Biochemical and Molecular Investigation of Hexosaminidase A Deficiency in G_{M2} Gangliosidosis Genotypes

by
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Abstract

The reliable diagnosis of Tay-Sachs disease (TSD) and detection of TS heterozygotes is dependent upon an accurate assessment of the catalytic activity of hexosaminidase A (Hex A). 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS) was synthesized in high yield by the sulfation of 4-methylumbelliferyl-ß-D-N-acetylglucosamine (4MUG) with chlorosulfonic acid, producing a substrate preferentially hydrolyzed by Hex A. By application of an empirical formula, 4MUGS assay values are transformed to allow the calculation of Hex A and Hex B activities without the requirement of thermal fractionation. The usefulness of this substrate for the detection of TS heterozygotes, by both manual and automated assay procedures, is demonstrated. The diagnosis of TSD genotypes, particularly the B^1 TSD and Hex A-deficient healthy adult variants, is significantly improved with clear implications for screening and prenatal diagnosis. Chromatographic separation of Hex isozymes and analysis of the profiles obtained by 4MUG and 4MUGS hydrolysis allows for the differentiation of later-onset forms of TSD. The kinetic characterization of the B^1 mutation using 4MUGS and the investigation of the TSD mutation in French Canadians by a DNA-based procedure demonstrates the heteroallelism within populations.

Resumé

Le diagnostic sûr de la maladie de Tay-Sachs et le dépistage des hétérozygotes de la maladie de Tay-Sachs sont dépendant de la mesure précise de l'activité de l'hexosaminidase A. J'ai synthétisé le substrat 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS) par sulfation de la 4-methylumbelliferyl-ß-D-N-acetylglucosamine (4MUG) avec l'acide chlorosulfonique. La sulfation de 4MUG produit un substrat spécifique pour l'hexosaminidase A. Par l'emploi d'une formule empirique, les montants de l'essai peuvent être transformer en activité des hexosaminidases A et B sans l'inactivation thermale. L'utilité du substrat 4MUGS pour le dépistage des porteurs de tare par les essai manuels et automatiques est démontré. Le diagnostic de la maladie de Tay-Sachs, en particulier les variante B^1 et les adulte en bonne santé sans l'activité de l'hexosaminidase A est amélioré, avec implication pour le dépistage et le diagnostic prénatal. Utilisant la chromatographie, les hexosaminidases sont isolées. Les maladies de Tay-Sachs avec une présentation tardive peuvent être distingués par l'examen des profils obtenus à l'hydrolyse enzymatique des 4MUG et 4MUGS. La caractérization cinétique de la mutation B^1 par 4MUGS et l'investigation de la mutation Tay-Sachs chez les Canadiens français démontrent la variation dans les populations.

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List of abbreviations

AMP	2-amino-2-methyl-1-propanol
AP	activator protein
bp	base pairs
BSA	bovine serum albumin
cpm	counts per minute
CRM	cross-reactive material
Da	daltons
DEAE	diethylaminoethyl
GAG	glycosaminoglycan
G_{M2}	G_{M2} ganglioside
Hex	ß-N-acetylhexosaminidase
HPLC	high performance liquid chromatography
HSA	human serum albumin
ICD	I-cell disease
kb	kilobase
kDa	kilodaltons
MCB	membranous cytoplasmic body
4MU	4-methylumbelliferone
4MUG	4-methylumbelliferyl-ß-D-N-acetylglucosamine
4MUGS	4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate
NANA	N-acetylneuraminic acid
NG	N-acetylglucosamine
NGP	N-acetylglucosamine-6-phosphate
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pNPG	para-nitrophenyl-ß-D-N-acetylglucosamine
pNPGS	para-nitrophenyl-ß-D-N-acetylglucosamine-6-sulfate
RER	rough endoplasmic reticulum
SD	Sandhoff disease
S.D.	standard deviation
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography

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TSD	Tay-Sachs disease
UV	ultraviolet

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I. INTRODUCTION

A. Tay-Sachs disease: historical perspective.

Tay-Sachs disease (TSD) is a degenerative disease of the nervous system. It was first described in 1881 by Dr. Waren Tay in the U.K. He examined a 12-month-old baby girl with progressive muscle weakness which had begun at the age of three weeks. He believed her cerebral development to be deficient and upon opthalmoscopic examination of both retina he discovered a brownish-red spot in each, surrounded by a large white patch. Tay felt the central spot "seemed a gap in the white patch, through which one saw healthy structure" (Tay, 1881). Four months later, the child's condition had deteriorated further and her optic discs were found to be atrophic; she died at 18 months of age (Tay, 1881).

Concurrently and independently, Dr. Bernard Sachs in the U.S. presented a detailed clinical and pathological account of the same disease. He described a baby girl born at full term to healthy parents. She was normal until the age of 2 months, at which time she had become weak and listless and was noticed to startle easily. Opthalmoscopic examination revealed a spot of cherry-red colour in each retina surrounded by "an intense grayish-white opacity" (Sachs, 1887). The child's condition continued to deteriorate until death intervened at 2 years of age. The major features noted at autopsy were the edematous nature of the entire brain tissue, the hardness of the cortex, and, histologically, grossly abnormal pyramid cells throughout the brain (Sachs, 1887). As Sachs continued his research of this disease he became aware of its familial character. By 1896 he had observed 19 cases, all of which occurred in families of Jewish extraction. In many of the cases he noted that the parents were related. He proposed to name the disorder 'amaurotic family idiocy' (Sachs, 1896).

Amaurotic family idiocy was subsequently found to comprise at least four distinct disorders based on the age of onset, clinical symptoms and ethnic ancestry of

the patients. One of these, infantile amaurotic idiocy, was later named Tay-Sachs disease after the two doctors who are credited with its discovery. Detailed reviews of the clinical findings appear in Frederickson and Trams (1966), Schettler and Kahlke (1967), and Schneck et al (1969).

TSD is inherited as an autosomal recessive trait. Male and female children are affected with equal frequency, consistent with this mode of inheritance. The parents of TSD children are clinically normal. Slome (1933) was perhaps the first to extensively study the pattern of inheritance of TSD in patients and their families. He observed ratios of affected to unaffected offspring consistent with autosomal recessive inheritance. He also noted a high rate of consanguinity in the parents of TSD children.

TSD is found predominantly, although certainly not exclusively, in individuals of Ashkenazi Jewish descent. The carrier frequency in Sephardic Jews and non-Jewish populations is estimated to be about 1/300 (Myrianthopoulos and Aronson, 1966). The heterozygote frequency in the Ashkenazi Jewish population has been estimated to be 0.033, or 1 individual in 30 (Aronson, 1964; Myrianthopoulos and Aronson, 1972). Recently, however, even higher heterozygote frequencies have been noted in Jews who trace their ancestors to central Europe. More specifically, Petersen et al (1983) found that the highest heterozygote frequencies are among Jews whose ancestors originated from Austria, Czechoslovakia, and Hungary. Heterozygote frequencies in these populations range between 0.072 and 0.109, values significantly higher than the carrier frequency of Jews of eastern European or Mediterranean descent. Very few Jews who trace their ancestry to the Near East have been found to be heterozygotes.

A brief account of the history of the Jewish people, reviewed by Myrianthopoulos and Melnick (1977) follows: The total Jewish population at the beginning of the Diaspora constituted about 14% of the Greco-Roman population of

70 million, most of whom resided in Palestine. The destruction of Jerusalem by the Romans (ca. 70 A.D.) was characterized by mass migrations of the Jews into Syria, Babylonia, and Europe. Jewish colonies were established in central Europe as early as the start of the 4th century A.D.; the earliest recorded was in Cologne, Germany in 321 A.D. By the 10th century Jewish communities were firmly ensconced in Germany and France. Widespread massacre of the Jews and expulsions during the Crusades (ca. 1100 A.D.) led to their gradual migration to eastern Europe. Religious intolerance brought continued expulsions which drove the Jews from the cities into the country. A large number of rural communities were established, most of which were destroyed in the mid-1600s by the Cossacks. Survivors of the massacres fled to central and western Europe. In the mid-1800s many central European Jews (primarily German Jews) began emigrating to the U.S., England, and Canada. Eastern European Jews followed in the late 1800s-early 1900s (Myrianthopoulos and Melnick, 1977). The difference in heterozygote frequencies for the Ashkenazim and Sephardim indicates that the mutation most likely originated after the separation of Ashkenazi and Sephardic communities (ca. 800 A.D.).

Two hypotheses have been proposed to explain how the TSD gene came to high frequency in the Ashkenazi Jewish population: genetic drift and heterozygote selective advantage. Chase and McKusick (1972) and Spyropoulos et al (1981) have proposed that genetic drift explains the high TSD gene frequency in Ashkenazi Jews. Myrianthopoulos and Melnick (1977) and Neel (1979) have rejected this as unlikely due to the number and scattered nature of Jewish communities throughout history. They contend that drift would have had to occur in parallel in many of the communities, which is unlikely. Also, the communities were neither isolated nor inbred and the continuous expulsions kept Jews constantly on the move; it is unlikely that drift would operate under these conditions.

Myrianthopoulos et al (1966, 1972, 1977) propose that the Jewish TSD

heterozygote has an overall reproductive advantage over the Jewish normal homozygote. They believe the selective agent to be tuberculosis (a pulmonary disease caused by Myobacterium tuberculosis) since Jews who emigrated to central and eastern Europe lived predominantly in cities and were exposed to infectious and contagious diseases, such as tuberculosis, more so than rural populations. This hypothesis has been supported by Petersen et al (1983), who noted that a high incidence of tuberculosis is found in the same region with a high frequency of the TSD gene, i.e., in Austria and Hungary. The mechanism by which TSD heterozygosity might protect an individual from tuberculosis is as yet unknown. However, that the heterozygote state of a recessive mutation might provide a compensating advantage has been established for at least two other autosomal recessive genes: sickle cell trait conferring resistance to Plasmodium falciparum malaria in populations of West African origin (Winslow and Anderson, 1983) and heterozygosity for congenital adrenal hyperplasia conferring resistance to Haemophilus influenzae type B infection in Yupik Eskimos (Petersen et al, 1984).

The prevalence of 3 other related autosomal recessive diseases in the Ashkenazi Jewish population lends further support to the heterozygote selection hypothesis. Besides TSD, type I Gaucher disease, type A Niemann-Pick disease, and type IV Mucolipidosis have attained high frequencies in Ashkenazi Jews (Myrianthopoulos and Melnick, 1977; Rotter and Diamond, 1987; Zlotogora et al, 1988). All are lysosomal storage disorders of sphingolipid metabolism and all four occur due to mutations at unlinked loci. Even Chase (1977) concedes that the prevalence of other neuromuscular disorders in Ashkenazi Jews is an argument in support of the selection hypothesis. However, the nature of the forces that might lead to such selection are unknown.

Since the turn of the century TSD has been found in other ethnic groups such as the Swiss (Hanhart, 1954), the Japanese (Murakami, 1957; Momoi et al,

1978), and the Pennsylvania Dutch (Kelly et al, 1975). There is also a high incidence of TSD in French Canadians of eastern Quebec, where the heterozygote frequency in some communities is at least as high as that found in Ashkenazi Jews (Andermann et al, 1977). The French Canadian TSD families have been found to originate primarily from small communities situated on the north and south shores of the St. Lawrence River, east of Quebec City. Many of these families can be traced to a founding population of about 2000 who came from France during the mid-1600s and colonized the Quebec City area (Charbonneau and Legaré, 1967). Genealogical histories indicate that common surnames occur in the pedigrees. This would seem to implicate founder effect as an explanation of the high incidence of TSD in these communities (Andermann et al, 1973, 1977).

TSD children appear normal at birth. The clinical symptoms of the disease usually begin between 3 and 6 months of age with motor weakness and lethargy. A characteristic feature of TSD is the persistence of the startle reflex, which normally disappears in infancy with cortical development. Mental and motor deterioration progress rapidly after the first year of life. Feeding becomes difficult due to problems in swallowing. The head becomes abnormally large. By 18 months of age the children are usually deaf and blind, the latter due to atrophy of the optic disc. In approximately 95% of those afflicted, the retina appears only as a cherry-red spot surrounded by a white halo. By 2 years of age the children are severely mentally retarded and a state of decerebrate rigidity ensues. Death invariably occurs between the ages of 2 and 4 years (O'Brien, 1983).

Klenk (1939) showed that the chemical composition of the TSD brain was changed from that of a normal brain. Extraction of brain lipids revealed a strongly polar glycolipid present in large quantity in the TSD brain that was destroyed by mild acid hydrolysis. Klenk proposed the name 'ganglioside' to describe this acidic glycolipid. Compositional studies of the ganglioside were performed by subjecting it

to strong acid hydrolysis and analyzing the lipid and sugar components (Klenk, 1941 and 1942). It was found to contain ceramide, composed of sphingosine and fatty acid in a molar ratio of 1:1, as well as hexose sugars, hexosamine, and sialic acid. The ganglioside derives its acidic character from the sialic acid. The molar ratio of lipid : sugar or sugar : sugar was found to be nonintegral in ganglioside from normal brain and Klenk concluded that the compound under analysis was not pure; rather, it consisted of a number of gangliosides, each with different sugar residues.

Svennerholm (1961 and 1962) used the technique of thin-layer chromatography to separate the brain gangliosides based on their polarity ; the more sialic acid residues, the more polar the ganglioside. He proposed a nomenclature system based on the number of sialic acids present. Thus, monosialic gangliosides are designated G_M, disialic G_D, trisialic G_T, etc. For further differentiation, numbers are assigned based on the order in which the compounds separate on chromatography (for example, G_{M3} ganglioside migrates farther than G_{M2} which, in turn, migrates farther than G_{M1}). The ganglioside accumulating to the greatest extent in the TSD brain was found to be a monosialo ganglioside, G_{M2}, which is similar in structure to the major monosialo ganglioside in the normal brain except that it is lacking the terminal galactose residue. G_{M2} ganglioside accounts for 70-90% of the total ganglioside fraction in the TSD brain. This contrasts with 0.1-0.3% in the normal brain.

The asialic derivative of G_{M2}, or G_{A2} glycolipid, also accumulates in the TSD brain, accounting for approximately 20% of the total ganglioside fraction (Svennerholm and Raal, 1961; Gatt and Berman, 1963). Recently, Neuenhofer et al (1986) demonstrated the lysosomal accumulation of small amounts of lysoganglioside lyso-G_{M2} in the postmortem brain of a TSD patient. The lyso-G_{M2}

was found to be a fatty acid-free derivative of G_{M2} and thus contains sphingosine with a free base rather than ceramide. Lysosphingolipids have been found to accumulate in other sphingolipid storage diseases as well. For example, glucosylsphingosine accumulates in the brains and spleens of Gaucher disease patients (Nilsson et al, 1982) and galactosylsphingosine (also called psychosine) accumulation has been demonstrated in the brains of Krabbe's disease patients (Svennerholm et al, 1980). Lysosphingolipids differ from their respective sphingolipids by the absence of the amide-linked fatty acid at the 2-amino position of sphingosine. It is thought that a sphingolipid and its lyso-derivative are hydrolyzed by the same enzyme, thus accounting for the accumulation of both compounds in sphingolipid storage diseases. Hannun et al (1986) found sphingosine to be a powerful inhibitor of protein kinase C activity and have subsequently shown many lysosphingolipids to similarly inhibit activity of this enzyme (Hannun and Bell, 1987). Protein kinase C has its highest concentration in the central nervous system, particularly at presynapses. It is believed to participate in the transduction of neurotransmitter chemicals and to play a role in neuronal differentiation. Hence, the accumulation of even small amounts of toxic lysosphingolipid compounds may contribute to the pathogenesis of sphingolipid storage diseases (Hannun and Bell, 1987; Sandhoff and Conzelmann, 1985). Lending support to this hypothesis, Kobayashi et al (1988) recently demonstrated a close association between galactosylsphingosine accumulation and the occurrence of lesions characteristic of Krabbe's disease in the brain, spinal cord, and peripheral nerves of affected infants.

The pathologic changes in TSD are confined primarily to the brain. The cerebrum and cerebellum appear to be the most severely affected. Postmortem studies of the brain show a profound decrease in the number of neurons in the cerebral cortex. The neuronal cytoplasm is ballooned and distended, with the nucleus

displaced to the periphery. Finely dispersed granules accumulate in the cytoplasm. The cells stain strongly with Periodate-Schiff and Sudan black B, indicative of large amounts of glycolipid. The brain is enlarged and quite often hard or leathery. Concomitant with the decreased number of axons is a proliferation of astrocytes and glial cells that serve as macrophages. It is this proliferation that is responsible for the macrocephaly. The glial cells are distended and filled with large granules which are similar in staining characteristics to those found in the neurons. Demyelination is consistently found and appears to be secondary to axonal degeneration (Aronson et al, 1955; Aronson and Volk, 1962).

Electron microscopic examination of the neurons, performed by Terry and Weiss (1963) and Samuels et al (1963), revealed the granules accumulating within the cytoplasm to be spirally-wound membranous structures. These membranous cytoplasmic bodies (MCBs) were observed only in the neurons of TSD children; no evidence of MCBs was found in the visceral organs.

MCBs are the site of intense acid phosphatase and thiolactate esterase activities, enzymes known to be associated with the lysosomal fraction of the cell. Thus, it was determined that the MCBs are of lysosomal origin (Wallace et al, 1964 and 1966). Isolation of the MCBs and analysis of their components revealed them to contain large quantities of G_{M2} ganglioside (Suzuki et al, 1969).

B. Characterization of the substance stored and the enzymatic defect in TSD.

1. G_{M2} ganglioside.

Approximately 80 different gangliosides have been identified. They occur primarily in vertebrates and have been detected in virtually all vertebrate tissues (Ledeen, 1983). The carbohydrate that defines this group of glycolipids, and which

gives it an acidic character, is sialic acid. Many different kinds of sialic acids exist, only a few of which have been found in gangliosides. The major sialic acid found in the mammalian brain is N-acetylneuraminic acid (NANA). Another sialic acid, N-glycolylneuraminic acid, occurs in conjunction with NANA in the brain and extraneural tissue gangliosides of some mammalian species. NANA, however, appears to be the exclusive sialic acid of human brain gangliosides (Ledeen, 1983).

The great majority of gangliosides belong to two families of glycosphingolipids: the ganglio- and neolacto- families. Both are synthesized from a lactosylceramide precursor. The ganglio- series contains N-acetylgalactosamine; most of the brain gangliosides belong to this series. The neolacto- family of gangliosides contains N-acetylglucosamine and tends to predominate in gangliosides of extraneural tissues. One glucosamine-containing ganglioside found in the human brain is sialosylparagloboside (also called G_{L4}). It is only a minor species of ganglioside in the human brain but a major ganglioside of human peripheral nerve and human erythrocytes. A third group of gangliosides is the hematosides which lack hexosamine. This group occurs only to a minor extent in the nervous system. G_{M3} and G_{M4} are the most prevalent hematosides (Ledeen, 1983).

Compositional studies of the gangliosides led to the elucidation of the structures of the four major brain gangliosides: G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} (Klenk and Gielen, 1963). The predominant ganglioside is G_{M1}. It contains a ceramide backbone, as do all sphingolipids, which is made up of sphingosine and fatty acid. In neutral glycosphingolipids and sphingomyelin, the fatty acid component of ceramide consists mostly of C_{20} - C_{24} fatty acids; stearate (a C_{18} fatty acid) predominates in ceramide from which gangliosides are derived. Gangliosides also contain an oligosaccharide chain linked to the ceramide. At least one of the sugars is the acidic

NANA. G_{M1} ganglioside was found to have the following structure : galactosyl-(β1—›3)-N-acetylgalactosaminyl-(β1—›4)-[(α2—›3)-N-acetylneuraminyl]-galactosyl-(β1—›4)-glucosyl-(β1—›1)-[2-N-acyl]-sphingosine. Cleavage of the terminal sugar residues of the oligosaccharide chain occurs to give different gangliosides (O'Brien, 1983). Makita and Yamakawa (1963) are credited with first reporting the correct structure of G_{M2} ganglioside. It was found to have the same structural formula as G_{M1} but is lacking the terminal galactose (Srivastava, 1977).

The distribution of gangliosides is different for different regions of the nervous system. The higher gangliosides G_{Q1b}, G_{T1b}, G_{D1b}, and G_{D1a} predominate in the gray matter of the brain while G_{D1a}, G_{M1} and G_{M4} predominate in the white matter. In addition, there are striking changes in the pattern and quantities of brain gangliosides during development with polysialogangliosides generally appearing later than monosialogangliosides (Suzuki, 1965).

Gangliosides are constituents of virtually all cell membranes but they are most abundant in neuronal membranes: neuronal cell perikarya, axons, and dendrites have been shown to contain gangliosides (Wolfe, 1961). Subcellular fractionation techniques have confirmed that the highest concentrations in the cell are found in synaptosomes and microsomes arising from nerve and dendritic endings, respectively (Hansson et al, 1977; Svennerholm, 1980). The hydrophobic moiety that anchors the ganglioside in the cell membrane is ceramide; the polar sugar residues are oriented toward the external fluid (Zwaal et al, 1973).

The high concentration of gangliosides in nerve tissue, and particularly at synaptic junctions, suggests that they may serve a special purpose. Some of the functions that have been ascribed to gangliosides include: synaptic transmission; receptor structures for bacterial toxins and viruses; and brain maturation. They will be

discussed briefly.

The release of neurotransmitter substances from vesicles into the synaptic cleft is known to be mediated by calcium. The demonstration of calcium-ganglioside complex formation (Behr and Lehn, 1973) led to the view that gangliosides might play a role in synaptic transmission. Svennerholm (1980) proposed that the dissociation of the complex, possibly initiated by the influx of potassium that occurs during nervous excitation, results in a release of calcium at the presynaptic membrane which, in turn, is thought to lead to a release of neurotransmitter chemicals.

Gangliosides present on the external surface of cell membranes have been shown to bind bacterial toxins. Most toxins have separate subunits for binding to the receptor and penetrating the cell. It is thought that binding to gangliosides on the cell surface might induce a conformational change in the toxin, promoting dissociation of the binding-effector complex (Fishman and Brady, 1976). Cholera and E. coli enterotoxins have been found to interact almost exclusively with G_{M1} ganglioside; tetanus and botulinum toxins appear to be most effectively bound by polysialogangliosides; furthermore, the location of the sialic acids appears to be important in binding to toxins. For example, G_{T1b} is more effective than G_{T1a} in binding botulinum toxin (Holmgren et al, 1980; Ledeen, 1983).

Haywood (1974) and Holmgren et al (1980) reported that gangliosides bind Sendai virus and are capable of inhibiting agglutination of erythrocytes by the virus; polysialogangliosides with at least one terminal sialic acid residue extending from the terminal galactose are a requirement for binding. Besançon and Ankel (1974) and Vengris et al (1980) have shown gangliosides to also bind interferon, inhibiting its antiviral activity.

Changes in ganglioside composition have been shown to accompany brain development and neuronal differentiation (Panzetta et al, 1980; Dreyfus et al, 1980).

Grunwald et al (1985) showed that the species of gangliosides, their abundance, and their distribution in chick retina change during neuronal development. The retina and brain are found to differ with respect to both the developmental age when polysialogangliosides appear and in their continued expression in the adult brain but not in the adult retina. While G_{M1} is the major ganglioside of both young and adult rat myelin, myelin from young rats has relatively more di- and trisialogangliosides than myelin from adults. Human myelin shows increasing G_{M4} with age, accounting for 15-20% of myelin ganglioside in the adult (Ledeen et al, 1980).

The biosynthesis of gangliosides occurs by the sequential addition of monosaccharides to a ceramide precursor. The activated nucleotide donors of the residues are UDP-glucose, UDP-galactose, UDP-N-acetylgalactosamine, and CMP-N-acetylneuraminic acid (Fishman and Brady, 1976; O'Brien, 1983). The addition of monosaccharide residues occurs at the nonreducing end of the oligosaccharide chain and is catalyzed by specific glycolipid glycosyltransferases (Roseman, 1970). Ganglioside biosynthesis was initially thought to occur in the neuronal plasma membrane. However, Keenan et al (1974) were among the first to show that the glycosyltransferases are actually localized in the Golgi apparatus. Gangliosides are transported from the site of synthesis in the Golgi to the neuronal plasma membrane and, in particular, to dendritic and synaptic membranes via vesicles (Forman and Ledeen, 1972; Yusuf et al, 1984).

In addition to gangliosides present at synaptic junctions, there are also enzymes capable of modifying the sialic acid content of gangliosides. Sialidase and sialyltransferase at the synaptic membrane have been shown to constitute 65% and 40%, respectively, of the total brain content of each enzyme in the calf brain (Tettamanti et al, 1980).

2. Enzymatic defect in TSD.

More than 40 disorders of sphingolipid metabolism have been identified. Most of the glycosidases responsible for the hydrolysis of sphingolipids have been identified as lysosomal in origin based in part on their acidic pH optima. The substances stored in these diseases have been found to accumulate within structures that have ultrastructural and histochemical characteristics of altered lysosomes (Gatt, 1980; Benson and Fensom, 1985).

The catabolism of gangliosides (shown in Figure 1) proceeds in a stepwise fashion with the removal of sugar residues from the nonreducing end of the oligosaccharide chain, in reverse order to biosynthesis. While ganglioside degradation may start at the synaptic membrane by the action of sialidase, the majority of the enzymatic steps in the degradation of gangliosides occurs within the lysosome. G_{M1} ganglioside produced at the synaptic membrane is transported from the axonal nerve ending to the lysosomes of the neuronal cell perikaryon for final degradation (Svennerholm, 1980). Transport of gangliosides to the lysosome probably occurs via endocytotic vesicles which fuse with the lysosomal membrane (Schwarzmann et al, 1984).

G_{M2} ganglioside is normally a very small component of neuronal plasma membranes. It is formed primarily as a metabolite of G_{M1} ganglioside degradation, catalyzed by the lysosomal enzyme ß-galactosidase. The accumulation of G_{M2} ganglioside in TSD results from a deficiency of the next glycosidase in the pathway of ganglioside metabolism. Since the nonreducing terminal sugar in G_{M2} ganglioside is a hexosamine the hydrolysis of this compound was expected to be catalyzed by a ß-hexosaminidase. However, when assayed with synthetic substrates, such as 4-methylumbelliferyl- or para-nitrophenyl- derivatives of N-acetylglucosamine, Hex

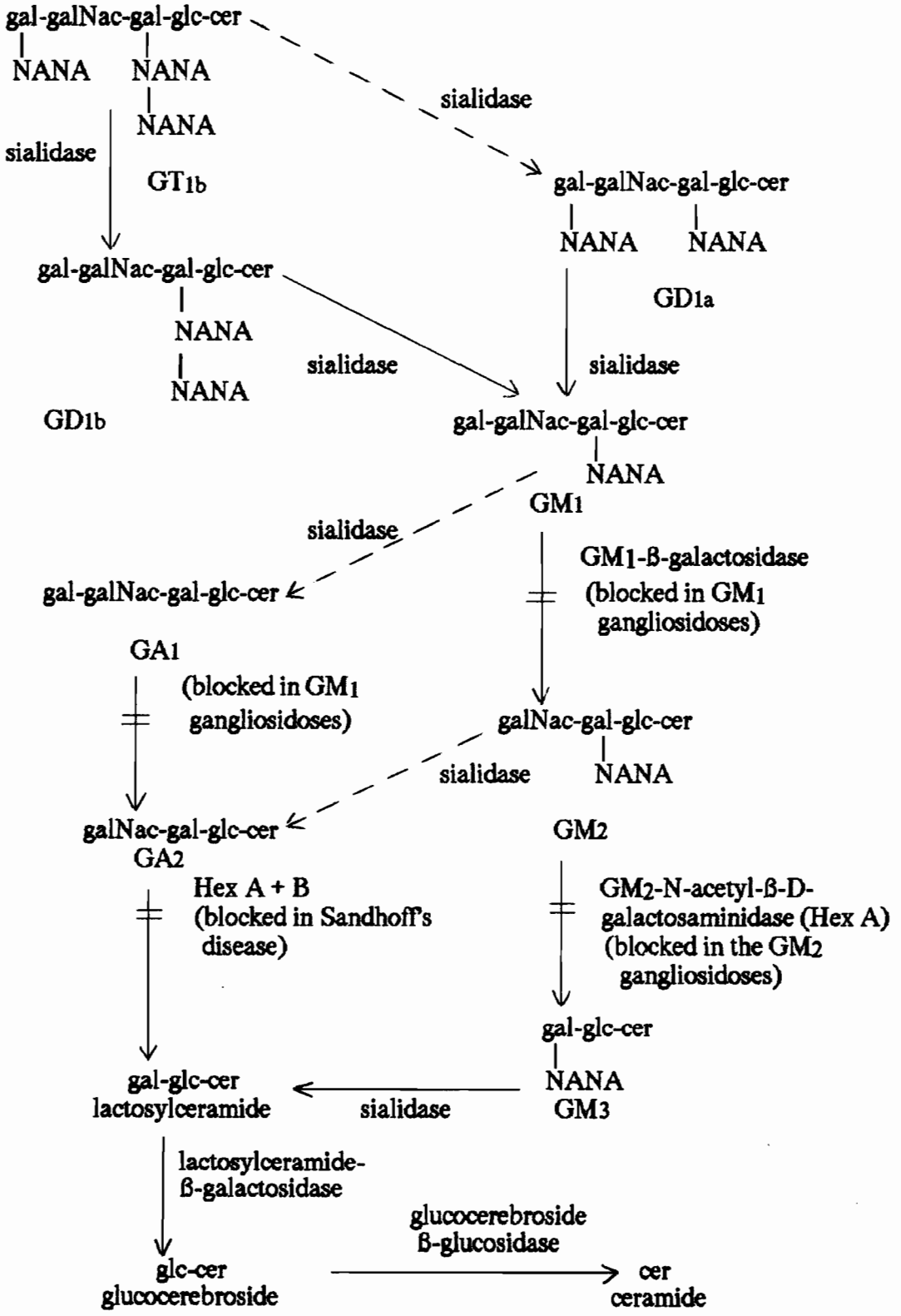

Figure 1. Pathway of ganglioside degradation. Minor pathways are indicated by dashed arrows. gal=galactose; glc=glucose; galNac=N-acetylgalactosamine; NANA=N-acetylneuraminic acid, cer=ceramide. *(From O'Brien, 1983)*

activity was found to be higher than normal in TSD brain tissue (Sandhoff et al, 1968). This problem was resolved when Robinson and Stirling (1968) separated two hexosaminidase isozymes from extracts of normal human spleen, both possessing ß-D-N-acetylglucosaminidase and ß-D-N-acetylgalactosaminidase activities. They reported that the two isozymes had similar Km values and were both present in the lysosome. Hexosaminidase A (Hex A) is acidic and heat-labile at 50° C; Hexosaminidase B (Hex B) is basic and stable to heating (Robinson and Stirling, 1968; O'Brien et al, 1971).

Okada and O'Brien (1969) demonstrated the presence of both hexosaminidase isozymes in all body tissues, fluids, and cells. They also showed that the more acidic, heat-labile isozyme (Hex A) was absent from all tissues of TSD patients while the Hex B component, as well as several other lysosomal enzymes, showed an increase of at least 10-fold in the TSD cerebral cortex. Sandhoff et al (1971) gave TSD patients the designation of G_{M2} gangliosidosis B variant because Hex B is present. Levels of Hex A in plasma, leukocytes, and cultured fibroblasts obtained from obligate TS heterozygotes were found to be intermediate between those of normal controls and TSD patients.

O'Brien et al (1970) developed a serum Hex A assay based on the differential thermolabilities of the Hex isozymes for the purpose of heterozygote detection. The first screening program for the detection of TSD heterozygotes in Jewish communities was begun by Kaback in 1970 (Desnick et al, 1976; Kaback et al, 1977) and became so widespread that an automated assay method for the mass screening of heterozygotes was established in those cities where the carrier frequency was high enough to make such screening possible (Lowden et al, 1973; Delvin et al, 1974).

The finding that amniotic fluid and cultured amniotic cells also express Hex

A and Hex B indicated that the detection of a homozygous TSD fetus in utero was possible. The first prenatal diagnosis of TSD by Hex A assay of both uncultured amniotic cells and amniotic fluid was performed by Schneck et al (1970). Prenatal diagnosis of TSD in a fetus by demonstration of an absence of Hex A activity in trophoblasts cultured from chorionic villi was first performed by Grebner et al (1983).

Hexosaminidases are expressed in nearly every tissue and cell type as well as body fluids. The discovery by Sandhoff et al (1968) of a deficiency of both Hex A and Hex B in a patient diagnosed as having TSD, as well as Robinson and Stirling's finding (1968) that acidic and basic forms of hexosaminidase exist, prompted inquiries into the structural relationship between Hex A and Hex B. Robinson and Stirling reported that incubation of Hex A with neuraminidase resulted in its conversion to Hex B. This was taken to indicate that Hex B might be a precursor of Hex A, formed by the addition of neuraminic acid to Hex B. It was soon discovered that the commercial preparation of neuraminidase contained merthiolate and that this was responsible for a rearrangement of protein structure. A second model proposed to explain the relationship between the two Hex isozymes suggested that they share a common subunit (Robinson and Carroll, 1972) and that TSD results from the absence of a subunit that is unique to Hex A.

Srivastava and Beutler (1972, 1974c) raised antibodies against purified placental Hex A and Hex B in rabbits. Antiserum raised against Hex A was found to cross-react with both Hex A and Hex B. Likewise, anti-Hex B antiserum cross-reacted with both isozymes. These results indicated antigenic determinants common to both. However, they found that anti-Hex A antiserum adsorbed with Hex B was no longer capable of cross-reacting with Hex B, yet retained the ability to precipitate Hex A. Antiserum raised against Hex B and treated with Hex A lost its ability to cross-react with both isozymes. These studies provided the first evidence that Hex A

and Hex B share a common subunit (ß) and, further, that Hex A has a subunit unique unto itself (α).

Srivastava et al (1974a, 1974b) studied the kinetic and structural properties of purified placental Hex isozymes. They reported Hex A and Hex B to have isoelectric points of 5.4 and 7.9, respectively. This finding is consistent with the earlier report by Robinson and Stirling (1968) that Hex A contains neuraminic acid residues and is, therefore, more acidic than Hex B. Srivastava and co-workers found Hex A and Hex B to have similar pH optima of 4.4 and similar Michaelis constants of 0.5 mM using 4-methylumbelliferyl-ß-D-N-acetylgalactosaminide as substrate. The molecular weight of native Hex A and Hex B by gel filtration was estimated at 140 kilodaltons (kDa) for both isozymes; using the sedimentation equilibrium technique, however, their molecular weights were determined to be 100 kDa.

Dissociation of the subunits of Hex A and Hex B and their separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Srivastava et al, 1974b; Geiger and Arnon, 1976) revealed both α and ß subunits to have an apparent molecular weight of 25,000 daltons (Da). Mahuran and Lowden (1980) subjected both Hex A and Hex B to extensive reduction and alkylation. Hex B was found to chromatograph on SDS-PAGE as a single band of 25,000 Da; Hex A, however, consistently chromatographed as two bands of 50,000 and 25,000 Da. They concluded that the subunit structures of Hex A and Hex B are $\alpha_1\beta_2$ and $2(\beta_2)$, respectively.

Subsequently, Mahuran et al (1982) found the ß subunit of Hex A and Hex B to be composed of two nonidentical polypeptide chains. Reduction and alkylation of the native enzymes followed by two-dimensional gel electrophoresis and peptide mapping, revealed two distinct ß chains with pI values that differ by one unit. One

polypeptide, β_a, has an isoelectric point of 4.7; the other, β_b, has a more basic pI of 5.9. The molecular weights of the two β polypeptide chains are similar at approximately 25 kDa, with the β_b species of a slightly lower molecular weight than β_a. They suggested a more accurate subunit structure of $\alpha\ \beta_a\beta_b$ for Hex A and $2(\beta_a\beta_b)$ for Hex B, based on these findings.

Lalley et al (1974), using human-mouse somatic cell hybridization and immunological techniques, demonstrated synteny between the genes coding for Hex A and two other enzymes, mannosephosphate isomerase and pyruvate kinase-3. Furthermore, they demonstrated that the expression of Hex A is dependent on Hex B expression; in contrast, Hex B expression is independent of Hex A . Similar work by Gilbert et al (1975) assigned the gene for Hex B to chromosome 5, while the gene for Hex A was assigned to chromosome 15. The gene encoding the β subunit of hexosaminidase was subsequently localized to region 5q13 by Dana and Wasmuth (1982). Thus, while a gene locus on chromosome 5 is responsible for Hex B expression, expression of Hex A requires gene loci on both chromosome 5 (encoding the β subunit of Hex common to both isozymes) and 15 (encoding the α subunit unique to Hex A). A mutation at the locus on chromosome 15 produces TSD due to an absence of Hex A; a mutation at the locus on chromosome 5, however, will result in an absence of Hex A and Hex B since they both contain β subunits. Such a deficiency of both isozymes produces Sandhoff disease (SD), another form of G_{M2} gangliosidosis, a discussion of which is presented later in this section.

3. Other hexosaminidase isozymes.

Since the initial discoveries of two major forms of hexosaminidase in body tissues and fluids, other forms of Hex electrophoretically intermediate to Hex A and

Hex B have been identified and designated as Hex I_1 and Hex I_2 (Price and Dance, 1972). Separation of normal serum Hex isozymes by ion-exchange chromatography reveals Hex I_1 to elute after Hex B, at the beginning of the salt gradient. Hex I_2, which is even more acidic than Hex I_1 but not as acidic as Hex A, elutes between hexosaminidases I_1 and A. The Hex I's are found to be stable to heating under conditions that inactivate Hex A and are thought to be composed solely of ß subunits. Furthermore, Hex I_2 is identical in electrophoretic and thermal characteristics to the form of hexosaminidase (Hex P) present in the sera of pregnant women. Geiger et al (1978) found the molecular weight of Hex P to be approximately 1.5 times that of Hex A and Hex B. Hex P (and Hex I) might represent a ß-chain polymer containing more ß subunits than contained in Hex B (Ben-Yoseph et al, 1985, 1988).

Hex S is yet another Hex isozyme, found primarily in patients with Sandhoff disease. It is more electronegative and more heat-labile than Hex A, has activity against ß-N-acetylgalactosamine and ß-N-acetylglucosamine substrates, and has a low optimum pH of 5.0 (Ikonne et al, 1975; Potier et al, 1979). Immunological studies performed by Beutler et al (1975) showed Hex S to react only with anti-Hex A antiserum. However, Geiger et al (1977) showed very weak cross-reactivity to anti-Hex B antiserum as well. This suggested that α and ß subunits might possess some immunological similarities and, hence, homology in their amino acid sequences (Sandhoff and Christomanou, 1979). The results of the immunological studies suggested that Hex S contains only α subunits. This is supported by the absence of Hex S in the chromatographic separation of hexosaminidases from TSD fibroblasts. Molecular weight determinations gave a value similar to that of Hex A and Hex B, indicating that Hex S is most likely a dimer of α subunits (Beutler et al, 1975;

Ikonne et al, 1975; Geiger et al, 1977; Potier et al, 1979).

Hex C has been found to have an electrophoretic mobility similar to that of Hex S (Poenaru and Dreyfus, 1973; Braidman et al, 1974; Penton et al, 1975). Characterization of Hex C demonstrated that it has a neutral pH optimum and larger molecular weight than Hex S and that it is located in the cytosol, rather than in the lysosomal fraction of the cell. Its failure to cross-react with antiserum raised against Hex A, as well as its presence in cells of TSD and SD patients, sets it completely apart from other hexosaminidase isozymes.

4. G_{M2}-specific activator protein.

Most lysosomal enzymes have a broad substrate specificity. Hexosaminidase A, for example, has been shown to hydrolyze N-acetylgalactosaminide and N-acetylglucosaminide residues from oligosaccharides, steroid hormones, paragloboside G_{L4}, G_{A2} glycolipid, and G_{M2} ganglioside. Hexosaminidase B is also capable of hydrolyzing these substrates, with the exception of G_{M2} ganglioside (Conzelmann and Sandhoff, 1979; O'Brien et al, 1978).

G_{M2} ganglioside hydrolysis occurs only by the action of hexosaminidase A. Early studies of hexosaminidase A were carried out using synthetic substrates, such as para-nitrophenyl- or 4-methylumbelliferyl- derivatives of glycosaminides. Defective metabolism of the natural substrate, G_{M2} ganglioside, in fibroblasts obtained from a patient with TSD was first demonstrated by Kolodny et al (1969) using G_{M2} ganglioside radiolabelled in the neuraminic acid moiety. Further in vitro studies of G_{M2} ganglioside hydrolysis, as well as the hydrolysis of other lipid substrates, entailed problems stemming from the relative insolubility of G_{M2} ganglioside in aqueous media and the resultant tendency of the molecules to aggregate to form micelles (Gatt et al, 1972). The formation of micelles makes the ganglioside

unavailable to the water-soluble hydrolase.

Detergents have been used widely to enhance the enzymic hydrolysis of G_{M2} ganglioside in vitro (Li et al, 1980; Hechtman et al, 1980). While they have been shown to greatly stimulate Hex A-catalyzed hydrolysis of G_{M2} some detergents, such as taurocholate and taurodeoxycholate, also stimulate G_{M2} ganglioside hydrolysis by Hex B (Li et al, 1981). The enhanced enzymic degradation of G_{M2} ganglioside in the presence of detergents is attributed to their ability to break down the large micelles, thus presenting individual monomers to the enzyme (Conzelmann and Sandhoff, 1980).

In vivo, a nonenzymic protein has been implicated in the hydrolysis of G_{M2} ganglioside by Hex A (Conzelmann and Sandhoff, 1978). Many other acid hydrolases have been shown to require such activator proteins for the hydrolysis of their sphingolipid substrates. These include arylsulfatase A (Fischer and Jatzkewtiz, 1975), glucocerebrosidase (Ho and O'Brien, 1971), galactocerebrosidase (Wenger et al, 1982), ß-galactosidase (Li et al, 1979), α-galactosidase (Gartner et al, 1983), and sphingomyelinase (Christomanou, 1980).

