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Tay-Sachs Disease with Hexosaminidase A: Characterization of the Defective Enzyme in Two Patients

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SUMMARY

Cases of infantile Tay-Sachs disease (TSD) with high residual hexosaminidase A (Hex A) activity have recently been described. The clinical presentation of the disease in these patients is identical to that found among Ashkenazi-Jewish patients. Fibroblasts from two such TSD patients had Hex A activity comprising 16% of total Hex when measured by thermal fractionation and quantitation with 4-methylumbelliferyl-β-D-*N*-acetylglucosamine (4MUG). Hydrolysis of 4-methylumbelliferyl-β-D-*N*-acetylglucosamine-6-SO_4 (4MUGS) by patient fibroblast extracts is catalyzed by an enzyme activity that comprises <1% of total Hex. Kinetic analysis of patient Hex A by using 4MUGS revealed K_m's similar to that of control Hex A but V_{max}'s significantly different from that of the control enzyme. The inhibitors *N*-acetylglucosamine and *N*-acetylglucosamine-6-PO_4 were used to distinguish between active sites associated with the two different subunits of Hex A. A β-subunit site with little activity toward 4MUGS is sensitive to *N*-acetylglucosamine but resistant to *N*-acetylglucosamine-6-PO_4. This site accounts for most of the hydrolysis of 4MUG. By contrast, an α-subunit site that is sensitive to *N*-acetylglucosamine-6-PO_4 but resistant to *N*-acetylglucosamine accounts for almost all of the hydrolysis of 4MUGS. In mutant cells, this site retains the ability to bind substrate but is deficient in catalytic activity toward 4MUGS. The pH optima of patients' Hex A is shifted to a more acidic range,

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and the enzymes are significantly more thermostable than control Hex A. By using the thermal fractionation procedure for serum isozyme discrimination, one parent of each patient is unambiguously classified as heterozygous for the TSD gene whereas the other parent has test values in the grey zone. When parents are tested by use of 4MUGS, however, all four parents are classified as heterozygotes. Comparison of the results of both assay procedures allows the carrier of the atypical TSD allele to be recognized and identifies the probands as compound heterozygotes.

INTRODUCTION

The G_{M2} gangliosidoses are a group of neurodegenerative diseases with an autosomal recessive mode of inheritance. In all forms of G_{M2} gangliosidosis cleavage of the β-*N*-acetylgalactosaminyl terminal linkage of G_{M2} ganglioside is reduced or absent usually owing to deficient hexosaminidase A (Hex A).

Two isozymes account for the majority of hexosaminidase activity in tissues: (1) Hex B, a thermostable isozyme with the subunit structure $2(\beta_a\beta_b)$, and (2) the more anionic form Hex A, a thermolabile enzyme with a subunit structure of $\alpha\beta_a\beta_b$ (Mahuran et al. 1985).

Defects in three gene loci lead to the accumulation of G_{M2} ganglioside. Mutations in the gene locus encoding the α subunit of Hex A, which map to chromosome 15, include Tay-Sachs disease (TSD) or variant B. Mutations in the gene encoding the β-subunit, which map to chromosome 5, include Sandhoff disease or variant O. Mutations at a locus encoding an activator protein are associated with a form of G_{M2} gangliosidosis termed the AB variant. This gene has recently been mapped to chromosome 5 (Burg et al. 1985). A number of variant forms of the G_{M2} gangliosidoses exist that are due to allelic mutations at the two subunit loci and that differ with respect to age of onset, severity of symptoms, and central nervous system involvement.

The infantile, or classical, TSD occurs with greatest frequency in Ashkenazi-Jewish and French-Canadian populations (Andermann et al. 1977). These patients, though recently shown to possess different mutant alleles (Myerowitz and Hogikyan 1986), do not synthesize the α-subunit of Hex A (Proia and Neufeld 1982; E. F. Neufeld, personal communication) and thus possess CRM-negative mutations. Infantile TSD cases with an enzymatically aberrant but neurologically classical form of TSD recently have been described. Such patients have significant Hex A activity against synthetic *N*-acetylglucosaminide substrates but no activity against the 6-sulfated derivates of these synthetic substrates. The use of unsulfated synthetic substrates for prenatal diagnosis has resulted in one known misdiagnosis of an ''atypical'' TSD fetus (Kolodny et al. 1983). Correct prenatal diagnosis, by use of an assay based on the sulfated synthetic substrate, of a fetus affected with this form of TSD also has been reported (Conzelmann et al. 1985).

In the present paper we report the characterization of the abnormal Hex A obtained from fibroblasts of two TSD patients who have significant Hex A activity. These patients are most likely to be compound heterozygotes having one classical (CRM-negative) TSD allele and another allele that produces a normal amount of a catalytically defective α-subunit. An abstract of this work has been presented elsewhere (Bayleran et al. 1985).

MATERIAL AND METHODS

Clinical Evaluation of Patients and Families

Patient 1 (Massachusetts).—This patient, who was misdiagnosed when amniocyte Hex A activity was measured by use of 4MUG, was the younger sib of a brother who was diagnosed as having TSD. The mother is of French-Canadian origin and the father of English, Irish, Scottish, and German ancestry. In the original report (Kolodny et al. 1983) both parents were found to be heterozygous on testing. Prenatal diagnosis based on measurement of thermolabile hexosaminidase indicated that the fetus was a heterozygote for the TSD allele and he was allowed to come to term. By 6 mo of age the exaggerated startle response and the macular cherry red spot anticipated the development of the classical stigmata of TSD. Retesting of the patient resulted in Hex A values of 20% in serum and 31.5% in fibroblasts. A more detailed account of the clinical findings will be reported shortly.

Patient 2 (California).—This patient was a female who presented with a typical course of TSD. Examination at 6 mo revealed the presence of a macular cherry red spot. By 12–14 mo seizures, psychomotor symptoms, and dementia were apparent. Serum Hex A was within the TSD-homozygote range, but in fibroblasts Hex A was found to be 12%–14% of total hexosaminidase activity.

The mother, who tested in the inconclusive range on the basis of both serum and leukocyte testing, is of Scottish-Irish ancestry, and the father, who was found to be a heterozygote, is of German-Scandinavian origin.

