrality. These results are in the range of subunit weights obtained with the beef spleen enzyme (4), and the presence of only a single band on the high pH gels when hexosaminidases A and B are run simultaneously indicates that the subunits of each enzyme are of identical mass. In separate experiments, when both enzymes were added to the same gel, no differences could be detected. An interesting difference was noted when urea and reducing agents were not included in the preincubation. In this case, only trace amounts of the 33,000 molecular weight unit were seen.

Hexosaminidase A had a dark band at the 65,000 level and trace amounts of a higher 120,000 to 140,000 molecular weight protein. In contrast, hexosaminidase B was rather resistant to breakdown into its subunits and displayed a pattern of proteins of molecular weight ranging from 120,000 to 140,000. This pattern was qualitatively like the trace amounts of high molecular weight A. From these data it would seem that disulphide bonds play an important part in the association of the two 65,000 units in B to form the tetramer. In hexosaminidase A, this type of bond does not yet exist between the units or is labile under the conditions employed; indeed, it is likely that the units are hydrophobically associated but not covalently linked. The heterogeneity of both enzymes on sodium dodecyl sulfate gels at the high molecular weight range is interesting because it may represent the association of varying amounts of sodium dodecyl sulfate with holoenzyme. Presumably the enzymes are quite close to their native state and may contain varying amounts of carbohydrates (4). Carbohydrate side chains change the interaction of sodium dodecyl sulfate with proteins (21).

This finding would also explain the ability to convert A into B by a mechanism in which the complementary —SH groups are in close apposition after heating and form one or more disulphide bridges. This may be due to an initial change in the hydrophobic interactions of the chains. The inability to convert B back into A is also explained by the presence of this new disulphide bond(s). Similar conformational changes have been seen in masking —SH groups in glyceraldehyde-3-P dehydrogenase (22). In dilute solution, where chain-chain interactions are of great importance, similar thermostability properties of the two enzymes may exist because of the conformational changes of the protein either preceding or accompanying the formation of the new disulphide bond(s). Similar conformational changes have been seen in masking —SH groups in glyceraldehyde-3-P dehydrogenase (22). In dilute solution, where chain-chain interactions are of great importance, similar thermostability properties of the two enzymes may exist because of the conformational changes of the protein either preceding or accompanying the formation of the new disulphide bond(s).
Hexosaminidase B might not be expected to show this denaturation. Other evidence supports this concept of the close relationship of the hexosaminidases. In the beef spleen study (4), the amino acid content of both enzymes was almost identical and the only differences between the two species was in their sialic acid and neutral carbohydrate content. Immunologic evidence (described below) also supports this close relationship (23, 24).

Using artificial (4-MU) substrates, various kinetic parameters of our enzymes were determined. They are essentially in agreement with the reports of other laboratories and the similar values obtained for each enzyme also point out their close relationship. In agreement with the values of Verpoorte (4), the $V_{\text{max}}$ of hexosaminidase B is slightly lower than that of hexosaminidase A. Studies on the hydrolysis of [3H]GalNAc-GM$_2$ indicate a pH optimum of 3.8, the same as that obtained with a partially purified liver preparation (17). A complex response to increasing amounts of sodium taurocholate was obtained with a maximal stimulatory effect at a concentration of 1.2 mg per ml of incubation. At this concentration of detergent, irregular response to increasing substrate (GM$_2$) concentration was observed. A maximal rate of hydrolysis was obtained at a concentration of 60 $\mu$M. No definitive $K_m$ could be calculated. This response to increasing concentrations of substrate is similar to that observed by Gatt et al. (25) and fits their type III kinetics. The dependence of each enzyme on detergent for GM$_2$ hydrolysis explains this anomalous kinetic situation, as the response of these enzymes to increasing artificial substrate concentration is classic.

Both normal human placental hexosaminidase A and B possess activity toward [3H]GalNAc-GM$_2$. Hydrolysis of this substrate was obtained only in the presence of sodium taurocholate. This dependence is in contrast to the earlier studies in crude brain tissue (10) where no detergent was required for GM$_2$ hydrolysis. In those studies we did not attempt to separate the activities of hexosaminidases A and B.