The G_{M2} activator protein has been found in all human tissues examined (such as liver, kidney, brain, cultured fibroblasts, etc.) as well as serum and urine (Mahuran et al, 1985). Its presence was first detected in human liver by Li et al (1973), Hechtman (1977), and Hechtman and LeBlanc (1977), who found that crude Hex preparations were able to hydrolyze G_{M2} ganglioside far better than purified preparations of Hex A. During the purification procedure, a low molecular weight activating protein was lost. Subsequently, Conzelmann and Sandhoff (1979) isolated the activator protein from human kidney; Hirabayashi et al (1983) isolated it from human brain. Molecular weight determinations of the G_{M2} activator protein by gel

filtration have varied. Li et al (1981) determined the activator to be 23 kDa by Sephadex G-75 gel filtration; Hechtman and LeBlanc (1977) arrived at a molecular weight of 36 kDa by Sephadex G-200; Conzelmann and Sandhoff (1979), using Sephadex G-100 gel filtration, determined the molecular weight to be 25 kDa. Burg et al (1985) studied the biosynthesis of the G_{M2} activator protein in cultured skin fibroblasts using affinity-purified antibodies to human kidney activator protein. They found normal, Tay-Sachs, and Sandhoff fibroblasts to secrete an activator protein of 24 kDa size; intracellularly these cell strains have G_{M2} acitvator protein with an apparent molecular weight of approximately 21,500 Da. They inferred that the 24 kDa protein is likely the biosynthetic precursor of the lower molecular weight form.

The G_{M2} activator protein was found to be fairly acidic, with a pI of about 4.8 (Li et al, 1981; Conzelmann and Sandhoff, 1979). It is stable in crude extracts at 60° C. Co-distribution with hexosaminidase in subcellular fractionation experiments of normal fibroblasts localized the activator protein to the lysosomal fraction of the cell. This is consistent with its function of promoting the interaction between Hex A and G_{M2} ganglioside delivered to the lysosome for degradation (Banerjee et al, 1984). The gene encoding the human G_{M2} activator protein has recently been mapped by Burg et al (1985), using an enzyme-linked immunoadsorbent assay for the activator in human-mouse somatic cell hybrids, to chromosome 5; the ß subunit of Hex also maps to chromosome 5 (Gilbert et al, 1975) in the region 5q13 (Dana and Wasmuth, 1982). The site of the activator protein locus on chromosome 5 has not yet been determined. It remains to be seen whether the genes encoding the activator protein and the ß subunit of hexosaminidase form a close linkage group.

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synthetic substrates by Hex A is unaffected in the presence of activator protein (Conzelmann and Sandhoff, 1980). More recently, Hechtman et al (1985) demonstrated activator protein binding to G_{M2} ganglioside by co-sedimentation in sucrose density gradients, lending further evidence to Conzelmann and Sandhoff's findings.

Neuenhofer and Sandhoff (1985) provided direct evidence of complex formation by affinity labelling of the activator protein with tritium-labelled lysoganglioside G_{M2}. The molar stoichiometry of the activator protein-G_{M2} ganglioside complex was found to be 1 : 1. The G_{M2} activator protein also forms complexes with G_{D1A}, G_{M1}, G_{M3}, and G_{A2}, although at physiological ionic strength complex formation occurs almost exclusively with G_{M2} ganglioside (Conzelmann and Sandhoff, 1979; Sandhoff, 1984).

Sandhoff and Conzelmann (1984, 1985) proposed a model for the lysosomal catabolism of G_{M2} ganglioside which assumes that the ganglioside delivered to the lysosome cannot be attacked directly by Hex A. Instead, the G_{M2} activator binds the ceramide moiety of the ganglioside at a hydrophobic groove of the activator protein. The water-soluble activator protein-G_{M2} ganglioside complex then binds to a specific site on the α subunit of Hex A (Kytzia and Sandhoff, 1985) in such a manner that the terminal N-acetylgalactosaminide residue of G_{M2} is correctly positioned at the α-subunit active site.

The physiological significance of the activator protein is apparent in the rare form of infantile G_{M2} gangliosidosis described by Sandhoff et al (1971), i.e. the AB variant. These patients accumulate large amounts of G_{M2} and G_{A2} ganglioside in spite of normal levels of Hex A and Hex B activity. The absence of the activator

protein for G_{M2} ganglioside hydrolysis in the AB variant was demonstrated by Conzelmann et al (1978) and Hechtman et al (1982).

5. Synthesis and processing of the α and ß subunits of hexosaminidase.

Much of the knowledge of how water-soluble acid hydrolases are synthesized and reach the lysosomal compartment of the cell is attributed to work on patients with I-cell disease. I-cell disease (ICD), also called mucolipidosis II, is a rare autosomal recessive condition in which excessive quantities of heterogeneous material accumulates within the lysosomes. ß-galactosidase, α-fucosidase, arylsulfatase A, α-mannosidase, α-iduronidase, ß-hexosaminidase, and ß-glucuronidase activities are severely deficient. The multiple enzyme deficiencies involve primarily connective tissue cells. Studies on cultured fibroblasts of ICD patients demonstrated that the lysosomal enzymes are synthesized, yet are secreted into the culture medium rather than transported to the lysosome (Hickman and Neufeld, 1972). Consistent with this finding is the presence of large quantities of these enzymes in the serum of ICD patients. The ability of cultured ICD fibroblasts to take up exogenous normal enzymes in the culture medium indicated that the defect does not reside with the cell membrane receptors. Instead it was believed that the defect involves a common recognition marker that targets newly-synthesized hydrolases for the lysosome. ICD patients were found to be deficient in an enzyme that phosphorylates acid hydrolases (Kornfeld, 1986; von Figura and Hasilik, 1986).

The α and ß polypeptide chains of hexosaminidase are synthesized on membrane-bound ribosomes in the rough endoplasmic reticulum (RER). The polypeptides are equipped with an N-terminal signal sequence of approximately 20 hydrophobic amino acids. The newly-synthesized chains are transported into the

lumen of the RER, where co-translational modifications occur. The signal sequence is cleaved almost immediately after transport of the polypeptides into the lumen by the action of a signal peptidase (Walter et al, 1984). Once inside the lumen the polypeptides are glycosylated by the transfer of mannose-containing oligosaccharides to asparagine residues of the nascent acid hydrolases (Kornfeld and Kornfeld, 1985).

The glycosylated α and ß polypeptide chains are transported to the Golgi via vesicles that fuse with the Golgi membrane. Other proteins, such as those destined for secretion or those to be inserted into membranes, are also transported to the Golgi. During their passage through the Golgi apparatus sialic acid residues are added. In contrast, mannose residues of proteins destined for the lysosome are phosphorylated through the action of two enzymes: the first reaction involves the transfer of N-acetylglucosamine-1-phosphate to the C6 hydroxyl of selected mannose residues and is catalyzed by N-acetylglucosamine-1-phosphotransferase. The second reaction involves the hydrolysis of N-acetylglucosamine by the action of N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (Reitman and Kornfeld, 1981; von Figura and Hasilik, 1986; Kornfeld, 1987). The resultant mannose-6-phosphate residues of the polypeptides serve as a recognition signal for receptor-mediated endocytosis of the lysosomal enzymes in the Golgi (Kaplan et al, 1977; Hasilik and Neufeld, 1980b; Creek and Sly, 1984). The enzymes involved in the formation of the phosphorylated recognition marker have been localized to an early Golgi compartment (Waheed et al, 1981; Pohlmann et al, 1982); the receptors that bind the recognition marker are thought to reside in the trans Golgi network (Geuze et al, 1985).

Proia et al (1984) demonstrated that α and ß polypeptide chains of Hex associate within the Golgi apparatus after the phosphorylation event and prior to the

binding of the precursor Hex A and Hex B enzymes to the receptors. Once bound, the ligand-receptor complexes exit the Golgi via clathrin-coated vesicles that bud off from the Golgi membrane (Mellman, 1984; von Figura and Hasilik, 1986) and are delivered to a prelysosomal compartment with which the vesicles fuse. The interior of the compartment is acidic; the low pH (≈4.5) is maintained by a Mg^{2+}-ATP-dependent proton pump (Reeves, 1984). The acidity of the prelysosome prompts the dissociation of the precursor Hex isozymes from their receptors, which are then either recycled back to the Golgi or move to the cell membrane and concentrate in coated pits to bind hydrolases that have been secreted.

Hasilik and Neufeld (1980a) demonstrated in pulse-chase experiments that the α and ß polypeptide chains of Hex are synthesized as high molecular weight precursors that are proteolytically processed to mature form. The α subunit was found to have an initial apparent molecular weight of 67 kDa. This is processed to a mature size of 54 kDa. The ß subunit, initially synthesized as a precursor polypeptide of 63 kDa, was found to be shortened to a 58 kDa intermediate. Further proteolytic processing of the ß subunit yields two fragments of 27 and 28 kDa linked by a disulfide bridge (Mahuran et al, 1985). Interestingly, Hasilik et al (1982) demonstrated that the precursor form of Hex A is also fully active against the G_{M2} ganglioside-activator protein complex.

The proteolytic processing of Hex A and Hex B was shown by Frisch and Neufeld (1981) to occur at acid pH and to be catalyzed by various enzymes such as acid proteases, trypsin, and chymotrypsin in a cell-free system. They concluded that processing to mature form probably occurs only after the precursor enzymes have reached the lysosome. The removal of the mannose-6-phosphate recognition marker was also shown to occur after the enzymes are taken up into the lysosomes (Kornfeld

and Sly, 1985).

While the great majority of lysosomal enzymes make their way to the lysosome by receptor-mediated endocytosis at the Golgi membrane, a small portion fail to bind to mannose-6-phosphate receptors and are instead secreted by the cell. Cells expressing the receptor in coated pits on their membrane will take up the secreted phosphorylated enzyme by pinocytosis and deliver it to the lysosome. This 'secretion and recapture' (proposed by Hickman and Neufeld, 1972) provides another route to the lysosome which is still dependent on the mannose-6-phosphate recognition signal and receptor. Attempts to demonstrate a pathway independent of the mannose-6-phosphate recognition marker have not been successful. However, it seems likely that such a pathway exists, based on two lines of evidence: (1) Cells of the liver, spleen, brain, and kidney of ICD patients, which are deficient in N-acetylglucosamine-1-phosphotransferase, do have some unphosphorylated acid hydrolases located within the lysosome (Owada and Neufeld, 1982; Waheed et al, 1982) and (2) Glucocerebrosidase, a nonsoluble lysosomal enzyme, is not phosphorylated and yet makes its way to the lysosome by a sorting mechanism that differs from that of water-soluble hydrolases (Erickson et al, 1985).

6. The structure of the genes encoding the α and ß subunits of hexosaminidase.

A cDNA clone for the α chain of Hex A was first isolated by Myerowitz and Proia (1984). The clone, containing a cDNA insert of 240 base pairs (bp), had sequences complimentary to an mRNA from normal human fibroblasts of approximately 1.9 kilobases (kb). The insert mapped to genomic sequences encoding the 3' end of the α-chain gene for Hex A. Subsequently, Myerowitz et al (1985) were able to isolate a cDNA clone from an adult human liver library that contained the

entire coding sequence of the precursor α-chain polypeptide of hexosaminidase. The full-length clone contains a cDNA insert that is 1944 bp in length with untranslated regions of 168 bp and 186 bp at the 5' and 3' ends, respectively. An open reading frame of 1587 bp corresponds to 529 amino acids with an apparent molecular weight of approximately 61,000 Da (smaller than the size estimated by gel electrophoresis of the biosynthetically labelled α polypeptide). The first 17-22 amino acids are predominantly hydrophobic and represent the signal sequence that is cleaved once the newly synthesized polypeptide chain is transported into the lumen of the ER. Myerowitz et al (1985) found the full-length cDNA to hybridize to two normal fibroblast mRNA species of 2.1 kb and 2.6 kb, differing only at their 3' ends. The larger mRNA species, which is much less abundant (representing only 5% of the total message detected), was found to have a different polyadenylation signal 453 bp downstream. The significance of this minor species of RNA is unknown; it was not detected in normal human placenta. Neither α-chain mRNA species were detected in Ashkenazi Jewish TSD fibroblasts. These findings were confirmed by Korneluk et al (1986). DNA sequences complementary to the cDNAs were localized to chromosome 15 in somatic cell hybrids confirming that they were, in fact, sequences of the precursor α gene (Myerowitz et al, 1985; Korneluk et al, 1986).

cDNA clones for the ß chain of Hex were isolated by O'Dowd et al (1985). The clones ranged in length from 1.4 to 1.7 kb. Restriction enzyme mapping indicated that their inserts were identical at the 3' end but differed in the 5' extensions. The cDNA clones hybridized to normal fibroblast RNA of approximately 2.2 kb and mapped to chromosome 5 in somatic cell hybrids, as expected for the ß gene of Hex.

O'Dowd et al (1985) determined the amino acid sequence of two ß-chain

peptides from the nucleotide sequence of the cDNA clones. Comparison of these sequences with the deduced amino acid structure of the α chain (Myerowitz et al, 1985; Korneluk et al, 1986) revealed strikingly similar sequences. A small ß-chain peptide of 20 amino acids showed 45% homology to the carboxyl terminus of the α-chain polypeptide, while a larger 63 amino acid ß-chain peptide was 70% homologous to the middle region of the α chain ; 65% homology of α- and ß-cDNA sequences was observed (Myerowitz et al, 1985). Likewise, Korneluk et al (1986) aligned precursor α- and ß-polypeptides and found 57% amino acid homology and 55% nucleotide homology. Most of the nonhomology of the amino acid sequence of the α and ß polypeptides occurs at the amino terminus. Both groups of investigators feel the high degree of homology indicates that the genes may have derived from a common ancestor gene and diverged after its duplication. Evidence of this was recently provided by Proia (1988) who isolated the ß-chain gene, characterized its intron-exon organization and compared it to that of the α-chain gene. He found the ß- and α-chain genes to be similar in their approximate size and number of exons. In addition, he found that the introns of the two genes interrupt the coding regions at homologous positions.

The intron-exon organization of the gene encoding the α subunit of Hex was elucidated by Proia and Soravia (1987). A schematic representation of the gene is shown in Figure 2. The gene was found to be over 35 kb in length and split into 14 exons. All of the introns have characteristic GT and AG dinucleotide sequences at their 5' and 3' ends, respectively. Most of the 13 introns are small (ranging from 0.2 to 2.1 kb in length); the exception is the first intron which has a length greater than 18

kb. At the 5' end of the gene there are sequences resembling the TATA box (TTATTTA) and CCAAT box (CCATC) with a GC-rich region between. These sequences are thought to form the promoter region of the α-chain gene. The first exon of the gene encodes both the signal sequence and the extension at the amino terminus of the precursor α polypeptide that are cleaved during the co- and post-translational modifications of the α chain. Little et al (1988) identified proteolytic cleavage sites in the α polypeptide to occur almost exclusively at the amino terminus; at most only a few amino acids are removed from the carboxyl terminus, as demonstrated by the ability of antibody directed against the last 15 amino acids at this terminus to precipitate both mature and precursor α chain.

Varki et al (1981) and Waheed et al (1982) demonstrated that the amino terminal extension of lysosomal enzymes does not appear to carry the signal by which they are recognized as substrate for N-acetylglucosaminyl phophotransferase, the enzyme that phosphorylates lysosomal enzymes at specific mannose residues. These findings were corroborated by Myerowitz et al (1985) who found no significant homology between the amino acid sequence of several lysosomal enzymes. Rather, Proia and Soravia (1987) suggest that since the pre- segment is removed following α- and ß-subunit association, the amino-terminus of the precursor polypeptides may play a role in this association.

Discovery of the structural organization of the α-chain gene has already led to the identification of the mutation in some patients with TSD. These include: a single base substitution producing a splice junction error in Ashkenazi Jewish infantile TSD (Arpaia et al, 1988; Myerowitz, 1988; Ohno and Suzuki, 1988b); a 4 bp insertion in exon 11 producing premature termination in Ashkenazi Jewish TSD

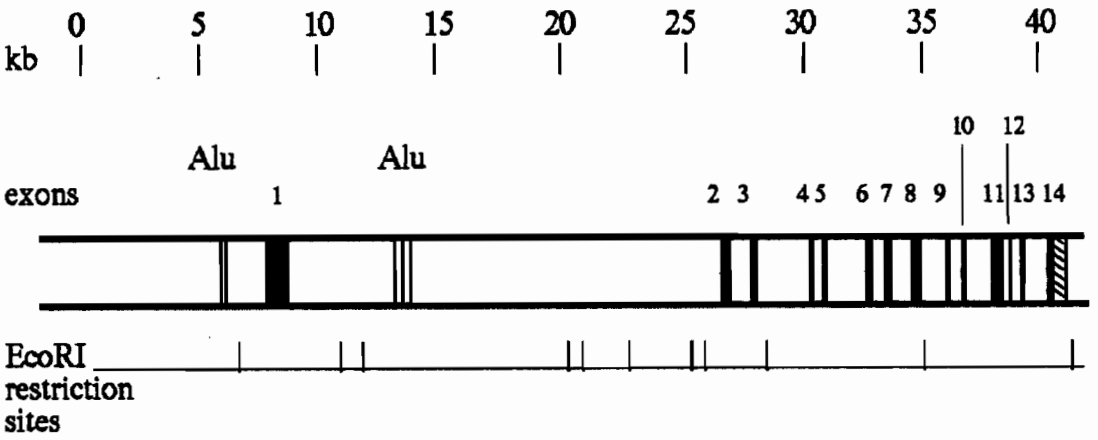

Figure 2. Intron-exon organization of the α-chain gene of hexosaminidase A. The bars at the top of the figure denote the α-chain gene from 5' (left) to 3' (right). Introns are represented by open areas within the bars. The enclosed boxes represent exons 1-14. The hatched area in exon 14 corresponds to the additional 3' untranslated sequence found in the larger of two mRNAs for the α chain. EcoRI restriction sites (pertinent to the study presented in this dissertation) are indicated below the α-chain gene structure map. Alu repetitive elements are indicated. (*From Proia and Soravia, 1987; Myerowitz and Hogikyan, 1987)*

(Myerowitz and Costigan, 1988); a deletion of a single base producing premature termination in an infantile TSD patient of Italian origin (Neufeld, 1988, personal communication); a partial 5' end gene deletion in French Canadian infantile TSD patients (Myerowitz and Hogikyan, 1986); and a single base substitution that produces an amino acid change near the amino terminus of the enzyme, significantly altering its secondary structure in a TSD B^1 variant (Ohno and Suzuki, 1988a). It is likely that many more mutations will be identified in the near future.

C. Variant alleles at the α-locus of Hex A.

Infantile TSD is most prevalent in Jews of Ashkenazi descent and French Canadians. It was once thought that the absence of Hex A in these two ethnic groups might be attributable to an identical mutation, perhaps arising independently or perhaps due to a Jewish ancestor in the French Canadian population (McKusick, 1986). However, TSD in these ethnic groups was shown by Myerowitz and Hogikyan (1986) to be due to at least two distinct mutant alleles which nevertheless result in an absence of Hex A production.

Fibroblasts of Ashkenazi Jewish TSD patients were not found to synthesize an α-chain precursor (Proia and Neufeld, 1982). Furthermore, they were shown to have undetectable mRNA for the α subunit (Myerowitz and Proia, 1984; Myerowitz et al, 1985; Korneluk et al, 1986) in spite of the fact that they do synthesize a primary RNA transcript (Paw and Neufeld, 1988). Arpaia et al (1988), Myerowitz (1988), and Ohno and Suzuki (1988b) identified a splice junction mutation (G—>C transversion) of the GT dinucleotide of intron 12 in PCR-amplified fragments of the α-chain gene from several Ashkenazi Jewish infantile TSD patients. Another mutation, a 4-base pair insertion in exon 11 that introduces premature termination 9

bp downstream from the site of the insertion, has recently been identified by Myerowitz and Costigan (1988). They estimate that this is the major defect causing classical infantile TSD in the Ashkenazi Jewish population since 70% of the carriers tested possessed this DNA lesion. It is likely that other mutations will be identified in the Ashkenazi Jewish population that give rise to the wide range of clinical phenotypes observed in the variant forms of TSD.

The mutation in two French Canadian TSD patients was demonstrated by Myerowitz and Hogikyan (1986) to be a 7.6 kb deletion at the 5' end of the α-chain gene. The deletion includes part of intron 1, all of exon 1, and extends 2 kb past the putative promoter (Myerowitz and Hogikyan, 1987; Proia and Soravia, 1987). The first exon was found to encode the amino-terminus of the precursor polypeptide that is proteolytically cleaved in the lysosome to produce mature α subunit (Proia and Soravia, 1987). Sequence analysis of the α-chain gene revealed Alu sequences in the normal gene (Proia and Soravia, 1987) that correspond to the 5' and 3' deletion boundaries in the French Canadian mutant gene, suggesting that the deletion may have arisen from unequal crossover between Alu sequences during meiosis (Myerowitz and Hogikyan, 1987). Though the mutations in the Ashkenazi Jews and French Canadians are different, the resultant clinical phenotype is identical.

Many phenotypic and genotypic variations exist within the G_{M2} gangliosidoses. Evidence has been accumulating that defective G_{M2} ganglioside metabolism results from mutations involving different gene loci as well as from multiple defects within a single gene locus. Mutations at three loci result in forms of G_{M2} gangliosidosis. These include the locus on chromosome 15 encoding the α subunit of Hex, the locus on chromosome 5 encoding the β subunit of Hex, and the

locus encoding the activator protein necessary for the in vivo hydrolysis of G_{M2} ganglioside, also mapped to chromosome 5. All forms of G_{M2} gangliosidosis have an autosomal recessive mode of inheritance. A discussion of the α-locus variants follows.

1. CRM-positive infantile G_{M2} gangliosidosis.

TSD has been identified in two unrelated families of Italian descent (Proia and Neufeld, 1982; Zokaeem et al, 1987). Both probands are the offspring of consanguineous parents and both fail to produce Hex A; their clinical phenotype is indistinguishable from that of CRM-negative infantile TSD patients. Biosynthetic labelling of cells in culture and immunoprecipitation of the Hex subunits demonstrated that both patients synthesize a precursor α polypeptide. The α chain enters the lumen of the ER, as indicated by the fact that it is N-glycosylated, but it fails to undergo posttranslational modifications that would result in the formation of a mature α chain. Proia and Neufeld (1982) demonstrated that an α chain of normal apparent molecular weight (67 kDa) is synthesized by the cells of one patient. This α chain is insoluble, however, and not transported from the ER to the Golgi apparatus. In contrast, the other patient synthesizes a shortened precursor α-chain of 65 kDa. This precursor is most likely trapped in the ER. It is not processed further and is degraded by proteolysis (Zokaeem et al, 1987). Neufeld (1988, personal communication) recently demonstrated that the shortened polypeptide synthesized by cells of this patient is due to a deletion of a cytosine nucleotide at position 1510 of the α-chain gene producing a frameshift mutation, followed by premature termination.

Both patients are most likely homozygous for their respective α-locus mutations; this has been demonstrated to be the case in the patient synthesizing a shortened α subunit, whose parents were shown to be heterozygous for the shortened α chain (Zokaeem et al, 1987).

Infantile TSD cases with an enzymatically aberrant but neurologically classical form of TSD have recently been described (Goldman et al, 1980; Li et al, 1981; Inui et al, 1983; Kytzia et al, 1983; Charrow et al, 1985; Bayleran et al, 1987; Besley et al, 1987; Gordon et al, 1988). These patients have significant Hex A activity against synthetic N-acetylglucosaminide substrates, but have severely deficient activity against the 6-sulfated derivatives of these substrates and G_{M2} ganglioside. Sonderfeld et al (1985) demonstrated by genetic complementation that this mutation (referred to as B^1 variant) is allelic to the classical TSD mutation.

Inui et al (1983) described two siblings, born to consanguineous parents, referred to as pseudo-AB or A^mB variants because they synthesize both Hex A and activator protein yet are unable to hydrolyze G_{M2} ganglioside. Unsulfated synthetic substrates were hydrolyzed normally by these individuals. This was taken to indicate that the α-subunit mutation affects the site at which the G_{M2}-activator complex binds Hex A, an interpretation that is no longer valid (Bayleran et al, 1987).

In spite of the normal or near-normal levels of Hex A activity measured by unsulfated synthetic substrates in the B^1 variants, their inability to hydrolyze G_{M2} ganglioside results in its storage in massive amounts. Most patients have a clinical and pathologic phenotype identical to that of infantile TSD. However, Inui et al (1983) and Charrow et al (1985) described patients who had a more juvenile presentation. With the exception of the patients described by Inui, the parents of the

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B^1 variants are unrelated.

Sonderfeld et al (1985) studied the processing of precursor α chains in the cells of a B^1 variant and found that the polypeptide was processed to a mature form that was indistinguishable from that observed in normal cells. However, the amount of mature α chain formed in the B^1 variant was decreased.

Ohno and Suzuki (1988a) recently cloned and characterized mutant cDNAs from fibroblasts of a B^1 variant - the same patient on whom Sonderfeld's work was based. The nucleotide sequence of a cDNA clone containing the entire coding sequence was found to be normal except for a single base substitution that resulted in a change from arginine to histidine at amino acid 178 near the amino-terminus. Computer analysis of the secondary structures of both normal and mutant enzymes indicated significant alterations in the structure as a result of the substitution. While the mutation in this patient apparently does not interfere with normal biosynthesis, processing, and maturation of Hex A or alter its immunologic properties, it may occur at or near the active site on the α subunit.

The significant activity of Hex A against N-acetylglucosaminide substrates makes prenatal diagnosis of the B^1 variant difficult. There is at least one documented case of misdiagnosis that occurred when amniocyte Hex A activity was assessed with the synthetic substrate 4-methylumbelliferyl-ß-D-N-acetylglucosamine. (Kolodny et al, 1983). Recently, however, Conzelmann et al (1985) correctly diagnosed a case of infantile G_{M2} gangliosidosis in a fetus with the B^1 variant form of the disease based on an inability of the fetus' Hex A to degrade radiolabelled G_{M2} in the presence of activator protein.

2. Juvenile G_{M2} gangliosidosis.

Juvenile G_{M2} gangliosidosis was first described by Bernheimer and Seitelberger (1968) and subsequently by Okada et al (1970), Suzuki et al (1970), Menkes et al (1971), and Brett et al (1973). Patients have symptoms similar to those of infantile TSD. However, the onset of symptoms is later, usually between 2 and 6 years of age (Okada et al, 1970). The clinical features of juvenile TSD include ataxia, spasticity, dysarthria, seizures, and the exaggerated startle reaction to sound. Seizures were notably absent in two juvenile TSD patients reported by Andermann et al (1977). As in infantile TSD, dementia is a characteristic feature of juvenile TSD but the deterioration is less rapid. Despite the slower progression of the disease death invariably occurs between the ages of 5 and 15 years (Okada et al, 1970; Brett et al, 1973; Meek et al, 1984).

Juvenile TSD is clinically similar to Batten-Spielmeyer-Vogt disease; however, the appearance of macular degeneration and retinitis pigmentosa early in the course of the latter disorder distinguishes it from juvenile G_{M2} gangliosidosis. Macular degeneration occurs only in some juvenile TSD patients and when it does it results in blindness late in the course of the disease (O'Brien, 1983).

Postmortem examination has revealed significant neuronal storage of G_{M2} ganglioside, though the quantity stored in the cortex is not as great as that stored in infantile TSD (the accumulation of G_{M2} ganglioside is 40-90 times normal in juvenile G_{M2} gangliosidosis) and there is slightly better preservation of myelin (Okada et al, 1970; Brett et al, 1973). The neuronal cytoplasm is ballooned and contains cytoplasmic inclusions of accumulated G_{M2} ganglioside, as seen in infantile TSD (Suzuki et al, 1970; Menkes et al, 1971; Brett et al, 1973).

A deficiency of Hex A has been demonstrated in brain, liver, spleen, leukocytes, fibroblasts, and serum of affected patients. The deficiency, measured by

heat inactivation, ranges from partial (Suzuki et al, 1970; Okada et al, 1970; Brett et al, 1973; Meek et al, 1984) to severe (Brett et al, 1973). Where Hex A activity has been measured with the natural substrate, it has been found to be severely deficient. The amount of G_{M2} ganglioside hydrolysis by juvenile TSD Hex A is only slightly greater than that seen in infantile TSD patients (O'Brien et al, 1977; Kolodny and Raghavan, 1983; Meek et al, 1984).

No ethnic predilection has been observed for juvenile G_{M2} gangliosidosis. Affected children are generally not the offspring of consanguineous parents. An exception is the finding of juvenile TSD in two Lebanese Canadian families (Andermann et al, 1977) in whom the parents are first cousins. Compound heterozygosity in a patient with the juvenile G_{M2} gangliosidosis phenotype was first demonstrated by Johnson et al (1980), who showed that the parents of the proband appeared different upon testing for heterozygote status.

d'Azzo et al (1984) studied the processing and association of α and β subunits in cells from a juvenile G_{M2} gangliosidosis patient. The results indicated that the mutation affects both the rate of synthesis of α polypeptide as well as its ability to associate with β subunits.

3. Chronic and adult-onset G_{M2} gangliosidosis.

Chronic and adult-onset G_{M2} gangliosidosis variants have been described in the literature (Rapin et al, 1976; Willner et al, 1981; Mantovani et al, 1985; Oates et al, 1986; Kaback et al, 1978; Navon et al, 1981, 1986; Johnson et al, 1982; Kolodny and Raghavan, 1983; Argov and Navon, 1984). It remains unclear whether these syndromes represent two distinct genotypes or whether phenotypic heterogeneity results from variable expression of the same genotype. Because there

are significant similarities between the two variants, they will be discussed in the same section.

Chronic G_{M2} gangliosidosis presents primarily as a progressive spinocerebellar degeneration, with symptoms of tremor, incoordination, and dysarthria first appearing between the ages of 3 and 15 years. Upper and lower motor neuron weakness and ataxia develop (Kolodny and Raghavan, 1983). Many patients develop dementia (Willner et al, 1981; Mantovani et al, 1985; Oates et al, 1986); some also experience recurrent psychotic episodes (Rapin et al, 1976). The clinical course of the disease resembles that of Friedreich ataxia. Willner et al (1981) reported nine chronic G_{M2} gangliosidosis patients who were initially misclassified as atypical variants of Friedreich ataxia.

Adult G_{M2} gangliosidosis has an even later age of onset. Motor difficulties first become apparent between the second and fourth decades of life. The symptoms are generally consistent with a spinal muscular atrophy reminiscent of Kugelberg-Welander disease (Johnson et al, 1982) or amyotrophic lateral sclerosis (Argov and Navon, 1984). Progressive proximal muscle weakness and dysarthria are common symptoms; dementia is not. Some patients experience psychotic episodes (Kaback et al, 1978; Navon et al, 1981).

Storage of G_{M2} ganglioside has been demonstrated by the appearance of MCBs in rectal ganglion and cultured skin fibroblasts of chronic and adult G_{M2} gangliosidosis patients (Navon et al, 1981). Postmortem examination of the brain of a chronic patient revealed cerebellar degeneration (Rapin et al, 1976). The neurons of the cerebral cortex appeared slightly swollen, but few had a ballooned appearance; the cerebral cortex is mostly unimpaired in patients with the adult form of the disease. In both variants, however, the gray matter of the spinal cord contains many MCBs consistent with anterior horn cell disease (Rapin et al, 1976; Willner, 1981; Oates,

1986; Navon et al, 1981; Johnson et al, 1982; Navon et al, 1986). The MCBs observed in the adult and chronic variants are indistinguishable from the inclusions observed in the neurons of infantile TSD patients, but their numbers are fewer, indicating a less severe accumulation of G_{M2} ganglioside in these patients. G_{M2} does not accumulate in macrophages, as it does in infantile TSD. Adult and chronic patients do not have seizures, nor is there macular degeneration (Kolodny and Raghavan, 1983).

The degree of Hex A deficiency appears to be comparable in the chronic and adult variants. Some patients have a severe, though measurable deficiency (Willner et al, 1981; Mantovani et al, 1985; Kaback et al, 1978; Johnson et al, 1981; Navon et al, 1981); others have only a partial deficiency (Oates et al, 1986). Deficient Hex A measured by thermal inactivation has been reported in sera, leukocytes, and fibroblasts. Rapin et al (1976) reported on a patient who had serum Hex A approaching that of classical TSD patients, yet the proportion of Hex A in the patient's leukocytes, though still abnormally low, was much higher than in the infantile form. Argov and Navon (1984) and Hechtman, Khoo, and Isaacs (1983) reported an increase of the Hex I isozyme in fibroblasts of chronic and adult patients. The ability of Hex A to hydrolyze G_{M2} ganglioside is severely affected in chronic and adult-onset G_{M2} gangliosidosis patients (Kolodny and Raghavan, 1983; Raghavan et al, 1985). Clearly the degree of enzyme deficiency does not correlate with the severity of the disease.

Chronic and adult-onset forms of G_{M2} gangliosidosis have been found primarily in individuals of Ashkenazi Jewish descent. Parents of affected individuals in most cases thus far studied are unrelated and the patients are thought to be compound heterozygotes (Navon et al, 1981). In some cases, infantile G_{M2} gangliosidosis in another family member has occurred (Navon et al, 1981; Johnson et

al, 1982).

The synthesis and processing of the subunits of Hex A in cells of chronic (d'Azzo et al, 1984) and adult (d'Azzo et al, 1984; Frisch et al, 1984) G_{M2} gangliosidosis patients was studied. Cells of adult-onset patients were found to synthesize an α polypeptide of normal 67 kDa size yet the amount was reduced to less than that synthesized by normal cells. In addition, very little mature α chain was observed, indicating defective association of the α and ß subunits. Cells obtained from chronic TSD patients were found to have the same association defect; however, α precursor was synthesized at a normal rate. In cells of both variants the amount of mature α subunit detected was only about 1/10 the normal level (d'Azzo et al, 1984).

Navon et al (1986) reported on the first successful diagnosis of adult-onset G_{M2} gangliosidosis in a fetus using heat inactivation of Hex A activity, separation of Hex isozymes by ion-exchange chromatography, and immunoprecipitation of biosynthetically labelled Hex subunits to observe the synthesis and processing of Hex A.

4. Hexosaminidase A-deficient healthy adults.

Apparently healthy individuals with deficient Hex A have been found mostly through family studies prompted by the occurrence of TSD in a family member. Vidgoff et al (1973) first described such an individual who was assessed when her child was discovered to have TSD. Measurement of heat-labile Hex A activity in serum and leukocytes revealed profoundly low levels, well below those established for other obligate heterozygotes. Vidgoff et al (1974) subsequently found levels of Hex A activity comparable to heterozygote levels in tears and cultured skin

fibroblasts, suggesting that the mutation in this individual may affect the distribution of Hex A. Similar cases have been reported in which a nonuniform distribution of Hex A has been found (Kelly et al, 1976; Thomas et al, 1982; Grebner et al, 1986) as well as healthy individuals in whom the deficiency of Hex A appears to be uniform throughout cells and fluids (Grebner et al, 1986).

Navon et al (1973 and 1976) reported on four healthy individuals with a severe deficiency of heat-labile Hex A activity in leukocytes, fibroblasts, serum and urine. They were members of the same family and were assessed when another member was diagnosed with TSD. A follow-up study of the four siblings revealed slowly progressive neurological symptoms resembling those associated with adult-onset G_{M2} gangliosidosis. The onset of symptoms in these patients occurred in their late 30's (Navon et al, 1980, 1981). It remains to be seen whether other healthy adults with low levels of Hex A are awaiting a neurological disease or will remain unaffected.

The deficiency of Hex A activity measured by thermal fractionation with the synthetic substrate, 4MUG, is not reflected in the ability of normal Hex A-deficient adults to hydrolyze G_{M2} ganglioside. Tallman et al (1974) found G_{M2} ganglioside hydrolysis by healthy Hex A-deficient individuals to be 50% of normal in leukocytes. Half-normal levels of G_{M2} ganglioside hydrolysis have also been observed by O'Brien et al (1977, 1978) using fibroblasts and leukocytes of healthy Hex A-deficient variants. Grebner et al (1986) demonstrated G_{M2} ganglioside metabolism that was closer to normal levels in three variants.

Healthy adults with profoundly low levels of Hex A activity measured by synthetic substrates yet normal or near-normal metabolism of G_{M2} ganglioside have been found in individuals of Ashkenazi Jewish as well as non-Jewish origin, born to nonconsanguineous parents. It is presumed that this phenotype is the result of

compound heterozygosity for an infantile TSD allele and another mutant α allele that affects the enzyme's ability to hydrolyze synthetic substrates while retaining the ability to hydrolyze G_{M2} ganglioside. Kelly et al (1976) demonstrated segregation of two mutant α alleles in a family of Pennsylvania Dutch extraction in whom TSD had occurred and in whom a healthy Hex A-deficient adult was identified, lending support to this hypothesis.

The synthesis and processing of the α and ß subunits of Hex A in two healthy siblings of Ashkenazi Jewish descent and in a healthy non-Jewish individual was studied by Grebner et al (1986). The siblings displayed a nonuniform deficiency of Hex A activity against 4MUG; the non-Jewish individual had uniformly low levels of Hex A. Cultured fibroblasts of all three persons were found to synthesize precursor α- and ß-polypeptides of normal size that were processed to mature form. There was, however, a difference in the amount of precursor α-chain synthesized. Cells from all three individuals showed a markedly reduced precursor α chain with a corresponding reduction in the amount of mature α subunit.

D. The ß-gene locus and Sandhoff Disease.

Sandhoff et al (1968) described the first case of G_{M2} gangliosidosis in an infant with a clinical phenotype, course, and pathologic changes in the nervous system identical to TSD, yet in whom large amounts of G_{A2} glycolipid were stored as well. Electron microscopic analysis (Sandhoff and Harzer, 1973) gave evidence of extraneurological involvement by the presence of vacuolated histiocytes in the lungs, liver, kidney, spleen, lymph nodes, and bone marrow containing cytoplasmic

lamellar inclusions of G_{M2} ganglioside and G_{A2} glycolipid - something not seen in TSD. A marked increase of globoside in the visceral organs was also noted (Sandhoff and Harzer, 1973; Suzuki et al, 1971). Subsequently, Okada et al (1972) demonstrated accumulations of cerebral G_{M2} to levels 300 times normal (comparable to that in the TSD brain); levels of G_{M2} were 8-30 times normal in the visceral organs. Accumulation of G_{A2} was 100 times normal in the Sandhoff disease (SD) brain (compared to 20 times normal in the TSD brain). Globoside accumulation in the visceral organs was significant, the highest amount observed in the liver where it was 200 times normal. The storage of sulfated glycosaminoglycans (GAG) in cultured fibroblasts (Cantz and Kresse, 1974) and branched-chain N-acetylglucosaminyl oligosaccharides in liver (Ng Ying Kin and Wolfe, 1974) of SD patients has been demonstrated. Warner et al (1986) demonstrated urinary excretion of these compounds in SD patients. Their metabolism is believed to be normally catalyzed by Hex B since they do not accumulate in TSD patients.

Sandhoff and Harzer (1973) found both Hex A and Hex B to be deficient in all tissues and body fluids of SD patients. Thus, these patients have also been referred to as O variants of G_{M2} gangliosidosis. They documented the deficiency using synthetic N-acetylgalactosaminide and N-acetylglucosaminide substrates, G_{M2} ganglioside, G_{A2} ganglioside, and globoside. Ikonne et al (1975) reported that the major component of residual Hex activity in SD patients is Hex S, the properties of which were discussed earlier in this section. Beutler et al (1975) demonstrated that Hex S is a dimer of α subunits, based on its apparent molecular weight and ability to cross-react with anti-α antiserum. The increase in Hex B observed in TSD cells has been attributed to excess β chains combining with one another in the absence of α

chains (Mahuran et al, 1985). In SD cells the intact α subunits also associate in the absence of ß subunits to form Hex S. However, only small amounts of Hex S are found in SD patients, suggesting that pre-α chain synthesis may in some way be linked to pre-ß chain synthesis or may be stabilized by ß chain (Tsui et al, 1983; Proia et al, 1983).

Rattazzi et al (1976) provided confirmation that TSD and SD result from mutations at different gene loci by demonstrating the appearance of Hex A in fibroblast heterokaryons formed between Tay-Sachs and Sandhoff cells. These findings were corroborated by d'Azzo et al (1984) who demonstrated the association of α and ß subunits in chronic TSD and SD cell heterokaryons. SD is the result of a mutation in the gene on chromosome 5 which encodes the ß subunit (Gilbert et al, 1975).

SD is extremely rare among Ashkenazi Jews, yet it is the most prevalent form of G_{M2} gangliosidosis in Lebanon (Der Kaloustian et al, 1981) and Argentina (Kremer et al, 1985). It has also been described in French Canadians of eastern Quebec by Melançon et al (1974) and Andermann et al (1977). The relative isolation of these communities indicates that the high incidence of SD may be attributable to genetic drift and/or inbreeding (Cantor et al, 1987). Autosomal recessive inheritance of SD was reported by Harzer et al (1971) and Okada et al (1972). The first successful diagnosis of SD prenatally was reported by Desnick et al (1973).

SD heterozygotes have a reduced level of total Hex activity in tissues throughout the body. Heterozygotes have been identified using a low percentage of serum Hex B as the criterion (Suzuki et al, 1973; Lowden et al, 1978). Thermolabile Hex B has been described in some SD heterozygotes (Lowden, 1979; Lane and Jenkins, 1978; Hechtman and Rowlands, 1979) who presumably synthesize a hybrid

enzyme of both normal ß chain and enzymatically active mutant ß chain that is unstable to heating. Abnormal ß subunits have also been found in a patient with classical TSD whose cells not only lacked Hex A activity, but the Hex B component showed an increased susceptibility to heat (Momoi et al, 1978), as well as healthy individuals in whom Hex B was heat-labile yet an active Hex A was synthesized (Hechtman and Rowlands, 1979; Navon et al, 1985). Dreyfus et al (1975 and 1977) described patients with the so-called 'Hexosaminidase Paris' mutation in whom mutant ß subunits preferentially associated with normal α subunits to form Hex A with increased heat lability.