Measurement of hexosaminidases.—Hexosaminidase activity against 4MUG and 4MUGS was measured fluorometrically by the method of Leaback and Walker (1961). In table 1 data are presented in the form of both percentage of Hex A and Hex A specific activity, as calculated by three methods. Column 1 of table 1, as well as figure 1*A*, show the values obtained by measurement of thermolabile hexosaminidase activity toward 4MUG. Hex A and Hex B activities against 4MUG are distinguished by their differential thermolabilities. Since the turnover numbers for 4MUG hydrolysis of the two isozymes are equal (Geiger and Arnon 1976), the percentage of Hex A and Hex B can be determined directly from assay values. Column 2 of table 1, and figure 1*B*, show the values obtained by measurement of Hex A activity toward 4MUGS. Since the V_{max} for hydrolysis of 4MUGS is very different for Hex A vis-à-vis Hex B, we have employed an empirical formula reported elsewhere (Bayleran et al. 1984) that transforms 4MUGS assay values into equivalent 4MUG units. This transformation makes it possible to compare the accuracy of genotypic classification of parents as based on the two substrate assay procedures (for the equation and

TABLE 1

DETERMINATION OF HEX A ACTIVITY IN HUMAN FIBROBLAST CELL EXTRACTS AND SERA

Source and Subject	% (Specific Activity) of Hex A as Determined by: 4MUGS and Thermal Fractionation	4MUGS and Formula	4MUGS
Fibroblasts:[a]			
Patient 1	16 (538)	0.7 (26)	3.6
Patient 2	16 (1,377)	0.7 (67)	2.2
Controls (N = 8)	47–79 (3,260–9,280)	46–89 (3,080–8,294)	230–460
TSD (N = 7)	1–5 (32–140)	0.4–2 (9–87)	1.1–6.7
Sera:[b]			
Patient 1	29 (1.2)	0.96 (0.04)	0.01
Mother	51 (2.5)	42 (2.1)	0.21
Father	57 (2.2)	35 (1.3)	0.13
Patient 2	NT	NT	NT
Mother	55 (4.6)	44 (3.7)	0.37
Father	51 (4.1)	47 (3.8)	0.39
Controls (N = 45)	60–81 (3.6–7.9)	60–88 (4.1–8.6)	0.43–0.87
Obligate heterozygotes (N = 28)	34–57 (2.2–4.6)	35–55 (1.3–3.8)	0.13–0.39
TSD (N = 6)	9–11 (0.53–1.2)	0–2 (0.01–0.28)	0.01–0.05

NOTE.—NT = not tested.

[a] Specific activity is expressed in nmol substrate hydrolyzed/h/protein and represents the mean of four determinations. Data presented in column headed "4MUGS and Formula" have been transformed to equivalent 4MUG units by application of the empirical formula (Bayleran et al. 1984).

[b] Specific activity is expressed in nmol substrate hydrolyzed/min/ml and represents the mean of four determinations. Data presented in column headed "4MUGS and Formula" have been transformed to equivalent 4MUG units by application of the empirical formula.

a definition of terms, refer to the Appendix). Column 3 of table 1 presents Hex A values as nanomoles of 4MUGS hydrolyzed, i.e., without application of the equation.

4MUG was purchased from Koch-Light (Edmonton, Alberta). 4MUGS was synthesized in our lab according to the method of Bayleran et al. (1984). *N*-acetylglucosamine and *N*-acetylglucosamine-6-PO_4, which were employed as enzyme inhibitors, were purchased from Sigma (St. Louis).

Biological samples.—Skin fibroblasts were cultured and prepared for assay as described elsewhere (Bayleran et al. 1984). Leukocytes were prepared from whole blood by the method of Kaback et al. (1977). Serum was prepared from clotted blood by centrifugation at 25 C. Hexosaminidase isozymes were separated by ion-exchange chromatography on DEAE-cellulose by the method of Nakagawa et al. (1977).

Protein estimation.—Protein was estimated by the method of Lowry et al. (1951), using crystalline bovine serum albumin as standard.

RESULTS

Hexosaminidase Activity of Patients and Parents

The evidence on the basis of which the two patients are classified as having biochemically atypical forms of TSD is presented in table 1 and figure 1. In the

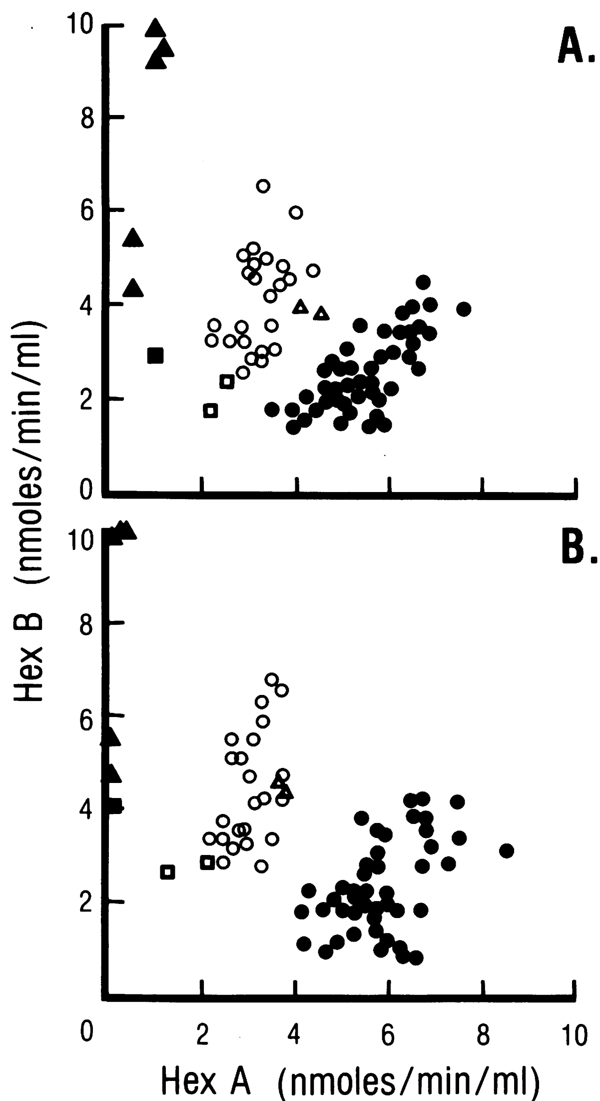

FIG. 1.—Determination of hexosaminidase isozymes in human serum. Hex A and B were determined (*A*) by thermolability, with use of 4MUG, and (*B*) by application of the equation, with use of 4MUGS. *A*, Thermal fractionation with 4MUG: mean ± SD Hex A = 5.53 ± 0.96 for controls and 3.28 ± 0.64 for heterozygotes; mean ± SD Hex B = 2.62 ± 0.83 for controls and 3.96 ± 1.17 for heterozygotes. *B*, 4MUGS employing equation: Hex A, mean ± SD for controls = 5.82 ± 0.92; for heterozygotes, 3.03 ± 0.60. Hex B, mean ± SD for controls + 2.33 ± 1.01; for heterozygotes, 4.21 ± 1.17. ● = Normal controls; ○ = obligate Tay-Sachs heterozygotes; ▲ = infantile Tay-Sachs homozygotes; ■ = patient 1; □ = parents of patient 1; △ = parents of patient 2.