Sandhoff (3, 26) using a purified preparation of hexosaminidase A from human liver demonstrated the hydrolysis of [3H]GM$_3$ (reductively tritiated in the sphingosine moiety) using long term incubations (24 hours) in the presence of sodium taurocholate. Only hexosaminidase A was capable of this hydrolysis. His purified B had no activity. However, because of the small amount of product GM$_3$ ($\sim$5%) formed and the complicated assay procedure necessitated by the nature of the labeling, a significant decrease in the activity of hexosaminidase B during the course of the incubation would make it difficult to detect activity present only in the initial stages of the reaction (i.e. a smaller amount of product). Such a decrease in activity was noted in our experiments (Fig. 7) and might account for his inability to demonstrate activity with B.

The use of frozen tissue as the purification source may account for the difficulties of another group (17) in demonstrating any GM$_3$ hydrolysis. We have been emphasizing the necessity of fresh tissue to study adequately the ganglioside catalytic enzymes (8-10, 27, 28); in light of such evidence, their inability to demonstrate activity is not surprising. However, in their experiments, the addition of a bacterial neuraminidase led to a significant conversion of GM$_3$ to the lactosylceramide. The pH of this incubation was 3.8, the optimum for the hydrolysis of GM$_3$ and far from the optimum of the neuraminidase. It is not clear from their data whether this is a two-step process involving GM$_3$ hydrolysis of GM$_3$ followed by the hydrolysis of this compound to lactosylceramide or an obstruct mechanism using a "neuraminidase-hexosaminidase" complex. Our attempts to recon-struct this experiment with our optimal substrate to detergent ratio were unsuccessful. We showed a slight inhibition of our active hexosaminidase A at pH 3.8 by the addition of neuraminidase (Sigma, type VI). At pH 5.0 we have achieved either stabilization of the hexosaminidase or some conversion of GM$_3$ to GM$_3$ followed by release of N-acetylgalactosamine from this compound. Based on this evidence and our previous negative results (9) in which we studied possible synergistic effects after the addition of purified mammalian neuraminidase to both crude lysosomal preparations from human brain and purified human hexosaminidases (27), we feel that the postulation of a "complex of neuraminidase and hexosaminidase" (17) is premature and clouds the mechanism of an otherwise "simple" hydrolysis.

There are a number of differences which we should mention between the present study with purified enzymes and past studies done in crude lysosomes from mammalian brain (10). In those studies, a higher ratio of natural to artificial substrate activity was obtained (1:600 versus 1:10$^5$ in the present study). The crude preparations exhibited a more basic pH optimum and a classical kinetic situation. There was no detergent requirement. It is certainly possible that within the intact lysosomes a different and more effective environment for GM$_3$ hydrolysis is obtained than with purified enzymes in dilute solution. It is notable that disruption of the lysosomes by sonication led to drastic reduction of the GM$_3$ hexosaminidase activity and the total loss of the multistep hydrolysis of [3H]GalNAc-GM$_3$ (12) which were able to observe in this system. Thus, a necessary cofactor or natural membrane component cannot be ruled out as being of physiological significance in GM$_3$ breakdown. The low levels of activity observed with purified hexosaminidases A and B in the present study cannot be considered physiologically significant.

Both hexosaminidases A and B have similar thermostability profiles as purified enzymes in dilute solution. In serum, under conditions of acidity (pH 4.4), hexosaminidase A is more readily inactivated than B in agreement with the generally accepted concept (5). However, not all A is inactivated. Although we have been able to successfully diagnose carriers and patients with the classical form of Tay-Sachs disease (29) by this procedure, its mechanism is still unclear and may be similar to that described above. It should also be noted that when freshly drawn plasma was examined by an independent method (30), it was clear that only trace amounts of hexosaminidase B were present; from this information we might conclude that some of the hexosaminidase A becomes B during the course of the preparation of serum for analysis.

The conversion of hexosaminidase A to B has been observed earlier (2, 31, 32) and was attributed by two of the groups (2, 31) to the removal of N-acetylneuraminic acid from hexosaminidase A to yield hexosaminidase B. At 37°C, we do get conversion of hexosaminidase A to B in the presence of neuraminidase or heat denatured neuraminidase but in the same experiment at 5°C where NeuAc is still cleaved, there is no conversion of A to B.