Many different SD phenotypes have been identified, associated with varying amounts of residual Hex activity and the age at which clinical symptoms appear. The later-onset forms that have been described include: juvenile (Kytzia et al, 1984; Wood and MacDougall, 1976; Goldie et al, 1977; MacLeod et al, 1977), adult-onset (Johnson et al, 1981; Warner and O'Brien, 1983; Barbeau et al, 1984; Federico et al, 1986) and Hex A- and Hex B-deficient normal adults (Dreyfus et al, 1975; Kytzia et al, 1984).

Hasilik and Neufeld (1980a) found no evidence of precursor ß chain synthesis in fibroblasts of SD patients. Precursor α chain synthesis was normal in SD cells but always appeared as free α chain of 67 kDa size (Proia et al, 1984). O'Dowd et al (1985) isolated human cDNA clones coding for the pre-ß chain of Hex. DNA isolated from fibroblasts of infantile and juvenile SD patients was probed with these clones (O'Dowd et al, 1986). Juvenile SD patients, who had some residual Hex activity, showed normal or reduced amounts of precursor ß-chain mRNA. No gross abnormalities in the gene encoding the ß subunit were found. Cells of some infantile SD patients were found to synthesize pre-ß mRNA. In some such patients a pre-ß

polypeptide was synthesized in normal amount and size, yet there was barely detectable Hex activity; in other patients pre-ß subunit was synthesized in levels of 5-10% normal, with or without measurable Hex activity. The cells of some infantile SD patients were found to synthesize no mRNA for the precursor ß chain. Of this latter group, the mutation in a few individuals was found to be a partial gene deletion at the 5' end of the gene. No individual in this group had Hex activity. (O'Dowd et al, 1986).

E. The AB variant.

Sandhoff et al (1971), Kolodny et al (1973) and deBaecque et al (1975) were among the first to describe a patient with normal Hex A and Hex B activities against a synthetic chromogenic substrate, yet with all the typical clinical signs of classical TSD (save for optic atrophy) and massive accumulation of G_{M2} ganglioside in the brain tissue. G_{A2} ganglioside was also found to accumulate. These patients were classified as AB variants since both Hex A and Hex B activities were present. Conzelmann et al (1978) found Hex A of an AB variant to be biochemically and immunologically identical to normal Hex A. More recently, Hasilik and Neufeld (1980a) demonstrated that the cultured fibroblasts of an AB variant synthesize precursor α- and ß-chains that are processed to mature form.

Conzelmann and Sandhoff (1978) found the AB variant to be defective in a factor necessary for the interaction of the glycolipid substrate and its water-soluble enzyme. The properties of this activator protein as well as its subcellular localization, the mechanism by which it exerts its action, and the chromosome encoding its production were discussed earlier.

Erzberger et al (1980) and Hechtman et al (1982) demonstrated the ability of the Hex A of AB variants to hydrolyze G_{M2} ganglioside in the presence of G_{M2}

activator protein isolated from normal kidney or liver.

An adult G_{M2} gangliosidosis patient with progressive muscle weakness and mental deterioration, yet with normal Hex A and Hex B activities, was described by O'Neill et al (1978) and is thought to represent an adult-onset AB variant.

Goldman et al (1980) described a patient diagnosed as an AB variant of G_{M2} gangliosidosis since Hex A and Hex B activities were apparently normal against synthetic substrates. Li et al (1981) subsequently demonstrated that levels of activator protein in cells of this patient were 3 times higher than normal. Since patient Hex A was unable to degrade G_{M2} ganglioside in the presence of normal activator protein, the investigators concluded that the disease in this patient was attributable to a defect in the enzyme rather than the activator protein. The defect resembles that described by Inui et al (1983) with the exception that Inui's patient had a juvenile presentation of the disease. The patient, originally described by Goldman and subsequently by Li, had an infantile-onset phenotype. More recently, these patients have been recognized as B^1 variants of TSD. Somatic cell fusion studies indicate that they are allelic to B variant mutations (Sonderfeld et al, 1985).

F. Measurement of Hex A for diagnosis of the G_{M2} gangliosidoses.

The elucidation of the structures of the genes encoding the α and β subunits of Hex is certainly fundamental to identifying the mutations in TSD. However, assessment of the amount and catalytic activity of Hex A is essential for the detection of carrier couples at risk for producing affected offspring as well as for pre- and post-natal diagnosis of affected children. The reliable diagnosis of TSD is dependent upon an assay method that accurately differentiates between the two major Hex isozymes (A and B) present in body tissues and fluids. Both isozymes are capable of hydrolyzing the nonreducing terminal N-acetylglucosamine or N-acetylgalactos-

amine residues on glycolipids, glycoproteins, and glycosaminoglycans (Conzelmann and Sandhoff, 1979; O'Brien et al, 1978) as well as p-nitrophenyl-, ß-naphthyl-, and 4-methylumbelliferyl- derivatives of artificial substrates (Leaback and Walker, 1961; Woollen et al, 1965; Hayashi, 1965).

1. Assays employing G_{M2} ganglioside and other natural substrates.

Because G_{M2} ganglioside is hydrolyzed only by Hex A it would seem the ideal substrate to employ for diagnostic purposes. G_{M2} ganglioside is usually labelled with tritium to achieve a satisfactory level of sensitivity in the assay. Various moieties have been labelled: the terminal N-acetylgalactosamine (Suzuki and Suzuki, 1972; Novak et al, 1979), the ceramide (Schwarzmann, 1978), and the sphingosine (Ghidoni et al, 1981) portions of the molecule have been labelled by reactions involving oxidation and/or reduction with sodium borotritiide. Kolodny et al (1970) described a procedure for biosynthetically labelling the sialic acid residue of G_{M2} ganglioside.

The most common assays employing G_{M2} ganglioside are those in which the ganglioside is labelled in the N-acetylgalactosamine residue. O'Brien et al (1977) assayed cell extracts from a number of G_{M2} gangliosidosis mutants employing the detergent, sodium taurocholate, as the activator of Hex. However, the specifities of both Hex A and Hex B have been shown to be altered in the presence of detergents (Conzelmann and Sandhoff, 1979). In an attempt to eliminate Hex B hydrolysis of G_{M2} ganglioside stimulated by detergents, Harzer (1983) separated Hex isozymes by ion-exchange chromatography prior to assay. Erzberger et al (1980) employed the natural activator protein as the specific activator of Hex A-catalyzed hydrolysis of G_{M2} ganglioside, permitting the use of an unfractionated extract as Hex A source.

Kolodny and Raghavan (1983 and 1985) described a method for measuring

G_{M2} ganglioside metabolism in intact fibroblasts. In this procedure G_{M2} ganglioside radiolabelled in the sphingosine moiety is added to the media of cultured cells, followed by HPLC analysis of their lipid content after 10 days of culture. This method has the advantage of being independent of exogenously supplied activator protein or detergents. It has been found particularly useful for diagnosis of atypical cases of Hex A deficiency (Raghavan et al, 1985). Charrow and Binns (1986) and Sonderfeld et al (1985) have employed a similar procedure.

While the prognostic value of G_{M2} ganglioside assays is indisputable, there are several disadvantages to its use. G_{M2} ganglioside must be obtained from the postmortem brains of infantile TSD children, since the levels to which it accumulates are 300 times greater than in normal brains. With the advent of carrier screening programs and prenatal diagnosis of affected fetuses, fewer affected children are being born in those communities with the highest incidence of TSD (i.e. Ashkenazi Jewish and French Canadian communities). Infantile TSD in other ethnic groups or variant forms of TSD are much rarer in their occurrence. Once a TSD brain is obtained, gangliosides are extracted and extensively purified to obtain G_{M2} ganglioside. The G_{M2} preparation to be radiolabelled must be uncontaminated by G_{A2} ganglioside (which is hydrolyzed by both Hex A and Hex B). Enzymatic assays of cell-free extracts employing G_{M2} ganglioside as substrate require an activator protein, which also must be extracted, usually from liver, and extensively purified. Alternatively, detergents used in place of activator protein are not specific activators of Hex A-catalyzed hydrolysis of G_{M2} ganglioside.

Other natural substrates that have been employed to assess Hex A activity include glycosaminoglycans (GAG), which are oligosaccharides that are usually sulfated. They also contain hexosamines which can be either α- or ß-linked at the

nonreducing end of the molecule. Only the ß-linked hexosamines serve as potential substrates for Hex (Mahuran et al, 1985). Some of the GAGs which have been employed in Hex A assays are hyaluronic acid (Bach et al, 1978; Yutaka et al, 1984), keratan sulfate (Ludolph et al, 1981; Yutaka et al, 1982), chondroitin-4-sulfate (Thompson et al, 1973), and chondroitin-6-sulfate (Yutaka et al, 1982, 1983). GAGs are radiolabelled at the hexosamine residues. They are hydrolyzed by both Hex A and Hex B, though Hex A appears to be predominantly active against these substrates.

N-acetylglucosamine and N-acetylgalactosamine residues of oligosaccharides on glycoproteins are also hydrolyzed by Hex A and Hex B (Bearpark et al, 1977). These oligosaccharides have been shown to accumulate in patients with SD due to the absence of both isozymes (Ng Ying Kin and Wolfe, 1974; Tsay and Dawson, 1976; Strecker et al, 1977).

2. Assays employing synthetic substrates.

Hex A-catalyzed hydrolysis of synthetic substrates occurs at a faster rate than hydrolysis of glycolipid substrates. Para-nitrophenyl-ß-D-N-acetylglucosamine (pNPG) has been used to assay Hex A activity. The colorimetric change that is produced when pNPG is hydrolyzed is measured spectrophotometrically (Aruna and Basu, 1975; Woollen et al, 1961). However its estimation is subject to interference from coloured cell extracts and body fluids such as serum.

Assay with the fluorogenic 4-methylumbelliferyl (4MU) derivative of N-acetylglucosamine, 4MUG, provides for a more sensitive assay. Cleavage of the N-acetylglucosaminyl linkage produces 4MU which is measured fluorometrically (Leaback and Walker, 1961). The disadvantage of most synthetic substrates, such as 4MUG or pNPG, for the determination of Hex A activity is that they are hydrolyzed by Hex B as well. Assays employing these substrates depend upon a two-step procedure based on the differential thermolabilities of the isozymes (O'Brien et al,

1970). However, the requirement for thermal fractionation introduces a tedious procedure as well as associated inaccuracies due to variability in the amount of Hex A remaining or the amount of Hex B inactivated. These substrates are capable of accurately diagnosing CRM-negative TSD genotypes as well as CRM-positive TSD genotypes with absent or severely diminished catalytic activity. However, some CRM-positive genotypes have been identified in which 4MUG hydrolysis was misleading. On rare occasions, mass screening for the TS gene reveals a healthy adult with low or absent Hex A. The clinical significance of these findings is not known because the assay of Hex A with 4MUG has no prognostic value in such cases. In addition, carrier detection of pregnant women by measurement of serum hexosaminidase activity using a thermal fractionation assay procedure presents special problems due to the increased amounts of heat stable Hex (Huddleston et al, 1971; Stirling, 1972). Like Hex I, Hex P is not present in cells and, therefore, assessment of carrier status in a woman who is pregnant is usually accomplished by assay of hexosaminidase activity in leukocytes by a tedious procedure. The source of increased levels of serum Hex I during pregnancy is not known; it has been suggested that the source is the liver, since electron microscopic studies have revealed that the liver undergoes ultrastructural changes during pregnancy that are associated with increased levels of protein synthesis (Pérez et al, 1971). In addition, patients with viral hepatitis also show increased levels of heat-stable Hex in serum (Woollen and Turner, 1965; Hultberg and Isaksson, 1983). That its production might be hormonally-stimulated is indicated by the observation that women taking oral contraceptives also exhibit higher levels of Hex I in serum (Kaback et al, 1973, 1974). Yet another factor complicating accurate carrier detection of pregnant women by assay of serum Hex activity is the appearance of increased levels of Hex A during pregnancy in addition to increased levels of heat-stable Hex (Navon et al, 1987; Ben-Yoseph et al, 1988). The increase of Hex A is probably attributable to leaking of

fetal Hex across the placenta into the mother's circulation and the amount of increase appears to be affected by the genotype of the fetus (Ben-Yoseph et al, 1988). Therefore, one cannot rule out the possibility that the increase of heat stable Hex in the sera of pregnant women is not also of fetal origin.

3. Sulfation of synthetic substrates.

Suzuki and Strominger (1960) and Lloyd (1959 and 1962) described reactions for the enzymatic and chemical sulfation of hexosamines, rexpectively. A reaction similar to that of Lloyd's was subsequently described by Ishihara et al (1976).

Kresse et al (1980) synthesized the sulfated derivative of pNPG using Ishihara's protocol and found it to be a substrate specific for Hex A (Kresse et al, 1981), thus offering the potential for an assay method for Hex A that does not rely on differential heat lability. para-nitrophenyl-ß-D-N-acetylglucosamine-6-sulfate (pNPGS) has been found to accurately diagnose TSD in a one-step assay (Fuchs et al, 1983) and to improve the classification of G_{M2} gangliosidosis genotypes (Li et al, 1983; Kytzia et al, 1983, 1984; Conzelmann et al, 1985). Different catalytic properties of Hex A and Hex B toward pNPG and pNPGS provided the first evidence for two distinct active sites on Hex A (Kytzia et al, 1983).

The specificity of pNPGS for Hex A found by Kresse et al (1981) indicated that similar results might be achieved by sulfation of the more sensitive substrate 4MUG.

The work presented in this dissertation includes: the synthesis of 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS) and its application in assay procedures for the diagnosis and classification of variant forms of G_{M2} gangliosidosis; the potential use of this substrate for screening of heterozygotes in populations at risk, including the testing of pregnant women for carrier status by

serum enzyme assay; the characterization of the defective Hex A synthesized in the B^1 variant of TSD; and an examination of the prognostic value of 4MUGS for adults with low Hex A. Much of this work has been published and since the initial disclosure of the synthesis of 4MUGS (Bayleran and Hechtman, 1983; Bayleran et al, 1984) it has been synthesized in other laboratories with comparable results (Inui and Wenger, 1984; Ben-Yoseph et al, 1985). An investigation of the homogeneity of the deletion mutation conferring TSD (Myerowitz and Hogikyan, 1986) in the French Canadian population of Quebec has been undertaken and is presented here.

II. MATERIALS AND METHODS

A. Preparation of 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS).

1. Synthesis.

4-methylumbelliferyl-ß-D-N-acetylglucosamine (4MUG) was purchased from Koch-Light (Edmonton, Alberta, Canada) or Sigma (St. Louis, MO, USA). Traces of free 4-methylumbelliferone (4MU) were removed by stirring 1.1 g of 4MUG in 450 ml of acetone overnight at 4° C. Washed 4MUG was filtered and stored in a vacuum dessicator.

One gram (2.67 mmol) of washed 4MUG was dissolved in 35 ml of pyridine (Fisher, Montreal, Quebec, Canada) that had been distilled and stored over KOH pellets (Fisher). Fifty mg of 4-dimethylaminopyridine (Sigma) were added and, once dissolved, the mixture was stirred in a sealed vial for 30 min. at 0° C. 266.5 µl (4.01 mmol) of chlorosulfonic acid (Fisher) were added to 3.5 ml of dichloromethane (Eastman, Rochester, NY, USA), that had been dried over Davison molecular sieves, 4 Å pore size (Fisher). The solution of chlorosulfonic acid/ dry dichloromethane was chilled over ice, taken up by syringe, and injected dropwise into the stirring 4MUG solution. The reaction mixture was allowed to stir at 0° C for 3-4 hours and then at 25° C for 2 hours. The reaction mixture was once again chilled over ice and 25 ml of cold distilled water were added slowly to stop the reaction.

2. Purification.

The mixture was taken to dryness in vacuo at 25° C. The residue was redissolved in 100 ml of distilled water and the step repeated. The residue was then taken up in 100 ml of water and lyophilized. The lyophilization procedure was repeated to remove final traces of pyridine.

The sulfated product was separated from unreacted starting material by ion-

exchange chromatography on Cellex D diethylaminoethyl (DEAE), OH^- form, purchased from Bio-Rad (Richmond, CA, USA). The DEAE-cellulose was prepared as follows: 100 g of DEAE (exchange capacity, 0.78 meq/g) were washed with 1 liter of 1 N HCl, followed by 2 l of distilled water. The DEAE-cellulose was then washed with 1 l of 0.5 N NaOH and washed again with distilled water to neutrality. The DEAE-cellulose was equilibrated in 10 mM sodium phosphate (NaH_2PO_4-NaOH), pH 6.0. A 300 ml column of DEAE, 61.2 x 2.5 cm, was prepared. The remainder of DEAE-cellulose was stored at 4° C.

400 mg of the lyophilized reaction material were redissolved in 10 ml of distilled water and applied to the DEAE-cellulose column. The column was washed with water to remove unreacted 4MUG and the eluate was monitored for absorption at 320 nm (A_{320}) on an LKB spectrophotometer (Biochrom Ultrospec, Model 4050) until the ultraviolet (UV) absorption of the eluate decreased to zero. Column fractions were collected on a Pharmacia Frac 100 fraction collector.

The DEAE column was then washed with 0.15 M ammonium acetate (Fisher) to elute the sulfated product. Column fractions eluting with the salt solution with high A_{320} were pooled, taken to dryness in a vacuum at 25° C, redissolved in 100 ml of water, and lyophilized, leaving a wet salt residue.

A 100 ml column (40 x 1.8 cm) of the cation exchange resin AG50W-X12, 200-400 mesh, H^+ form (Bio-Rad) was converted to Na^+ according to the manufacturer's instructions. The residue containing the ammonium salt of 4MUGS was dissolved in 5 ml of water and NH_4^+ exchanged for Na^+ by passage of the sample through the resin. Fractions with high A_{320} in the distilled water eluate were pooled and lyophilized.

Inorganic salts were removed by passage of the redissolved residue through

a gel filtration column of Sephadex G-10 (Pharmacia). 60 g of Sephadex G-10 were swollen overnight in 400 ml of water and a 300 ml column was poured. The residue containing the sodium salt of 4MUGS was redissolved in 5 ml of water and applied to the column. Fractions were eluted with distilled water. Column fractions with high A_{320} were pooled and lyophilized. The purified product was stored in a vial with dessicant (Dri-Rite, W. Hammond Dri-rite Co., Xenia, OH, USA) at -20° C.

3. Analysis of 4MUGS.

Sulfur analysis was performed by Dr. R.N. Pandey, Guelph Chemical Laboratories (Guelph, Ontario, Canada).

Nuclear Magnetic Resonance (NMR) was performed by Dr. A.S. Perlin, Pulp and Paper Institute, McGill University (Montreal, Quebec, Canada) in D_2O/ pyridine at 200 and 400 mHz.

Ultraviolet spectra of 4MUG and 4MUGS were determined with 0.25 mM solutions of the substrates in distilled water.

Thin layer chromatography (TLC) was performed by spotting 20 μg of 4MUG and 4MUGS (dissolved in pyridine) in different lanes on a Silica gel plate (Eastman). The plate was placed in solvent containing 2-propanol: chloroform: water (6: 2:1) and allowed to run until the solvent was 0.5 cm from the top of the plate. The plate was observed under UV light and the fluorescing spots marked on the plate with pencil.

B. Measurement of hexosaminidases.

1. Measurement of hexosaminidase activity with 4MUG using thermal fractionation.

Hexosaminidase activity was determined fluorometrically according to the method of Leaback and Walker (1961). Reaction mixtures contained 0.05 M sodium

a gel filtration column of Sephadex G-10 (Pharmacia). 60 g of Sephadex G-10 were swollen overnight in 400 ml of water and a 300 ml column was poured. The residue containing the sodium salt of 4MUGS was redissolved in 5 ml of water and applied to the column. Fractions were eluted with distilled water. Column fractions with high A_{320} were pooled and lyophilized. The purified product was stored in a vial with dessicant (Dri-Rite, W. Hammond Dri-rite Co., Xenia, OH, USA) at -20° C.

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citrate-sodium phosphate buffer, pH 4.4, 2.5 mg/ml human serum albumin (HSA, purchased from Connaught Labs, Toronto, Ontario, Canada), 0.5 mM 4MUG, and enzyme in a volume of 200 µl. Enzyme was omitted in blank tubes.

Reaction mixtures were incubated for 30 min. at 37° C and reactions terminated with 5 ml of 0.2 M 2-amino-2-methyl-1-propanol (AMP), pH 10.2 (Aldrich Chemical Company, Milwaukee, WI, USA). Fluorometric units were recorded on a Turner fluorometer with excitation wavelength at 365 nm and emission wavelength at 460 nm. Neutral density filters were not used. 4MU standards were purchased from Koch-Light. One unit of enzyme activity is that amount of enzyme which hydrolyzes 1 nanomole of 4MUG/minute at 37° C.

Thermal fractionation of hexosaminidase activity was performed by heating enzyme preparations for 2 hours at 47° C for fibroblasts (30 min. at 52° C for sera) in a volume of 100 µl containing 0.1 M sodium citrate-sodium phosphate buffer, pH 4.4, and 5 mg/ml HSA.

2. Hexosaminidase A determinations with 4MUGS.

Reaction mixtures contained 0.04 M sodium citrate buffer, pH 4.2, 2.5 mg/ml HSA, 0.5 mM 4MUGS, and enzyme in a volume of 200 µl. The substrate concentration used resulted in a maximum ratio of Hex A/Hex B activities. Reaction mixtures were incubated at 37° C for 30 min. and terminated with 5 ml of 0.2 M AMP, pH 10.2.

3. Hexosaminidase assays employing both 4MUG and 4MUGS.

Hexosaminidase A specific activity and percentage of hexosaminidase A in a cell extract or serum are parameters that have routinely been measured for the purpose of classifying G_{M2} gangliosidosis genotypes. In a thermal fractionation assay, these

parameters can be determined directly from the assay values since the turnover numbers for 4MUG hydrolysis by Hex A and Hex B are equal (Geiger and Arnon, 1976).

To facilitate comparison of genotype classification by 4MUGS with that obtained by thermal fractionation, it was desirable to determine the quantity '% Hex A' as measured by 4MUGS. However, this substrate is not hydrolyzed to an equal extent by Hex A and Hex B.

Comparison of genotypic classification by the two substrate procedures necessitated the development of the following empirical equation which converts fluorometric measurements obtained with 4MUGS to equivalent enzyme units based on 4MUG hydrolysis:

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

g_A = fluorometric units attributable to Hex A when 4MUGS is used as substrate.

S = total fluorometric units measured by 4MUGS.

G = total fluorometric units measured by 4MUG.

r_A = ratio of the rate of hydrolysis of the two substrates (4MUGS/ 4MUG) by pure Hex A.

r_B = ratio of the rate of hydrolysis of the two substrates (4MUGS/ 4MUG) by pure Hex B.

The derivation of this formula appears in Appendix A.

4. Separation of Hex A and Hex B from fibroblast cell extract and serum.

Resolution of the ß-hexosaminidases was achieved by ion-exchange chromatography on DEAE-cellulose (prepared as described on page 55) according to the procedure of Nakagawa et al (1977).

Each column fraction was assayed for hexosaminidase activity and enzyme-containing fractions were pooled, dialyzed against 10 mM NaH_2PO_4-NaOH, pH 6.0, and concentrated using Aquacide II (Calbiochem, La Jolla, CA, USA).

5. Inhibition of 4MUG and 4MUGS hydrolysis.

N-acetylglucosamine (NG) and N-acetylglucosamine-6-phosphate (NGP), which were employed as enzyme inhibitors, were purchased from Sigma.

C. Automated Tay-Sachs screening procedure.

The protocol for the identification of TSD heterozygotes by an automated Hex assay procedure employing the synthetic substrate, 4MUG, was described by Delvin et al (1974). This procedure is used routinely at the Montreal Children's Hospital for genetic screening of those individuals in the community who are at risk for carrier status.

In order to determine the ability of the serum Hex A assay procedure which employs the sulfated synthetic substrate, 4MUGS, to classify heterozygotes by an automated procedure it was necessary to modify the autoanalyzer (Technicon Instruments Corp., Terrytown, NY, USA) for use with two substrates. An aliquot from each serum sample to be tested was taken up by a sampler probe and split into two lines of red and green tubing. The lines were passed into a 37° C water bath, and were connected to two fluorometers (Turner, Model 111) which were calibrated independently.

The serum samples in the red line were diluted with 0.04 M sodium citrate-sodium phosphate buffer, pH 4.4. 4MUG (0.94 mM), dissolved in buffer, 2% HSA, and 1 mg/l Brij wetting agent (Sigma), was added.

The sample in the green tubing was diluted in 0.04 M sodium citrate buffer, pH 4.2 and 0.94 mM 4MUGS in buffer, HSA, and Brij was added. Both samples

entered the 37° C water bath simultaneously and were incubated for 5 minutes. Reactions were terminated with AMP, pH 10.2.

Fluorescence detected by the fluorometers was registered on a recorder which traced two curves, one for total Hex (measured with 4MUG) and the other for 4MUGS hydrolysis (predominantly due to Hex A). With the use of 4MU standard solutions, chart units were converted into nanomoles of 4MU released. The quantity '% Hex A' by 4MUGS was obtained by application of the empirical formula, as stated on page 59.

D. Biological samples.

1. Fibroblasts.

a. Culture of skin fibroblasts.

Human fibroblasts were grown from skin biopsies in 175 cm^2 flasks (Becton Dickinson Labware, Lincoln Park, NJ, USA) using Eagle's Minimal Essential Medium (Gibco, Grand Island, NY, USA) supplemented with antibiotic and 10% fetal calf serum (Flow Labs, Mississauga, Ontario, Canada).

Confluent monolayer cultures were harvested by trypsinization (Difco Labs, Detroit, MI, USA) and the cell suspension transferred to 50 ml plastic centrifuge tubes. The tubes were centrifuged at 25° C for 10 min. at 1000 rpm. The cell pellets were resuspended and washed three times in 1X phosphate-buffered saline. The cell pellets were stored at -20° C.

b. Disruption of cell membranes for measurement of Hex activity.

Cell pellets were suspended in 1.0 ml of 10 mM NaH_2PO_4-NaOH, pH 6.0/ 0.02% Triton X-100 (Sigma) and transferred to Corex tubes. The cells were disrupted by three cycles of freezing in dry ice/ isopropanol and thawing in a 37° C

water bath. The tubes were centrifuged at 4° C for 10 min. at 10,000 rpm in a Sorvall Superspeed SS-34 rotor. The supernatants were transferred to fresh tubes and hexosaminidase assays were performed.

c. Source and classification of fibroblast cell strains.

Fibroblasts were obtained from the Repository for Mutant Cell Strains, Montreal, Quebec, Canada. Cell strains WG93, WG103, WG1105, WG1114 (obtained from patients of Ashkenazi Jewish ancestry), WG107, WG733, WG884, WG1065, WG1499 (from French Canadian patients), WG1051 (from a patient of Italian descent), and WG1108 and WG1110 (from patients of French Canadian/Irish and German/Irish ancestry, respectively) were classified as having the infantile form of TSD. Cell strain WG534 was obtained from a French Canadian patient with Sandhoff Disease (SD). Cell strain WG802 (the patient described by Sandhoff et al, 1971) was obtained from an infantile G_{M2} gangliosidosis patient, AB variant. In most cases, G_{M2} gangliosidosis was confirmed by thin layer chromatography of brain gangliosides.

Cell strains WG306 and WG312 (from siblings of Lebanese ancestry), WG928 and WG1115 (from individuals of Irish and English descent, respectively) were diagnosed as having the juvenile TSD phenotype.

WG1047 (obtained from an Ashkenazi Jewish patient) and WG1048 (from a patient of English descent) were diagnosed with the chronic form of G_{M2} gangliosidosis.

WG1116, WG1107, and WG1111 cell strains were obtained from patients of Ashkenazi Jewish (WG1116 and WG1111) and Welsh (WG1107) ancestry. WG1116 was diagnosed as having the adult-onset form of TSD; WG1107 and WG1111 were found to be clinically normal individuals despite a deficiency of Hex A as measured with the synthetic substrate, 4MUG.

Cell strain WG1117 was obtained from an adult who is clinically normal despite a deficiency of Hex A and Hex B activities. She is also the maternal aunt of a SD patient.

Diagnosis of the α-locus variants was made on the basis of clinical criteria, age of onset, and reduced Hex A activity in skin fibroblasts.

The following cell strains, obtained from patients diagnosed at the Montreal Neurological Hospital, were initiated from biopsies taken at the Montreal Children's Hospital: WG93, WG103, WG107, WG733, WG884, WG1065, WG534, WG306, WG312, WG928, WG1047, and WG1048. WG1051 was diagnosed and biopsied at the Montreal Children's Hospital. WG1105, WG1108 and WG1107 were biopsied at the Eunice Kennedy Shriver Center for Mental Retardation (Waltham, MA, USA) and sent to us by Dr. E.H. Kolodny. Cell Strains WG1114, WG1110, WG1115, WG1116, WG1111, and WG1117 were diagnosed and biopsied by Dr. M.M. Kaback at the University of California, La Jolla. WG1499 was diagnosed by Dr. S. Verret at the Hôpital Enfant-Jésus (Quebec City, Quebec, Canada) and sent to us by Dr. R. Gagné at Le Centre Hospitalier de L'Université Laval, Sainte-Foy, Quebec, Canada.

Other cell strains are the obligate heterozygotes WG885 and WG886 (the parents of WG884), WG1066 (the mother of WG1065), and WG1113 (parent of Ashkenazi Jewish descent whose child has classical TSD). WG885, WG886, and WG1066 were biopsied at the Montreal Children's Hospital; WG1113 was biopsied at Dr. M.M. Kaback's laboratory.

2. Liver.

Postmortem livers from adults who had died of causes other than hepatic disease were obtained within 4 hours of death. The liver was homogenized, centrifuged and the supernatant dialyzed. Enzyme activity was enriched 1100-fold by

adsorption onto concanavalin A-sepharose (Pharmacia) prior to resolution of Hex A from Hex B by DEAE-Sephadex chromatography (according to the procedures of Hechtman, 1977 and Hechtman et al, 1982). Samples of separated liver Hex isozymes were stored at -20° C.

3. Sera.

Serum samples were obtained for the purpose of determining carrier status. Blood samples from Jewish individuals were obtained at screening clinics in Montreal. Samples from French Canadian individuals were collected at clinics held in Rimouski and Sayabec, in the Gaspé region of Quebec. Sera of pregnant obligate heterozygotes and normal controls were obtained from Dr. J.T.R. Clarke at the Hospital for Sick Children (Toronto, Ontario, Canada) and from the Prenatal Diagnosis Clinic at the Montreal Children's Hospital.

Five ml of blood were collected into vacutainer tubes and allowed to stand at 25° C for 30 min. Serum was obtained from the clotted blood by centrifugation at 1200 rpm for 10 min. The serum was transferred to plastic scintillation vials and stored at -20° C.

E. Estimation of protein.

Protein was estimated according to the procedure of Lowry et al (1951) using a 1 mg/ml solution of crystalline bovine serum albumin (BSA, purchased from Sigma) as the standard. 2 N Folin-Phenol reagent was purchased from Fisher. The samples were read on an LKB spectrophotometer at a wavelength of 725 nm.

F. Biosynthetic labelling studies.

Biosynthetic labelling of proteins with ^{3}[H] leucine or $^{32}PO_4$, and immunoprecipitation, electrophoresis, and autoradiography of protein subunits were

adsorption onto concanavalin A-sepharose (Pharmacia) prior to resolution of Hex A from Hex B by DEAE-Sephadex chromatography (according to the procedures of Hechtman, 1977 and Hechtman et al, 1982). Samples of separated liver Hex isozymes were stored at -20° C.

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F. Biosynthetic labelling studies.

Biosynthetic labelling of proteins with [^{3}H] leucine and immunoprecipitation, electrophoresis, and autoradiography of protein subunits were

performed according to the method of Proia et al (1984). Goat antisera directed against purified Hex A, Hex B, and α-chain were a gift of Dr. E.F. Neufeld. The following modifications were used: fibroblast lysates were prepared in a buffer containing 1.4 M NaCl and prior to the preclearing step, lysates were centrifuged at 145,000 x g for 45 min at 4° C. The biosynthetic labelling studies were performed by B. Boulay, a technician at the deBelle Laboratory for Biochemical Genetics (Montreal Children's Hospital).

G. Analysis of genomic DNA.

1. Extraction of genomic DNA.

DNA was obtained from human whole blood by the isolation of nuclei. The extraction was performed at 25° C according to the following procedure: Ten ml of blood were collected into two 5 ml vacutainer tubes containing EDTA as anticoagulant. The tubes were centrifuged at 25° C for 10 min. at 1800 rpm. All subsequent centrifugations were at 2000 rpm for 10 minutes. Most of the top plasma layer was removed leaving the buffy coat, containing the leukocytes, undisturbed. The blood was transferred to a 50 ml plastic tube and 16 ml of RS buffer (10 mM Tris-HCl, pH 7.6/ 10 mM KCl/ 10 mM $MgCl_2$) were added and mixed well by gentle pipetting with a plastic transfer pipette (Sarstedt, W. Germany).

Nucleated cells were lysed by the addition of 60 µl of the non-ionic detergent, Nonidet P40 (NP40, purchased from BRL, Gaithersburg, MD, USA). The tube was inverted several times to mix thoroughly and centrifuged. The supernatant was discarded and the leukocyte nuclei pellet was resuspended in 3 ml of SDS solution (10 mM Tris-HCl, pH 7.6/ 10 mM KCl/ 10 mM $MgCl_2$/ 0.5 M NaCl/ 2 mM EDTA/ 0.5% SDS, w/v) to lyse the nuclei and release the DNA.

An equal volume of distilled phenol, equilibrated in 1 M Tris-HCl, pH 8.0,

was added to extract protein. The tube was inverted several times to mix well and centrifuged. The aqueous upper phase was transferred to a fresh tube and 3 ml of chloroform/ isoamyl alcohol (24:1, v/v) were added and mixed well to extract the phenol. The tube was centrifuged. The aqueous layer was transferred to a glass scintillation vial, two volumes of ice-cold ethanol (99%, v/v) were added, and the solution was mixed well to precipitate the DNA. The DNA fibres were transferred to a sterile 1.5 ml Eppendorf tube (Sarstedt) using a glass micro-sampling pipette (Corning) fused at one end. The DNA was allowed to dry overnight and was then dissolved in 200-400 µl of sterile water. The yield from 10 ml of blood, was 300-600 µg of DNA.

High molecular weight DNA was prepared in the same manner from blood stored for several days at 4° C, with yields comparable to that of DNA extracted from fresh samples of blood.

DNA was obtained from cultured skin fibroblasts by a procedure similar to that described for blood with the following modifications: human fibroblasts were cultured and harvested as described on page 61. Three 175 cm^2 flasks of harvested cells were suspended in 5 ml of RS buffer (10 mM Tris-HCl, pH 7.5/ 10 mM NaCl/ 1.5 mM $MgCl_2$). A 10 µl aliquot was added to 20 ml of Isoton II (Coulter Electronique, Montreal, Quebec, Canada) and the number of cells counted (Coulter Counter). 175 cm^2 was found to contain 5.0-7.0 x 10^6 cells.

The cells were transferred to a conical graduated plastic tube and centrifuged. The pellet was resuspended in 5 ml of RS buffer + 1% NP40 (v/v), kept on ice for 3 min. to lyse the cells, and centrifuged. The supernatant was discarded and the step repeated. The nuclei pellet was then resuspended in 1.2 ml of SDS solution (10 mM Tris-HCl, pH 7.5/ 1.5 mM $MgCl_2$/ 0.5 M NaCl/ 2 mM EDTA/ 0.5% SDS, w/v).

The cell suspension was transferred to a 5 ml Sarstedt tube and phenol/ chloroform extractions were performed. The DNA was precipitated with ethanol, the DNA fibres were spooled out, and transferred to a sterile Eppendorf tube. Once dry, the fibres were dissolved in 100-500 µl of sterile distilled water. 175 cm^2 of cells yielded 100-200 µg of DNA.

High molecular weight DNA was generally obtained from freshly-harvested cells. Ocassionally, DNA extractions were performed on cell pellets that had previously been washed three times in 1X PBS and stored at -20° C. This was not the preferred method, however, since the yield of DNA was lower.

2. Restriction enzyme digestion of genomic DNA.

DNA was isolated from the cells of individuals of French Canadian descent for the purpose of analyzing the TSD mutation in this ethnic group. Genomic DNA was digested with the restriction enzyme, EcoRI (purchased from BRL) at a concentration of 10 Units EcoRI/ µg of genomic DNA.. Reactions typically contained 10 µg of genomic DNA in a total volume of 44 µl. The reaction mixture was incubated for a minimum of 2 hours at 37° C. Digested samples not to be electrophoresed immediately were stored at 4° C until use.

Glass plates were prepared as horizontal slabs for gel electrophoresis and 0.8% agarose gels (0.6 x 19 x 19 cm) were prepared and poured according to the procedure of Maniatis et al (1982). Agarose (Ultrapure, electrophoresis grade) was purchased from BRL. 0.75 µg/ ml ethidium bromide (Sigma) was added to the gel prior to pouring. Once set, the gel was submerged in an electrophoresis tank containing 1.5 l of TAE buffer (0.04 M Tris-acetate/0.001 M EDTA).

The digested genomic DNA was mixed with 6 µl of 6x gel-loading buffer (0.25% bromophenol blue/ 40% sucrose, w/v, in water). For accurate size estimation of the DNA fragments obtained from gel electrophoresis, a Hind III digest of

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bacteriophage lambda DNA (purchased from BRL), which contains fragments of known size, was used. Hind III-digested lambda and EcoRI-digested genomic DNA samples were applied to the wells of the gel using an automatic micropipettor (Gilson). An electrical current of 35 mA was applied for 16 hours at 25° C.

The gel was transferred to a Spectroline transilluminator and visualized under UV light at a wavelength of 302 nm. The positions of the lambda Hind III fragments were marked by cuts made into the gel; excess gel was cut away. A picture of the gel was taken using a Polaroid camera and Polaroid 4 x 5 Land Film, Type 52. The time of exposure was 1 sec.

The gel was transferred to a Pyrex dish and the DNA denatured according to the method of Southern (1975).

3. Southern blotting.

The protocol used for the transfer of electrophoresed DNA fragments onto nitrocellulose filter (0.45 μm pore size, Schleicher and Schuell, Inc., Keene, NH, USA) was essentially that of Southern (1975), with modifications: Three sheets of 0.3 mm Whatman filter paper (purchased from Fisher) were cut larger than the dimensions of the trimmed gel allowing for a 10 cm border around the gel. Three layers of 0.1 mm Whatman filter paper soaked in 2x SSC were cut to the same size as the gel and placed over the nitrocellulose filter. A 1 kg weight was placed on top of the apparatus and the DNA was allowed to transfer for 3.5 hours. Complete transfer of DNA to the nitrocellulose filter was confirmed by staining the dehydrated gel in a solution of 0.75 μg/ml ethidium bromide for 1 hour and visualizing the gel under UV light.

The filter was immersed in 500 ml of prehybridization solution, prepared according to the protocol described in Maniatis et al (1982), for 2 hours at 25° C. Denatured salmon sperm DNA, Ficoll, PVP, and BSA were purchased from Sigma;

Formamide was purchased from BDH.

4. Probe.

The genomic probe used in this study was kindly donated by Dr. Rachel Myerowitz at the NIH (Bethesda, MD, USA). The probe contains a 300-bp intronic sequence mapping 7.6 kb downstream from the first exon of the gene encoding the α subunit of Hex A, ligated into the plasmid, pUC18. The plasmid arrived as a vial of agar with a stab culture of E. coli containing the recombinant plasmid.

L-Broth was prepared as described in Maniatis et al (1982) and 200 µl of 25 mg/ml ampicillin (Sigma) were added and mixed well. The bacteria were grown, and the plasmid amplified and isolated by lysis of the E. coli with SDS, according to the protocol described by Maniatis et al (1982).

Aliquots of the culture sample were frozen for long-term storage according to the following procedure: 50 ml of glycerol were transferred to a small bottle and sterilized by autoclaving. 800 µl of sterile glycerol were pipetted into small plastic culture vials. 600 µl of culture were added and mixed well. The vials were sealed and stored at -80° C.

The plasmid DNA was purified by centrifugation to equilibrium in a cesium chloride gradient (Sigma) as described by Miller (1972) with the following modifications: 135 µg/ml ethidium bromide were added to visualize the plasmid DNA in the cesium chloride gradient after centrifugation. The DNA was centrifuged at 22° C for 40 hours at 37,000 rpm in a Beckman 50T1 rotor. The lower band, containing the closed circular DNA of interest, was carefully pipetted into a plastic centrifuge tube. An equal volume of NaCl-saturated isopropanol was added to remove ethidium bromide. The contents were mixed well, and the upper phase was discarded. The extraction procedure was repeated until the solution was colorless.

The DNA solution was dialyzed overnight against 2 l of 0.01 M Tris-HCl, pH 7.5/ 0.2 M NaCl to remove cesium chloride and the plasmid precipitated with ethanol. It was then centrifuged for 30 min. at 15,000 rpm in an SS-34 rotor. The DNA pellet was dissolved in 2 ml of twice-distilled sterile water. The DNA was quantitated, and its purity determined, by taking optical density readings of an aliquot at wavelengths of 260 nm (DNA) and 280 nm (protein). The plasmid DNA solution was transferred to a sterile Eppendorf tube and stored at 4° C until use.

One μg of plasmid DNA was labelled with 250 μCi of dCT^{32}P (purchased from NEN, Boston, MA, USA) using a nick-translation kit purchased from Amersham. The nick-translation reaction was performed according to the manufacturer's instructions (Amersham Technical Bulletin, 1980), as described by Rigby et al (1977). TCA-precipitable DNA generally represented 80-87% of the total amount of radioactively-labelled DNA. Specific activity of the probe DNA was 300-500 x 10^6 cpm/ μg.

0.1-0.3 μg of radiolabelled probe (representing approximately 100 x 10^6 cpm) was transferred to a sterile Eppendorf tube and the probe DNA denatured by immersion in a boiling water bath for 3-5 min. Hybridization of the nitrocellulose filter to the radiolabelled probe was performed according to Maniatis et al (1982) with the following modifications: hybridization was carried out in a 42° C water bath for 16-20 hours. Following hybridization excess counts were removed from the blot in three successive washes of 2x SSC, 2x SSC + 0.1% SDS at 50° C, and 0.1x SSC.