laboratories at which the patients were identified, levels of Hex A were significantly greater than expected for classical TSD. Confirmation of these anomalous results appears in table 1, which reports 16% Hex A for fibroblasts of both patients. These values, as well as the corresponding specific activities for Hex A, are significantly higher than the 0%–5% Hex A expected for classical TSD. The most frequent mutant alleles associated with the classical form of the disease (i.e., those occurring in the Ashkenazi-Jewish and French-Canadian patients) are now understood to be the result of CRM-negative mutations (Proia and Neufeld 1982; E. F. Neufeld, personal communication). Hex A values greater than zero in cells of this genotype are usually considered to be an artifact of thermal fractionation.

With serum testing, the Hex A value obtained for patient 1 is 29%, which is within the heterozygote range and would result in misdiagnosis of the patient. However, when the 4MUGS assay for determination of the activity of this enzyme is used, both patients are unambiguously diagnosed as Hex A deficient. As measured by this procedure, Hex A values for patient 1 serum and fibroblasts of both patients are indistinguishable from those found in serum or fibroblasts of patients with the classical form of the disease.

The parents' sera were assayed for Hex A both by thermal fractionation with 4MUG and by direct estimation with 4MUGS. Two of the parents (the mother of patient 1 and the father of patient 2) test comparably by both substrate procedures and fall within the heterozygote range for both percentage of Hex A and Hex A specific activity. Comparable values by the two substrate procedures were not obtained for the father of patient 1 and the mother of patient 2. Values of Hex A and Hex B measured by thermal fractionation and plotted as a two-discriminant test (fig. 1*A*) place these two parents slightly outside the range for other heterozygotes for the TSD gene. When Hex A is measured by the 4MUGS assay procedure (fig. 1*B*), all four parents are unambiguously classified as carriers. Both couples would most likely be advised of their risk for having an affected fetus, based on the results of the thermal fractionation assay. However, the 4MUGS assay procedure more clearly identifies them as couples at risk.

The observation that the father of patient 1 and the mother of patient 2 fall into the grey zone for classification on the basis of 4MUG hydrolysis yet are clearly heterozygotes on the basis of 4MUGS hydrolysis may indicate that the probands are compound heterozygotes for the CRM-negative TSD allele and a mutant allele that produces a defective but not deficient form of the α subunit of Hex A. The presence of the former type of allele can be detected equally well by both 4MUG and 4MUGS. The allele that produces the defective α subunit with altered catalytic properties is accurately detected only by 4MUGS, a substrate that is specific for the α-subunit catalytic site.

Fractionation of Hexosaminidases from Normal and Mutant Fibroblasts

Both probands produce a form of hexosaminidase that is thermolabile and that hydrolyzes 4MUG but does not hydrolyze 4MUGS and, presumably, does not hydrolyze G_{M2} ganglioside. To understand the biochemical basis for this

type of mutation, we undertook a fractionation of the hexosaminidase isozymes of patient and control fibroblasts and a study of the catalytic properties of the Hex A obtained from the fibroblasts of both patients.

Figure 2 shows the ion-exchange chromatography of hexosaminidases obtained from control fibroblasts (fig. 2*A*) and from patient 1 (fig. 2*B*). The chromatographic profile of hexosaminidases obtained from patient 2 was virtually identical to that of patient 1 and therefore is not shown. Assay of column fractions with 4MUG revealed activity peaks corresponding to Hex A and Hex B in control fibroblasts (fig. 2*A*, open circles), whereas in the fibroblasts of patient 1 a third species intermediate between Hex A and Hex B was seen (fig. 2*B*, open circles). This form of hexosaminidase is more anionic than Hex B yet is thermostable under conditions that completely inactivate Hex A. We have speculated that this intermediate species is a homopolymer of β subunits of a form that are usually incorporated preferentially into Hex A. In the absence of α subunits, this intermediate accumulates in TSD cells. No Hex A peak was seen following fractionation of TSD fibroblast extracts obtained from Ashkenazi-Jewish or French-Canadian patients (Hechtman et al. 1983).

Significant hexosaminidase activity toward 4MUGS was associated only with the Hex A peak from control cells (fig. 2*A*, closed circles). The ratio of Hex A hydrolysis of 4MUGS divided by its hydrolysis of 4MUG was 0.082 (using 1 mM concentrations of both substrates). We have found this ratio for fresh preparations of both serum and fibroblast Hex A. Serum-derived Hex A retains this ratio after either prolonged storage at −20 C or multiple freezing and thawing. Fibroblast Hex A suffers a threefold to fourfold decrease in the ratio under these conditions. Storage in the presence of human serum albumin (5 mg/ml) stabilized the fibroblast Hex A preparations.

The ratios associated with patient Hex A were 0.002 for patient 1 and 0.003 for patient 2. These ratios are within the range obtained for normal Hex B preparations.

Hex A activity (as defined by 4MUG hydrolysis) measured after chromatography of the control-cell supernatant accounted for 60% of the total recovered hexosaminidase activity (fig. 1*A*). This value is in accord with the estimation of percentage of Hex A in the unfractionated control-cell supernatant as measured after thermal fractionation (table 1). By contrast, Hex A recovered after chromatography of patient 1 fibroblast supernatant (fig. 2*B*) accounted for 30% of the eluted hexosaminidase activity, or twice the percentage estimated by means of thermal fractionation (table 1).