Model of Hexosaminidases—We submit a working model for the interrelationship of the hexosaminidases which represents to us the clearest explanation of the data obtained for normal enzymes and the enzymes from the various clinical forms of Tay-Sachs disease. Hexosaminidases A and B represent different conformational states of the same enzyme. Evidence for this theory is based on the almost identical kinetic patterns ob-

3 J. F. Tallman, unpublished observation.
tained when the catalytic activity with various substrates is investigated, identical thermal denaturation curves in dilute solution near neutrality, identical number of subunits per holoenzyme and identical molecular weights of these units, a "strikingly similar" amino acid composition of purified beef spleen enzymes (4), and immunological evidence (23, 24) which indicates that A and B cross-react extensively. Adsorption of anti-A by hexosaminidase B leads to the production of specific anti-A antisemur. However, B has no antigens which A lacks. The specific antisemur A may result from an antigen characteristic such bonds has been shown to change the antigenic properties of other proteins (33). What is particularly significant for the chymotrypsin (4), albeit immunological evidence (23, 24) which indicates that A and B cross-react extensively. Absorption of anti-A by hexosaminidase B leads to the production of specific anti-A antisemur. However, B has no antigens which A lacks. The specific antisemur A may result from an antigen characteristic such bonds has been shown to change the antigenic properties of other proteins (33). What is particularly significant for the model is the finding that there are no specific anti-B antibodies which might be expected if there were one common and one unique subunit for A and B. In addition, the presence of this unique subunit in each enzyme would be expected to render the amino acid composition of each enzyme much more variant than it appears (4).

The distinct possibility exists that the early folding of the subunit is followed by their association to form a tetramer which is a metastable A in spite of the fact that the thermodynamically more stable form may be hexosaminidase B. In fact, an energy barrier between the two forms may exist and this barrier is overcome by heating. If extrusion of newly synthesized enzyme after glycosylation into the extracellular medium is followed by uptake to form lysosomes (34), a high proportion of the A isoenzyme might be expected in tissue culture medium and extracellular fluids such as fresh plasma where A is found almost exclusively (30).

In patients with classic Tay-Sachs disease, the initial folding of the enzyme in the A conformation is less stable than usual or the energy barrier between the two forms is lower because of a nonsense mutation which results in an amino acid substitution at a point critical for the folding of the subunits and their association (hydrophobic region). In this case, the disulfide bond necessary for the formation of the stable B is readily formed. Thus, hexosaminidase B is formed almost immediately at the moderate temperatures of the body. It is not the normal B which would be necessary if the common and unique subunit theory held but an enzyme which moves more slowly toward the anode in neutral pH electrophoretic schemes; the residual A moves similarly (23). Concurrent with this mutation, but not as a result of the loss of the A conformation above, there is a loss of the ability of either form (A or B) to catalyze the breakdown of Gm4G. This loss of Gm4G-cleaving ability is the real defect in classic Tay-Sachs disease.

In the “O” variant (Sandhoff’s disease) both hexosaminidases A and B cross-reacting proteins are said to be present (24) although this has been disputed (23). The mutation, different from that in classic Tay-Sachs disease, would be at a site which is important in the actual mechanism of hydrolysis of N-acetylated-hexosamine from any of the substrates. Thus, a mechanistically important amino acid is implicated in this disease and not an amino acid involved in conformational aspects.

In the “AB” variant where both isozymes are present, Gm4G degradation is decreased (35). Here we have a mutation which involves Gm4G hydrolysis but does not affect the conformational stability of the A form; accordingly, A is present.

One might predict, on theoretical grounds, that since both A and B have activity against Gm4G, there may exist another benign condition in which patients might lack the A isozyme yet their B may retain Gm4G-hydrolyzing ability. These patients would present no clinical signs and would only be noticed as a result of the mass screening programs presently based on differential heat denaturation of hexosaminidase A (36, 37). Recently, such patients have been described (38). We hope that this model will stimulate additional conceptualization about the enzymatic basis of Tay-Sachs disease and the interrelationship of these hexosaminidases.

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