The blot was air-dried and taped to a piece of 0.3 mm Whatman filter paper. The positions of the wells and lambda Hind III standards were marked on the filter paper with ^{14}C-labelled ink (purchased from NEN). The blot was covered in Saran Wrap and placed in a Kodak X-ray exposure holder (20.3 x 25.4 cm, Eastman Kodak Company) with a sheet of Kodak Diagnostic Film-X-Omat AR. The film was

exposed for 2-4 days at -80° C and developed to visualize the radioactive fragments.

5. PCR-amplification of DNA.

Genomic DNAs were amplified by the polymerase chain reaction technique, digested with Dde I, and the fragments eletrophoresed according to previously published procedures (Saiki et al, 1985; Arpaia et al, 1988). This work was kindly performed by E. Arpaia (Hospital for Sick Children, Toronto, Ontario, Canada).

III. RESULTS

A. Synthesis, purification, and analysis of 4MUGS.

My initial attempts to synthesize 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS), a substrate that would provide for the measurement of Hex A in a one-step assay, involved the 6-sulfation of 4MUG with a 5 molar excess of chlorosulfonic acid (as described by Lloyd, 1959). This procedure yielded disulfated product.

To reduce the chance of disulfation and yet produce a high yield of monosulfated product, 4MUG was sulfated with a 1.5 molar excess of chlorosulfonic acid at low temperature and under anhydrous conditions, as suggested by Dr. A.S. Perlin at the Pulp and Paper Institute, McGill University (Montreal, Quebec, Canada). The chemical reaction for the synthesis of 4MUGS by this procedure is shown in figure 3.

Purification of the product of the reaction was achieved using three chromatographic steps. Ion-exchange chromatography on DEAE-cellulose resolved the negatively-charged 4MUGS from unreacted 4MUG, as shown in figure 4. Column fractions were monitored for absorbance at 320 nm, the wavelength at which maximum absorbance of 4MUG occurs. The uncharged starting material was eluted with distilled water. 4MUGS bound to the column and was eluted with 0.15 M ammonium acetate. 4MUGS represented approximately 80-85% of the UV-absorbing material applied to the column. This eluate, which contained the ammonium salt of 4MUGS, was lyophilized and redissolved in water three times. It was then applied to a column of Dowex-50 Na^+ resin to produce the sodium salt of 4MUGS. The substrate was further purified by gel filtration on Sephadex G-10 to eliminate inorganic salts such as sodium chloride and sodium sulfate. After lyophilization the 4MUGS recovery was 76% by weight and 77% by absorbance of the theoretical

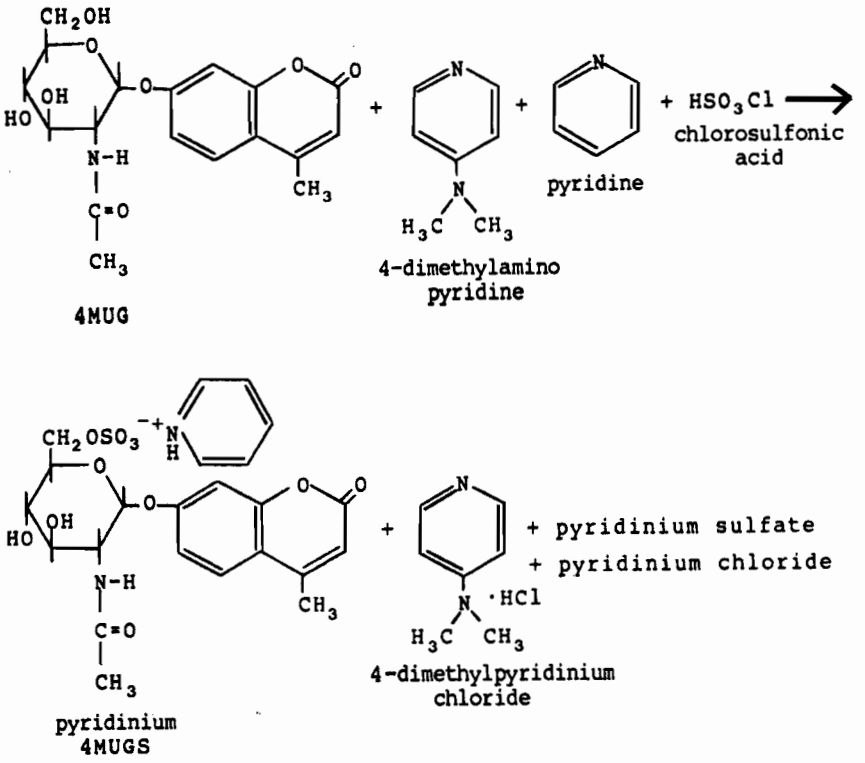

Figure 3. Synthesis of 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS). One gram (2.67 mmoles) of 4-methylumbelliferyl-ß-D-N-acetyl-glucosamine (4MUG) was dissolved in 35 ml of distilled pyridine and sulfated with a 1.5 molar excess (4.01 mmoles) of chlorosulfonic acid dissolved in dry dichloromethane. The reaction was stirred for 3-4 hours at 0° C and 2 hours at 25° C. The reaction was stopped with cold H_2O.

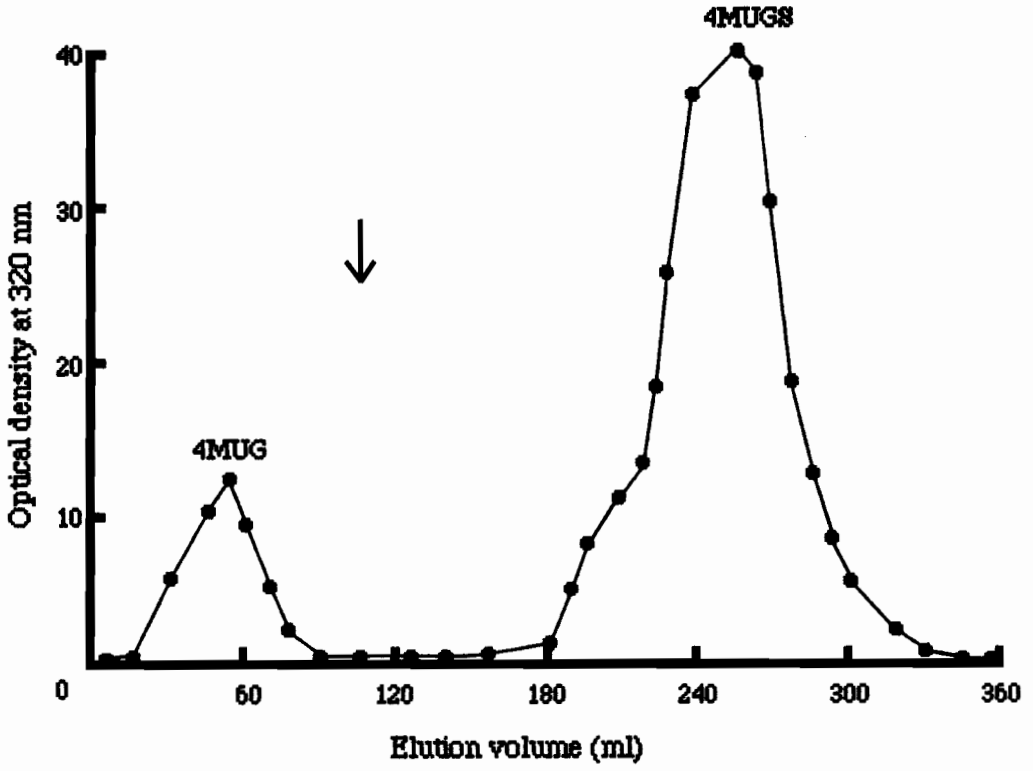

Figure 4. Separation of 4MUGS from unreacted 4MUG by ion-exchange chromatography. Unreacted 4MUG was eluted with distilled H_2O and monitored for absorbance until A_{320} was zero. Negatively-charged 4MUGS was eluted with 0.15 M ammonium acetate. A_{320} O.D. units in the ammonium acetate eluate accounted for 85% of the total O.D. units applied to the column. The arrow indicates the start of elution with ammonium acetate.

expectation.

The UV absorption spectra of 4MUG and 4MUGS are shown in figure 5. The spectra were nearly identical, with the absorption maximum occurring at 320 nm for both compounds, indicating that the umbelliferone ring was not modified by the sulfation reaction. The molar extinction coefficients of 4MUG and 4MUGS were found to be 8780.

Thin layer chromatography of 4MUG and 4MUGS is shown in figure 6. It was performed by spotting approximately 20 μg of these substrates in different lanes on a silica gel plate and running them in a solvent of 2-propanol : chloroform : water (6:2:1). 4MUG migrated as one spot approximately 4.4 cm from the origin. 4MUGS, synthesized in two separate reactions, was spotted in two different lanes. The preparations of 4MUGS migrated as one spot 1.6 and 1.8 cm from the origin, consistent with a more polar compound.

Nuclear magnetic resonance (NMR) spectra were performed by Dr. A.S. Perlin at McGill University. 4MUG and 4MUGS were dissolved in D_2O/ pyridine and the spectra performed at 200 mHz. The spectra are shown in figures 7A and 7B. Comparison of the original glycoside (7A) and the sulfated product (7B) showed the only significant change to be the position of the two 6-hydrogens that were displaced downfield, as expected on O-sulfation at the 6-position. Not only were the positions of the two 6-hydrogens characteristic of O-6 sulfation but their splitting patterns indicated sulfation only at position 6. The pattern of hydrogens 3 and 4 in the 4MUG and 4MUGS spectra indicated that there was no substitution at these positions; thus, disulfation had not occurred. All the protons were accounted for in the 4MUGS spectrum indicating the presence of only one compound and, therefore, the absence of unreacted starting material.

Based on the chemical structure and molecular mass the theoretical percent

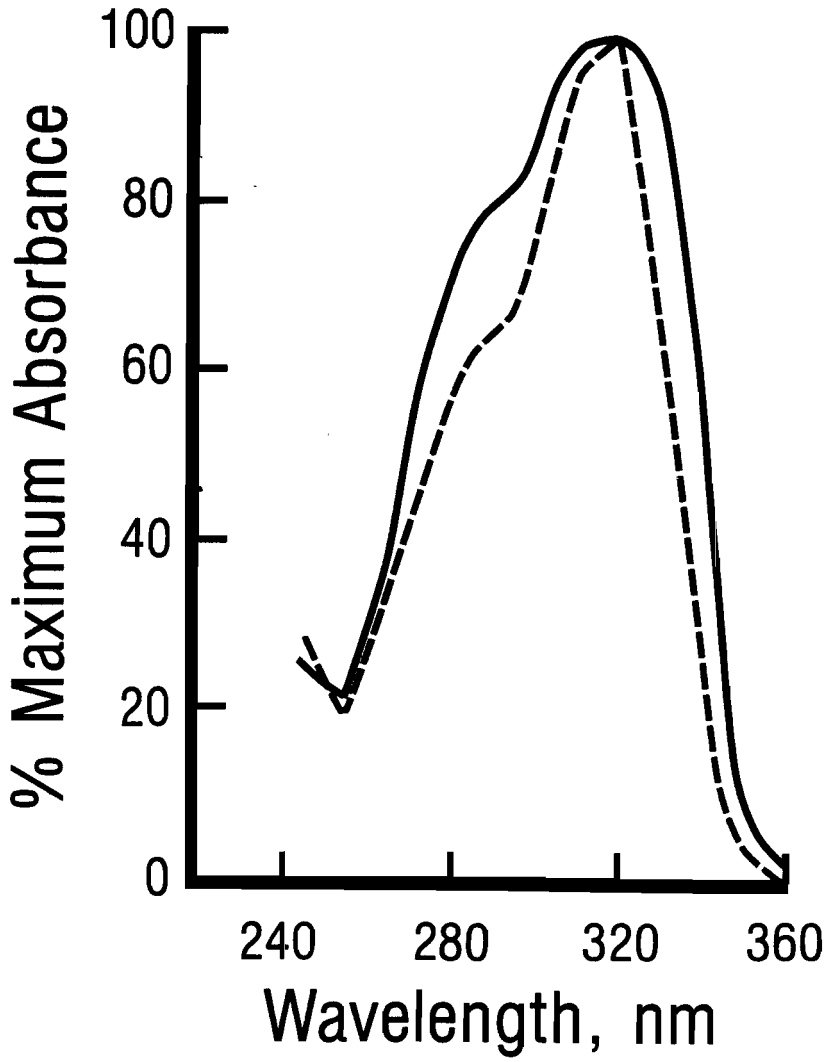

Figure 5. UV-absorption spectra of 4MUG (solid line) and 4MUGS (dashed line). 1 mM solutions of 4MUG and 4MUGS were monitored for absorbance between 240 and 360 nm. Maximum absorbance occurred at a wavelength of 320 nm for both compounds.

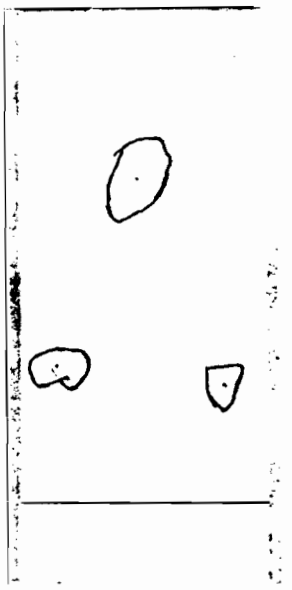

Figure 6. Thin layer chromatography of 4MUGS and 4MUG. 20 μg of 4MUGS (synthesized in two different reactions) and 4MUG were spotted on a silica gel plate and run in a solvent of 2-propanol : chloroform : water (6:2:1). When the solvent was 1 cm from the top of the plate, it was removed from the solvent and allowed to dry. Spots were visualized under UV light. The left and right lanes contain the 4MUGS preparations; the center lane contains 4MUG.

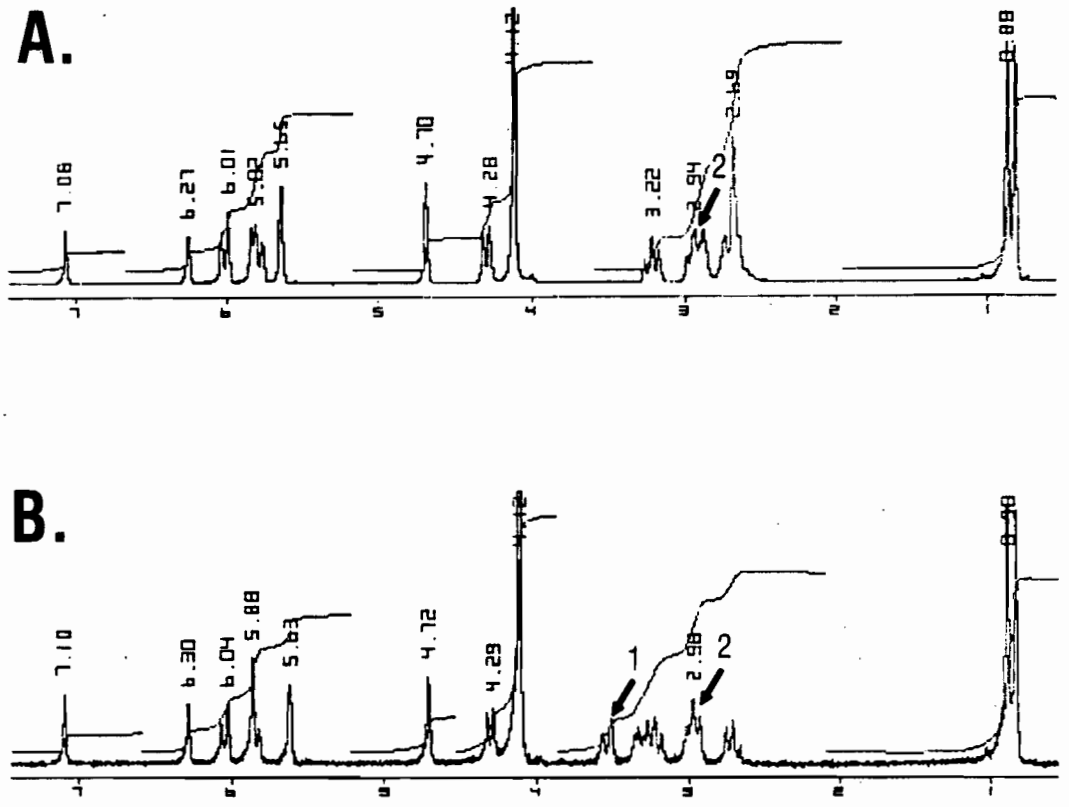

Figure 7. NMR spectra of 4MUG (A) and 4MUGS (B). Compounds were dissolved in D_2O/ pyridine and NMR spectra performed at 200 mHz. Arrow 1 indicates downfield displacement of C6 hydrogen atoms in 4MUGS. Arrow 2 indicates the identity of C3 and C4 hydrogen atoms in both 4MUG and 4MUGS.

sulfur was calculated to be 10.97 for a disulfated product and 6.65% for 4MUGS, a monosulfated compound. Elemental analysis, performed by Dr. R.N. Pandey at Guelph Chemical Laboratories (Guelph, Ontario, Canada), gave a value of 6.41% sulfur, consistent with a monosulfated product.

Thus, the results of the absorption and NMR spectra, thin layer chromatography, and elemental analysis clearly indicated the product of the reaction to be monosulfated at position 6 of the glycosidic ring and free of disulfated substrate as well as unreacted starting material. In addition, the glycosidic bond was found to be intact and the aromatic ring structure unmodified by the reaction. This work has appeared in publication (Bayleran and Hechtman, 1983; Bayleran et al, 1984, refer to Appendix B).

B. Activity of isolated Hex A and Hex B toward 4MUGS.

Hex A and Hex B from normal human fibroblasts and Hex A, Hex B, and Hex I from normal human sera were separated by ion-exchange chromatography. The chromatographic profiles are shown in figures 8A (fibroblasts) and 8B (sera). Column fractions were assayed for hexosaminidase activity with both 4MUG and 4MUGS. All three isozymes had activity toward 4MUG, while 4MUGS was preferentially hydrolyzed by Hex A; Hex B and serum Hex I were barely active against 4MUGS. Human liver isozymes were also fractionated by ion-exchange chromatography after enrichment of Hex activity by concanavalin A-sepharose chromatography. The extraction of Hex from liver tissue, adsorption on concanavalin A-sepharose, and chromatographic separation of liver Hex isozymes were performed by Wayne Saray. DEAE column fractions of liver isozymes were assayed only with 4MUG. Pooled, dialyzed, and concentrated liver Hex isozymes were then employed to measure kinetic parameters of 4MUGS hydrolysis.

Enzyme-containing column fractions were pooled, dialyzed against column

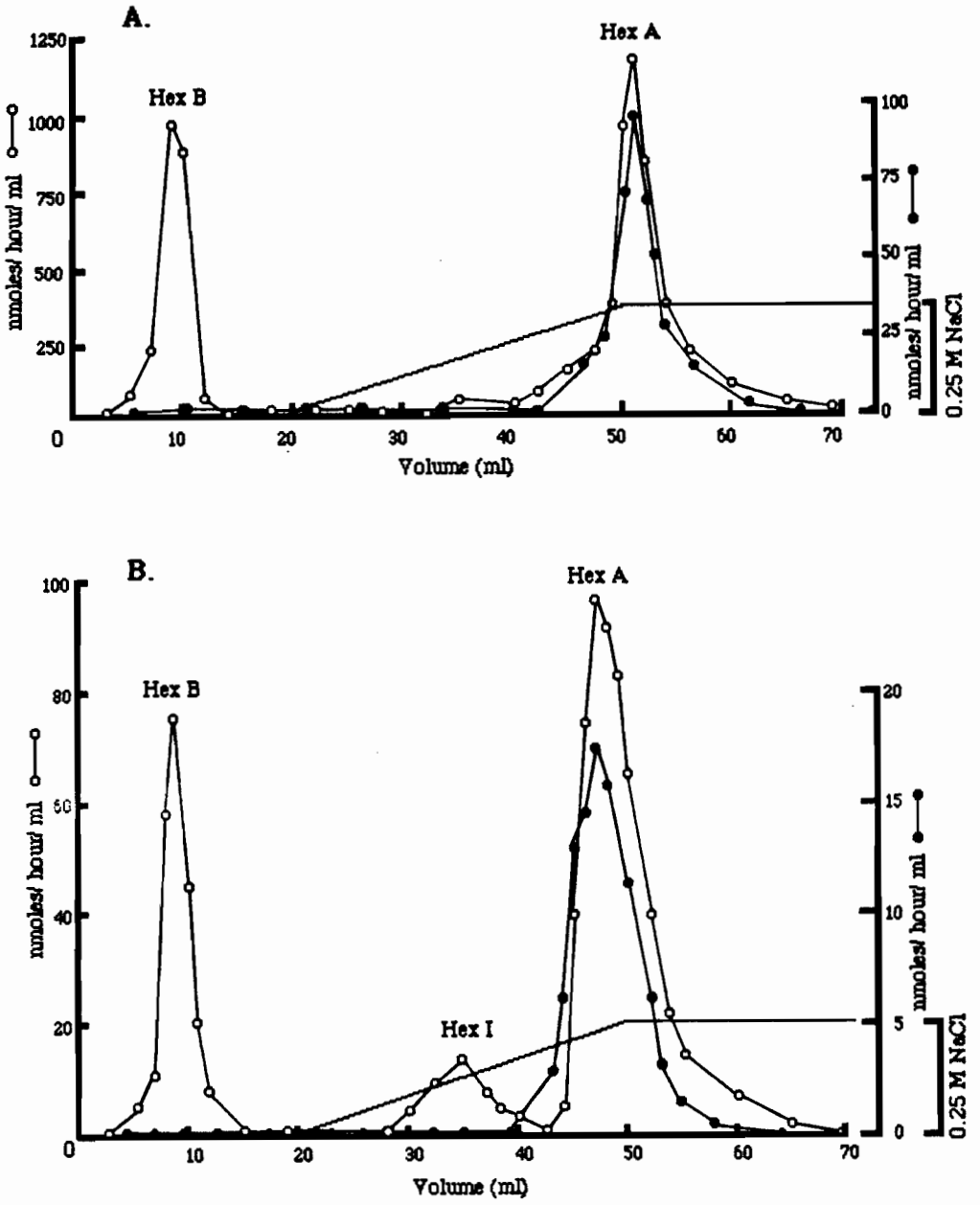

Figure 8. Ion-exchange chromatography of normal human fibroblast (A) and serum (B) hexosaminidases. Fibroblast pellets obtained fom cell cultures grown to confluence in 175 cm^2 flasks were extracted and the supernatants were chromatographed on DEAE-cellulose equilibrated in 10 mM NaH_2PO_4-NaOH, pH 6.0. Enzyme activities were normalized to fibroblast supernatant applied to the column. Control serum (1.3 ml) was dialyzed against 300 ml of the same column buffer and chromatographed on DEAE. The procedure of Nakagawa et al (1977) was used. Column fractions were assayed with 4MUG (o—o) and 4MUGS (●—●).

buffer, and concentrated by Aquacide II. The relative rates of hydrolysis of 4MUGS/ 4MUG by Hex A and Hex B (separated from normal fibroblast, serum, and liver) and Hex I (obtained from serum only), shown in table 1, were as follows: for Hex A, 0.066 (fibroblast), 0.10 (serum), and 0.10 (liver); for Hex B, 0.0015 (fibroblast), 0.0014 (serum), and 0.0034 (liver), and for Hex I, 0.0012 (serum). These relative rates of hydrolysis corresponding to constants r_A, r_B, and r_I for Hex A, Hex B, and Hex I, respectively, were employed in the empirical formula (page 59 of Materials and Methods) for the determination of Hex A specific activity and % Hex A using the sulfated synthetic substrate. Serum-derived Hex A retained the same r_A ratio after either prolonged storage at -20° C or multiple freezing and thawing. Fibroblast Hex A suffered a 3-fold to 4-fold decrease in the ratio under these conditions. Storage in the presence of human serum albumin (5 mg/ml) stabilized the fibroblast Hex A preparation.

pH-activity curves of Hex A- and Hex B-catalyzed hydrolysis of 4MUGS are shown in figures 9A and 9B. The pH optima of Hex A and Hex B were 3.9 and 3.2, respectively, and were the same for Hex isozymes isolated from normal liver (9A) and fibroblast (9B). Srivastava et al (1974b) demonstrated that the pH optima of both Hex A and Hex B were 4.4 when assayed with the synthetic substrate 4MUG. The pH optima of the two isozymes were not only different when assayed with 4MUGS, but the value of 3.9 for Hex A is similar to the pH optimum of 4.1 found for Hex A-catalyzed hydrolysis of G_{M2} ganglioside (O'Brien et al, 1977). In order to provide assay conditions that maximally select for Hex A-catalyzed hydrolysis of 4MUGS, citrate buffer at pH 4.2 was used in all assays employing the sulfated substrate.

Lineweaver-Burk plots for normal liver and fibroblast Hex A and Hex B, determined with 4MUGS, are shown in figures 10A and 10B, respectively. Liver

Table 1. Relative rates of hydrolysis of 4MUGS/ 4MUG by fibroblast, serum, and liver hexosaminidase isozymes. *

	r_A	r_B	r_I	Selectivity (r_A/r_B)
Fibroblast	0.066	0.0015	---	44
Serum	0.10	0.0014	0.0012	67
Liver	0.10	0.0034	---	29

* The values of r_A, r_B, and r_I were obtained under assay conditions and do not represent a comparison of Vmax values. Enzyme assay values are the average of four determinations.

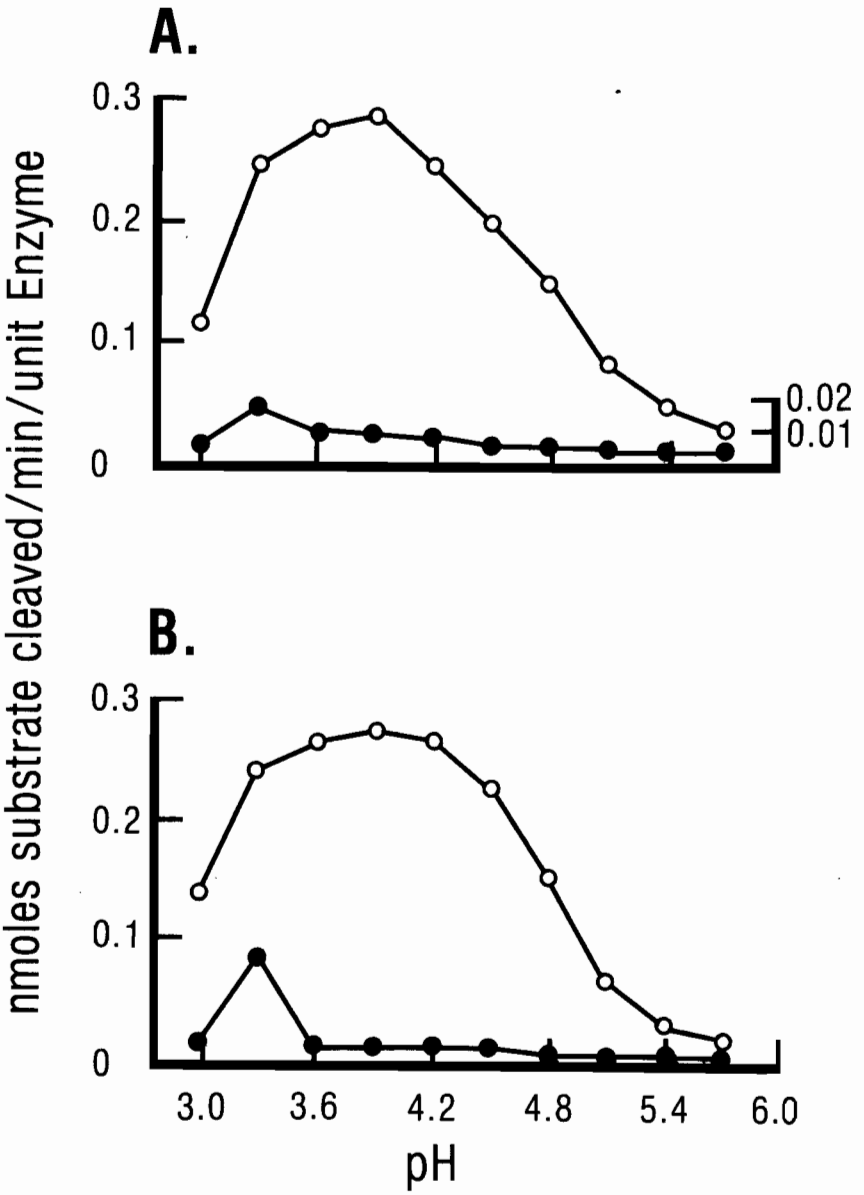

Figure 9. pH optima of hexosaminidase-catalyzed hydrolysis of 4MUGS. Liver (A) and fibroblast (B) Hex A (o—o) and Hex B (•—•) were assayed in 0.04 M sodium citrate buffer at the indicated pH's with 1.5 mM substrate. The scale on the left in figure 9A corresponds to Hex A-catalyzed hydrolysis of 4MUGS; Hex B-catalyzed hydrolysis of 4MUGS is represented on the right scale in figure 9A. One unit of enzyme activity is that amount of enzyme which hydrolyzes 1 nanomole of 4MUG/ minute.

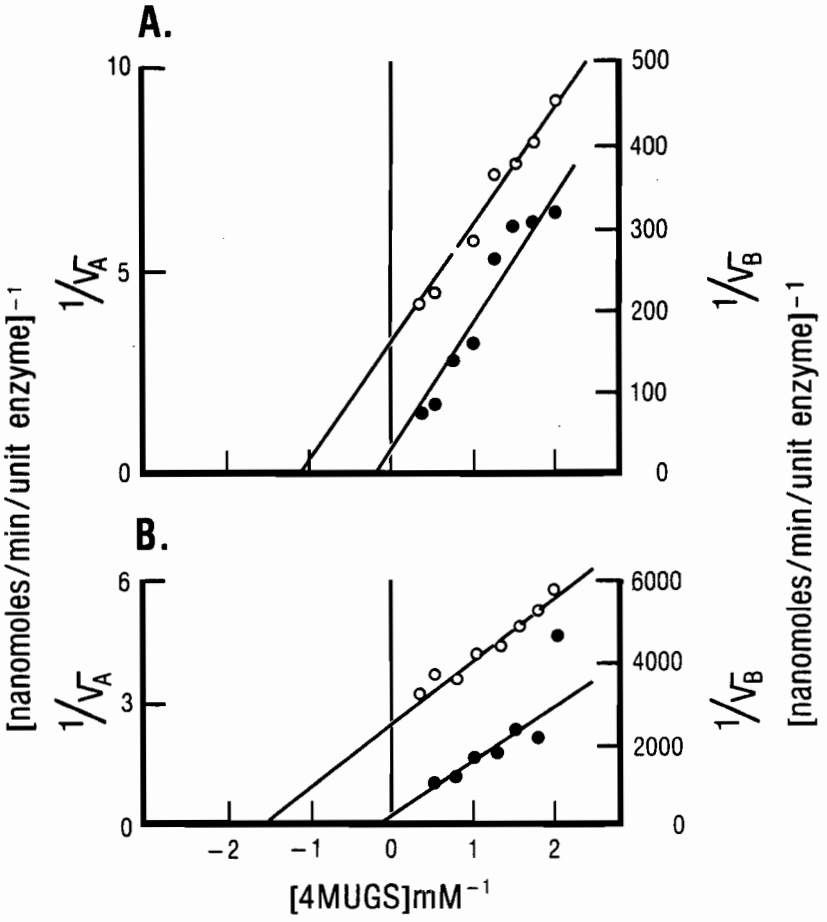

Figure 10. Lineweaver-Burk plots of normal human liver (top) and fibroblast (bottom) Hex A (o—o) and Hex B (●—●) for the hydrolysis of 4MUGS. Liver Hex A Km=0.94 mM, Vmax=0.31 nmoles/ min/ unit of enzyme; Liver Hex B Km=5.6 mM, Vmax=0.03 nmoles/ min/ unit of enzyme. Fibroblast Hex A Km=0.65 mM, Vmax=0.40 nmoles/ min/ unit of enzyme; Fibroblast Hex B Km=5.9 mM, Vmax=0.004 nmoles/ min/ unit of enzyme.

Hex A had a Km of 0.94 mM and a Vmax of 0.31 nmoles/ minute/ unit of enzyme while liver Hex B had a Km of 5.6 mM and a Vmax of 0.03 nmoles/ minute/ unit of enzyme. The Km of Hex A from a normal fibroblast cell strain was found to be 0.65 mM with a Vmax of 0.40 nmoles/ minute/ unit of enzyme. Fibroblast Hex B had a Km of 5.9 mM while its Vmax was 0.004 nmoles/ minute/ unit of enzyme. The Vmax for liver Hex A was found to be 10-fold greater than the Vmax for liver Hex B whereas a 100-fold difference was observed for the Vmax values of the fibroblast isozymes. The difference in the ratio of the relative rates of hydrolysis of 4MUGS by liver and fibroblast isozymes (i.e., the value of r_A/r_B) was attributable to a difference in Hex B-catalyzed hydrolysis of this substrate. The reason for the difference is unknown. However, it was thought that perhaps the partially purified liver Hex B preparation contained a sulfatase that converted 4MUGS to 4MUG, which is easily hydrolyzed by Hex B. Phosphate is known to be a competitive inhibitor of the activity of most sulfatases. Its presence in reaction mixtures containing 4MUGS should inhibit the release of 4MU by liver Hex B if the hydrolysis of the glycosidic linkage was dependent on the action of a sulfatase. As shown in table 2, in the presence of sodium citrate-sodium phosphate buffer (100 mM NaH_2PO_4) the release of 4MU by partially purified liver Hex B was inhibited 20% compared to the reaction mixture containing no phosphate ion. When either normal fibroblast Hex B or an unfractionated fibroblast extract of a homozygous TSD cell strain was used, phosphate inhibition of 4MU release from 4MUGS was not observed. Thus, in the liver Hex B preparation some sulfatase activity contributed to the hydrolysis of the glycosidic linkage of 4MUGS, although the phosphate inhibitable component did not appear to account for the higher Vmax for liver Hex B compared to the fibroblast Hex B preparation.

Table 2. Inhibition of sulfatase activity by phosphate.

	nmoles 4MUGS hydrolyzed/ ml/ minute	
Source of Hex B	+ phosphate	- phosphate
Unfractionated normal fibroblast cell extract	69.7	63.6
Unfractionated infantile TSD fibroblast cell extract	2.67	2.18
Isolated normal fibroblast Hex B	34.9	33.4
Isolated normal liver Hex B	54.4	66.9

Normal fibroblast and liver Hex B were isolated by ion-exchange chromatography as previously described. The source of phosphate as an inhibitor of possible sulfatase activity was sodium citrate/ sodium phosphate buffer, 0.04 M, pH 4.5. The concentration of phosphate was 0.06 M. Sodium citrate, 0.04 M, pH 4.5 was the buffer used in the absence of phosphate.

C. Hexosaminidase activity against 4MUG and 4MUGS.

1. Determination of Hex A in human fibroblast cell extracts.

An investigation of the value of 4MUGS for the diagnosis of TSD was undertaken by assay of fibroblast cell extracts with both 4MUG and 4MUGS. The results of the Hex A assays are shown in table 3 for a number of G_{M2} gangliosidosis variants. Hex A specific activities and % Hex A values were determined for each cell strain tested using two assay procedures: (i) thermal fractionation of hexosaminidase activity and assay of total and heat-stable Hex activities with 4MUG and (ii) assay of Hex activity with both 4MUG and 4MUGS and application of the formula. The range for % Hex A in the normal controls (N=9) and obligate heterozygotes (N=3) was 47-79% and 27-47%, respectively, when 4MUG was used as substrate and 46 -89% and 38-44%, respectively, by applying the formula to values obtained for hydrolysis of both 4MUG and 4MUGS. The two assay procedures yielded comparable results for all controls and heterozygotes tested.

Twelve infantile TSD fibroblast cell extracts were assayed. A description of the ethnic origin of each coded cell strain has been given on pages 62-63 of Materials and Methods. The first 9 cell strains are from patients with CRM-negative mutations. Cell strain WG1051 is from a patient with a CRM-positive mutation whose cells synthesize an α-chain polypeptide, yet do not produce catalytically active Hex A. The range for % Hex A in these 10 cell strains was 1-5% by thermal fractionation with 4MUG and 0.4-2% by the two-substrate assay procedure that employs 4MUGS. Because no Hex A is synthesized in these patients it should be possible, in theory, to obtain zero activity for Hex A measurements in tissues and fluids from affected individuals by using the constant r_B to determine the portion of 4MUGS activity attributable to Hex B. Although the source of the observed residual Hex A activity in homozygous TSD fibroblasts was not determined, two possibilities may account for

Table 3. Determination of Hex A activity in human fibroblast cell extracts.

	Hex A by thermolability using 4MUG		Hex A by application of formula using 4MUGS	
Cell strain	Hex A sp. act.	% Hex A	Hex A sp. act.	% Hex A
NORMALS				
N=9	3260-9280	47-79 %	3380-8290	46-89 %
OBL. HTZ.				
N=3	1342-7660	27-47%	2214-6077	38-44 %
INFANTILE TSD				
WG 93	140	4 %	69	2 %
WG 103	32	1	45	1.4
WG 1105	64	5	9	0.7
WG 1114	86	2	16	0.4
WG 107	139	4	19	0.5
WG 733	82	1	87	1
WG 884	120	5	33	1.4
WG 1499	58	2	15	0.5
WG 1065	103	2	36	0.7
WG 1051	120	5	33	1.4
WG 1108	538	16	26	0.7
WG 1110	1377	16	67	0.7
JUVENILE TSD				
WG 306	634	10 %	108	1.7 %
WG 312	464	13	157	4
WG 928	422	11	108	3
WG 1115	535	15	111	3
CHRONIC GM2 GANGLIOSIDOSIS				
WG 1047	170	9 %	43	2 %
WG 1048	281	8	90	3
ADULT GM2 GANGLIOSIDOSIS				
WG 1116	180	8 %	56	2 %
HEX A-DEFICIENT HEALTHY ADULT				
WG 1107	278	15 %	1030	56 %
WG 1111	661	18	2604	71
SANDHOFF				
WG 534	242	89 %	995	370 %
WG 1117	705	91	1558	200
AB VARIANT				
WG 802	4949	42 %	7167	61 %

Specific activity is expressed as nanomoles of substrate hydrolyzed/ mg protein/ hour and represents the mean of four determinations.

it. The activity of Hex B toward the sulfated substrate is much lower than Hex B activity toward 4MUG. Consequently, greater amounts of this enzyme are required to obtain measurements of 4MUGS hydrolysis. This may introduce inaccuracies into the calculation of r_B. Alternatively, the apparent Hex A activity observed in TSD fibroblasts against 4MUGS may be due to the action of a sulfatase. If present, a sulfatase would hydrolyze the O-6 sulfate linkage of 4MUGS to yield 4MUG which can rapidly be hydrolyzed by Hex B. Although phosphate, a potent inhibitor of most sulfatases, did not inhibit the hydrolysis of 4MUGS to 4MU using extracts of TSD cells, it is impossible to rule out completely that the low levels of 4MUGS hydrolysis in TSD cell extracts are due to the action of a sulfatase not inhibited by phosphate. In spite of this, both assay procedures accurately identified these patients as severely affected when compared to the values obtained for normal controls and obligate heterozygotes. It is important to note, however, that Hex A specific activities and % Hex A values determined using the two-substrate assay procedure were consistently lower than those measured by thermal fractionation.

Cell strains WG1108 and WG1110 are from patients with a CRM-positive mutation in which Hex A is synthesized. These patients have been classified as B^1 variants of TSD. Fibroblasts from these patients had Hex A activity comprising 16% of total Hex when measured by thermal fractionation with 4MUG yet they had < 1% Hex A when measured by the two-substrate assay procedure that employs 4MUGS. Assay with the sulfated substrate represents a clear improvement in the ability to diagnose patients with the B^1 variant form of TSD. A detailed analysis of the Hex A synthesized by cells of these patients has been published (Bayleran et al, 1987, refer to Appendix B) and will be presented later in this section.

Juvenile TSD cell strains WG306 and WG312 were obtained from siblings

born to consanguineous parents. They are, therefore, likely to be homozygous for an α-locus mutant allele. The clinical defect in juvenile TSD patients was described by Andermann et al (1977) and these two siblings correspond to family N in that publication. A partial deficiency of Hex A activity representing 10% and 13% of total Hex activity by the thermal fractionation assay procedure was found for WG306 and WG312, respectively. However, they were found to be severely deficient in Hex A activity by the two-substrate assay procedure that employs both 4MUG and 4MUGS. Percent Hex A values obtained by application of the formula in this assay procedure gave 1.7% and 4% for WG306 and WG312, respectively. A manuscript on the Hex A synthesized by the cells of these patients was recently accepted for publication (Hechtman et al, 1989, refer to Appendix B). Immunoprecipitation experiments, performed by Bernard Boulay, revealed that cells of both siblings synthesize a precursor α subunit of normal size that is not phosphorylated and, thus, no mature α subunit is detected. Exposure of cells to NH_4Cl-containing medium resulted in the secretion of β chains but not α chains, indicating that α-β subunit association does not occur. The finding of Hex activity that co-chromatographed with Hex A by ion-exchange chromatography with an optimum pH identical to that of control Hex A indicates that a small amount of Hex A survives the targeting pathway and is probably functional within the lysosomes. However, the residual activity of Hex A within the lysosome is most likely below a critical threshold level (Sandhoff and Conzelmann, 1985) and thus G_{M2} ganglioside accumulates significantly to produce a severe disease. The reduced Hex A activity measured with 4MUGS (table 3) thus represents an improvement in the ability of this assay procedure to diagnose these patients as affected with a severe form of TSD.