Catalytic Properties of Patients' Hexosaminidase A

Thermal inactivation of hexosaminidase is shown in figure 3. Hex B recovered from the two atypical TSD fibroblasts was heated for intervals up to 2 h and assayed with 4MUG. The probands' Hex B behaved identically to control Hex B. Thermal inactivation of control and patient Hex A at 42 C for 2 h and assay with 4MUGS revealed Hex A from both patients to be more thermostable than control Hex A. For two normal cell strains, the T_{50} values for Hex A inactivation were 19.5 min and 20 min. The T_{50} values for inactivation of

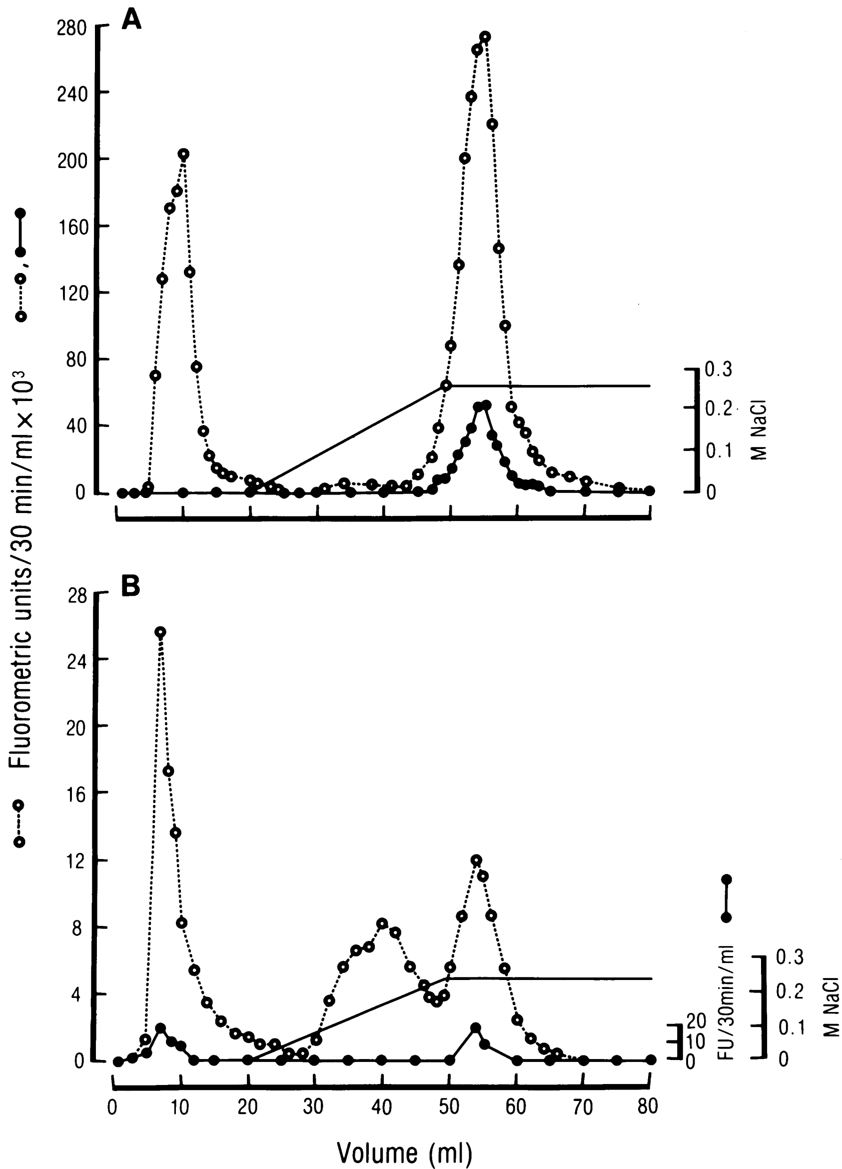

FIG. 2.—DEAE cellulose chromatography of fibroblast hexosaminidases in (*A*) normal control and (*B*) patient 1. Hexosaminidases were resolved by salt-gradient elution according to the procedure of Nakagawa et al. (1977) Fractions were assayed with 4MUG (○- - -○) and 4MUGS (●——●).

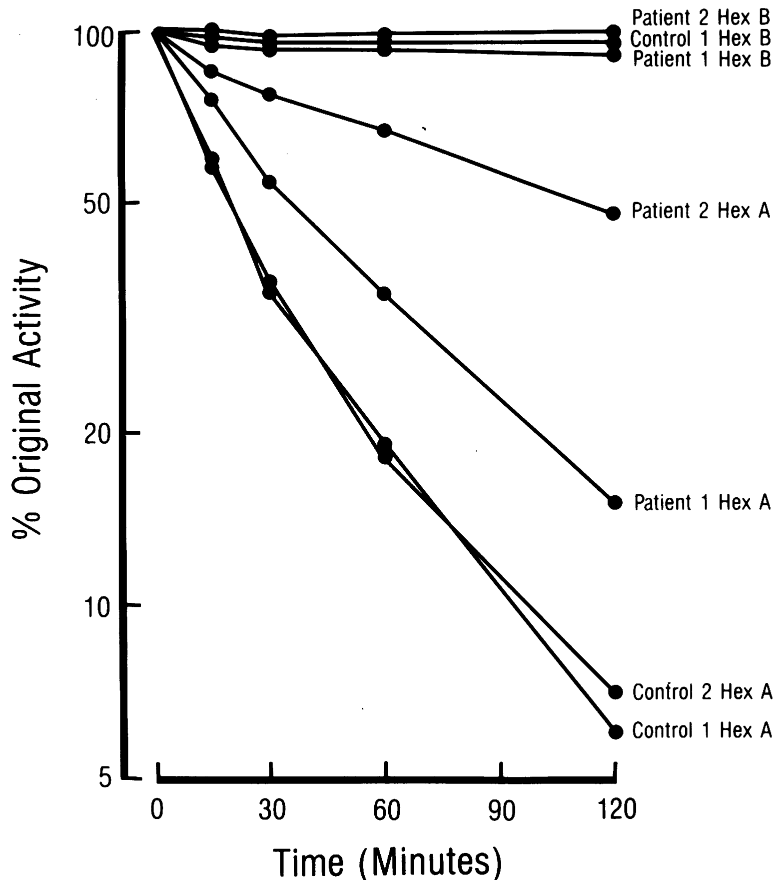

FIG. 3.—Thermal inactivation of control and patient Hex A and Hex B. Hex A was denatured at 42 C and assayed with 4MUGS at 37 C. Hex B was denatured at 47 C and assayed at 37 C with 4MUG.

the probands' Hex A were 36 min (patient 1) and 111 min (patient 2), indicating a significant difference from that of control Hex A as well as from each other. The increased thermostability of patient Hex A accounts for the discrepancy in percentage of Hex A values obtained by thermal fractionation (16%) versus chromatographic fractionation (30%) of patient fibroblast supernatants.

pH Activity curves of Hex A–catalyzed hydrolysis of 4MUGS are shown in figure 4. The pH optimum of control Hex A, at 3.9, was significantly different from that of the patients' enzymes, which occurred at a pH of 2.3.

Kinetic Analysis of Proband Hex A

Evidence has been accumulating (Kytzia and Sandhoff 1985) that supports the existence of two distinct catalytic sites on Hex A. A site on the β-subunit has preference for 4MUG and other substrates that carry no electric charge,

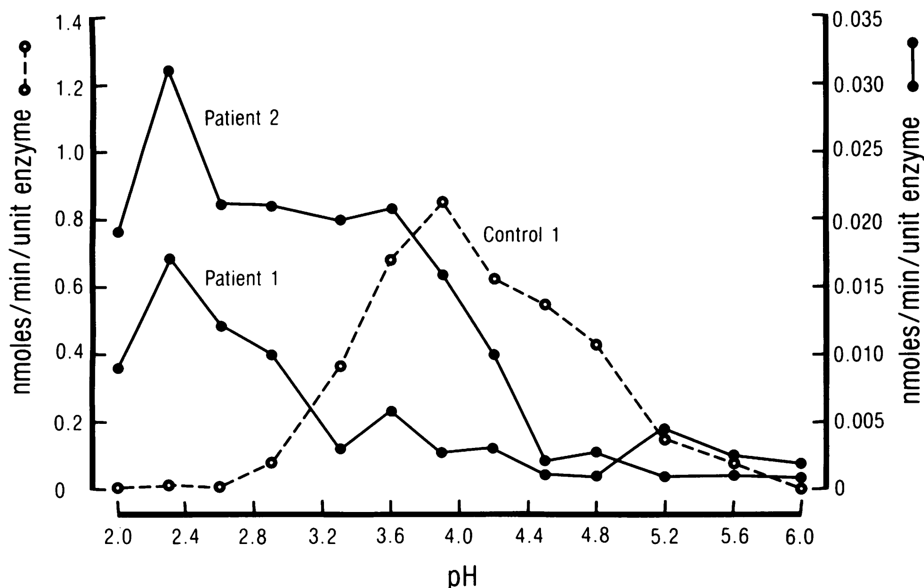

FIG. 4.—pH activity curve of Hex A–catalyzed hydrolysis of 4MUGS. Normal control and patient fibroblast Hex A were assayed in 0.04 M sodium citrate buffer at the indicated pH's with 1.5 mM substrate. One unit of enzyme activity is that amount of enzyme which catalyzes the hydrolysis of 1 nmol 4MUG/min under standard conditions of temperature and pH.

whereas the α-subunit has a site that possesses affinity for 4MUGS, G_{M2} ganglioside, and substrates carrying a negative charge.

Although hydrolysis of 4MUGS by patient Hex A was severely reduced, it was not completely absent. This observation alone made it impossible to distinguish between the following two possibilities: (1) that the mutant α-subunits retained active sites that functioned at a severely reduced rate of hydrolysis or (2) that the mutant α-subunits did not possess active sites and that the residual catalytic activity of proband Hex A toward 4MUGS was, therefore, entirely due to hydrolysis occurring at the active site on the β-subunit.

Lineweaver-Burke plots for normal fibroblast Hex A and Hex B, determined with 4MUGS, are shown in figure 5. Control Hex A has a K_m of 1.3 mM and a V_{max} of 0.91 nmol/min/4MUG unit of enzyme. Control Hex B (fig. 5, insert) has a K_m of 5.9 mM with a V_{max} of 0.004 nmol/min/4MUG unit of enzyme. This kinetic analysis clearly indicates that an active site on the α subunit of Hex A has a much greater affinity for the sulfated substrate than does the active site on the β subunit (this latter active site being common to both isozymes).

Figures 6 and 7 show the results of studies of the inhibition of 4MUG and 4MUGS hydrolysis by *N*-acetylglucosamine and *N*-acetylglucosamine-6-PO_4. The upper graphs in both figures demonstrate the specificity of the two inhibitors for the β- and α-subunit active sites, respectively. Thus, comparison of figures 6*A* and 6*B* reveals that the hydrolysis of 4MUG (open circles) by either control Hex A or control HEX B is inhibited to the same extent by *N*-

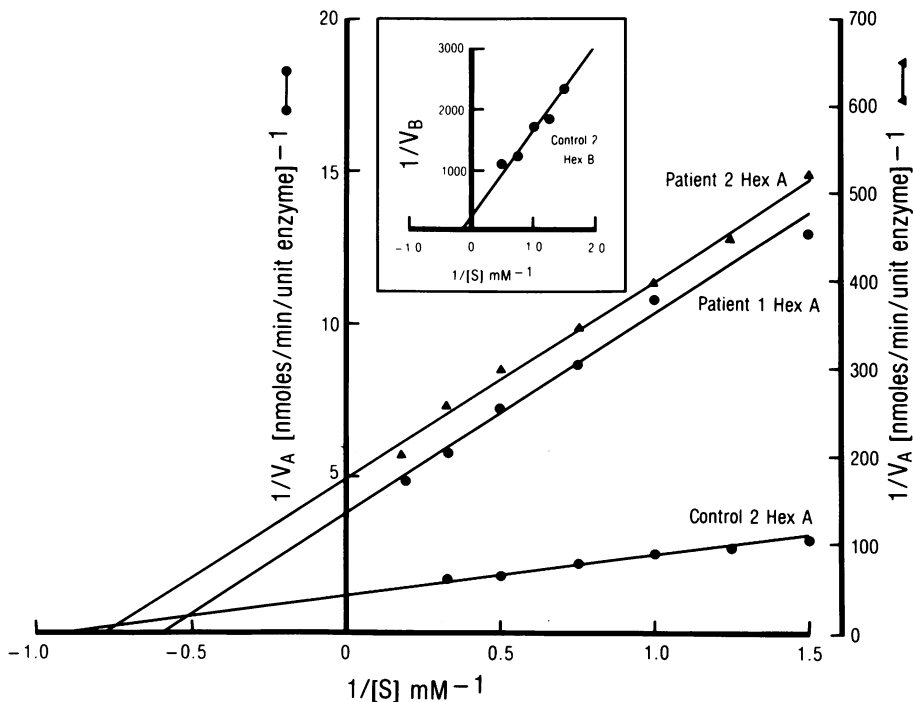

FIG. 5.—Lineweaver-Burke plots of control and patient fibroblast Hex A and control fibroblast Hex B (*insert*) for hydrolysis of 4MUGS. K_m for control Hex A = 1.3 mM; V_{max} = 0.91 nmol/min/unit enzyme. K_m for patient 1 Hex A = 1.7 mM; V_{max} = 0.26 nmol/min/unit enzyme. K_m for patient 2 Hex A = 1.5 mM; V_{max} = 0.006 nmol/min/unit enzyme. K_m for control Hex B = 5.9 mM; V_{max} = 0.004 nmol/min/unit enzyme.

acetylglucosamine. This suggests that this substrate is hydrolyzed by an identical active site on both enzymes. By contrast, hydrolysis of 4MUGS (closed circles) by control HEX A is inhibited only 18% at a concentration of 12 mM *N*-acetylglucosamine, whereas 4MUGS hydrolysis catalyzed by control HEX B is 95% inhibited at a concentration of 6 mM *N*-acetylglucosamine.