Cells from the other juvenile TSD patients, WG928 (whose clinical and biochemical phenotype have been reported elsewhere by Meek et al, 1984) and WG1115, were virtually identical to WG306 and WG312 in the residual Hex A activity measured by both thermal fractionation with 4MUG and the two-substrate assay procedure with 4MUG and 4MUGS. WG928 is the offspring of non-Jewish parents. The father of the proband was adopted and no information regarding his natural family was available (Meek et al, 1984). However, it is unlikely that the parents of WG928 are related. WG1115 is also the offspring of nonconsanguineous parents. The father of WG1115 is of Jewish descent; the mother is not Jewish. Since the parents of WG928 and WG1115 are unrelated it is likely that the probands are compound heterozygotes for two mutant alleles at the α locus. The four juvenile TSD cell strains presented here have a severe deficiency of Hex A measured by the assay procedure that employs the sulfated substrate. A similar severe deficiency based on hydrolysis of G_{M2} ganglioside has been measured in juvenile TSD cell strains (O'Brien et al, 1977; Kolodny and Raghavan, 1983; Meek et al, 1984).

Patients with the chronic and adult-onset forms of G_{M2} gangliosidosis were indistinguishable from each other based on the ability of their Hex A to hydrolyze both 4MUG and 4MUGS. Cell strains WG1047 and WG1048, from patients diagnosed with the chronic form of G_{M2} gangliosidosis, and WG1116, from a patient diagnosed with the adult-onset form of the disease (described by Kaback et al, 1978), had reduced Hex A activity by thermal fractionation representing 8-9% of total Hex activity. The two-substrate assay procedure employing 4MUGS gave values of 2-3% Hex A that were indistinguishable from earlier-onset forms of TSD. A similar severe deficiency of G_{M2} ganglioside hydrolysis by chronic and adult G_{M2} gangliosidosis cells has been demonstrated (Kolodny and Raghavan, 1983;

Raghavan et al, 1985). Compared to normal controls, chronic and adult G_{M2} gangliosidosis patients hydrolyze the natural substrate at a greatly reduced rate. However, the rate of degradation of G_{M2} ganglioside must be greater than that which occurs in the brain tissue of infantile-onset TSD patients in order to sustain life past the early childhood years. The lack of correlation between the degree of enzyme deficiency, as measured by various synthetic substrate assays, and the severity of the disease often makes accurate diagnosis based solely on the measurement of Hex A activity in a patient's cells difficult.

Cells from healthy individuals with an apparent deficiency of Hex A, WG1107 (described by Grebner et al, 1986) and WG1111, were found to have 15% and 18% Hex A, respectively, by thermal fractionation. They appeared indistinguishable from the B^1 and juvenile TSD variants based on 4MUG hydrolysis. However, by the two-substrate assay procedure and application of the formula WG1107 and WG1111 were found to have 56% and 71% Hex A, respectively. Thus, the assay procedure that employs 4MUGS accurately identifies these individuals as unaffected. An investigation of the prognostic value of the sulfated substrate for adults with low Hex A activity will be presented later in this section.

Cell strains from patients with G_{M2} gangliosidosis attributable to mutations at sites other than the α-locus were also cultured and cell extracts assayed by both procedures. WG534 is from a patient with the infantile form of Sandhoff disease, a G_{M2} gangliosidosis that is due to a mutation at the gene locus that encodes the β subunit of hexosaminidase. The finding of severely reduced total hexosaminidase in the Sandhoff disease cell strain together with the observation that 89% of the hexosaminidase activity was thermolabile was not unexpected. What was surprising, however, was the nearly 4-fold greater specific activity measured by the two-

substrate assay procedure than that determined by thermal fractionation. Hex S, an isozyme more thermolabile and more anodal than Hex A, accumulates in cells which are homozygous for the Sandhoff mutation (Beutler et al, 1975; Potier et al, 1979). If this isozyme is composed entirely of α subunits (Geiger et al, 1977), then Hex S is most likely the source of this elevated rate of 4MUGS hydrolysis.

Cell strain WG1117 is from a clinically normal Hex A- and Hex B-deficient adult who is the maternal aunt of an infantile SD patient. Total hexosaminidase activity, as measured by 4MUG, was reduced in cells of this individual with an increased proportion of thermolabile Hex (91%). Specific activity measured by the two-substrate assay procedure and application of the formula was 2-fold greater than that measured by thermal fractionation. Fibroblasts of SD heterozygotes have been found to have similarly low levels of total hexosaminidase activity measured with 4MUG, with an increased percentage of heat-labile Hex (Molzer and Bernheimer, 1976). This was initially thought to be attributable to increased Hex A. However, thermal inactivation of serum hexosaminidase isozymes of SD heterozygotes separated by ion-exchange chromatography, revealed that both Hex B and Hex I were thermolabile (Lowden, 1979). This suggested that SD heterozygotes might produce a ß-subunit polypeptide that is sensitive to heat.

Finally, the AB variant form of G_{M2} gangliosidosis is attributable to a mutation at the gene locus that encodes the activator protein necessary for the in vivo hydrolysis of G_{M2} ganglioside by Hex A. Patients with this form of the disease have been shown to synthesize normal Hex A and Hex B (Conzelmann et al, 1978; Hechtman et al, 1982). Because hydrolysis of synthetic substrates such as 4MUG and 4MUGS is not dependent upon the action of an activator protein, these substrates will be hydrolyzed normally by the Hex A of AB variant patients. Hex A of cell strain WG802, Sandhoff's original patient (Sandhoff et al, 1971), represented 42% of total

Hex by thermal fractionation and 61% by the assay procedure that employs the sulfated substrate.

Accurate diagnosis of some of the variant forms of TSD is often difficult due to a lack of correlation between the degree of enzyme deficiency and the severity of the disease. Use of the 4MUG substrate to measure Hex A activity further complicates the problem since this substrate is not specific for Hex A and, as such, does not directly assess the product of the mutant gene. The requirement for thermal fractionation in an assay employing 4MUG has been associated with inaccuracies due to variable amounts of Hex B inactivated or Hex A remaining; the reproducibility of heating conditions from one assay to the next presents a problem. Not all of these problems have been alleviated by the use of 4MUGS to measure Hex A. However, this substrate is preferentially hydrolyzed by Hex A. The elimination of the requirement for thermal fractionation does represent an improvement. While both assay procedures detect Hex A activity in cell extracts obtained from infantile TSD patients, these values are considerably lower by the two-substrate procedure that employs the sulfated substrate. More importantly, improved diagnosis has been achieved for those variant forms of TSD in which Hex A is synthesized but is catalytically inactive. The improved diagnosis of the B^1 variant form of TSD is an example of the value of the 4MUGS assay procedure. The two B^1 variants studied here are believed to be compound heterozygotes. The reliance upon the thermal fractionation assay to measure amniocyte Hex A activity resulted in the misdiagnosis of one of the B^1 probands (Kolodny et al, 1983). Hex A activity by the thermal fractionation assay approaches carrier levels in the B^1 compound heterozygotes. However, Hex A values are clearly in the range of other severely affected infantile TSD patients when the two-substrate assay procedure is used to assess Hex A activity. Thus, use of this assay procedure would not have resulted in misdiagnosis

of the B^1 TSD proband. B^1 homozygotes who have a juvenile presentation of TSD have also been identified. Thermolabile Hex activity against 4MUG is within the normal range in these patients (Goldman et al, 1980; Li et al, 1981; Inui et al, 1983) and they would most definitely be misdiagnosed prenatally by the thermal fractionation assay procedure. The two-substrate assay procedure would accurately diagnose them as severely affected.

The 4MUGS assay procedure is also able to differentiate between adult patients with a G_{M2} gangliosidosis (e.g. patients with the chronic or adult-onset forms) and those adults who are asymptomatic in spite of very low levels of Hex A by thermal fractionation. Adults with low Hex A activity are predominantly, though not exclusively, of Ashkenazi Jewish descent. They are usually ascertained through routine TS carrier screening by measurement of thermolabile Hex activity with 4MUG. Many of these individuals have reported the occurrence of infantile TSD in a member of their family and are thought to be compound heterozygotes for a classical TSD allele and a milder mutant allele. The two-substrate assay procedure offers the potential to predict the development of a neurological disease in adults with low Hex A activity detected through carrier screening.

The inability to distinguish between classical TSD carriers and carriers of milder mutant alleles at the α locus as well as the appearance of these variant alleles in infantile TSD families makes prenatal diagnosis particularly difficult. The two-substrate assay procedure may prove useful in prenatal diagnosis, since it clearly differentiates between a fetus affected with a neurological disorder and one which is not affected. However, the two-substrate assay procedure is not able to clearly differentiate between those fetuses which will have a severe form of TSD and those which will be only mildly affected with a later-onset form.

2. Determination of Hex A activity in human sera.

Serum hexosaminidase activities were measured by both thermal fractionation of activity toward 4MUG and the two-substrate assay procedure that employs 4MUG and 4MUGS in order to compare the usefulness of the two assay procedures for classification of Tay-Sachs carriers. The reliability of the two assay procedures was assessed by comparing the coefficients of variation (S.D./ mean) for eight determinations performed on single serum samples obtained from a normal control and an obligate heterozygote. The coefficients of variation for the control were 0.010 for total hexosaminidase (4MUG), 0.020 for heat-labile hexosaminidase (4MUG), and 0.0084 for hexosaminidase activity against 4MUGS. The coefficients of variation for the obligate heterozygote were 0.0073 for total hexosaminidase, 0.032 for heat-labile Hex, and 0.012 for 4MUGS Hex activity. Thus, the reliability of Hex A measurements was improved approximately 3-fold by the use of 4MUGS for the measurement of Hex A. The increased reliability of the 4MUGS assay is presumably a reflection of the difficulty in reproducing heating conditions in the 4MUG assay.

Figure 11 presents the results of Hex A and Hex B determinations using sera obtained from 45 normal controls, 24 obligate heterozygotes, and 5 infantile TSD probands. Each point represents the mean of four determinations on a single serum sample. The values plotted on the ordinates of figures 11A and 11B represent the sum of Hex B and Hex I. However, since these isozymes behave identically in the thermal fractionation procedure and since both species have identical ratios of activity toward the two substrates, the ordinates have been labelled 'Hex B' for convenience. Assistance was provided by David Sovetts and Norbert Laschic in the collection of blood samples from individuals tested and the centrifugation of the samples to obtain serum, respectively.

Figure 11A shows the values of Hex A and Hex B, obtained from

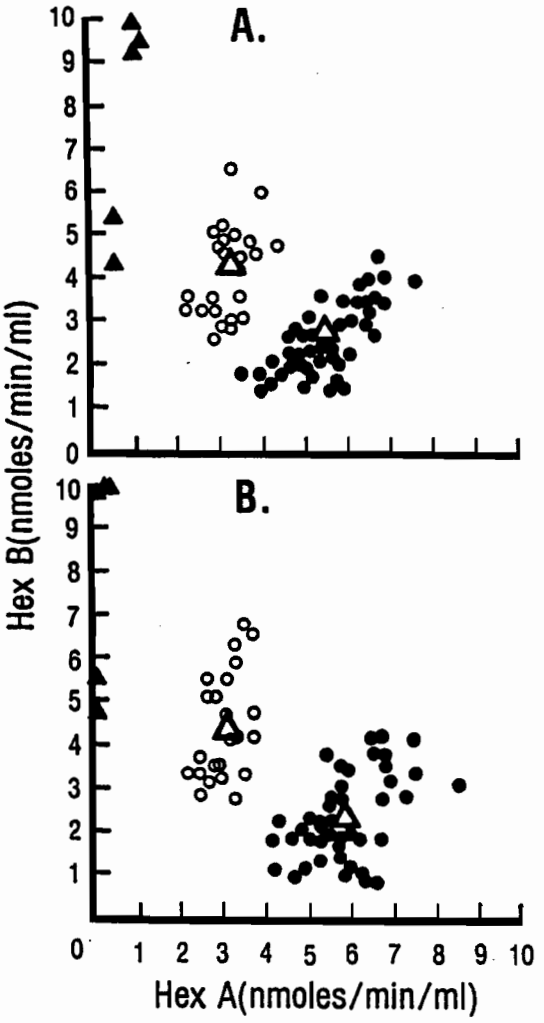

Figure 11. Determination of Hex A activity in human sera. Hex A was determined by thermolability using 4MUG (A) and by application of the equation using 4MUGS (B). Thermal fractionation with 4MUG: Hex A, mean ±S.D. for controls 5.53±0.96, for heterozygotes 3.28±0.64. Hex B, mean±S.D. for controls 2.62±0.83, for heterozygotes 3.96±1.17. 4MUGS procedure: Hex A, mean±S.D. for controls 5.82±0.92, for heterozygotes 3.03±0.60. Hex B, mean±S.D. for controls 2.33±1.01, for heterozygotes, 4.21±1.17.
● = normal controls, o = obligate TS heterozygotes, ▲ = infantile TSD probands,
Δ = mean values for normal control and carrier groups.

measurements of heat-labile and heat-stable hexosaminidases, plotted as a two-discriminant test. Figure 11B shows Hex A and Hex B values obtained by measurement of total Hex activity with 4MUG and transformation of 4MUGS assay values by application of the equation derived in Appendix A. Using this transformation, the values for Hex A and Hex B can be plotted as a two-discriminant test, comparable to that of figure 11A. While the two graphs look essentially similar, closer inspection of the distribution of data points reveals a slight statistical improvement in classification by the two-substrate assay procedure. This is indicated by the greater separation of means for each group as well as smaller S.D. values obtained with the 4MUGS assay procedure (11B) than when heat-labile hexosaminidase activity is measured with 4MUG (11A). In addition, no overlap occurs between the range of Hex A values for controls and heterozygotes determined by the two-substrate assay procedure whereas there is considerable overlap between the range of these values for the control and carrier groups by the thermal fractionation assay with 4MUG.

The classification of genotypes based on two discriminants has been shown to allow greater resolution of test values for the two groups than can be obtained with a single test (Gold et al, 1974). However, figure 12 provides a comparison of serum Hex A values for the three genotypes plotted as a single discriminant and without reference to total Hex activity. The data indicate that the range of Hex A values measured as 4MUGS-cleaving activity (without transformation of 4MUGS assay values into equivalent 4MUG enzyme units) for control sera does not overlap with the range of heterozygote values, whereas considerable overlap is observed between the control and heterozygote groups when serum Hex A activity is based on thermal fractionation. In theory, therefore, a one-step assay procedure could be used for the detection of Tay-Sachs heterozygotes by measurement of 4MUGS hydrolysis. However, the superior resolution offered by the use of two discriminants should not

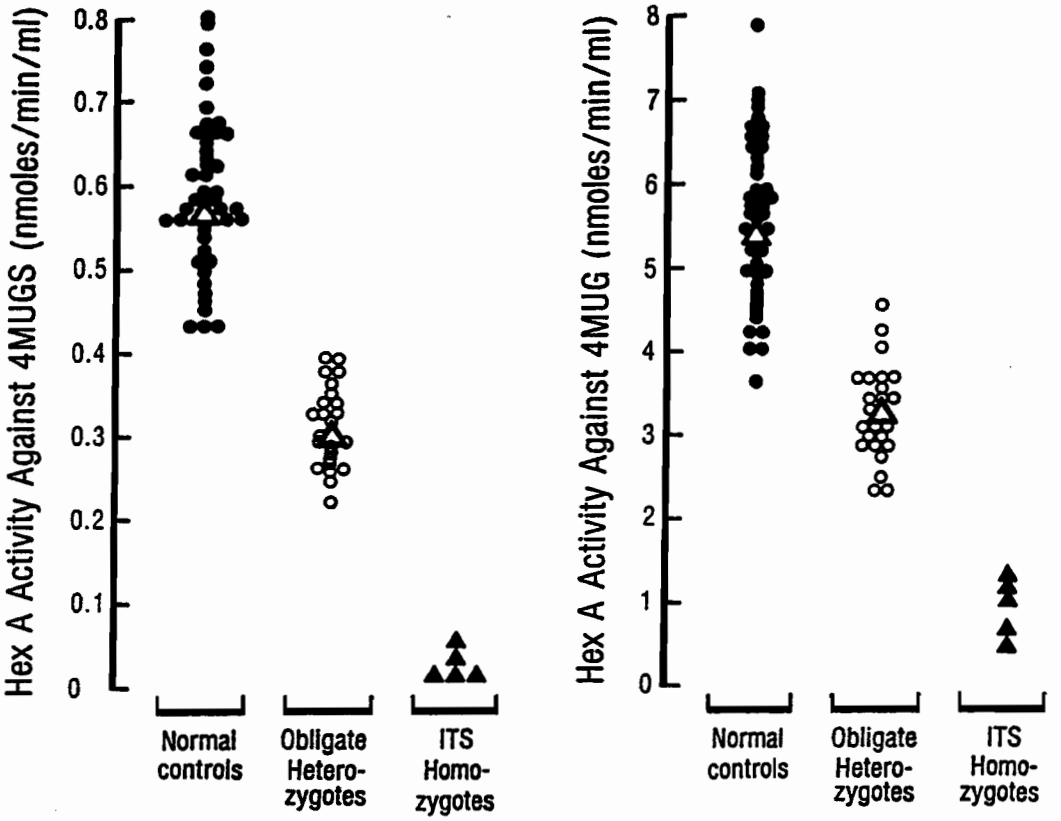

Figure 12. Activity of sera toward 4MUGS (left) compared to heat-labile activity toward 4MUG (right). Hexosaminidase A measurement using 4MUGS: mean±S.D. 0.59±0.10 for controls, 0.31±0.05 for heterozygotes.
● = normal controls, o = obligate TS heterozygotes, ▲ = infantile TSD probands, Δ = mean values for normal control and carrier groups.

be abandoned in favor of the increased speed of the one-step 4MUGS procedure. The data represented in figures 11 and 12 are also presented in tabular form (table 4) to facilitate comparison between the Hex A and Hex B values obtained for controls and heterozygotes in this study with those values obtained for other controls and carriers in studies presented later in this section.

In figure 13, % Hex A values determined by both assay procedures are plotted for each individual tested. Percent Hex A plotted on the ordinate was obtained by measuring total activity against both 4MUG and 4MUGS and transforming these values by the equation. The % Hex A values plotted on the abscissa were obtained by measurement of heat-labile Hex activity with 4MUG. The correlation coefficient ($r=0.78$) indicates a high degree of correlation between the two assay procedures in identifying TS carriers by the quantity '% Hex A'. Thus, both assay procedures resolved the control and carrier genotypes into well-separated clusters. Furthermore, for each individual tested there was good correlation between the % Hex A values obtained by the thermal fractionation and two-substrate assay procedures. The control and heterozygote groups had ranges of 61-81% Hex A and 34-54% Hex A, respectively, measured as heat-labile hexosaminidase. Using the two-substrate procedure, the range for controls was 60-88% Hex A and for heterozygotes 35-55% Hex A. Among the five infantile TSD probands (two of Jewish ancestry, two of French Canadian descent, and one of Italian origin) whose serum Hex A was determined, the range for % Hex A (9-11%) by thermolability was much higher than the range (0-2%) measured by the assay procedure that employs 4MUGS. The measurement of thermolabile hexosaminidase in fluids obtained from TSD homozygotes frequently gives values as high as 15% Hex A (Saifer and Perle, 1977). This artifact is presumably due to loss of variable amounts of Hex B during the thermal fractionation procedure.

Table 4. Determination of serum Hex A activity. *

	Hex A		Hex B	
	range	mean±S.D.	range	mean±S.D.
Thermal fractionation with 4MUG				
Controls (N=45)	3.63-7.90	5.53±0.96	1.28-4.72	2.62±0.83
Obligate TS Heterozygotes (N=24)	2.17-4.60	3.28±0.64	2.63-6.63	3.96±1.17
4MUGS with application of formula				
Controls (N=45)	4.06-7.61	5.82±0.92	0.70-4.19	2.33±1.01
Obligate TS Heterozygotes (N=24)	2.10-3.80	3.03±0.60	2.65-6.53	4.21±1.17
4MUGS without application of formula				
Controls (N=45)	0.43-0.87	0.59±0.10		
Obligate TS Heterozygotes (N=24)	0.22-0.39	0.31±0.05		

*The results presented here are the same as those shown graphically in figures 11 and 12. They are presented in tabular form to facilitate comparison with values shown in tables 5, 6, and 12.

Hex A and Hex B activities are expressed as nanomoles of substrate hydrolyzed/ml/minute and represent the mean of four determinations.

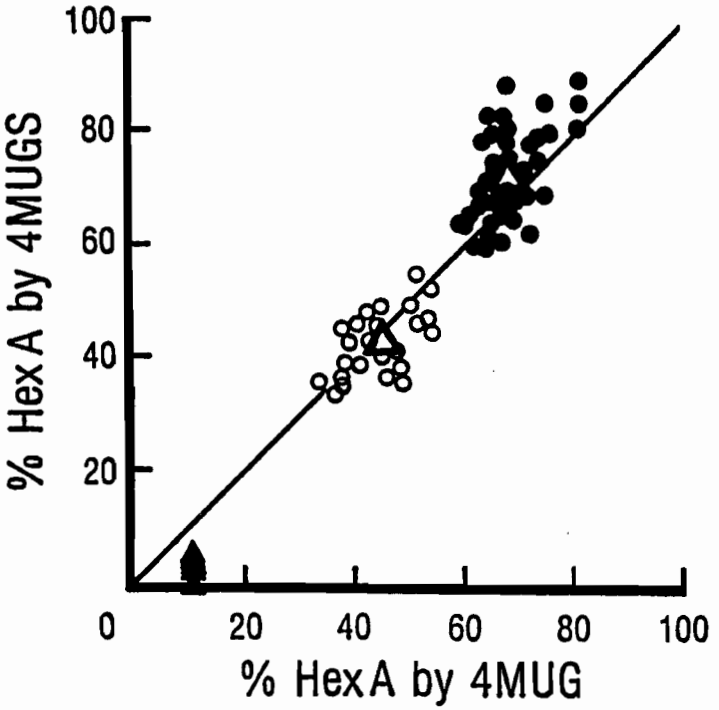

Figure 13. Correlation of % Hex A determined with 4MUGS and by thermal fractionation with 4MUG. Percent Hex A mean by thermolability 68% for controls and 46% for obligate heterozygotes. Percent Hex A mean by the 4MUGS procedure 72% for controls and 42% for obligate heterozygotes.
● = normal controls, o = obligate TS heterozygotes, ▲ = infantile TSD probands,
Δ = means for normal control and carrier groups.
The line represents the expected agreement between pairs of assay values. Correlation coefficient = 0.78.

3. Heterozygote screening by an automated assay procedure.

In the two-discriminant plot of Hex A and Hex B activities measured with both 4MUG and 4MUGS and application of the formula (figure 11B), the absence of overlap of Hex A values between the control and heterozygote groups indicated that this substrate procedure could be used for the detection of Tay-Sachs carriers, thus eliminating the requirement for thermal fractionation of hexosaminidase activity. With that in mind, the potential of the two-substrate assay for mass Tay-Sachs carrier screening by an automated procedure was investigated. The automated assay procedure currently employs 4MUG as substrate and is dependent upon thermal fractionation. This work was performed by Maria diLorenzo, a 2nd year medical student at McGill University, under the co-supervision of Dr. Charles Scriver and myself. Norbert Laschic adapted the apparatus to use with the sulfated substrate as per our specifications (refer to Materials and Methods, page 60) and ran the samples for us by the automated procedure. The preparation of 4MUGS used in this investigation was purchased from HSC Corporation (Toronto, Ontario, Canada). Because preliminary tests with this substrate indicated a high degree of impurity as evidenced by significant Hex B activity against the 4MUGS preparation, we purified the substrate by ion-exchange chromatography and gel filtration. A significant amount of unreacted starting material, 4MUG, was eliminated by the purification procedure and represented approximately 25% of the material applied to the ion-exchange column.

An aliquot of each serum sample to be tested was taken up by a sampler probe and split into two lines containing the appropriate buffers and substrates, as described on page 60. Hydrolysis of 4MUG and 4MUGS produced fluorescence which was read on two fluorometers. In order to obtain peak heights within the readable range of the charts (i.e. between 10-90 chart units) it was necessary to adjust the sensitivity of the fluorometer reading 4MUGS hydrolysis to a setting that was

10-fold greater than the setting of the fluorometer reading 4MUG hydrolysis. This necessitated, in turn, calibrating the fluorometers using two different sets of standard solutions. Fluorescence read on the fluorometers was registered on a recorder which traced two curves. Figure 14 is a sample of the tracings obtained with the automated assay procedure. The left figure shows the tracings obtained by thermal fractionation with 4MUG. Peaks '1' and '2' represent total and heat-stable hexosaminidase activities, respectively. Tracings obtained from the 4MUGS assay procedure are shown in the right figure. Peak '3' represents total Hex as measured with 4MUG; peak '4' represents 4MUGS-cleaving activity. In both automated procedures the peak heights, measured in chart units, were converted into an amount of enzyme activity with the use of 4MU standards. The concentration of each 4MU standard solution was divided by 5 (which is the number of minutes the serum samples were incubated with substrate in the 37° C water bath) and by the molecular weight of 4MU. This gave a constant value for each standard solution, the units of which were nanomoles 4MU/ ml/ minute. Average peak heights for each standard solution were then plotted against the constant value for that solution to generate a standard curve, the ordinate of which was in nanomoles 4MU/ ml/ minute while the abscissa was in chart units. The slope of the standard curve, in nanomoles 4MU/ ml/ minute/ chart unit, was calculated for each fluorometer. The number of chart units in the peak heights for each serum sample was multiplied by the slopes to yield a value of enzyme activity. Hexosaminidase A activity and the quantity of '% Hex A' by 4MUGS were determined by application of the empirical formula. Hex A activity against 4MUGS alone, i.e. without the formula, was also determined for each serum sample tested.

Table 5 shows the results of screening of 31 normal controls, 8 obligate TS heterozygotes, and 16 nonobligate TS carriers by the automated assay procedure employing 4MUG to measure total Hex activity and 4MUGS to measure primarily Hex A activity. The hexosaminidase activities generated by the automated procedure

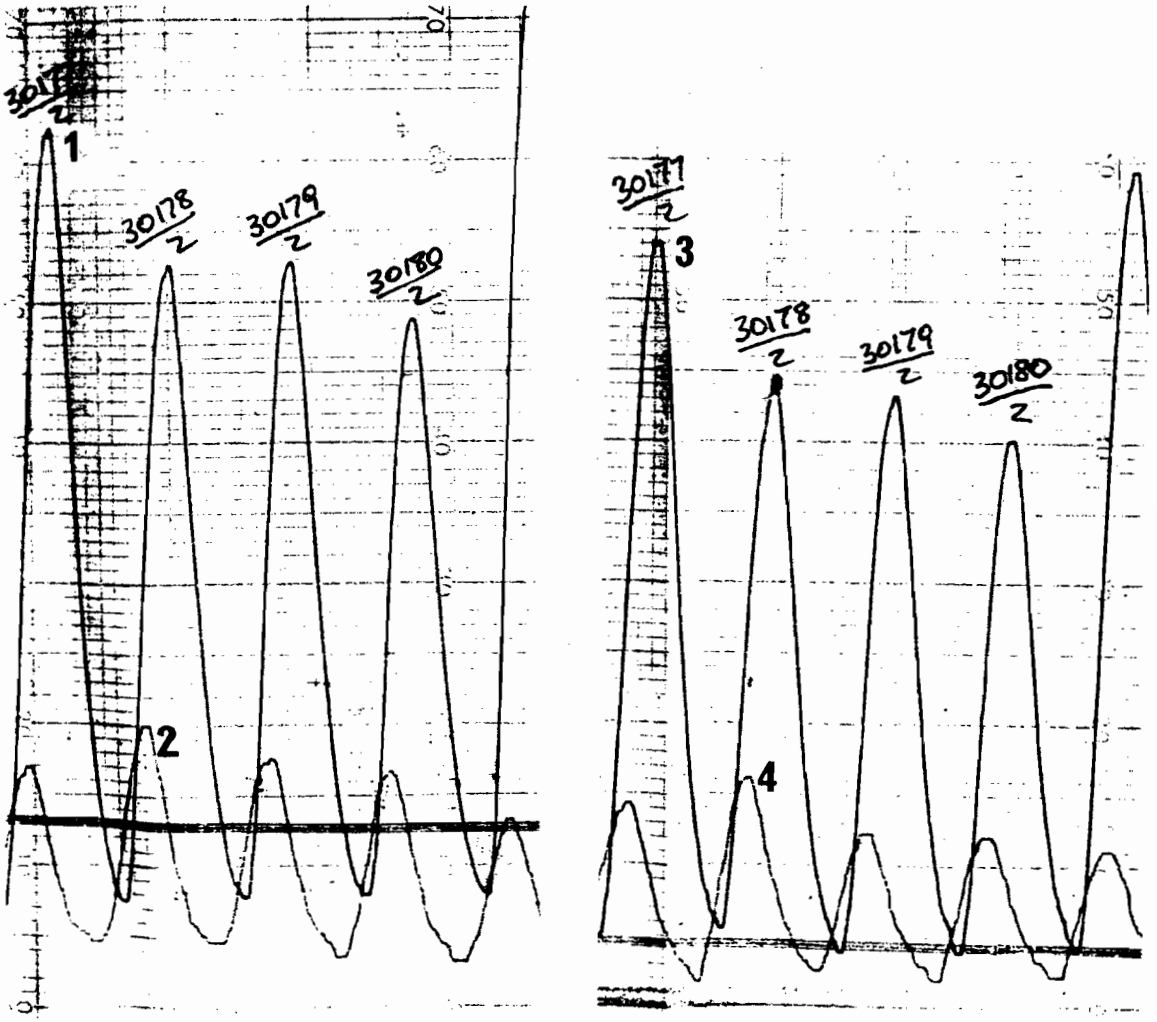

Figure 14. Samples of tracings obtained from the automated assay procedure. The curves traced by the recorder are one peak out of phase.The left figure is a typical tracing obtained in the automated assay employing thermal fractionation and 4MUG. Peak '1' corresponds to total Hex activity; Peak '2' represents heat-stable Hex activity. The right figure is a tracing obtained in the automated assay procedure, adapted for use with both 4MUG and 4MUGS. Peak '3' corresponds to total Hex activity as measured with 4MUG; Peak '4' represents Hex activity against 4MUGS.

Table 5. Heterozygote screening by an automated assay procedure.

	Hex A		Hex B	
	range	mean ± S.D.	range	mean ± S.D.
Thermal fractionation with 4MUG				
Controls (N=31)	8.20-15.2	10.7±1.61	2.50-10.1	5.53±1.81
Obligate TS Heterozygotes (N=8)	3.80-5.90	4.85±0.84	7.30-16.9	8.51±4.62
Nonobligate TS Heterozygotes (N=16)	3.70-8.81	6.74±1.32	5.60-10.9	7.93±1.61
4MUGS with application of formula				
Controls (N=31)	9.50-16.4	12.2±1.90	1.00-10.8	4.91±1.98
Obligate TS Heterozygotes (N=8)	5.40-7.20	6.38±0.82	6.40-17.2	9.20±3.76
Nonobligate TS Heterozygotes (N=16)	4.40-8.90	6.91±1.28	4.40-12.3	7.78±2.37
4MUGS without application of formula				
Controls (N=31)	1.60-2.70	1.96±0.33		
Obligate TS Heterozygotes (N=8)	0.94-1.20	1.09±0.13		
Nonobligate TS Heterozygotes (N=16)	0.70-1.40	1.10±0.21		

Hex A and Hex B activities are expressed as nanomoles of substrate hydrolyzed/ml/minute and represent the mean of four determinations.

(table 5) were higher than those obtained by the manual procedure (table 4) for both controls and heterozygotes. Interesting differences were revealed when ranges and means for Hex A activity determined by the automated assay procedure were compared for the obligate and nonobligate TS heterozygotes. By both thermal fractionation with 4MUG and the two-substrate assay employing 4MUG and 4MUGS, there was a greater separation of means between the obligate carriers and normal controls than between the nonobligate TS carriers and normal controls. While the separation of obligates and normal controls was comparable by both assay procedures, better discrimination between the nonobligate TS heterozygote group and the normal controls was achieved by the assay procedure employing the sulfated substrate. Improved discrimination was revealed by means that were significantly farther apart by the two-substrate assay procedure as well as the absence of overlap in the range of Hex A activities for the nonobligate TS carrier and normal control groups. Such overlap was observed in the thermal fractionation assay employing 4MUG. Thus, it would appear that while both assay procedures are comparable in the ability to discriminate normal controls and obligate TS heterozygotes, the ability to detect TS carriers whose genotypes are not known with certainty by virtue of their having an affected child is improved by the assay procedure that utilizes the sulfated substrate. More samples need to be tested, but the results are very encouraging and indicate the potential of 4MUGS for the identification of Tay-Sachs carriers by an automated assay procedure independent of thermal fractionation. However, great care must be taken in the preparation of 4MUGS to ensure a pure product free of contaminants that might reduce the specificity of this substrate for Hex A.

4. Determination of carrier status during pregnancy by serum Hex A assay.

Table 6 shows the results of serum Hex A assays performed manually on

Table 6. Determination of carrier status during pregnancy by serum Hex A assay.

	Hex A		Hex B	
	range	mean±S.D.	range	mean±S.D.
Thermal fractionation with 4MUG				
Controls (N=18)	4.50-11.1	7.13±1.57	2.70-12.0	7.68±2.96
Obligate TS Heterozygotes (N=7)	1.30-5.30	3.76±1.25	2.30-17.4	7.76±5.30
4MUGS with application of formula				
Controls (N=18)	4.60-13.4	7.66±1.99	2.90-12.5	7.17±2.85
Obligate TS Heterozygotes (N=7)	1.20-4.40	2.86±0.96	2.40-18.3	8.66±5.37
4MUGS without application of formula				
Controls (N=18)	0.66-1.41	0.94±0.20		
Obligate TS Heterozygotes (N=7)	0.14-0.59	0.41±0.16		

Hex A and Hex B activities are expressed as nanomoles of substrate hydrolyzed/ml/minute and represent the mean of four determinations.

18 pregnant normal controls and 7 pregnant obligate TS heterozygotes. Total hexosaminidase activity is increased in the sera of pregnant women, mostly attributable to increased synthesis of the heat-stable isozyme, Hex P (Ben-Yoseph et al, 1988). The presence of this intermediate Hex isozyme is evident when one compares the values for Hex A and Hex B activities for pregnant controls and obligate heterozygotes presented in this study with those values for controls and obligate heterozygotes presented earlier in table 4 (page 101). A significant increase in the amount of 'Hex B' (which actually represents the sum of heat-stable Hex activities) was evident in the sera of pregnant normal controls and carriers. The synthesis of Hex P during pregnancy presents special problems for the accurate detection of TS carriers by serum Hex A assay. The increased levels of total hexosaminidase result in low % Hex A values with the consequent risk of the misclassification of pregnant women as carriers. As shown in table 6, the range of Hex A activities in the sera of pregnant controls and obligate TS heterozygotes overlapped when measured by the thermal fractionation assay with 4MUG. However, the overlap was attributable to a high value for Hex A activity in only one of the heterozygotes tested. No overlap of Hex A activities between the pregnant control and obligate heterozygote groups was observed when Hex A was measured by the two-substrate assay employing 4MUGS and application of the formula. Percent Hex A values were low, as in the thermal fractionation assay, since the determination of % Hex A by application of the formula is dependent upon total Hex as measured with 4MUG, a value to which Hex P contributes.

Assessment of Hex A activity with the sulfated substrate alone is perhaps preferable to the other assay procedures since hydrolysis of this substrate is independent of the heat-stable isozymes, Hex B and Hex P. Indeed, the Hex A values of pregnant controls and obligate heterozygotes based on 4MUGS hydrolysis did not overlap. However, it must be noted that only a small number of controls and

heterozygotes were tested.

Ben-Yoseph et al (1988) evaluated the usefulness of sulfated fluorogenic substrates for the detection of carriers by serum assay during pregnancy. They found that two changes in serum Hex activity occur simultaneously in pregnant women. One is the increase in the thermostable Hex P isozyme, which hydrolyzes only 4MUG. They also noted, however, a significant rise in the level of Hex A which was found to gradually increase with gestational age. The increased Hex A is thought to be of fetal origin, attributable to leakage of fetal Hex A across the placenta into the mother's circulation. If this is an accurate assessment, then measurement of Hex A with the sulfated substrate would also be considered inaccurate since it would be influenced by the genotype of the fetus. The greatest danger in such a case would be the misclassification of a carrier mother as a noncarrier. Since Hex P is not normally present in cells and fetal Hex A would only be observed in the serum of the mother, it is possible that accurate detection of TSD heterozygosity in pregnant women by assay of serum Hex isozymes is not possible and should only be performed on leukocytes. Clearly, more testing is necessary.

D. Infantile TSD in an Italian family.

Infantile TSD was discovered in a 12-month-old child of Italian origin when she was brought to the Montreal Children's Hospital for evaluation. A skin biopsy obtained from this child corresponds to cell strain WG1051 (Materials and Methods, page 62) and was recently described by Zokaeem et al (1987; refer to Appendix B). The child was noted to have the typical signs of TSD: she startled easily, appeared unaware of her surroundings, was unable to sit unsupported, etc. Macular cherry red spots were observed. Hex A assay of serum and fibroblasts confirmed the diagnosis of TSD.

1. Pedigree of Italian TSD family.

The child's parents originated from the same small village in Italy. Pedigree analysis (performed by Dr. Paige Kaplan, Montreal Children's Hospital) revealed that the parents of the proband were related through both their fathers and mothers. A partial pedigree of this family is shown in figure 15. The proband's grandmothers were first cousins while her grandfathers were third cousins. The proband was likely a homozygote.

2. Measurement of Hex A activity in TSD proband and family.

The results of Hex A assays, confirming the diagnosis of TSD, are presented in table 7. By the thermal fractionation assay with 4MUG, % Hex A values of proband sera and fibroblasts were found to be 10% and 5%, respectively. This was in the range characteristic for other infantile TSD patients. By the two-substrate assay procedure that employs 4MUGS, proband Hex A values in sera and fibroblasts were 0% and 1.4%, respectively. Thus, the sulfated substrate assay procedure yielded significantly lower values for Hex A specific activity and % Hex A than the thermal fractionation assay procedure. The parents of the proband were clearly identified as heterozygotes by both assay procedures, while the sister of the proband was identified as a normal homozygote. Sera obtained from 11 second and third cousins of the proband (paternal relatives of the proband's father) were also tested to determine carrier status. Six of them were assigned such status by both assay procedures.

3. Fractionation of proband Hex isozymes by ion-exchange chromatography.

Ion-exchange chromatography of proband fibroblast cell extract revealed no peak associated with Hex A activity when column fractions were assayed with either 4MUG or 4MUGS. The chromatographic profile of proband Hex isozymes is shown

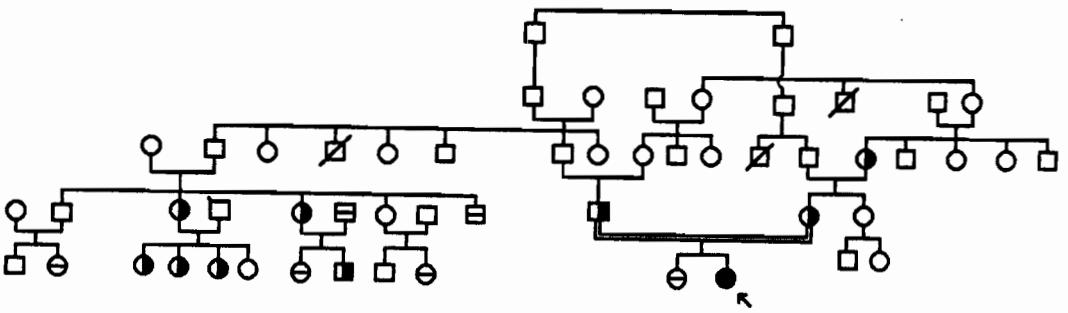

Figure 15. Partial pedigree of an Italian TSD family. The proband (WG1051, indicated by the arrow) was born to consanguineous parents who are related through both their mothers and fathers. The proband's grandmothers were first cousins; her grandfathers were third cousins. □, ○= not tested; ⊟, ⊖= tested, and normal homozygote; ◨ , ◑= tested, and heterozygote.

Table 7. Determination of Hex A activity in an Italian TSD proband and her family.

Tissue and Subject	Hex A specific activity (%) by thermolability		Hex A specific activity (%) by 4MUGS *	
Serum:				
Proband	64	(10%)	0.6	(0%)
Mother	147	(45)	131	(40)
Father	242	(39)	230	(38)
Sister	389	(65)	410	(68)
Controls (N=45)	218-474	(60-81)	243-457	(60-88)
Obligate TS Heterozygotes (N=24)	130-276	(34-54)	126-228	(35-55)
Tay-Sachs patients (N=5)	32-74	(9-11)	0.6-17	(0-2)
Fibroblasts:				
Proband	120	(5%)	33	(1.4%)
Controls (N=9)	3260-9280	(47-79)	3380-8290	(46-89)
Tay-Sachs patients (N=9)	32-140	(1-5)	9-87	(0.4-2)

For serum, activity is expressed as nmoles of substrate hydrolyzed/ ml/ hour; for fibroblast cell extract, specific activity is expressed as nmoles of substrate hydrolyzed/ mg protein/ hour.

* Fluorometric units measured with 4MUGS have been converted to equivalent 4MUG enzyme units by means of the formula of Bayleran et al (1984, refer to Appendix B).

in figure 16. While no Hex A activity eluted with the salt buffer, an intermediate isozyme did elute in the salt gradient. This isozyme is not normally present in cells. Its appearance in TSD cells is thought to be due to an association of excess ß subunits that occurs in the absence of α-subunit polypeptides with which they would normally associate (Mahuran et al, 1985).

Biosynthetic labelling of proband fibroblast cell proteins in culture and immunoprecipitation of the Hex subunits with goat antisera directed against purified Hex A, Hex B, and isolated α chain was performed by Zokaeem et al (1987, Appendix B). Cells of the proband were found to synthesize an α-polypeptide precursor that is shorter than normal α chain by approximately 2-3 kDa. The failure to immunoprecipitate mature α chain with antisera directed against both Hex A and Hex B showed that the shortened α subunit never associates with ß subunit. In addition, the absence of incorporation of ^{32}P into the α chain of WG1051 indicated that the shortened subunit fails to reach the Golgi for processing. Rather, it is degraded in the ER. Both parents were found to be heterozygous for this shortened subunit, confirming that the child is a homozygote rather than a compound heterozygote for two mutant alleles. More recently, Neufeld (1988, personal communication) found the shortened α chain in WG1051 cells to be due to a homozygous deletion of a single nucleotide of the α-chain gene producing a frameshift mutation leading to premature termination 12 nucleotides downstream from the site of the mutation.