Similar results were obtained with the inhibitor *N*-acetylglucosamine-6-PO_4. Figure 7*A* shows that this compound is an inhibitor of the control α-subunit active site but has little effect on hydrolysis occurring at the β-subunit active site.

Lineweaver-Burke plots for patient Hex A hydrolysis of 4MUGS (fig. 5) were compared with those for control Hex A and Hex B. The K_m's of both patients' Hex A, at 1.7 and 1.5 mM for patients 1 and 2, respectively, were similar to that of control Hex A. The V_{max} of patient 1 Hex A was 0.26 nmol/min/4MUG unit of enzyme. The V_{max} of patient 2 Hex A was 0.006 nmol/min/4MUG unit of enzyme. The hydrolysis of 4MUGS by patients' Hex A therefore occurs at a site with a binding affinity that is more characteristic of an α-subunit active site than of a β-subunit active site.

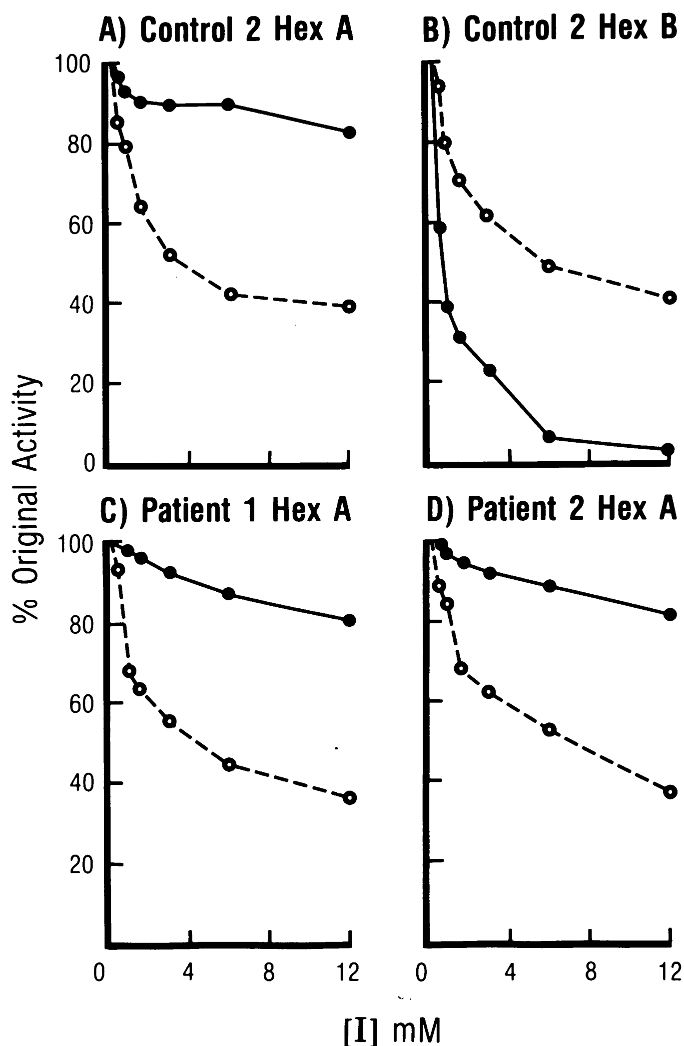

FIG. 6.—Inhibition of 4MUG (○---○) and 4MUGS (●—●) hydrolysis by *N*-acetylglucosamine. Concentration of both substrates was 0.5 mM.

Hydrolysis of 4MUG by patients' Hex A is inhibited by *N*-acetylglucosamine to the same extent as is that of control Hex A and Hex B (fig. 6*C*, 6*D*). However, hydrolysis of 4MUGS by Hex A preparations from both patients is reduced by only 19% in the presence of 12 mM *N*-acetylglucosamine, establishing that the catalytic site of the patients' Hex A has characteristics associated with an α-subunit site.

Figures 7*C* and 7*D* show inhibition of 4MUG and 4MUGS hydrolysis by patients' Hex A. Hydrolysis of 4MUGS by patient 1 Hex A (fig. 7*C*, closed circles) is as sensitive to *N*-acetylglucosamine-6-PO_4 inhibition as is that by

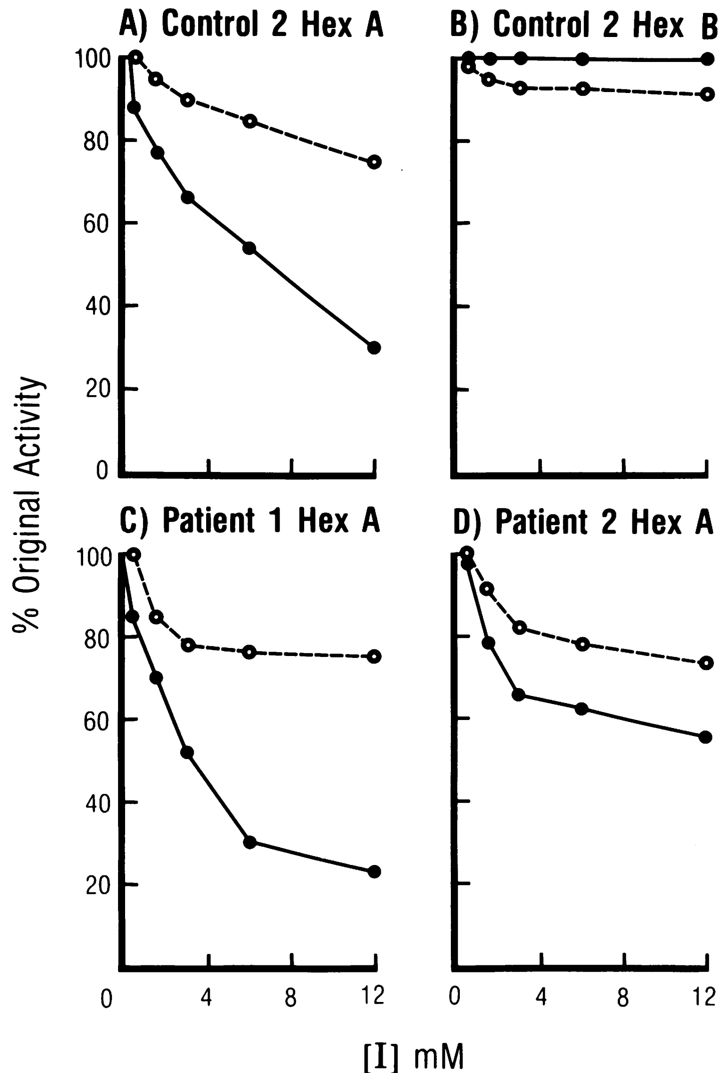

FIG. 7.—Inhibition of 4MUG (o- - -o) and 4MUGS (●——●) hydrolysis by *N*-acetylglucosamine-6-PO_4. Concentration of both substrates was 0.5 mM.