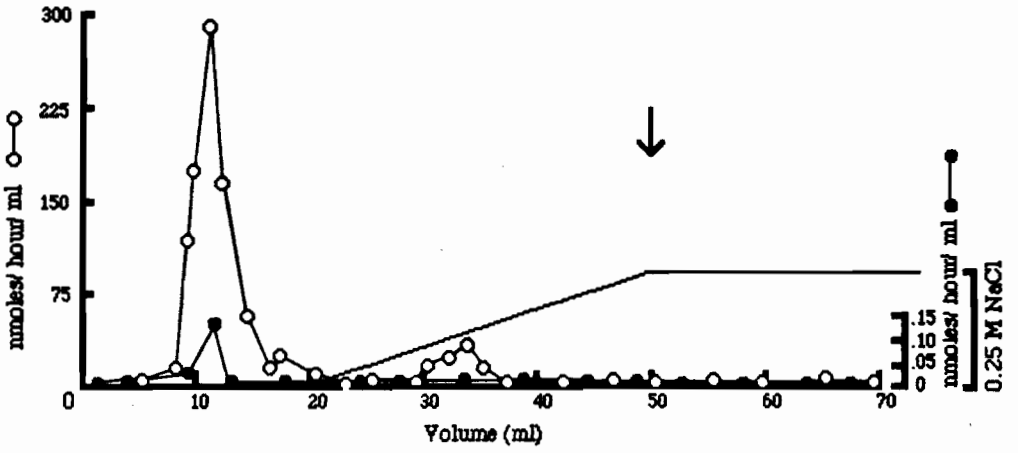

Figure 16. Ion-exchange chromatography of Italian TSD proband (WG1051) Hex isozymes. Fibroblast pellets were cultured to confluence in 175 cm^2 flasks. Cell extracts were chromatographed according to the procedure of Nakagawa et al (1977) on DEAE-cellulose. Column fractions were assayed with 4MUG (o—o) and 4MUGS (●—●). Arrow indicates the elution position of Hex A in ion-exchange chromatography of normal cells.

E. Infantile TSD, B^1 variant.

Cases of infantile TSD with high residual Hex A activity have been described. The clinical presentation of the disease in these patients is identical to that found among Ashkenazi Jewish and French Canadian patients. A detailed analysis of the Hex A synthesized by the cells of two such patients, designated WG1108 (referred to as patient 1) and WG1110 (referred to as patient 2), was undertaken (Bayleran et al, 1987; Appendix B) and is presented following a description of the clinical evaluation and biochemical findings of the probands and their parents.

1. Clinical evaluation of patients and families.

Patient 1, who was misdiagnosed when amniocyte Hex A activity was measured with 4MUG, was the younger sibling of a brother who was diagnosed as having TSD. The mother is of French Canadian descent and the father of English, Irish, Scottish, and German ancestry. In the original report by 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 months 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.

Patient 2 was a female who presented with a typical course of TSD. Examination at 6 months of age revealed the presence of a macular cherry red spot. By the age of 12-14 months seizures, psychomotor symptoms, and dementia were apparent. Serum Hex A activity was within the range for TSD homozygotes, 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. The father, whose serum Hex A

values placed him in the heterozygote range, is of German-Scandinavian descent.

2. Hexosaminidase activity in cells and sera of probands and parents.

The evidence upon which the two patients were classified as having biochemically atypical forms of TSD was presented in table 3 (page 88). In the 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 3, which reported 16% Hex A in fibroblasts of both patients by the thermal fractionation procedure with 4MUG. These values, as well as the corresponding specific activities for Hex A, were significantly higher than the 0-5% Hex A expected for infantile TSD. The most frequent alleles associated with the infantile form of TSD (i.e. those occurring in the Ashkenazi Jewish and French Canadian patients) are understood to be the result of CRM-negative mutations (Proia and Neufeld, 1982; Myerowitz and Proia, 1984; Myerowitz and Hogikyan, 1986). Hex A values greater than zero in cells of these genotypes are usually considered to be an artifact of thermal fractionation. Both patients were unambiguously diagnosed as Hex A-deficient by the assay procedure that employs both 4MUG and 4MUGS. The value of 0.7% Hex A found in fibroblasts of both patients was clearly within the range found for other infantile TSD patients.

Figure 17 shows serum Hex A activity determined with 4MUG after thermal fractionation (17A) and determined with the two substrates (17B) for patient 1 and the parents of patients 1 and 2, plotted as a two-discriminant test. These values are plotted against the same group of normals, obligate heterozygotes, and infantile TSD patients presented in figure 12 (page 99). The Hex A value obtained for patient 1 by thermal fractionation was 29%, which approaches the heterozygote range and would result in misdiagnosis of the patient. However, the two-substrate assay for determination of Hex A activity yields 1% Hex A, a value within the range of other

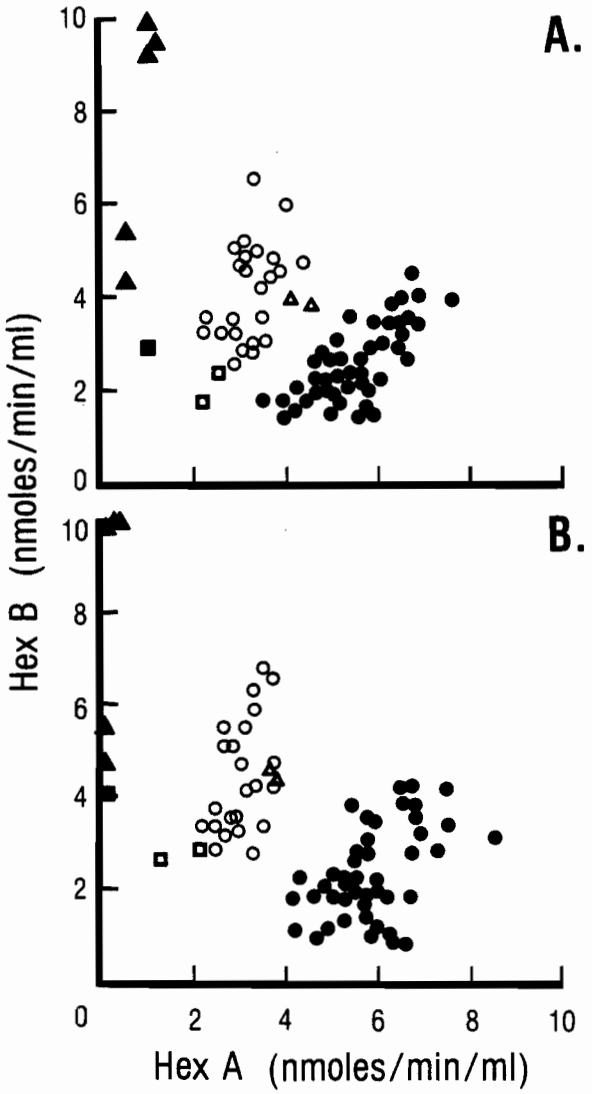

Figure 17. Determination of serum Hex activity in parents of B^1 variant TSD probands. Hex A and Hex B activities were measured by thermal fractionation with 4MUG (A) and by application of the formula with 4MUGS (B). Values are plotted as a two-discriminant test. Mean and S.D. values for the 45 normal controls and 24 obligate heterozygotes previously tested are as stated in figure 12 on page 99.
● = normal controls, o = obligate TS heterozygotes, ▲ = infantile TSD homozygotes, ■ = patient 1, □ = parents of patient 1, Δ = parents of patient 2.

TSD patients and clearly identifying this patient as severely affected.

The parents' sera were assayed for Hex A by thermal fractionation with 4MUG and by the two-substrate procedure that employs 4MUGS. Two of the parents (the mother of patient 1 and the father of patient 2) tested comparably by both assay procedures and fell within the heterozygote range for both percentage of Hex A and Hex A activity. Comparable values by the two assay 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 (figure 17A) placed these two parents slightly outside the range for other obligate heterozygotes. Measurement of Hex A activity by the two-substrate assay procedure (figure 17B) unambiguously identified all four parents 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. These results may indicate that the parents who test comparably by both assay procedures are carriers for an allele that does not produce an α-subunit polypeptide or, alternatively, are carriers for an allele that produces an α subunit which is incapable of complexing with ß subunits. These types of mutant alleles are detected accurately by both thermal fractionation and the two-substrate assay procedure. The parents who are assigned heterozygote status only by the assay procedure that employs the sulfated substrate are probably heterozygous for an allele that produces a defective α subunit capable of complexing with ß subunits to form Hex A. This defective subunit is accurately detected only by 4MUGS, a substrate that is specific for the α-subunit catalytic site.

The ability to diagnose patients with the B^1 variant form of infantile TSD, as

well as to detect carriers for the B^1 allele, has been significantly improved by the use of 4MUGS for the measurement of Hex A activity. Charrow et al (1985), Besley et al (1987a and 1987b), and Gordon et al (1988, Appendix B) have achieved a similar improvement in diagnosis of B^1 TSD probands using the sulfated derivative of 4MUG. These findings have clear implications for the prenatal diagnosis of fetuses affected with the B^1 variant form of TSD and for heterozygote screening, particularly as it reaches out to non-Jewish communities. Most of the cases that have been reported in the literature of infantile TSD patients who have neither Ashkenazi Jewish nor French Canadian ancestry are of the B^1 variant type.

3. Fractionation of B^1 variant Hex isozymes by ion-exchange chromatography.

Both probands produce a form of hexosaminidase that is detected by the thermal fractionation procedure yet does not hydrolyze 4MUGS. To understand the biochemical basis for this type of mutation hexosaminidase isozymes of patient and control fibroblasts were fractionated and the catalytic properties of the isolated Hex A were studied.

Figure 18 shows the ion-exchange chromatographic profiles of hexosaminidases obtained from control (figure 18A) and patient 1 (figure 18B) fibroblasts. The chromatographic profile of hexosaminidases obtained from fibroblasts of patient 2 was virtually identical to that of patient 1 and, therefore, is not shown. Assay of column fractions with 4MUG (represented by open circles) revealed activity peaks corresponding to Hex A and Hex B in control fibroblasts, whereas in the fibroblasts of patient 1 a third species intermediate between Hex A and Hex B was also observed. This form of hexosaminidase is more anionic than Hex B yet is thermostable under conditions that completely inactivate Hex A. It is thought to be a homopolymer of ß subunits of a form that are usually incorporated preferentially

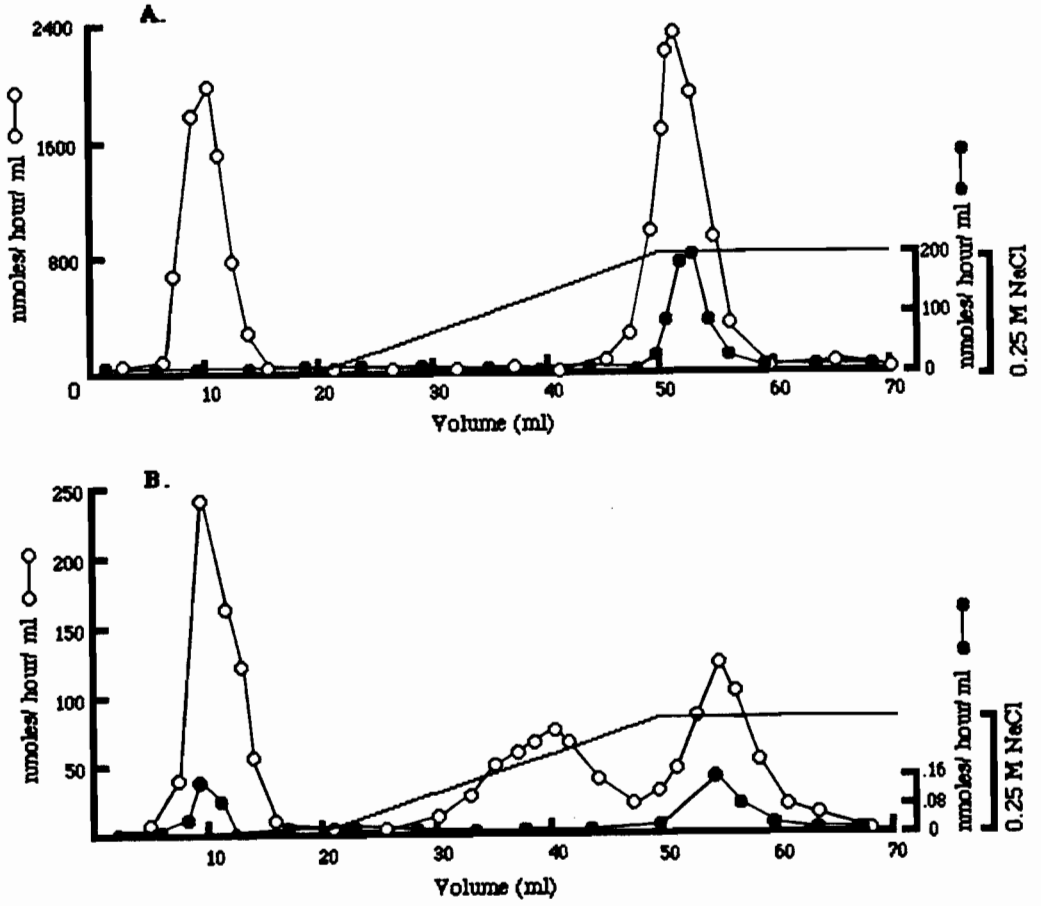

Figure 18. Ion-exchange chromatography of fibroblast hexosaminidases of normal control (A) and patient 1 (B). Hexosaminidases were resolved by salt-gradient elution according to the procedure of Nakagawa et al (1977). Fractions were assayed with 4MUG (o—o) and 4MUGS (●—●). Enzyme activities were normalized to fibroblast supernatant applied to the column.

into Hex A (Geiger et al, 1978; Mahuran et al, 1985). In the absence of α subunits, this intermediate accumulates in TSD cells. No Hex A peak was observed following fractionation of a fibroblast extract obtained from WG1051, the TSD patient of Italian descent (figure 16, page 115). However, the intermediate Hex isozyme was present.

Hexosaminidase A activity (as defined by 4MUG hydrolysis) measured after chromatography of the control fibroblast cell extract accounted for 60% of the total recovered hexosaminidase activity (figure 18A). This value is in accord with the estimation of percentage of Hex A in the unfractionated control cell extract as measured by the thermal fractionation procedure with 4MUG (table 3). By contrast, Hex A recovered after chromatography of patient 1 fibroblast supernatant (figure 18B) accounted for 30% of the total eluted hexosaminidase activity, or roughly twice the percentage estimated by assay of unfractionated cell extract by thermal fractionation. Likewise patient 2 Hex A activity, recovered after ion-exchange chromatography of Hex isozymes, accounted for 29% of the total eluted hexosaminidase activity (not shown).

Significant Hex A activity toward 4MUGS was associated with a Hex A peak only in control cells. A small though detectable peak that co-chromatographs with Hex A was observed in the 4MUGS profiles of Hex isozymes obtained from fibroblasts of the B^1 TSD probands. The catalytic activity of the patients' Hex A against the sulfated substrate was similar to Hex B-catalyzed hydrolysis of 4MUGS.

4. Thermal inactivation of proband Hex A and Hex B.

Thermal inactivation of isolated hexosaminidases is shown in figure 19. Hex B recovered from the two B^1 variant fibroblasts was heated at 47° C for intervals up to 2 hours and assayed with 4MUG. The probands' Hex B behaved identically to control Hex B. Therefore, the amount of heat-labile Hex activity measured in the

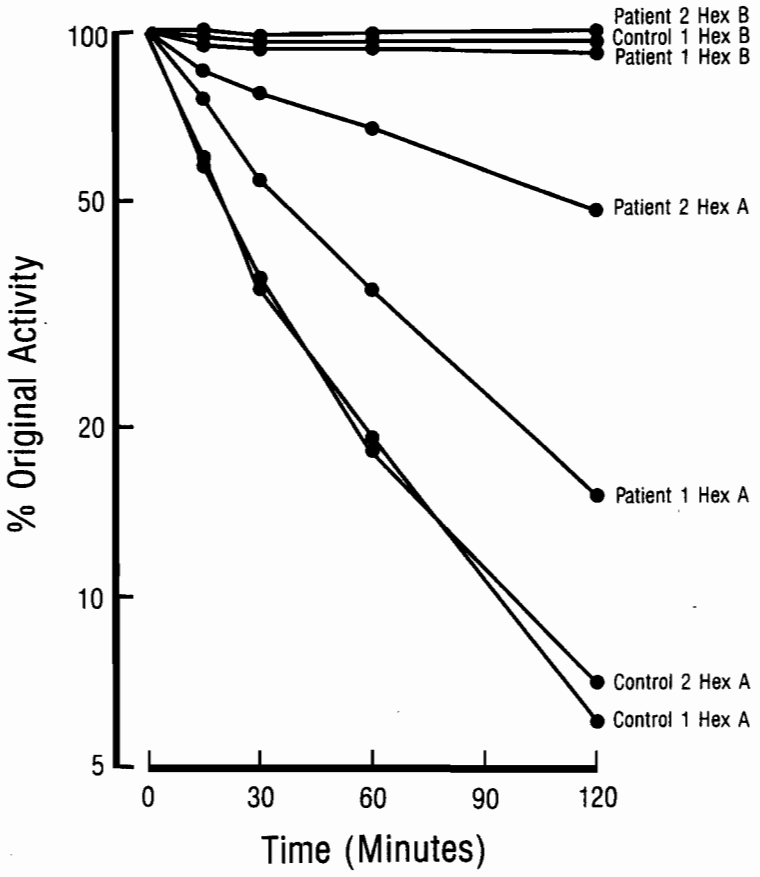

Figure 19. Thermal inactivation of normal control and B^1 variant TSD Hex A and Hex B. Hex A was denatured at 42° C and assayed with 4MUGS at 37° C. Hex B was denatured at 47° C and assayed at 37° C with 4MUG.

thermal fractionation assay with 4MUG (16% in fibroblasts of patients 1 and 2, and 29% in serum of patient 1) was not attributable to a heat-labile form of Hex B. Thermal inactivation of control and patient Hex A at 42° C for intervals up to 2 hours and assay with 4MUGS revealed Hex A from both patients to be more thermostable than control Hex A. For two normal control cell strains, the T_{50} values for Hex A inactivation were 19.5 and 20 minutes. The T_{50} values for inactivation of the probands' Hex A were 36 minutes for patient 1 and 111 minutes for patient 2. These results indicate that the probands' Hex A differed significantly from 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 (29-30%) of patient fibroblast supernatants.

5. pH optima of proband Hex A.

pH-activity curves of Hex A-catalyzed hydrolysis of 4MUGS are shown in figure 20. The pH optimum of control Hex A was found to be 3.9. The Hex A of both B^1 variant TSD patients showed greatly reduced activity and had a much lower optimum pH of 2.3. The shift in the optimum pH of the patients' Hex A to a more acidic range may indicate that the enzymes are nonfunctional within the lysosome.

6. Kinetic analysis of B^1 variant Hex A.

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

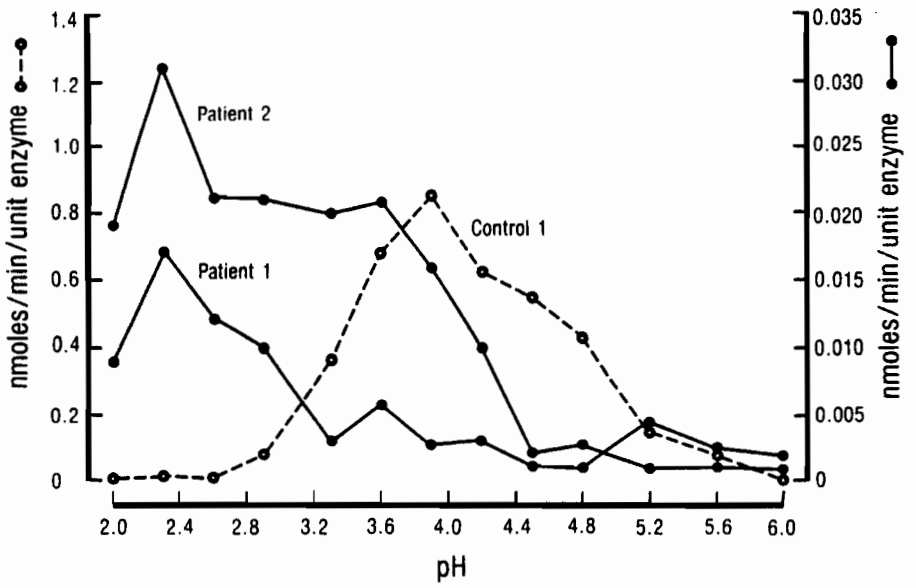

Figure 20. pH-activity curves of Hex A-catalyzed hydrolysis of 4MUGS. Normal control and B^1 variant TSD fibroblast Hex A were assayed in 0.04 M sodium citrate buffer at the indicated pH's with 1.5 mM substrate. One unit of enzyme activity is that amount of enzyme which catalyzes the hydrolysis of 1 nmole of 4MUG/ minute under standard conditions of temperature and pH.

subunits did not possess active sites and the residual catalytic activity of proband Hex A toward 4MUGS was, therefore, entirely attributable to hydrolysis occurring at the active site on the ß subunit.

Kinetic analysis of the probands' Hex A activity against 4MUGS (shown in table 8) revealed Km values that were very similar to that of control Hex A. The Km values of patient 1 and patient 2 Hex A were 1.7 and 1.5 mM, respectively; the Km of control Hex A was 1.3 mM. This suggests that hydrolysis of 4MUGS by the probands' Hex A occurs at a site with a binding affinity that is more characteristic of an α-subunit active site than a ß-subunit active site. Maximum velocities of the probands' Hex A were quite different from that of control as well as from each other. Control Hex A had a Vmax of 0.91 nmoles/ min/ unit of enzyme. The Vmax values of the patients' Hex A were 0.26 nmoles/ min/ unit of enzyme for patient 1 and 0.006 nmoles/ min/ unit of enzyme for patient 2. Since Vmax values are expressed as nanomoles of 4MUGS hydrolyzed per unit of enzyme (measured by 4MUG hydrolysis), rather than per mg of protein, they reflect reduced catalytic activity at the α-subunit active site rather than a reduced amount of enzyme.

7. Inhibition of Hex A and Hex B activity against 4MUG and 4MUGS.

Figures 21 and 22 show the results of studies of the inhibition of 4MUG and 4MUGS hydrolysis by N-acetylglucosamine (NG) and N-acetylglucosamine-6-phosphate (NGP). The upper graphs (A and B) in figures 21 and 22 demonstrate the specificity of the two inhibitors for the ß- and α-subunit active sites, respectively. Thus, comparison of figures 21A and 21B reveals that the hydrolysis of 4MUG (open circles) by either isolated control Hex A or control Hex B was inhibited to the same extent by NG. This suggests that 4MUG was hydrolyzed by an active site

Table 8. Kinetic analysis of B^1 variant TSD Hex A activity against 4MUGS.

	Km (mM)	Vmax (nmoles/ min/ unit of enzyme)
Normal control Hex A	1.3	0.91
Normal control Hex B	5.9	0.004
Patient 1 Hex A	1.7	0.26
Patient 2 Hex A	1.5	0.006

One unit of enzyme activity is that amount of enzyme that hydrolyzes one nmole of 4MUG/ minute.

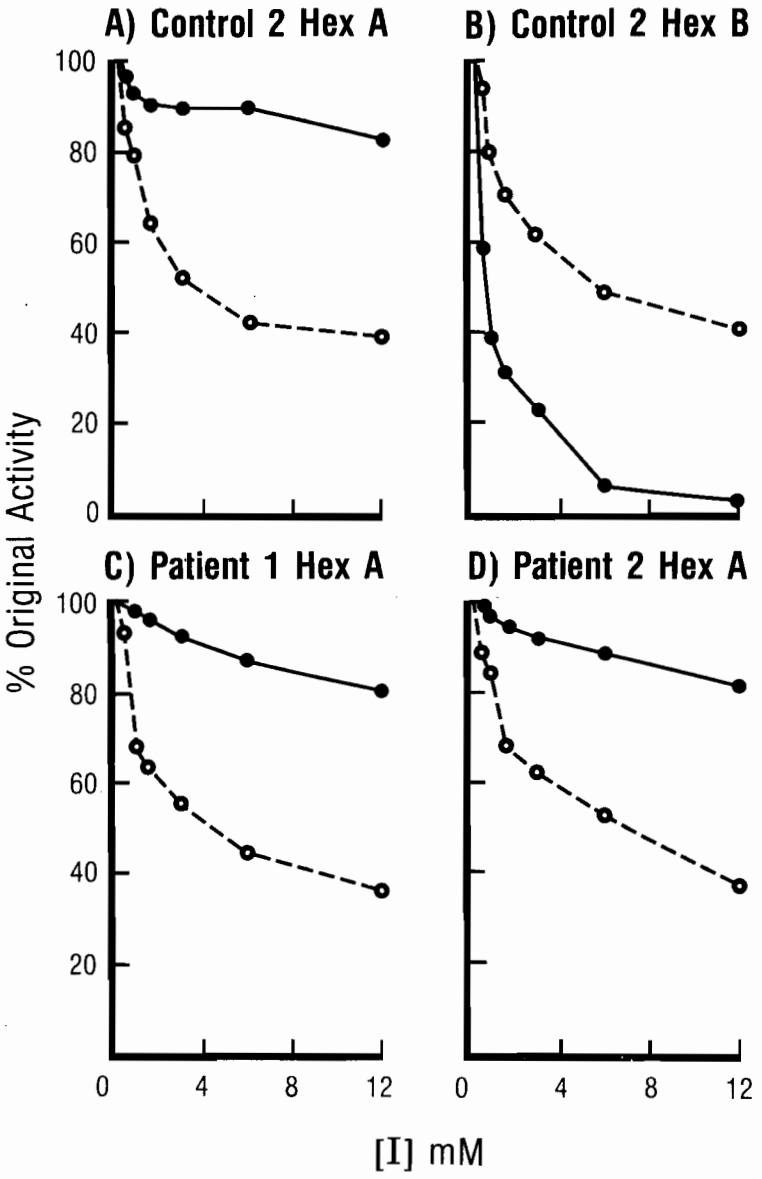

Figure 21. Inhibition of 4MUG (o---o) and 4MUGS (●—●) hydrolysis by N-acetyl-glucosamine. Concentration of both substrates was 0.5 mM. Hex A and Hex B were isolated by ion-exchange chromatography according to the procedure of Nakagawa et al, 1977.

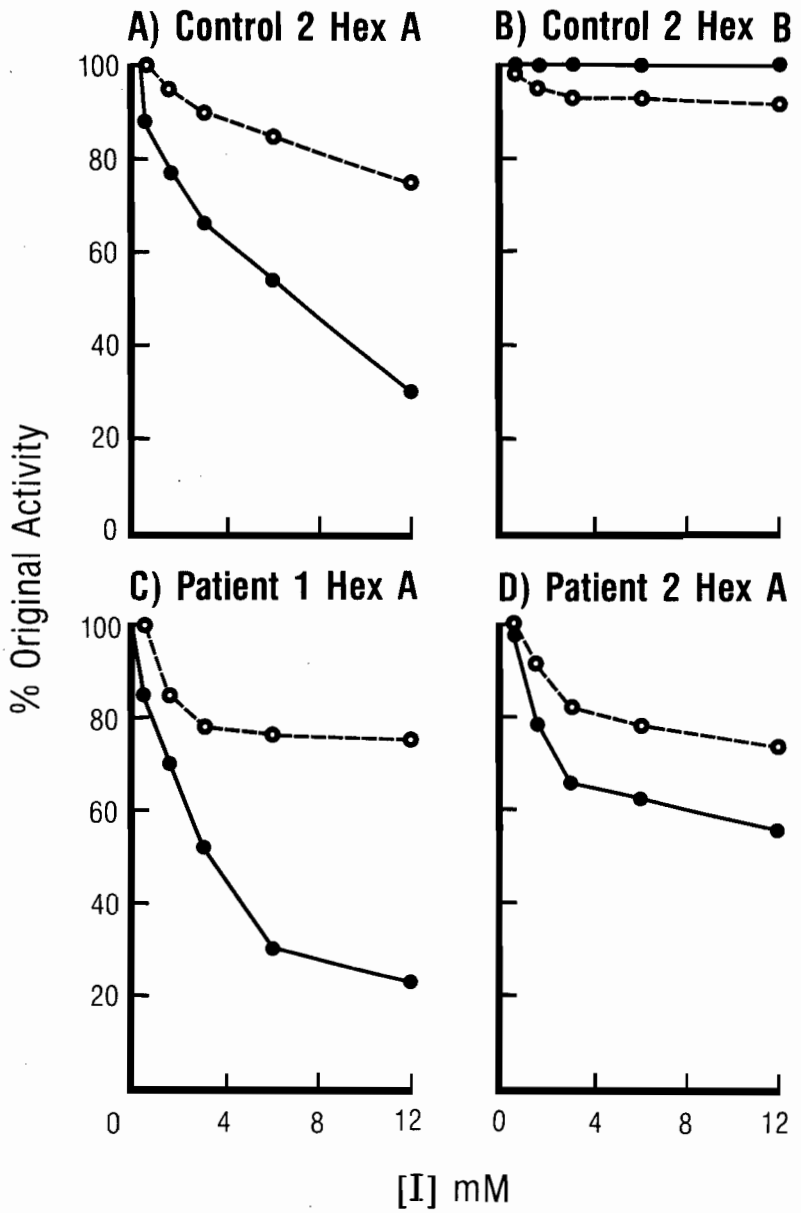

Figure 22. Inhibition of 4MUG (o---o) and 4MUGS (●—●) hydrolysis by N-acetylglucosamine-6-phosphate. Concentration of both substrates was 0.5 mM. Hex A and Hex B were isolated by ion-exchange chromatography according to the procedure of Nakagawa et al, 1977.

common to both isozymes which, therefore, must be associated with the ß subunit. In contrast, hydrolysis of 4MUGS (closed circles) by control Hex A was inhibited only 18% at a concentration of 12 mM NG, whereas the small amount of 4MUGS hydrolysis catalyzed by control Hex B was 95% inhibited at a concentration of 6 mM NG.

Hydrolysis of 4MUG by both patients' Hex A (figures 21C and 21D) was inhibited by NG to the same extent as was that of control Hex A and Hex B. However, hydrolysis of 4MUGS by the patients' Hex A was reduced by only 19% in the presence of 12 mM NG, establishing that the patients' Hex A had characteristics associated with an intact α-subunit active site capable of binding 4MUGS. Thus, if the α-subunit active site of patient Hex A was not able to bind the sulfated substrate and the residual catalytic activity toward 4MUGS was due to hydrolysis at the ß-subunit active site, an inhibition profile similar to that of Hex B would have been observed.

Hex A-catalyzed hydrolysis of 4MUGS was strongly, and most likely competitively, inhibited in the presence of NGP (figure 22A). However, the very small amount of Hex B-catalyzed hydrolysis of 4MUGS was not inhibited by NGP (figure 22B). This indicates that 4MUGS is hydrolyzed preferentially by an active site unique to Hex A. The results of the inhibition studies thus clearly establish the existence of two distinct catalytic sites on hexosaminidase A, one on the α subunit and one on the ß subunit, corroborating the findings of Kytzia and Sandhoff (1985).

Figures 22C and 22D show inhibition of 4MUG and 4MUGS hydrolysis by the patients' Hex A in the presence of NGP. Hydrolysis of 4MUGS by patient 1 Hex A (figure 22C, closed circles) was as sensitive to NGP inhibition as was that by control Hex A (figure 22A). However, the inhibition curve for patient 2 Hex A

(figure 22D) was intermediate between that of control Hex A and control Hex B. This suggests that both the α- and ß-subunit active sites of patient 2 Hex A may contribute to the residual 4MUGS hydrolysis, a finding that is consistent with the much lower Vmax of this proband's Hex A.

Classification of the two infantile TSD patients reported here was vastly improved when Hex A activity was measured with 4MUGS. Similarly, the unambiguous classification of all four parents as heterozygotes was achieved only when serum Hex A activity was measured by the assay procedure that employs 4MUGS. This indicates that the thermal fractionation procedure does not accurately detect B^1 alleles in the heterozygous state. Both infantile TSD patients are likely compound heterozygotes. One of the mutant alleles either does not encode an α subunit or does produce α subunit which is incapable of complexing with ß subunits; the other mutant allele (i.e., the B^1 allele) encodes an α subunit that associates normally with ß subunits. Proband Hex A binds to and hydrolyzes 4MUG because the mutation does not affect the ß subunit. The similarity in Km values of normal and patient Hex A as well as the results of the inhibition studies implicate an α-subunit active site capable of binding 4MUGS and NGP. Thus, while the catalytic site binds 4MUGS it 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. Significant - though probably functionally irrelevant - differences in the T_{50} for thermal inactivation and in the Vmax for the two probands' Hex A's suggest that the two B^1 mutant alleles might be different. The mutation in a B^1 variant TSD patient was found by Ohno and Suzuki (1988a) to be a base substitution resulting in a

changed amino acid that, in turn, produces a change in the conformation of the α-subunit polypeptide at or near the catalytic site. Such a mutation might be expected to affect the thermal characteristics of the enzyme as well as its catalytic activity. Whether the B^1 TSD patients presented here have a similar mutation or whether they do indeed possess different B^1 mutant alleles at the α-locus remains to be seen.

F. Adult G_{M2} gangliosidosis and Hex A-deficient healthy adults.

On rare occasions mass screening for the Tay-Sachs gene reveals a healthy adult with low or absent Hex A. The significance of these findings is not known because the assay of Hex A with 4MUG has no prognostic value in such cases. Some individuals with a similar biochemical phenotype develop adult-onset forms of TSD (Navon et al, 1981; Johnson et al, 1982; Conzelmann et al, 1983) whereas others remain free of neurological symptoms (Vidgoff et al, 1973; Kelly et al, 1976; O'Brien et al, 1978; Kolodny and Raghavan, 1983; Grebner et al, 1986). Assays which employ the natural substrate, G_{M2} ganglioside, offer greater prognostic value than those which measure heat-labile hexosaminidase activity. However, G_{M2} ganglioside is not used routinely to measure Hex A activity for the reasons outlined earlier (pages 48-51). An evaluation of the prognostic value of the sulfated fluorogenic substrate, 4MUGS, for adults with low Hex A activity was undertaken. An abstract on this work has been presented elsewhere (Bayleran et al, 1986).

1. Clinical evaluation of patients.

The adult-onset G_{M2} gangliosidosis patient (corresponding to cell strain WG1116) was described by Kaback et al (1978). The patient was a clinically normal 19-year-old who was ascertained through participation in a Tay-Sachs screening

program. Hex A assay with 4MUG revealed that his serum was totally deficient in Hex A. Fibroblasts and leukocytes showed 8-12% Hex A. Hex A values for his parents and sister were in the range for TSD heterozygotes.

The onset of symptoms occurred at the age of 21. The first symptom to appear was dysarthria. Nerve conduction time was diminished, accompanied by muscle weakness and tremor. The general clinical picture was consistent with amyotrophic lateral sclerosis. Electron microscopic examination of neurons obtained from rectal biopsy showed MCBs. G_{M2} ganglioside hydrolysis by fibroblast cell extracts was deficient. There has been no noticeable intellectual deterioration. The patient does, however, manifest a bipolar affective disorder which is being treated with Lithium. Both of his parents are of Jewish descent and the patient's father reported a case of infantile TSD on his side of the family.

The Hex A-deficient healthy adult (corresponding to cell strain WG1107) was reported as patient 3 by Grebner et al (1986). He is presently 32 years old. He is of Welsh extraction and was ascertained because his wife, who is of Jewish ancestry, requested testing for Tay-Sachs heterozygosity. Routine screening of his serum by the thermal fractionation assay with 4MUG revealed severely deficient Hex A activity. Leukocytes and fibroblasts were also found to have severely deficient Hex A activity. G_{M2} ganglioside was hydrolyzed normally by his cells. The individual is completely free of any neurological symptoms. Biosynthetic labelling studies conducted on fibroblasts obtained from this individual showed that his cells synthesize the α-subunit precursor which associates with ß-subunit precursor and is processed to mature form. However, the amount of α-subunit precursor synthesized was markedly decreased over that observed in normal cells, with a corresponding reduction in the amount of mature α subunit (Grebner et al, 1986).

The Hex A-deficient adult corresponding to cell strain WG1111 is a clinically normal female of Ashkenazi Jewish extraction. Fibroblasts obtained from this individual were biopsied at the laboratory of Dr. M. Kaback. Assessment of Hex A activity by the thermal fractionation assay procedure, performed in his laboratory, yielded values comparable to those of infantile TSD patients in serum and leukocytes. In contrast, a small amount of Hex A activity was detected in fibroblasts.

2. Determination of Hex A activity.

Table 9 shows the results of Hex A assays on fibroblasts of three adult variants and sera of the parents of the adult affected with a neurological disorder. The parents of WG1116, who are not related, tested comparably as heterozygotes by both the thermal fractionation assay with 4MUG and the two-substrate assay procedure that employs 4MUGS. The adult variants were essentially indistinguishable based on the thermal fractionation assay using 4MUG. The three variants had Hex A specific activities that were significantly lower than the normal controls or obligate heterozygotes tested. Such individuals ascertained through routine screening would probably be diagnosed as likely to develop a disease. The sulfated substrate assay procedure did distinguish between the adult affected with a neurological disease and the healthy adults. By application of the empirical formula, Hex A specific activity was very low in the adult TSD cell extract yet was in (or approached) the heterozygote range in the two Hex A-deficient healthy adult cell strains. While the 4MUGS assay procedure did discriminate between the adult TSD patient and the two clinically normal adults, it did not clearly distinguish between adult and infantile forms of TSD. This finding has serious implications for prenatal diagnosis.

3. Ion-exchange chromatography of adult variant Hex isozymes.

Figure 23 shows the chromatographic separation of Hex isozymes from

Table 9. Determination of Hex A activity in fibroblasts of adult variants and sera of parents.

Tissue and subject	Hex A sp. act. (%) by thermolability		Hex A sp. act. (%) by 4MUGS *	
Fibroblasts:				
Adult-onset TSD WG1116	180	(8%)	56	(2%)
Hex A-deficient healthy adult WG1107	278	(15)	1030	(56)
Hex A-deficient healthy adult WG1111	661	(18)	2604	(71)
Controls (N=9)	3260-9280	(47-79)	3380-8290	(46-89)
Heterozygotes (N=3)	1342-7660	(27-47)	2214-6077	(38-44)
Infantile TSD (N=10)	32-140	(1-5)	9-87	(0.4-2)
Sera:				
Mother of adult-onset TSD	184	(38%)	167	(35%)
Father of adult-onset TSD	199	(53)	178	(48)
Controls (N=45)	218-474	(60-81)	243-457	(60-88)
Heterozygotes (N=28)	130-276	(34-57)	79-228	(35-55)
Infantile TSD (N=5)	32-74	(9-11)	0.6-17	(0-2)

For serum, activity is expressed as nmoles of substrate hydrolyzed/ hour/ ml; for fibroblasts, specific activity is expressed as nmoles of substrate hydrolyzed/ hour/ mg protein. All values represent the mean of four determinations.

* Fluorometric units measured with 4MUGS have been converted to equivalent 4MUG enzyme units by application of the formula of Bayleran et al (1984).

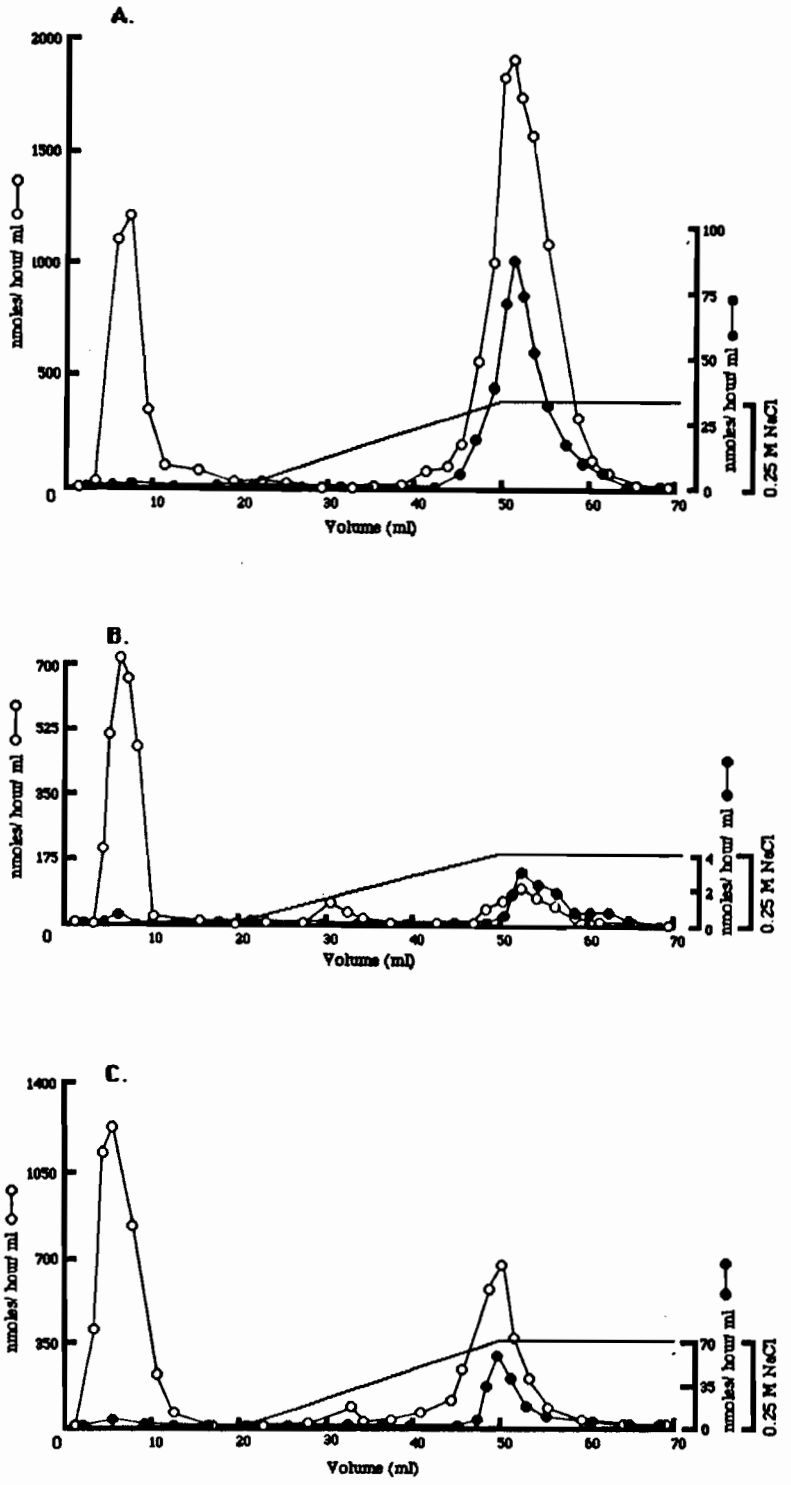

Figure 23. Ion-exchange chromatography of normal control (A), adult-onset G_{M2} gangliosidosis WG1116 (B), and Hex A-deficient healthy adult WG1107 (C) Hex isozymes. Hexosaminidases were resolved according to the procedure of Nakagawa et al (1977) on DEAE-cellulose. Column fractions were assayed with 4MUG (o—o) and 4MUGS (•—•). Enzyme activities were normalized to fibroblast supernatant applied to the column.

fibroblast cell extracts of a normal control (23A), the adult-onset TSD patient WG1116 (23B), and one of the Hex A-deficient normal adults, WG1107 (23C). Column fractions were assayed with both 4MUG and 4MUGS. The chromatographic profiles of hexosaminidases obtained from fibroblasts of both adult variants revealed an intermediate isozyme when column fractions were assayed with 4MUG. This was not seen in the chromatographic profile obtained from fibroblasts of the normal control. Argov and Navon (1984) reported observing Hex I in the chromatographic profiles of fibroblast cell extracts obtained from adult G_{M2} gangliosidosis patients. Grebner et al (1986) also observed this intermediate isozyme in fractionated cell extracts of several Hex A-deficient healthy individuals.

Biosynthetic labelling and immunoprecipitation studies of fibroblasts obtained from adult G_{M2} gangliosidosis patients (d'Azzo et al, 1984; Frisch et al, 1984; Navon et al, 1986) and Hex A-deficient normal individuals (Grebner et al, 1986) have shown that both variants synthesize α-subunit precursor of normal size, yet the amount is reduced over that synthesized by normal cells. The appearance of Hex I in the chromatographic profiles of cell extracts obtained from the two adult variants studied here is consistent with reduced α subunit synthesis. Hex I produced from the association of excess β subunits has been observed in cell extracts of other TSD variants.

A small though measureable amount of Hex A activity against 4MUGS was detected in the adult TSD chromatographic profile. This contrasts with the virtual absence of a Hex A peak in the chromatographic profiles of Hex isozymes obtained from fibroblasts of infantile TSD patients (figures 16 and 18) and might provide a useful means of discriminating these genotypes in a prenatal diagnosis. The chromatographic profile depicted in figure 23C was obtained from cells of the same

Hex A-deficient healthy adult described as patient 3 by Grebner et al (1986). Somewhat disconcerting is the striking difference in the profiles obtained from this patient. Grebner et al (1986) observed high Hex B activity, low Hex I activity, and very low, though measureable, Hex A activity against 4MUG; Figure 23C shows significant Hex A activity against 4MUG. In addition, 4MUGS assay of column fractions yielded activity consistent with a Hex A peak. Grebner et al (1986) fractionated isozymes obtained from plasma of this Hex A-deficient healthy adult; figure 23C depicts the isozyme pattern obtained from fractionation of fibroblast isozymes. Clinically healthy adults have been reported with uniformly low levels of Hex A activity in tissues and fluids (Kelly et al, 1976; Grebner et al, 1986) as well as though individuals who appear to have a nonuniform deficiency. In the latter case, serum is usually severely deficient of Hex A activity (as assessed with 4MUG) while a small amount of activity is detected in leukocytes and fibroblasts (Vidgoff et al, 1974; Thomas et al, 1982; Kolodny and Raghavan, 1983). It is possible that the patient reported here has such a nonuniform deficiency of Hex A activity. Alternatively, the possibility that an inhibitor of Hex A activity is present in unfractionated cell extracts obtained from this individual cannot be ruled out.

Table 10 shows the % Hex A values measured with 4MUG (10A) and the r_A values of Hex A (10B) obtained from the chromatographic separation of the normal control, adult-onset TSD, and Hex A-deficient healthy adult cell extracts. The % Hex A for the normal control (10A) was 70%. This is in agreement with the % Hex A determined by thermolability in the unfractionated cell extract. The % Hex A values determined after chromatographic separation of adult variant Hex isozymes, however, were significantly higher than % Hex A determined by assay of unfractionated cell extracts with 4MUG. By the thermal fractionation assay with 4MUG, the adult-onset TSD patient was found to have 8% Hex A (table 9).

Table 10. Percent Hex A and r_A values of Hex A isolated from fibroblasts of adult variants.

A.

	% Hex A by 4MUG (from chromatographic profiles)
Control	70%
Adult-onset TSD	22%
Hex A-deficient healthy adult	34%

B.

	r_A values (on isolated Hex A)
Control	0.0866
Adult-onset TSD	0.0279
Hex A-deficient healthy adult	0.1000

$$r_A = \frac{\text{rate of hydrolysis of 4MUGS by Hex A}}{\text{rate of hydrolysis of 4MUG by Hex A}}$$

r_A values were determined on pooled, dialyzed, and concentrated Hex A isolated by ion-exchange chromatography using 0.5 mM 4MUG and 4MUGS.

In contrast, Hex A by chromatographic separation accounted for 22% of total Hex activity applied to the column. Likewise, the Hex A-deficient healthy adult had 15% Hex A against 4MUG by thermolability and 34% by assay of ion-exchange column fractions with 4MUG. Increased activity following the separation of Hex isozymes was also observed in the chromatographic profiles of the two B^1 variant TSD cell strains discussed earlier (page 121). The adult variants probably result from compound heterozygosity for rare α-locus mutations in combination with an allele that either does not produce an α subunit or produces one that cannot associate with ß subunits. This is indicated both by the lack of consanguinity in the parents of the probands and the occurrence of infantile TSD in a family member. The increased Hex A activity observed after chromatographic separation of Hex isozymes obtained from fibroblasts of these adult variants is thus attributable to the rare α-locus allele.

In the case of the B^1 variants, the % Hex A after ion-exchange separation of fibroblast Hex isozymes was half that of normal. This was taken to indicate that the B^1 allele produces a normal amount of α-subunit, the other mutant allele producing no α-ß subunit association. Percent Hex A after chromatography of adult TSD fibroblast Hex isozymes was approximately 1/3 that of normal. This is consistent with the reduced α-subunit synthesis observed in other adult TSD patients by d'Azzo et al (1984), Frisch et al (1984) and Navon et al (1986). Chromatographic fractionation of the healthy adult's fibroblast Hex isozymes yielded 34% Hex A which was approximately 1/2 that of normal. This is in accord with Grebner et al's (1986) finding that the cells of this clinically normal individual show a reduced amount of α-subunit synthesis.

The ratio of the relative rates of hydrolysis of 4MUGS and 4MUG by control and adult variant Hex A are shown in table 10B. The ratio of 4MUGS/ 4MUG hydrolysis by the adult TSD patient's Hex A was approximately 1/3 that of normal, while Hex A of the healthy adult hydrolyzed both substrates with a ratio comparable to that of control Hex A.

4. Thermal inactivation of adult variant Hex A.

Thermal inactivation of isolated Hex A of the normal control, adult G_{M2} gangliosidosis patient, and Hex A-deficient healthy adult is shown in figure 24. Hex A was heated for intervals up to 2 hours at 42° C and 48° C and assayed at 37° C with 4MUGS (24A) and 4MUG (24B), respectively. Hex A of the adult TSD patient was found to be significantly more stable to heating than normal Hex A. This difference in thermostability was obvious only when inactivated Hex A was assayed with 4MUG. These results indicate that the discrepancy between % Hex A obtained after chromatographic separation of adult TSD Hex isozymes (22%) and % Hex A determined in the thermolability assay of unfractionated cell extract (8%) might be attributable to Hex A that is less heat-labile than normal Hex A. Hex A of the healthy adult was virtually identical to that of normal Hex A in its thermolability. Thus, the increase in % Hex A observed after separation of Hex isozymes isolated from cells of this variant over that obtained by assay of unfractionated cell extract cannot be explained by a heat-stable form of Hex A.

The adult TSD and Hex A-deficient healthy adult cell strains were found to be similar with respect to total Hex and % Hex A measured with 4MUG. In both cell strains Hex A constituted a greater proportion of total Hex activity by chromatographic fractionation than by thermal fractionation when assayed with 4MUG. This appears to be explained, at least for the adult TSD patient's cells, by an

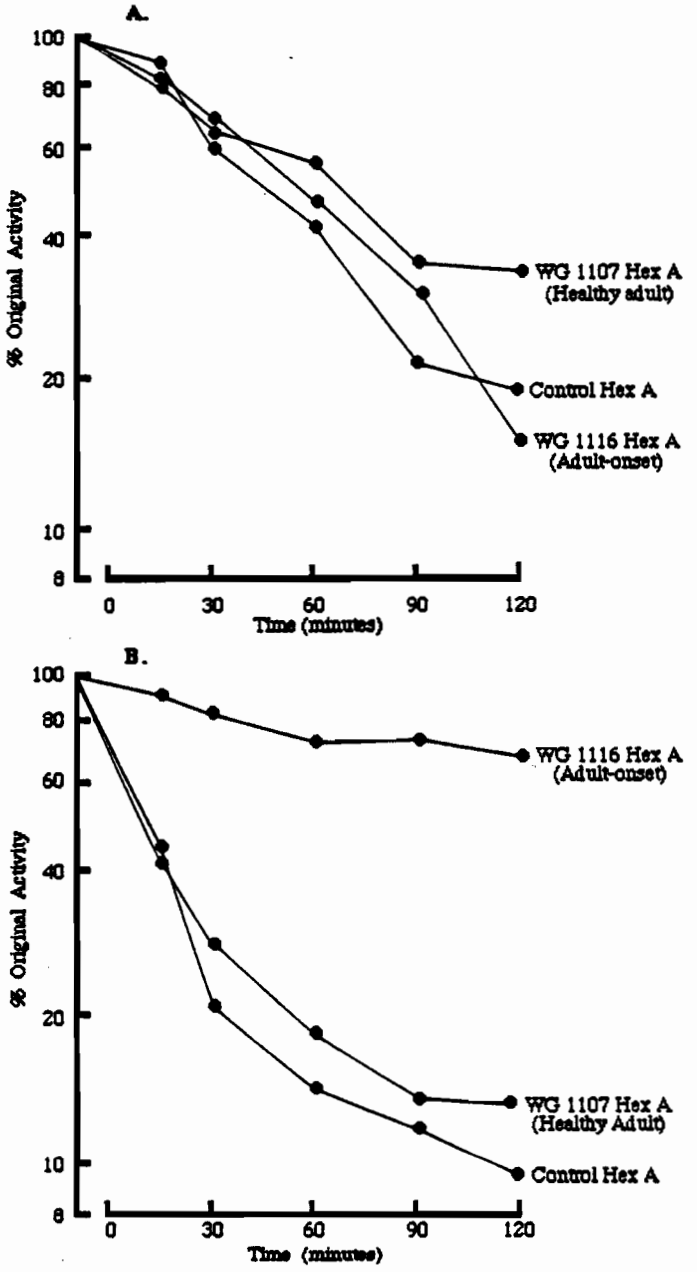

Figure 24. Thermal inactivation of control and adult variant Hex A. Hex A was denatured at 42° C and assayed with 4MUGS at 37° C (A). Hex A was denatured at 48° C and assayed with 4MUG at 37° C (B).

increased resistance of Hex A to heating. Percent Hex A measured by thermal fractionation of Hex A activity and assay with 4MUG was not a discriminant of clinical phenotype. However, assay of Hex A using 4MUGS was a satisfactory discriminant. Cells from the Hex A-deficient healthy adult had activity approaching the heterozygote range whereas the adult TSD variant had severely deficient 4MUGS cleaving activity. While the two adult variants are distinguished based on 4MUGS hydrolysis by unfractionated cell extracts, the results clearly indicate that the adult TSD variant is indistinguishable from more severely affected infantile TSD patients. This could have serious implications in the prenatal diagnosis of a fetus with a genotype that would confer an adult-onset form of TSD. Ion-exchange chromatography of Hex isozymes and assay of the column fractions with 4MUGS greatly improved the ability to differentiate between adult-onset TSD and infantile TSD. Comparison of the chromatographic profile of adult G_{M2} gangliosidosis Hex isozymes (figure 23B) with the profile of Hex isozymes obtained from cells of an infantile TSD patient (figure 16, page 115) clearly shows a species of Hex co-chromatographing with Hex A based on 4MUGS hydrolysis (closed circles) in the adult-onset profile, yet no Hex A-associated peak in the infantile TSD profile. Chromatographic separation of Hex isozymes and assay of column fractions with 4MUGS might, therefore, prove useful in distinguishing these two genotypes. However, many cases will have to be studied to assess the prognostic value of 4MUGS.

G. Infantile TSD in the French Canadian population of Quebec.

Infantile TSD in French Canadians was first described by Andermann et al (1973). The heterozygote frequency in French Canadians of eastern Quebec has been estimated to be approximately equal to the carrier frequency in the Ashkenazi Jewish

population (Andermann et al, 1977). The high incidence of TSD in communities along the north and south shores of the St. Lawrence River in eastern Quebec has been attributed to the founder effect. Many families can be traced to a founding population of about 2000 who came from France in the mid-1600s and colonized the area around Quebec City. However, a common ancestor has not been identified in five extensively examined pedigrees from southeastern Quebec (Andermann, unpublished).

It was thought that the mutation in French Canadians was identical to that in Ashkenazi Jewish TSD patients (McKusick, 1986). Fibroblasts from both Ashkenazi Jewish and French Canadian patients fail to synthesize an α-subunit polypeptide. The first indication that these mutations were different was the demonstration that a primary transcript is synthesized by the cells of Ashkenazi Jewish patients though no mature message is produced (Proia and Neufeld, 1982; Myerowitz and Proia, 1984; Paw and Neufeld, 1988), while a primary transcript does not appear to be synthesized by the cells of French Canadian TSD patients (Neufeld, personal communication). Myerowitz and Hogikyan (1986) demonstrated that the mutation in two French Canadian patients was a deletion at the 5' end of the gene encoding the α-subunit of Hex A.

1. Distribution of TSD families in Quebec.

Figure 25 shows a partial map of Quebec indicating the locations of the TSD families. To date, 13 families have been found most of whom live in towns along the north and south shores of the St. Lawrence River. The two probands whose DNA was analyzed by Myerowitz and Hogikyan (1986) correspond to families 1 and 2 on the map. I have analyzed DNA from six probands and their parents. Attempts are currently underway to obtain DNA samples for Southern

analysis on the remaining families.

Table 11 lists the TSD families corresponding to the numbers on the map of Quebec. The cell strain of the proband (where it is available), the town in which the family resides, and the known family relationships are indicated. Five of these patients came from the region of the lower south shore of the St. Lawrence River encompassing the cities of Rimouski, Sayabec, and Edmundston, New Brunswick. The sixth French Canadian TSD patient comes from Quebec City. The family of proband 7 currently resides in the upper penninsula of Michigan in the U.S. The proband, who was heterozygous for the B^1 allele at the α-locus, was described earlier (page 116). The TSD gene in this individual is traceable to French Canadian ancestry on the maternal side through great-grandparents who emigrated from St. Germain, Quebec.

French Canadian pedigrees with TSD probands have been identified in three other regions of Quebec, geographically remote from the Rimouski/ Sayabec/ Edmundston regions. These include families 8 and 9 from the lower north shore of the St. Lawrence River, families 10 and 12 from Montreal, and families 11 and 13 from the Gatineau region of Quebec.

2. Collection of samples.

An investigation of the TSD gene in French Canadians was undertaken utilizing samples obtained at several screening clinics. Dr. Eva Andermann was instrumental in organizing the clinic. Dr. Andermann (from the Montreal Neurological Hospital) and Carol Clow, Marietta Bardanis, and Norbert Laschic (from the Biochemical Genetics Department at the Montreal Children's Hospital) travelled to Rimouski and Sayabec, collecting and coding blood samples and taking family histories from individuals who requested testing. Blood samples were

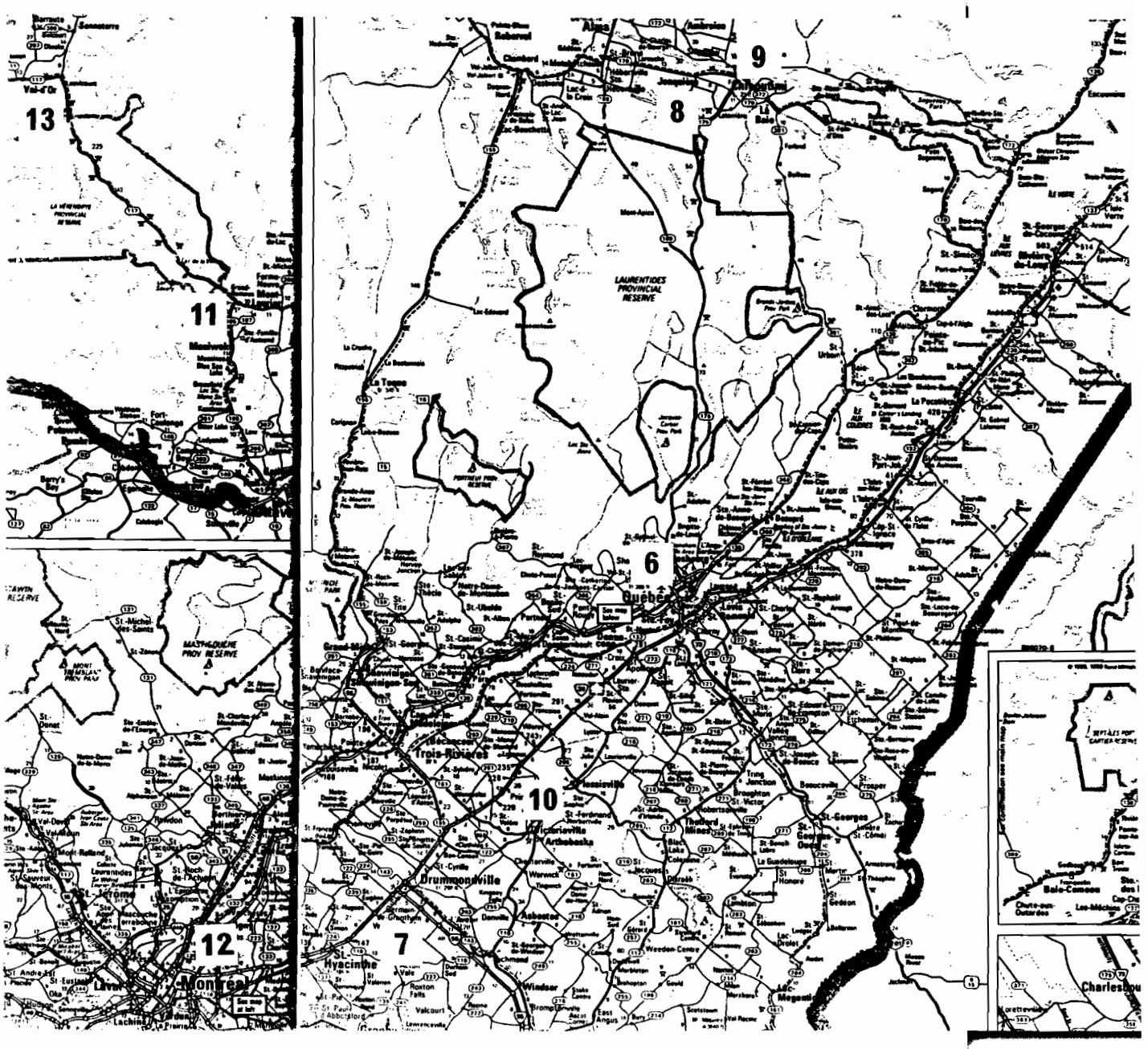

Figure 25. Map of Quebec indicating the locations of 13 TSD families.

Table 11. Tay-Sachs disease families in Quebec.

Family	Cell strain of proband	Location	Family interrelationships
1	WG107	Temiscouta	Parents are 2nd cousins
2	WG733	Notre Dame du Lac	
3	WG884	Edmundston, New Brunswick	
4		Sayabec	Mother is the aunt of mother of 5
5	WG1065	Sayabec	Mother is the niece of mother of 4
6	WG1499	Quebec City	Parents are 1st cousins
7	WG1108	St. Germain	
8		Arvida	Fathers of 8 and 9 are brothers and the mothers of 8 and 9 are sisters
9		Chicoutimi	
10		Montreal	
11		Gatineau	Family emigrated from France in the early 20th century
12		Montreal	
13		Val d'Or	Grandparents were from Quebec City

obtained from individuals for the purpose of assessing carrier status by assay of serum Hex A activity as well as for the purpose of analyzing DNA for the 5' end gene deletion. Blood to be tested for Hex A activity was immediately centrifuged and the serum collected and stored, frozen. The serum samples were transported to the lab at the Montreal Children's Hospital on dry ice. Blood obtained for Southern analysis of DNA was stored and transported to the lab at room temperature. Assistance in isolating DNA from the samples obtained at the clinic was provided by Dr. Andrea Richter and Louise Smith-Jones.

Blood samples were also obtained from the parents of the TSD proband from Quebec City (family 6 on the map) for the purpose of serum Hex A assay and Southern analysis of DNA. Only serum was obtained from the mother of proband 7; she was not available to obtain blood for DNA analysis. Samples from the parents of probands 6 and 7 were obtained from Drs. R. Gagné and E. Kolodny, respectively.

Southern analysis of the DNA of six probands (WG107, WG733, WG884, WG1065, WG1499, and WG1108) was performed using DNA isolated from cultured fibroblasts. Thus, while I was unable to obtain DNA from the mother of proband 7, I did isolate DNA from the proband's fibroblasts (WG1108).

3. Determination of serum Hex A activity in French Canadians.

The results of serum Hex A assays, performed manually, are shown in table 12. Hex A activity was assayed by the thermal fractionation procedure using 4MUG as well as by the two-substrate procedure that employs 4MUG and 4MUGS and depends upon application of the empirical formula. 4MUGS hydrolysis without application of the formula was also measured. Comparable classification of all individuals tested was achieved by the three assay procedures. A slight statistical improvement in classification was obtained by the 4MUGS assay procedure which classifies genotypes using a two-discriminant analysis. Sera from a total of 63

Table 12. Determination of serum Hex A activity in French Canadians.

	Hex A		Hex B	
	range	mean±S.D.	range	mean±S.D.
Thermal fractionation with 4MUG				
Controls (N=45)	3.94-8.65	6.02±1.26	1.82-8.03	3.68±1.35
Obligate TS Heterozygotes (N=10)	2.43-4.50	3.26±0.74	3.02-5.81	4.62±1.26
Nonobligate TS Heterozygotes (N=8)	2.44-4.55	3.03±0.68	1.02-6.01	4.03±1.64
4MUGS with application of formula				
Controls (N=45)	4.52-10.0	6.07±1.39	1.05-6.44	3.15±1.24
Obligate TS Heterozygotes (N=10)	2.30-4.21	2.98±0.65	2.93-7.00	4.52±1.37
Nonobligate TS Heterozygotes (N=8)	1.82-3.47	2.83±0.55	1.63-5.11	3.70±1.16
4MUGS without application of formula				
Controls (N=45)	0.55-1.50	0.85±0.18		
Obligate TS Heterozygotes (N=10)	0.35-0.52	0.41±0.05		
Nonobligate TS Heterozygotes (N=8)	0.27-0.52	0.43±0.08		

Hex A and Hex B activities are expressed as nmoles of substrate hydrolyzed/ ml/ minute and represent the mean of four determinations.

individuals were assayed. Forty-five individuals tested as normal homozygotes and 18 individuals were classified as heterozygotes. Ten of those who tested as carriers by serum Hex A assays are obligate heterozygotes. The remaining 8 individuals who were assessed as carriers by the three assay procedures are not obligates, yet all are related to infantile TSD probands. The ranges of Hex A activity and the mean and standard deviation values for the nonobligate carriers were virtually identical to the statistical domain established for the obligate TS heterozygotes. Heterozygote status in the 8 nonobligate carriers was confirmed by Southern analysis of genomic DNA.

4. Southern analysis of genomic DNA.

Genomic DNAs isolated from leukocytes and fibroblasts of TS heterozygotes and TSD probands, respectively, were analyzed by Southern blotting to determine the diversity of TSD alleles in the French Canadian population. A map of the intron-exon organization of the α-locus gene of Hex A is shown on page 31. The probe used in this study (kindly donated by Dr. R. Myerowitz) contains a 300-bp intronic sequence that maps 7.6 kb downstream from the first exon of the α-locus gene. An abstract on this work has been presented (Bayleran et al, 1988).

Southern analysis of genomic DNAs digested with EcoRI, electrophoresed, and hybridized with the 300-bp intronic probe are shown in figure 26. By Southern analysis, genomic DNAs obtained from all normal homozygotes (such as E.M. in figure 26) were found to possess one fragment of 9.5 kb. This was also observed in DNA isolated from cells of a TSD proband of Ashkenazi Jewish descent (WG93 in figure 26). The probands from southeastern Quebec and northern New Brunswick (WG107, WG884, WG1065, and WG733) were found to be homozygous for the 5' end gene deletion. This was revealed as a single 23 kb band by Southern analysis. The larger fragment is generated from the abolishment of three EcoRI restriction sites

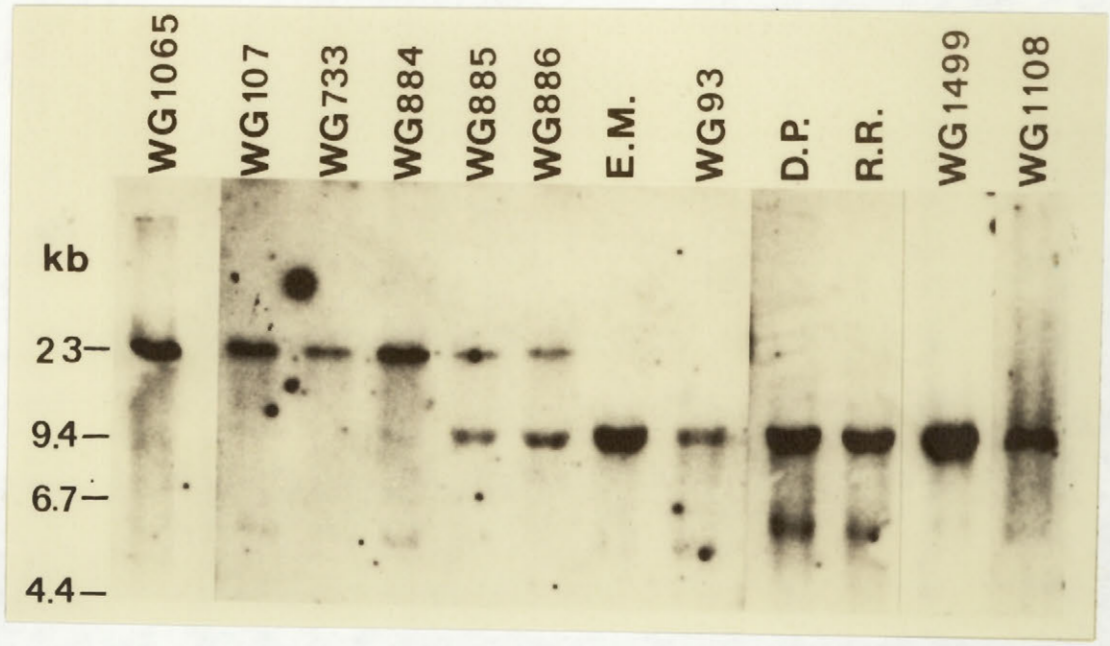

Figure 26. Southern analysis of digested genomic DNAs. 10-15 μg of EcoRI-digested genomic DNA were electrophoresed and hybridized with 0.1-0.3 μg of radiolabelled probe (representing approximately 100 x 10^6 cpm). Radioactive fragments were visualized by autoradiography. DNA from WG1065, WG107, WG733, and WG884 correspond to French Canadian TSD probands; WG885 and WG886 are from French Canadian obligate TS heterozygotes; E.M. is DNA obtained from a French Canadian normal homozygote; WG 93 is from an Ashkenazi Jewish TSD proband; D.P. and R.R. are French Canadian obligate TS heterozygotes; WG1499 is DNA obtained from their daughter, a TSD patient; WG1108 corresponds to a B^1 variant TSD patient whose mother is of French Canadian descent.

as a consequence of the deletion. All of the individuals from the eastern south shore who tested as heterozygotes by serum Hex A assay, both obligates (such as WG885 and WG886 in figure 26) and nonobligates, were also found to be heterozygous for the 5' end gene deletion, possessing both 9.5 and 23 kb fragments. Thus, the 8 nonobligates are as certain to be carriers as the obligates, based on analysis of their DNA. The absence of the 23 kb band in the proband and parents from Quebec City (WG1499, D.P. and R.R.) indicates that a different type of mutant allele is segregating in this family. Likewise, Southern analysis of DNA isolated from fibroblasts of the B^1 variant (WG1108), whose mother is of French Canadian ancestry, revealed only a 9.5 kb band. This was interesting since one would expect to see both 9.5 and 23 kb bands representing the mutant alleles inherited from the proband's father and mother, respectively. It is clear that the mutant α-locus allele for which the mother of WG1108 is heterozygous is not a deletion mutation, at least not of the type characterized by Myerowitz and Hogikyan (1986).

5. Pedigrees of two French Canadian TSD families.

The pedigrees of families 6 and 7 are shown in figures 27 and 28, respectively. The parents of proband 6 (cell strain WG1499) are from Quebec City. Family histories obtained from the parents indicated that they were unrelated to the TSD families along the north and south shores of the St. Lawrence River. Both parents tested as heterozygotes by serum Hex A assay. Southern analysis of DNA isolated from cells of both the proband and her parents revealed a single band of 9.5 kb. Examination of the pedigree revealed that the parents are first cousins.

The pedigree of family 7 is shown in figure 28. The family currently resides in Michigan's upper penninsula. The proband (cell strain WG1108) is a compound heterozygote for the B^1 allele (inherited from his father) and an allele which either

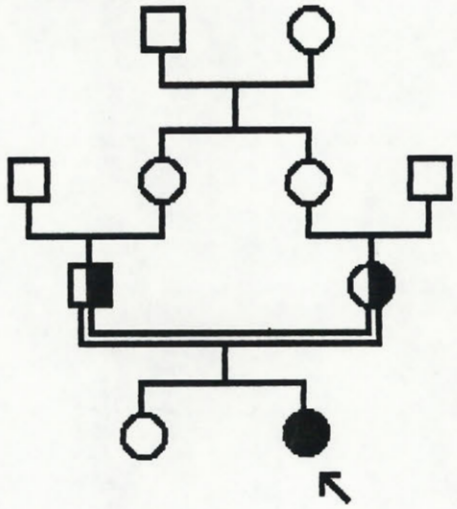

Figure 27. Pedigree of a French Canadian TSD family (number 6 on map). Family 6 is from Quebec City and has no known relationship to other TSD families. The proband is indicated by the arrow. The parents of the proband are first cousins.

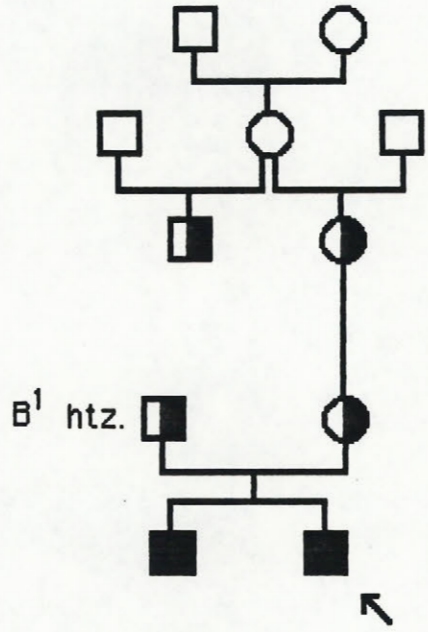

Figure 28. Pedigree of B^1 variant TSD family. The proband (indicated by the arrow) is a compound heterozygote. The CRM-positive B^1 allele was inherited from his father. The other allele, possibly CRM-negative, was inherited from his mother who is of French Canadian descent and traces her origins to St. Germain, Quebec.

does not produce α subunit or produces α subunit incapable of complexing with ß subunits (inherited from his mother). Analysis of the proband's DNA was included in this investigation because his mother is of French Canadian descent and traces her origins to St. Germain, Quebec (number 7 on the map) through her great-grandparents, both of whom were French Canadian. Southern analysis of the proband's DNA revealed a single band of 9.5 kb.

6. Biosynthetic labelling and immunoprecipitation of hexosaminidase subunits.

The B1 TSD proband (WG1108) was born to unrelated parents and is a compound heterozygote for two different mutant α-locus alleles. Cells of the proband synthesize Hex A as indicated by the chromatographic profile of the proband's fibroblast Hex isozymes (figure 18). Compound heterozygosity for two different mutant alleles at the α locus presents problems when attempting to determine the contribution of each allele to the synthesis and maturation of the α subunit. For this reason it is difficult to determine whether the mutant α-locus allele which the proband inherited from his French Canadian mother is CRM-positive or CRM-negative. However, the parents of proband WG1499 are first cousins and are likely heterozygous for an identical mutant α-locus allele. Since it was not known if the cells of WG1499 produce an α subunit of Hex A, fibroblast cell proteins of the proband were biosynthetically labelled and immunoprecipitated with antisera directed against the Hex isozymes as well as isolated α chain. Figure 29 shows the results of

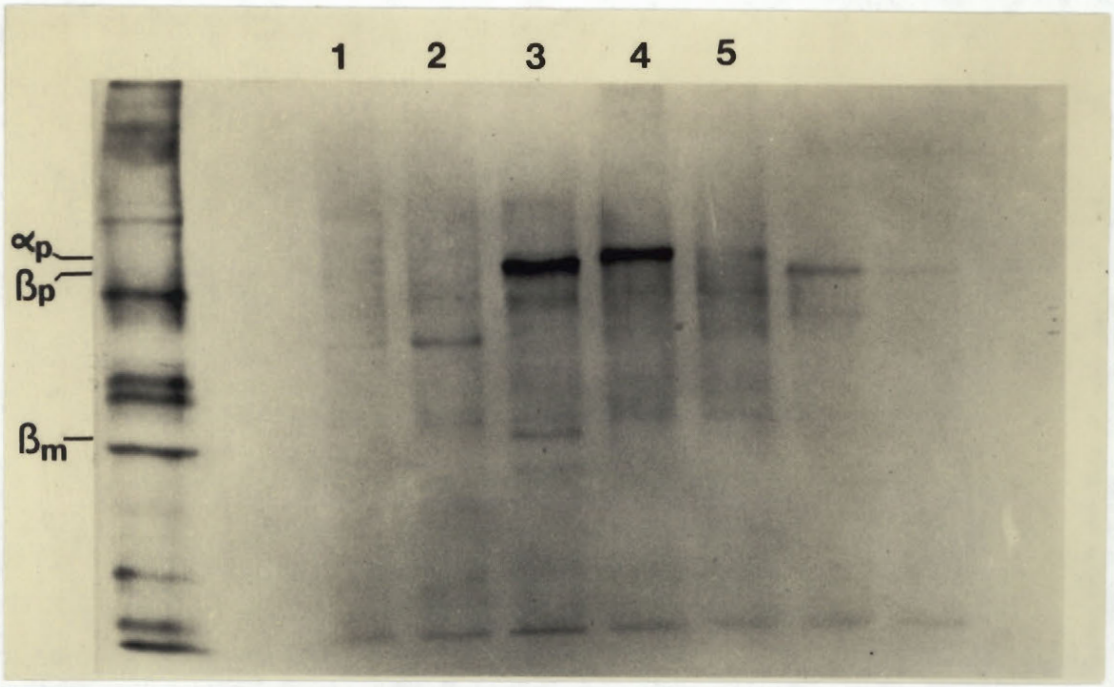

Figure 29. Immunoprecipitation and electrophoresis of hexosaminidase subunits biosynthetically labelled with [^{3}H] leucine from cell lysates. Cell proteins were pulsed for 1 hour. Radiolabelled antigens were precipitated with anti-α antiserum with the exception of cell antigens represented in lane 3, which were precipitated with antiserum directed against Hex A. Lane 1: Infantile TSD fibroblasts (WG1499), 1 hour chase. Lane 2: Infantile TSD fibroblasts (WG1499), 16 hour chase. Lane 3: Infantile TSD fibroblasts (WG1499), 1 hour chase. Lane 4: normal control fibroblasts, 1 hour chase. Lane 5: normal control fibroblasts, 16 hour chase.

α_p = 67 kDa; β_p = 63 kDa; β_m = 29 kDa.

these studies. Cell proteins of a normal control and the proband (WG1499) were labelled with [^{3}H] leucine and immunoprecipitated with antiserum directed against the α subunit of Hex A. The proband's cell proteins were also precipitated with anti-Hex A antiserum. Bands were visualized by fluorography. Anti-α antiserum precipitates only unassociated α-subunit polypeptides. Precipitation of control cell antigens with anti-α antiserum following a one hour chase with cold leucine revealed a band at 67 kDa, consistent with an α-subunit precursor polypeptide (lane 4). After a 16 hour chase with unlabelled leucine, very little free α subunit was detected (lane 5). In contrast, a 67 kDa band was clearly absent when the French Canadian TSD proband cell antigens were precipitated with anti-α antiserum and chased for one and 16 hours (lanes 1 and 2, respectively). Thus, no α-subunit precursor is synthesized in cells of proband WG1499. Antiserum directed against Hex A precipitates all free or associated α and ß subunits. After a one hour chase of labelled WG1499 cells, only antigens of 63 and 29 kDa were precipitated with antiserum directed against Hex A (lane 3). This is consistent with production, association, and maturation of ß subunits only.

Figure 30 shows the results of immunoprecipitation of labelled normal control and proband cell proteins with anti-Hex B antiserum. Antiserum directed against Hex B detects all ß subunits, free or associated, as well as α subunits associated with ß subunits; it does not detect unassociated α chains. Lanes 3 and 4 of figure 30 clearly show that normal control cells produce both Hex A and Hex B.

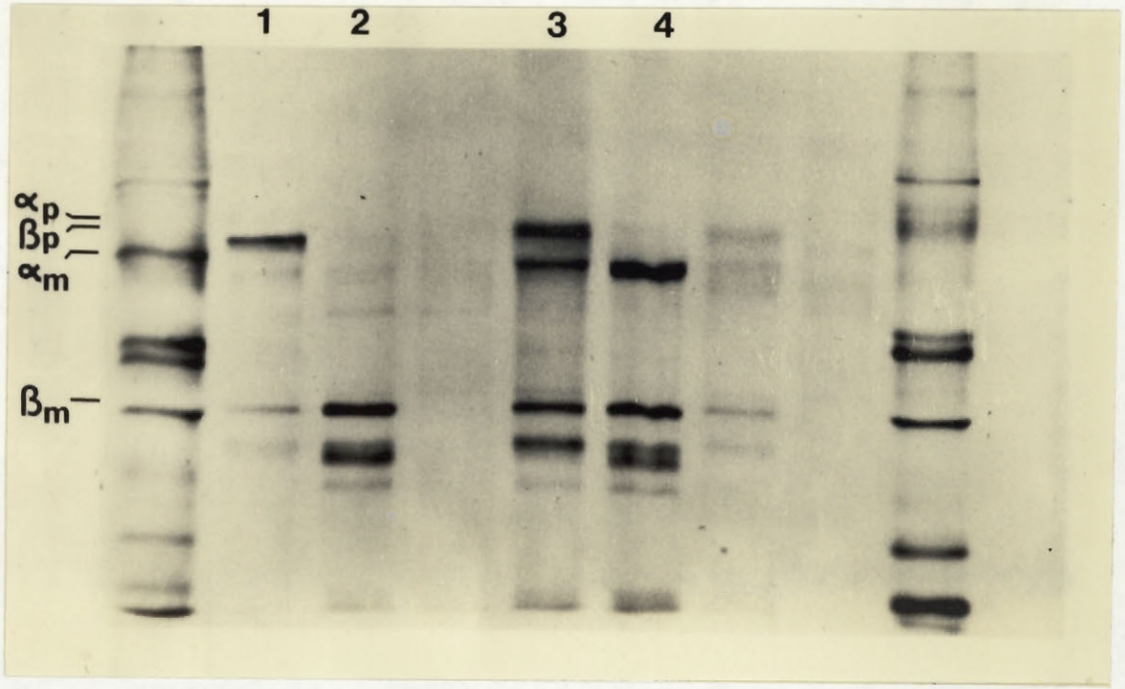

Figure 30. Immunoprecipitation and electrophoresis of hexosaminidase subunits biosynthetically labelled with ^{3}[H] leucine from cell lysates. Cell proteins were pulsed for 1 hour. Radiolabelled antigens were precipitated with anti-Hex B antiserum. Lane 1: Infantile TSD fibroblasts (WG1499), 1 hour chase. Lane 2: Infantile TSD fibroblasts (WG1499), 16 hour chase. Lane 3: normal control fibroblasts, 1 hour chase. Lane 4: normal control fibroblasts, 16 hour chase.

α_p = 67 kDa; α_m = 54 kDa; β_p = 63 kDa; β_m = 29 kDa.