control Hex A (fig. 7*A*). However, the inhibition curve for patient 2 Hex A (fig. 7*D*) is intermediate between that of control Hex A and control Hex B, suggesting that, for this patient's Hex A (which has a much lower V_{max} than patient 1 Hex A), both active sites may contribute significantly to the residual 4MUGS hydrolysis.

DISCUSSION

For carrier detection and diagnosis, hexosaminidase activity is most often measured by use of the synthetic substrate 4MUG. Because 4MUG is hydro-

lyzed by both Hex A and Hex B, the assay requires a two-step procedure based on the differential thermolabilities of the two isozymes. Since 4MUGS is hydrolyzed primarily by Hex A, with Hex B activity against 4MUGS being negligible, assays employing this substrate have been shown to improve classification and diagnosis of the G_{M2} gangliosidoses (Fuchs et al. 1983; Bayleran et al. 1984; Ben-Yoseph et al. 1985).

Recently, cases of TSD have been described with significant Hex A activity against 4MUG but severely deficient activity against 4MUGS and G_{M2} ganglioside (Goldman et al. 1980; Li et al. 1981; Inui et al. 1983; Kytzia et al. 1983; Charrow et al. 1985). These patients have been referred to as B1 variants since they have been shown to be allelic (Sonderfeld et al. 1985) to classical TSD, i.e., to be variant B. Alternatively, these mutations have been referred to as pseudo-AB or A^mB variants (Inui et al. 1983) because the cells synthesize both Hex A and the activator protein yet are unable to hydrolyze G_{M2} ganglioside. As a consequence of their inability to cleave G_{M2} ganglioside, they store this substance in massive amounts and have a clinical phenotype identical to that of either infantile TSD (Goldman et al. 1980; Li et al. 1981; Kytzia et al. 1983) or juvenile-onset (Inui et al. 1983; Charrow et al. 1985).

Classification of the two infantile TSD patients reported here is vastly improved when Hex A activity is measured with 4MUGS. Similarly, the unambiguous classification of all four parents as heterozygotes when serum Hex A is measured with 4MUGS indicates that the thermal fractionation procedure is not detecting atypical TSD alleles in the heterozygous state. Our results are similar to those reported by Charrow et al. (1985) for a family with a proband who had late-onset G_{M2} gangliosidosis and near-normal Hex A activity against 4MUG but deficient activity against 4MUGS. This improvement in classification of genotypes by use of 4MUGS for measurement of Hex A has clear implications for prenatal diagnosis and heterozygote screening, particularly as it reaches out to non-Jewish communities.

The characterization of the mutant phenotype at the enzymatic level in the two probands extends the range of known mutation mechanisms that result in G_{M2} ganglioside storage. These defects include both deletion and nondeletion (Myerowitz and Hogikyan 1986) CRM-negative mutations, as well as CRM-positive mutations without and with catalytic activity. Among reports of patients in the latter group, ours is not the first report of an altered catalytic site (Conzelmann et al. 1983; Kytzia et al. 1983). However, to our knowledge, it does represent the first case in which the effect of the mutation on the catalytic site of the enzyme has been studied in detail by use of a synthetic substrate specific for this site. Previous attempts to demonstrate kinetic differences in proband Hex A employed 4MUG and therefore did not directly assess the product of the mutant gene. A large number of cases of the variant forms of G_{M2} gangliosidoses should now be reexamined for possible defective catalytic activity, using sulfated synthetic substrates to determine whether any correlations exist between clinical phenotypes and enzymatic alterations.

The model that we propose as a means of explaining the genotypes of the two probands encompasses the following features: (1) Both patients are compound

heterozygotes. (2) One allele is CRM negative for the α-subunit (Raghavan et al., personal communication). (3) The other allele encodes an α-subunit that is produced in normal amounts and associates normally with β-subunits. (4) Proband Hex A binds to and hydrolyzes 4MUG normally because the mutation does not affect the β subunit. (5) The catalytic site on the α-subunit binds 4MUGS but has a diminished capacity to hydrolyze this substrate. The catalytic alteration is revealed as a shift in the pH-activity curve of the mutant enzymes. (6) Significant—but probably functionally irrelevant—differences in T_{50} for thermal inactivation and in V_{max} for the two probands' Hex A's suggest that the two CRM-positive mutations represent different alleles. The frequency of such CRM-positive alleles in the population is not at present known.

A crucial assumption in the model presented above is the presence of two distinct active sites on Hex A. In an earlier paper we had proposed, on the basis of the inability of G_{M2} ganglioside to competitively inhibit hydrolysis of 4MUG, that distinct sites for 4MUG and G_{M2} ganglioside exist (Hechtman and Kachra 1980). More recently, Kytzia and Sandhoff (1985) have provided kinetic data in support of a two-site model. In the present report, the different responses of Hex A– and Hex B–catalyzed hydrolysis of 4MUG and 4MUGS to inhibition by *N*-acetylglucosamine and *N*-acetylglucosamine-6-PO_4 also point to the likelihood of Hex A being a bifunctional enzyme.

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APPENDIX

EMPIRICAL FORMULA FOR CALCULATION OF Hex A UNITS BY MEANS OF TWO SUBSTRATE ASSAYS, AND DEFINITION OF TERMS

$$g_A = \frac{S - (G \times r_B)}{r_A - r_B}; \%\text{Hex A} = \frac{g_A}{G} \times 100 .$$

G = Total fluorometric units when 4MUG is used as substrate; S = total fluorometric units when 4MUGS is used as substrate; g_A = fluorometric units attributable to Hex A when 4MUG is used; r_A = ratio of the rate of hydrolysis of the two substrates (4MUGS/4MUG) by pure Hex A; and r_B = ratio of the rate of hydrolysis of the two substrates (4MUGS/4MUG) by pure Hex B.

REFERENCES

Andermann, E., C. R. Scriver, L. S. Wolfe, L. Dansky, and F. Andermann. 1977. Genetic variant of Tay-Sachs disease: Tay-Sachs disease and Sandhoff's disease in French-Canadians, juvenile Tay-Sachs disease in Lebanese-Canadians, and a Tay-Sachs screening program in the French-Canadian population. Prog. Clin. Biol. Res. **18**:161–188.

Bayleran, J., P. Hechtman, and E. E. Kolodny. 1985. Atypical Tay-Sachs disease: characterization of the defective hexosaminidase A. Am. J. Hum. Genet. **37**:A6.

Bayleran, J., P. Hechtman, and W. Saray. 1984. Synthesis of 4-methylumbelliferyl-β-D-*N*-acetylglucosamine-6-sulfate and its use in classification of Gm_2 gangliosidosis genotypes. Clin. Chim. Acta **143**:73–89.

Ben-Yoseph, Y., J. E. Reid, B. Shapiro, and H. L. Nadler. 1985. Diagnosis and carrier detection of Tay-Sachs disease: direct determination of Hex A using 4-methylumbelliferyl derivatives of *N*-acetylglucosamine-6-sulfate and *N*-acetylgalactosamine-6-sulfate. Am. J. Am. Genet. **37**:733–748.

Burg, J., E. Conzelmann, K. Sandhoff, E. Solomon, and D. M. Swallow. 1985. Mapping of the gene coding for the human Gm_2 activator protein to chromosome 5. Ann. Hum. Genet. **49**:41–45.

Charrow, J., K. Inui, and D. A. Wenger. 1985. Late-onset Gm_2 gangliosidosis: an α-locus genetic compound with near normal hexosaminidase activity. Clin. Genet. **27**:78–84.

Conzelmann, E., H.-J. Kytzia, R. Navon, and K. Sandhoff. 1983. Ganglioside G_{M2} *N*-acetyl-β-D-galactosaminidase activity in cultured fibroblasts of late-infantile and adult G_{M2} gangliosidosis patients and of healthy probands with low hexosaminidase level. Am. J. Hum. Genet. **35**:900–913.

Conzelmann, E., H. Nehrkorn, H.-J. Kytzia, K. Sandhoff, M. Macek, M. Lehovsky, M. Elleder, A. Jirasek, and J. Kobilkova. 1985. Prenatal diagnosis of Gm_2 gangliosidosis with high residual hexosaminidase A activity (variant B^1; pseudo AB). Pediatr. Res. **19**:1220–1224.

Fuchs, W., R. Navon, M. M. Kaback, and H. Kresse. 1983. Tay-Sachs disease: one-step assay of β-*N*-acetylhexosaminidase in serum with sulfated chromogenic substrate. Clin. Chim. Acta **133**:253–261.

Geiger, B., and R. Arnon. 1976. Chemical characterization and subunit structure of human *N*-acetylhexosaminidases A and B. Biochemistry **15**:3484–3492.

Goldman, J. E., Y. Yamanaka, I. Rapin, M. Adachi, K. Suzuki, and K. Suzuki. 1980. The AB-variant of Gm_2 gangliosidosis: clinical, biochemical and pathological studies of two patients. Acta Neuropathol. **52**:189–202.

Hechtman, P., and Z. Kachra. 1980. Interaction of activating protein and surfactants with human liver hexosaminidase A and Gm_2 ganglioside. Biochem. J. **185**:583–591.

Hechtman, P., K. Khoo, and C. Isaacs. 1983. A new form of residual hexosaminidase activity in infantile Tay-Sachs disease fibroblasts. Clin. Genet. **24**:206–215.

Inui, K., E. E. Grebner, L. G. Jackson, and D. A. Wenger. 1983. Juvenile G_{M2} gangliosidosis (A^mB variant): inability to activate hexosaminidase A by activator protein. Am. J. Hum. Genet. **35**:551–564.

Kaback, M. M., L. J. Shapiro, B. S. Hirsch, and C. Roy. 1977. Prog. Clin. Biol. Res. **18**:276–277.

Kolodny, E. E., S. S. Raghavan, T. A. Lyerla, R. L. Proia, E. F. Neufeld, and E. E. Grebner. 1983. Misdiagnosis in a fetus with an unstable hexosaminidase A catalytically inactive toward G_{m2} ganglioside. Am. J. Hum. Genet. **35**:47A.

Kytzia, H.-J., U. Hinrichs, I. Maire, K. Suzuki, and K. Sandhoff. 1983. Variant of Gm_2 gangliosidosis with hexosaminidase A having a severely changed substrate specificity. EMBO J. **2**:1201–1205.

Kytzia, H.-J., and K. Sandhoff. 1985. Evidence for two different active sites on human β-hexosaminidase A. J. Biol. Chem. **260**:7568–7572.

Leaback. D. H., and P. G. Walker. 1961. The fluorometric assay of *N*-acetyl-β-glucosaminidase. Biochem. J. **78**:151–156.

Li, S.-C., Y. Hirabayashi, and Y.-T. Li. 1981. A new variant of type-AB Gm_2 gangliosidosis. Biochem. Biophys. Res. Commun. **101**:479–485.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193**:265–275.

Mahuran, D., A. Novak, and J. A. Lowden. 1985. The lysosomal hexosaminidase

isozymes. Pp. 229–288 *in* Isozymes: current topics in biological and medical research. Vol. **12.** Alan R. Liss, New York.

Myerowitz, R., and N. D. Hogikyan. 1986. Different mutations found in Tay-Sachs patients of Ashkenazi-Jewish and French-Canadian origin. Science **232:**1646–1648.

Nakagawa. A., S. Kumin, and H. M. Nitowsky. 1977. Human hexosaminidase isozymes: chromatographic separation as an aid to heterozygote identification. Clin. Chim. Acta **75:**181–191.

Proia, R. L., and E. F. Neufeld. 1982. Synthesis of β-hexosaminidase in cell-free translation and in intact fibroblasts: an insoluble precursor α chain in a rare form of Tay-Sachs disease. Proc. Natl. Acad. Sci. USA **79:**6360–6364.

Sonderfeld, S., S. Brendler, K. Sandhoff, H. Galjaard, and A. T. Hoogeveen. 1985. Genetic complementation in somatic cell hybrids of four variants of infantile Gm_2 gangliosidosis. Hum. Genet. **71:**196–200.