Precursor and mature α and ß subunits were detected after a one hour chase, while only α and ß subunits of mature size were detected after a chase of 16 hours with unlabelled leucine. This is in clear contrast to cells of the TSD proband, WG1499. Cell proteins were labelled with [3H] leucine for one hour and chased for one hour with cold leucine. Proteins of 63 kDa and 29 kDa were precipitated with anti-Hex B antiserum (lane 1). After a chase of 16 hours only mature ß subunit of 29 kDa was observed (lane 2). The results of immunoprecipitation studies with antiserum directed against Hex B confirm the findings of immunoprecipitation studies with antiserum directed against both free and associated α chain. The French Canadian TSD proband cells (WG1499) produce ß subunit of normal size which associates with other ß subunits to form Hex B and is proteolytically processed to mature size. In contrast, an α-subunit polypeptide is not synthesized by cells of this proband and she is thus homozygous for a CRM-negative mutation at the gene locus encoding the α subunit of Hex A. The biosynthetic labelling and immunoprecipitation experiments were performed by Bernard Boulay, a technician at the deBelle Laboratory for Biochemical Genetics (Montreal Children's Hospital, Montreal, Quebec, Canada); the antisera used in the study were kindly donated by Dr. Elizabeth Neufeld (UCLA, Los Angeles, CA).

7. Analysis of French Canadian TSD DNA by a PCR-amplified technique.

The 300-bp genomic probe used in Southern analysis of DNA isolated from French Canadian infantile TSD probands failed to detect the 5' end deletion mutation in two such affected probands (WG1108 and WG1499). The mutation in some TSD patients of Ashkenazi Jewish descent was recently found by Arpaia et al

(1988), Myerowitz (1988), and Ohno and Suzuki (1988b) to be a G —› C transversion in the 5' splice junction site of intron 12, detected by nucleotide sequencing of normal control and Ashkenazi Jewish TSD DNAs. The nucleotide change in the mutant DNA was found to generate a Dde I restriction site. A 151-bp segment of DNA surrounding this site was amplified by the polymerase chain reaction, followed by digestion of the PCR product with Dde I and separation of the restriction fragments by PAGE (Arpaia et al, 1988). Genomic DNA that I isolated from WG1108 and WG1499 fibroblasts was amplified by the PCR technique to determine if the mutation in these two patients is similar to the mutation found in the Ashkenazi Jewish infantile TSD patients. The amplification and electrophoresis of WG1108 and WG1499 DNAs were performed by E. Arpaia (Hospital for Sick Children, Toronto, Ontario, Canada). The results are shown in figure 31.The lanes labelled 124 and 822 are from plasmids containing the normal and mutant sequences, respectively. Digestion with Dde I yielded a 120-bp fragment of DNA in the normal controls (124 and MCH41) and two fragments of 85- and 35-bp in the Ashkenazi Jewish infantile TSD mutant (822). These two fragments are due the generation of a Dde I site in the mutant DNA as a consequence of the mutation. PCR-amplified genomic DNA from the two infantile TSD patients with French Canadian ancestry, WG1108 and WG1499, yielded only one fragment of 120-bp when digested with Dde I and electrophoresed. Thus, the α-locus mutation(s) inherited from their French Canadian parents, for which WG1108 is heterozygous and WG1499 is homozygous, is (are) not the intron 12 splice junction transversion.

The results of Southern analysis of DNA obtained from 6 TSD families with French Canadian ancestry clearly indicate the existence of more than one mutation in this population. The deletion mutation at the 5' end of the α-chain gene, initially discovered by Dr. R. Myerowitz, was found in four families from the south

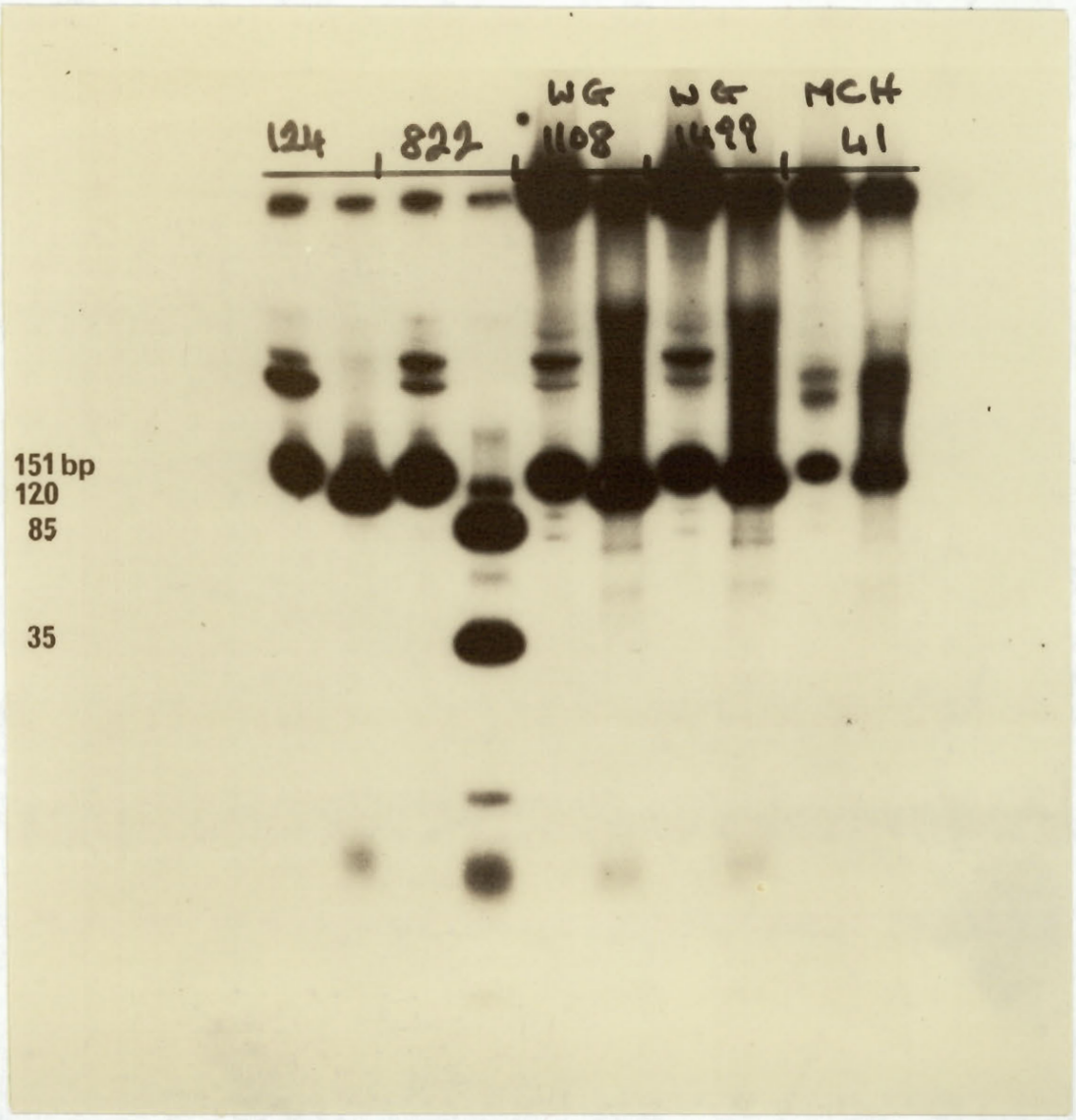

Figure 31. Polyacrylamide gel electrophoresis of PCR-amplified DNA. A 151-bp sequence surrounding the first nucleotide of the 5' splice junction site of intron 12 was amplified by PCR. [^{32}P] dCTP was incorporated during priming throughout the PCR cycles. 124 and MCH 41 represent normal controls. 822 represents an Ashkenazi Jewish infantile TSD patient. WG1108 and WG1499 represent two infantile TSD patients with French Canadian ancestry who do not possess the 5' end gene deletion. The first lane of each pair contains the amplified 151-bp sequence. The second lane of each pair contains the same material after Dde I digestion. The gels were exposed without drying to X-ray film overnight.

shore of the St. Lawrence River encompassing the cities of Rimouski and Sayabec (in Quebec), and one family from Edmundston, New Brunswick. Two families originating from regions of Quebec geographically remote from these communities do not possess the deletion mutation. Fibroblasts from one of the probands (WG1108) produce an α subunit which complexes with ß subunit to form Hex A. The Hex A has activity against unsulfated synthetic substrates yet is unable to hydrolyze sulfated synthetic substrates and G_{M2} ganglioside. The proband is a compound heterozygote for the B^1 allele and, most likely, a CRM-negative mutant allele. This latter allele was inherited from the proband's mother who is of French Canadian descent. The second proband of French Canadian descent (WG1499) was born to consanguineous parents. Fibroblasts obtained from this patient have no Hex A activity nor do they synthesize an antigen which cross-reacts with antiserum directed against the α subunit of Hex A. Analysis of PCR-amplified DNA obtained from fibroblasts of both patients (WG1108 and WG1499) indicates that the mutation differs from the intron 12 splice junction error observed in amplified DNA of some Ashkenazi Jewish TSD patients. The nature of the mutation in these two patients has yet to be characterized. Thus, while the founder effect may explain the high incidence of TSD in a single highly inbred region of the southeastern province of Quebec, it cannot account for all cases of TSD in the French Canadian population.

IV. DISCUSSION

A. Overview.

Tay-Sachs disease was described a century ago by Drs. Waren Tay (1881) and Bernard Sachs (1887) as a progressive disease affecting children in infancy and characterized by dementia, seizures, and macular cherry-red spots. Since that time, much has been learned about the substance stored in the brains of affected individuals, its pathophysiological effects, and the enzyme normally responsible for its degradation. A complex picture has emerged.

Neuronal storage of G_{M2} ganglioside results from mutations at three gene loci encoding two different kinds of polypeptides necessary for the production of hexosaminidase A and one activator protein required for the Hex A-catalyzed hydrolysis of G_{M2} ganglioside (Johnson, 1981). A wide variety of clinical disorders result from multiple mutant alleles at the two subunit loci. Thus, TSD is actually a group of recessively-inherited diseases that result from mutations at the gene locus encoding the α-subunit polypeptide of Hex A on chromosome 15. Affected individuals may be homozygous for a particular α-locus mutant allele. More often, however, they are heterozygous for two different mutant alleles at this locus.

The range of known mutation mechanisms that result in the storage of G_{M2} ganglioside include both deletion and nondeletion CRM-negative mutations (Myerowitz and Hogikyan, 1986; Arpaia et al, 1988; Myerowitz, 1988) as well as CRM-positive mutations with (Ohno and Suzuki, 1988) and without (Zokaeem et al, 1987) Hex A-associated catalytic activity. The effects of neuronal storage of G_{M2} ganglioside may become apparent anywhere from infancy, with a rapid progression of the disease resulting in death within the first few years of life, to adulthood, with a slow progression and no apparent effects on longevity. The variant forms of TSD

were reviewed previously (pages 32-43).

There is currently no treatment for TSD. Studies aimed at enzyme replacement therapy, however, have been encouraging but are still in the experimental stage. Brooks et al (1980) demonstrated that cultured cerebellar cells of a TSD fetus incorporated Hex A after exposure of the cells to concanavalin-A. Neuwelt et al (1984) demonstrated the delivery of catalytically active Hex A to rat brain after osmotic blood brain barrier disruption. They found that approximately 76% of the Hex A was packaged in a subcellular organelle. Dobrenis et al (1987, 1988) reported the enhanced uptake of Hex A conjugated to tetanus toxin fragment C by neurons in culture. As a result of this upake, neural cell cultures from a neonatal kitten with G_{M2} gangliosidosis showed a reduction in stored G_{M2} ganglioside. These studies indicate that enzyme replacement therapy may someday be feasible. Until then, however, clinicians must rely on the ability to identify couples at risk for producing affected offspring and the prenatal detection of affected fetuses.

The reliable diagnosis of affected individuals and the detection of TS carriers in the population depends upon an accurate measurement of the amount and catalytic activity of Hex A. Methods employing the natural substrate, while undeniably providing the most precise diagnostic information, are impractical for widescale use. The problems inherent in isolating and labelling G_{M2} ganglioside as well as isolating the activator protein are not insignificant and many laboratories are unequipped for such work.

Commercially available synthetic substrates used to measure Hex A activity include the para-nitrophenyl- and 4-methylumbelliferyl- derivatives of N-acetylglucosamine, the latter generally preferred because it fluoresces when hydrolyzed and provides for a more sensitive assay. The disadvantage of assays employing these substrates is that they are hydrolyzed by both Hex A and Hex B.

The contribution of each isozyme to Hex activity present in tissues and fluids must be determined by an assay method that exploits the differences between the two isozymes. The method most often employed - thermal inactivation of Hex A - is associated with inaccuracies due to variable amounts of Hex A remaining or Hex B inactivated. Additionally, since the hydrolysis of 4MUG occurs at a catalytic site on the ß-subunit common to both isozymes (Kytzia and Sandhoff, 1985; Bayleran et al, 1987), it does not directly assess the product of the mutant TSD gene. A synthetic substrate that is specifically hydrolyzed by Hex A is required.

Kytzia and Sandhoff (1985) demonstrated that Hex A possesses two active sites with different substrate specificities. A site on the ß subunit (common to both Hex A and Hex B) hydrolyzes N-acetygalactosaminyl- and N-acetylglucosaminyl-linkages from substrates that carry no electric charge. A site on the α subunit (unique to Hex A) possesses an affinity for substrates carrying a negative charge. The α subunit is also provided with an activator protein-binding site. Thus, the active site on the α subunit of Hex A hydrolyzes the terminal N-acetylgalactosaminyl linkage of the negatively-charged G_{M2} ganglioside. The hydrolysis of G_{M2} occurs only in the presence of G_{M2} activator protein, which presents the ganglioside to Hex A as an activator/lipid complex (Banerjee et al, 1984) with a stoichiometric ratio of 1:1. The AP portion of the complex positions itself in the AP-binding site on the α subunit of Hex A.

G_{M2} ganglioside is not a substrate for Hex B, a homopolymer of ß subunits. Neither is it a substrate for Hex S, a dimer of α subunits, even though the α subunits of this isozyme possess the AP-binding site. This indicates that α subunits require the

assistance of ß subunits in order to bring about degradation of G_{M2} ganglioside (Kytzia et al, 1984).

Although Hex B is unable to hydrolyze G_{M2} ganglioside, both Hex A and Hex B hydrolyze the asialic derivative of G_{M2}. Thus, the negatively-charged NANA residue present in G_{M2} and absent in G_{A2} glycolipid also plays a role in the unique ability of Hex A to hydrolyze G_{M2} ganglioside (Li et al, 1984). Mahuran et al (1985) have proposed that the hydrolysis of natural substrates carrying negatively-charged groups near the terminal nonreducing sugar residue occurring exclusively by the action of Hex A is suggestive of a positively-charged binding pocket in the α subunit that is not present in the ß subunit. Hydrolysis of G_{A2} glycolipid occurs at the ß subunit active site common to both isozymes.

Evidence that sulfated derivatives of synthetic glucosaminide substrates are specifically hydrolyzed by Hex A was first provided by Kresse et al (1981) who synthesized the 6-sulfated derivative of pNPG. Sulfation produced a substrate with a negatively-charged group in the vicinity of the terminal N-acetylglucosaminyl linkage that is cleaved. This negatively-charged group promotes binding preferentially to the α subunit catalytic site of Hex A.

B. Sulfation of 4MUG produces a substrate preferentially hydrolyzed by Hex A.

Sulfation of 4-methylumbelliferyl glucosaminide was undertaken to produce a fluorogenic substrate that would be preferentially hydrolyzed by Hex A. Optimum conditions for 4MUG sulfation include a 1.5 molar excess of chlorosulfonic acid at low temperature and under anhydrous conditions. These conditions favor monosulfation at the primary hydroxyl group on C6 of the hexosamine ring (Bayleran

and Hechtman, 1983; Bayleran et al, 1984). The protocol used to synthesize 4MUGS produces a high yield of product. Extensive purification of the reaction product by ion-exchange chromatography and gel filtration eliminates unreacted starting material and inorganic salts, respectively. The identity of the product is confirmed by comparison of the UV absorption spectra of 4MUG and 4MUGS, as well as by TLC, NMR, and elemental analysis, all of which demonstrate the presence of a single compound that is monosulfated at position 6 of the ring. There is no unreacted starting material (i.e. 4MUG) present. Nor is there any disulfated substrate. Purification of the reaction product is admittedly time-consuming since it involves three chromatographic steps and multiple lyophilizations. An alternative to the multiple chromatographic and lyophilization procedures might be desirable. However, the steps that I employ yield a very pure product as well as a high recovery. The 4MUGS recovery by my protocol is 75-77% of the theoretical expectation. 4MUGS has subsequently been synthesized by Inui and Wenger (1984) and Ben-Yoseph et al (1985) by protocols different from mine. Both groups have encountered problems of producing di- and trisulfated compounds. Inui and Wenger (1984) have obtained yields of monosulfated product that are 30% and Ben-Yoseph et al (1985) that are 39-45% of the theoretical yield. The first group to synthesize a sulfated derivative of glucosaminide was Kresse et al (1981) and Fuchs et al (1983) who synthesized pNPGS. They reported obtaining small amounts of di- and trisulfated compounds that were easily separated from unreacted starting material and monosulfated product by ion-exchange chromatography. The final yield of pNPGS after purification was 46% of the theoretical yield (Fuchs et al, 1983). Thus, the yields of monosulfated product that have been achieved by others using different protocols for the sulfation of glucosaminides have been considerably lower than mine.

The specificity of 4MUGS for the α subunit active site of Hex A was

demonstrated using Hex A and Hex B isolated from normal control fibroblast cell lysates, sera, and liver homogenates. Kinetic analyses, pH optimum determinations, and inhibition studies of Hex A- and Hex B-catalyzed hydrolysis of 4MUG and 4MUGS were undertaken.

Previous reports of the kinetic parameters of 4MUG hydrolysis by purified control Hex A and Hex B gave similar Km and Vmax values, as well as similar pH optima (Srivastava et al, 1974b; Tallman et al, 1974; Kytzia and Sandhoff, 1985; Tommasini et al, 1985). In contrast, these parameters are found to differ significantly for the two enzymes when measured with 4MUGS. Figure 9 indicates that the pH optima of Hex A and Hex B activities differ when measured with the sulfated substrate. The optimum pH of Hex A against 4MUGS, 3.9, is very similar to the value of 4.1 found by O'Brien et al (1977) for Hex A-catalyzed hydrolysis of G_{M2} ganglioside. Kresse et al (1981) determined pH activity profiles for the hydrolysis of pNPG and pNPGS by isolated Hex A. They found optimal Hex A activity against pNPG to occur at a pH of 4.3 whereas optimal activity against the sulfated derivative of pNPG occurred at a pH of 3.9.

Kinetic analysis of normal control Hex A and Hex B, depicted in figure 10, strongly suggests 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 common to both isozymes. This finding is confirmed by a comparison of the relative rates of hydrolysis of 4MUGS/ 4MUG by control Hex A and Hex B (table 1). The constant values, r_A and r_B, which express these ratios are employed in the empirical formula (Appendix A) that facilitates comparison of assay values obtained using 4MUG and 4MUGS substrates. These ratios are an indication of the substrate specificity of Hex A and Hex B. It is clear that Hex A-catalyzed hydrolysis of 4MUGS relative to 4MUG (represented by the constant, r_A) occurs at a much faster rate than Hex B-

catalyzed hydrolysis of the sulfated substrate relative to 4MUG (represented by r_B). The determination of these constants can be utilized as a measure of the purity of the sulfated substrate preparation and it is particularly advisable that an r_B value be determined for each substrate preparation. The r_B constant for my preparations of 4MUGS, measured using Hex B isolated from normal fibroblasts or sera, was consistently low, at a value of 0.0015. Ben-Yoseph et al (1985) and Kytzia and Sandhoff (1985) synthesized 4MUGS preparations which have r_B values of approximately 0.0014 and 0.0027, respectively. A rough approximation of the relative rates of hydrolysis of pNPGS/ pNPG by isolated Hex B from the chromatographic profile presented by Fuchs et al (1983) gives a low r_B value comparable to those obtained for my preparations of 4MUGS as well as Ben-Yoseph's. In contrast, I found a significant amount of serum Hex B activity against a commercial preparation of 4MUGS (purchased from HSC Corporation, Toronto, Ontario) and felt further purification of the preparation was required before it could be used reliably in an assay. The r_B value prior to purification was 0.0184. Purification by the three chromatographic steps described earlier eliminated a significant amount of unreacted starting material and produced a much lower ratio of Hex B activity against the two substrates. Thus, the r_B constant after further purification of the commercially prepared 4MUGS was 0.0012. Likewise, Besley et al (1987b) measured the relative rates of hydrolysis of 4MUGS/ 4MUG by isolated Hex B using a commercial preparation of sulfated substrate (obtained from Koch-Light). They measured an r_B of 0.007. These examples illustrate the variation in purity of different preparations of sulfated substrate and demonstrate the usefulness of determining an r_B value for every preparation of sulfated substrate as a measure of its purity.

Inhibition of 4MUG and 4MUGS hydrolysis by control Hex A and Hex B in the presence of N-acetylglucosamine (NG) and N-acetylglucosamine-6-phosphate (NGP) demonstrate the specificity of the two inhibitors for the ß- and α-subunit active sites, respectively. Hydrolysis of 4MUG by either control Hex A or control Hex B is inhibited to the same extent by NG (figures 21A and 21B). This suggests that this substrate is hydrolyzed by an identical active site on both enzymes. In contrast 4MUGS hydrolysis by control Hex A is strongly inhibited by NGP, yet this inhibitor has little effect on the small amount of hydrolysis that occurs at the ß-subunit active site (figures 22A and 22B). Thus, the different responses of Hex A- and Hex B-catalyzed hydrolysis of 4MUG and 4MUGS to inhibition by NG and NGP point to the likelihood that Hex A is a bifunctional enzyme. These results corroborate similar findings by Kytzia and Sandhoff (1985), though their approach was to simultaneously present 4MUG and 4MUGS to Hex A at varying partial concentrations but keeping total substrate concentration constant and to determine whether total substrate hydrolysis was additive (as expected for two different active sites) or competitive (as expected if there was only one active site on Hex A).

The specificity of 4MUGS for the α subunit predicts a high r_S value, since Hex S consists solely of α subunits. This is suggested by the results I obtained from Hex assays using Sandhoff disease cell extract (table 3). Sandhoff disease is due to a mutation at the gene locus encoding the ß subunit of the hexosaminidases. As such, patients with this disease are deficient in both Hex A and Hex B. The gene locus encoding the α subunit is unaffected in SD. In the absence of ß subunits, the α subunits complex to form Hex S. 4MUG is hydrolyzed to a small extent by the active site on the α subunit and thus the small amount of 4MUG hydrolysis by the SD cell

extract is almost completely attributable to the Hex S isozyme in cells of this patient. 4MUGS, however, is preferentially hydrolyzed at the α-subunit active site. Since cells of the infantile SD patient are deficient in both Hex A and Hex B, the 4MUGS hydrolysis is attributable to the action of Hex S. In the assay procedure that employs the two substrates and application of the empirical formula, hydrolysis of 4MUGS by SD cell extract is greater than the total Hex activity measured by hydrolysis of 4MUG, suggesting a high r_S value. It should be noted that I used only unfractionated extract of SD cells and did not attempt to isolate Hex S whereas others did isolate this isozyme. Hex S isolated by ion-exchange chromatography from fibroblasts (Kytzia and Sandhoff, 1985; Besley et al, 1987b) and sera (Ben-Yoseph et al, 1988) of infantile SD patients was assayed with both 4MUG and 4MUGS. All three groups presented data that indicate the r_S value to be approximately 0.33-0.57.

C. Tay-Sachs heterozygote screening.

The high incidence of TSD in Ashkenazi Jewish populations and the lethal nature of the infantile form of the disease, as well as the inability to provide treatment, has led to the development of procedures aimed at carrier detection and prenatal diagnosis. Screening for TS carriers is currently performed using an assay procedure that is dependent upon the thermal inactivation of Hex A activity. The substrate most often employed is 4MUG. It has been stated previously that this assay procedure is tedious and is often associated with inaccuracies due to variable inactivation of the Hex isozymes. Other problems encountered in Hex assays employing 4MUG include the following: (1) Plotting enzyme activities in two dimensions resolves normal controls and TS heterozygotes into two distinct clusters. However, there is overlap between the range of low and high Hex A activities for the controls and carriers, respectively. A certain percentage of individuals tested will fall within this 'grey

zone', i.e., into an inconclusive zone for classification. Usually, retesting these individuals by the more accurate, although more tedious, leukocyte assay will improve genotype assignment. (2) Determination of carrier status by serum Hex assay in pregnant women is not possible using the thermal fractionation assay. During pregnancy, a heat-stable isozyme (Hex P) which has activity against 4MUG is present in serum and results in false-positive testing. This isozyme is not present in cells and thus assessment of carrier status in women who are pregnant must rely on leukocyte assay. (3) Many variant forms of TSD are believed to represent compound heterozygosity for two mutant α-locus alleles, one of which is usually a CRM-negative mutation. The other allele is a mutation with milder neurological consequences, often producing an α subunit that is capable of associating with ß subunits to form a species of Hex A with altered catalytic activity. The carriers of such 'milder' mutant alleles may not be accurately detected by the 4MUG substrate, particularly if the mutation does not influence the formation of the α-ß dimer. (4) Occasionally, routine screening or investigation of pedigrees with TSD probands reveals healthy adults who are enzymatically indistinguishable from affected infantile TSD probands. The significance of these findings is unknown since 4MUG has no prognostic value in determining whether an adult with low Hex A activity is awaiting a neurological disorder or whether he/she will remain symptom-free. (5) The prenatal misdiagnosis of an affected fetus who is a B^1 compound heterozygote for variant alleles at the α-locus is attributed to the reliance on the thermal fractionation assay procedure to assess Hex A activity in amniocytes (Kolodny et al, 1983). A B^1 homozygous fetus would also be at risk for misdiagnosis in utero based on the thermal fractionation assay procedure to assess Hex A activity. Most of the

disadvantages associated with use of the 4MUG substrate to assess Hex A activity stem from the fact that this substrate is hydrolyzed by an active site on the ß subunit of the Hex isozymes and thus does not provide for a direct assessment of the mutant α-locus gene product.

Because 4MUGS is hydrolyzed specifically by an active site on the α subunit of Hex A, assay with this substrate was expected to resolve some of the problems of carrier detection and enzymatic diagnosis of affected probands. Serum was analyzed using two different assay procedures for the discrimination of obligate TS heterozygotes and normal controls. The two assay procedures employed were: (i) assay of total and heat-stable Hex activities with 4MUG following thermal inactivation of Hex A and (ii) assay of Hex activity with both 4MUG and 4MUGS substrates and application of an empirical formula (Appendix A) to determine Hex A and B activities expressed as nanomoles of 4MUG hydrolyzed. The empirical formula was developed to facilitate comparison of the two-substrate assay procedure with the thermal fractionation assay procedure routinely used to assess Hex A activity. It transforms fluorometric units measured with 4MUGS into equivalent 4MUG enzyme units and incorporates constants for the relative rates of hydrolysis of 4MUGS/4MUG by isolated Hex A and Hex B. A value for % Hex A can be easily determined using total Hex activity measured with 4MUG. This equation is routinely employed for TS heterozygote screening in at least one other laboratory (G.D. Vladutiu, personal communication). Ben-Yoseph et al (1985) also routinely employ a two-substrate assay procedure to assess Hex A activity using the sulfated substrate. They employ the ratic of hydrolysis of 4MUG/4MUGS by isolated Hex A to convert 4MUGS activity to the corresponding 4MUG activity of Hex A. They then calculate a % Hex A value for each individual tested using total Hex activity as measured with

4MUG. The calculation of an r_B constant in the equation that I employ, however, has the advantage of subtracting the small amount of Hex B activity against 4MUGS.

While both methods are satisfactory for classifying 'classical' TSD genotypes using a two-discriminant plot (represented in figure 11 and in table 4) the procedure employing 4MUGS does offer a slight statistical improvement in classification as indicated by the greater separation of enzyme activity means and the smaller standard deviations for the control and obligate TS carrier groups. In addition, there is no overlap in Hex A activities between the control and carrier groups by the two-substrate assay procedure whereas considerable overlap exists in the range of Hex A activities for these two groups when determined by the thermal fractionation assay with 4MUG. While no individuals were found to fall in an inconclusive zone for classification by the two-substrate assay procedure, it is possible that testing of more individuals than the numbers tested in this study would reveal some who cannot be reliably classified.

Percent Hex A values determined by the thermal fractionation assay employing 4MUG were plotted against the same values determined by application of the equation using the two-substrate assay for each individual tested (figure 13). The results indicate that not only do individuals resolve into two distinct clusters representing controls and carriers, but there is a high degree of correlation between the % Hex A value obtained by 4MUG and the % Hex A value determined by the sulfated substrate assay procedure for each individual tested.

A third assay procedure employed in serum testing was the measurement of 4MUGS hydrolysis alone, i.e. without any reference to total Hex activity and without application of the empirical formula. These values are shown in the single-discriminant plot (figure 12 and table 4). No overlap between the control and carrier groups occurs when 4MUGS hydrolysis is measured, while there is a significant

amount of overlap between these two groups when thermolabile Hex activity is assessed with 4MUG. In theory, therefore, a one-step assay procedure could be used for the detection of TS heterozygotes by measurement of 4MUGS hydrolysis alone. While assay of Hex activity by this procedure would certainly provide for a rapid assessment of carrier status, the superior resolution offered by the use of two discriminants should not be abandoned in favor of the increased speed of the one-step 4MUGS procedure.

Some laboratories in larger cities across North America employ an automated procedure for their mass TS screening programs (Delvin et al, 1974; Lowden et al, 1973; Desnick et al, 1976). While the automated assay procedure still employs 4MUG as substrate and is, therefore, dependent upon thermal fractionation, it is preferred by some to manual assays for its speed and increased reliability. The reproducibility of the automated assay has been reported to be about two-fold greater than assays performed manually (Lowden et al, 1973; Delvin et al, 1974). However, even by an automated procedure most of the disadvantages associated with the use of 4MUG to assess carrier status still apply.

The usefulness of the two-substrate assay procedure for the larger numbers of samples screened by an automated assay method was investigated. The automated assay procedure currently employed in the mass TS screening program in Montreal (deBelle Laboratory for Biochemical Genetics, Montreal Children's Hospital) is dependent upon thermal fractionation of serum Hex A activity and assay with 4MUG. The apparatus used in the automated assay procedure was changed to accomodate two substrates (as previously described, page 60). 4MUG measures total Hex activity while 4MUGS measures hydrolysis occurring primarily at the α-subunit catalytic site. Application of the empirical equation to transform 4MUGS assay values into equivalent 4MUG enzyme units yields an amount of Hex A activity and a % Hex A

value for each individual that can be compared to those values obtained by the automated thermal fractionation assay.

Hexosaminidase activities generated by the automated assay are higher than those measured manually. Kolodny (1977) attributed a similar finding in his laboratory to a higher final substrate concentration in the automated procedure than that employed in the manual procedure. The ability to identify TS heterozygotes by an automated assay procedure employing the sulfated substrate was compared to the ability of the automated thermal fractionation assay to detect carriers (table 5). To my knowledge, this is the first such attempt to employ sulfated glucosaminides to an automated carrier detection assay procedure. Thirty-one normal controls, 8 obligate TS heterozygotes and 16 nonobligate TS carriers were tested by the automated assay procedures. The range, mean, and standard deviation values of Hex A activities for the obligate and nonobligate TS heterozygotes were surprisingly different by the thermal fractionation assay employing 4MUG. The range of Hex A activity was narrower and the mean and standard deviation values smaller for the obligate heterozygotes than for the nonobligate carriers. In contrast, these values were nearly identical by the automated two-substrate assay procedure that employs both 4MUG and 4MUGS.

The results in table 5 clearly demonstrate that the ability to detect TS carriers is improved when serum Hex A activity is assessed by the automated assay procedure that employs 4MUGS than by the automated assay dependent upon thermal inactivation of Hex A activity. Adapting the automated assay procedure to use with two substrates has the advantage of measuring Hex A activity several ways. By application of the formula, 4MUGS fluorometric units can be converted into equivalent 4MUG enzyme units and from this a % Hex A value can be determined using total Hex activity as measured by 4MUG. Alternatively, Hex A activity against 4MUGS alone, without application of the formula, can be assessed. Furthermore,

this adaptation can be achieved with an absolute minimum of changes to the auto-analyzer. Hydrolysis of 4MUG by the Hex isozymes occurs at a faster rate than hydrolysis of 4MUGS. Fuchs et al (1983) believed this to be an indication that the sulfated substrates were unsuited to an automated procedure. However, this problem is handled quite effectively by increasing the sensitivity setting of the fluorometer which reads 4MU fluorescence generated by hydrolysis of the sulfated substrate.

The importance of TS carrier screening programs is the identification of couples at risk for having an affected fetus and, ultimately, the prevention of TSD. Occasionally, a woman who is already pregnant requests testing for TS heterozygosity. The determination of carrier status during pregnancy presents problems due to a significant increase in the amount of heat-stable Hex isozyme present in serum. This increase is attributable to the synthesis of large amounts of Hex P, an enzyme that has electrophoretic and thermal characteristics identical to Hex I_2 (Geiger et al, 1978) and is also a β-chain oligomer (Beutler et al, 1975; Geiger et al, 1978). Most laboratories still employ the heat inactivation assay for TS heterozygote screening. The increased levels of heat-stable Hex in serum during pregnancy thus give the impression of a reduced Hex A component. Since Hex P is present in sera but not cells of pregnant women, O'Brien et al (1970) proposed that the assay of Hex A activity from leukocytes would more reliably identify carriers during pregnancy. Navon et al (1973) demonstrated that leukocyte assays did reliably classify genotypes of pregnant women and, as a result, when a pregnant woman requests testing the assay is routinely performed on leukocytes (Kaback et al, 1974). However, the isolation of leukocytes from whole blood involves a tedious procedure.

I investigated the usefulness of the sulfated substrate, 4MUGS, for the classification of pregnant women as TS heterozygotes or normal homozygotes by assay of serum Hex A activity. The working assumption was that the specificity of

4MUGS for the α subunit active site would provide for a direct assessment of serum Hex A activity and, thus, the increased levels of heat-stable isozyme would not interfere with accurate genotype classification of the pregnant women. The results of this investigation (presented in table 6) indicate that the measurement of 4MUGS hydrolysis alone, without reference to total Hex activity, differentiates between pregnant normal homozygotes and pregnant TS heterozygotes. Hex A activity measured by application of the formula also appears to be a useful discriminant of genotypes. However, the '% Hex A' statistic determined by application of the formula in the two-substrate assay procedure is still inaccurate since Hex P contributes to the total Hex activity measured by 4MUG. Since the sera of only 25 pregnant women were tested, 7 of whom were TS carriers, it is clear that more testing is required before an accurate assessment can be made of the reliability of the sulfated substrate to identify carriers by serum during pregnancy.

Ben-Yoseph et al (1985) reported that the 6-sulfated derivatives of 4MU-ß-D-N-acetylglucosamine and 4MU-ß-D-N-acetylgalactosamine were especially useful for the detection of TS heterozygotes by serum assay during pregnancy, which he based on the ability to clearly differentiate between pregnant normal controls and non-pregnant TS heterozygotes. They provided no data on sera obtained from pregnant TS carriers. More recently, however, Ben-Yoseph et al (1988) presented their findings on the usefulness of sulfated substrates for the detection of carriers using serum samples obtained from pregnant and nonpregnant normal homozygotes and obligate TS heterozygotes. They provide evidence that assays with these substrates cannot reliably detect carrier status in pregnant women when serum is utilized as the enzyme source. Ben-Yoseph et al (1988) found that the increased serum Hex activity during pregnancy is attributable not only to an elevation of Hex P, but that there is also an increase in Hex A activity during pregnancy which is evident upon

chromatographic isolation of serum Hex isozymes. They reported that serum Hex A activity gradually increases during pregnancy and that the increase appears to be influenced by the fetus' genotype since Hex A activities were found to be higher in pregnant TS heterozygotes who carried homozygous normal fetuses than in those carrying fetuses who were TS heterozygotes. Navon et al (1987) also observed a rise in levels of serum Hex A activity of pregnant women particularly after the first trimester of pregnancy. They tested the sera of 19 pregnant women at various stages throughout their pregnancies. They reported no change in the Hex A levels of three pregnant obligate TS heterozygotes who were found by amniocenteses to be carrying affected TSD fetuses. In contrast, they found a 4-fold increase of Hex A activity in the sera of two women affected with the adult-onset form of TSD who were found to be carrying unaffected fetuses. Hex A activites in the affected mothers returned to very low levels following delivery of their normal children. Thus, the increase of Hex A activity in the sera of pregnant women appears to be of fetal origin. The leakage of fetal enzymes into maternal serum has been reported in women carrying fetuses affected with I-cell disease (Hug et al, 1984). In addition, increases in the maternal serum of α-fetoprotein (Milunsky et al, 1980) and iduronate sulfate sulfatase activities (Zlotogora and Bach, 1984), both of which are of fetal origin, have been reported.

While the source of Hex P in the sera of pregnant women is not known with certainty, it is believed to be the liver (Pérez et al, 1971). Heat-stable Hex activity is also increased in the sera of women taking oral contraceptives (Kaback et al, 1973 and 1974; Lowden et al, 1973). For this reason, the production of Hex P is believed to be hormonally-stimulated. Since only the heat-stable component of Hex is elevated in the sera of women taking oral contraceptives the sulfated substrate assay procedure might prove useful in the determination of carrier status. By the current thermal

fractionation assay procedure utilized in screening programs, these women frequently fall into the inconclusive zone for classification or are misclassified as normal homozygotes. However, during pregnancy serum Hex A as well as Hex P levels are elevated. It is important to note that the passage of Hex A across the placenta appears to be unidirectional, from the fetus to the mother, since the sera of newborn TSD infants lack Hex A activity (Perry et al, 1979; Kaback, personal communication, in Ben-Yoseph et al, 1988). Thus the sulfated substrates can be relied upon to accurately diagnose fetuses prenatally. It appears that they cannot be relied upon, however, to accurately classify the genotypes of pregnant women by assay of serum Hex activities. Further testing might bear out Ben-Yoseph et al's (1988) conclusion that accurate classification of pregnant women can only be achieved by assay of leukocyte Hex activities which are influenced neither by Hex P nor by fetal Hex A.

D. Diagnosis of TSD using 4MUGS.

Fibroblast cell extracts from patients with variant forms of G_{M2} gangliosidosis were assayed for the purpose of determining the diagnostic value of the sulfated substrate. The two assay procedures employed in this study were: (i) the thermal fractionation of Hex activity toward 4MUG and (ii) an assay procedure employing both 4MUG and 4MUGS and based on the differential activities of Hex A and Hex B toward the sulfated substrate. Hex A specific activities and % Hex A values were determined in this assay procedure by application of the empirical formula. The results are presented in table 3.

Infantile TSD in Ashkenazi Jewish and French Canadian populations is associated with mutations that result in a complete absence of the α subunit of Hex A (Proia and Neufeld, 1982). The infantile TSD phenotype has also been described in individuals who are neither of Jewish nor French Canadian ancestry. Most of these

cases of infantile TSD are of the B1 variant type(s) in which Hex A is synthesized but with altered substrate specificity (Goldman et al, 1980; Kytzia et al, 1983; Inui et al, 1983; Conzelmann et al, 1985; Besley et al, 1987a; Gordon et al, 1988). Interestingly, most of the patients described in the literature appear to have either Puerto Rican or mixed Irish ancestry. Other cases of infantile TSD in non-Jewish, non-French Canadian individuals that have been reported in the literature include two unrelated TSD probands of Italian descent. Both patients have CRM-positive α-locus mutations but do not produce Hex A. The mutations in these two patients have been shown to be different (Proia and Neufeld, 1982; Zokaeem et al, 1987; Neufeld, 1988, personal communication).

The first ten infantile TSD cell strains reported in table 3 were obtained from patients of Ashkenazi Jewish, French Canadian, or Italian descent who do not exhibit catalytic activity associated with Hex A. It should, therefore, be possible to obtain zero activity for Hex A measurements in tissues and fluids obtained from these individuals by using the constant, r_B, to determine the portion of 4MUGS hydrolysis attributable to Hex B (see Appendix A). However, by both assay procedures activity is detected in extracts obtained from cells of these patients. The source of observed residual Hex A activity in the sulfated substrate assay procedure was not determined. It is likely that it results from inaccuracies in the calculation of the constant r_B since even with the greater amounts of Hex B required to obtain measurements of 4MUGS hydrolysis by this isozyme, a small number of fluorometric units are recorded. Alternatively, it cannot be completely ruled out that the apparent Hex A activity observed in infantile TSD fibroblasts against 4MUGS may be due to the action of a sulfatase. If present, a sulfatase would hydrolyze the 6-O sulfate linkage of 4MUGS to yield 4MUG which can be hydrolyzed by Hex B. Although phosphate, a powerful inhibitor of most sulfatases, does not inhibit the hydrolysis of 4MUGS to 4MU using

extracts of TSD cells (shown in table 2) it is impossible to rule out that the low levels of 4MUGS hydrolysis are due to the action of a sulfatase not completely inhibited by phosphate.

It is important to note that while there is detectable Hex A activity by both assay procedures, the values for Hex A specific activity and % Hex A are significantly lower when measured by the two-substrate procedure that employs 4MUGS than by thermal fractionation. This is also evident in Hex A values for the sera of 5 infantile TSD probands assayed by both procedures (figure 11). An improvement in diagnosis is achieved by the sulfated substrate assay, narrowing the range for % Hex A in TSD sera from 9-11% determined by the thermal fractionation assay to 0-2% by assay with both 4MUG and 4MUGS and application of the empirical formula.

The value of the sulfated substrate for the diagnosis of TSD is most dramatically evident in the assessment of Hex A activity of the B^1 variant form(s) of TSD. The Hex A synthesized by cells of these patients has significant activity against 4MUG but is unable to hydrolyze G_{M2} ganglioside and, thus, most patients present with a clinical phenotype indistinguishable from that of infantile TSD patients. A few patients have been described with a juvenile presentation of the disease (Inui et al, 1983; Charrow et al, 1985). At least one case of prenatal misdiagnosis of a B^1 TSD variant has been documented (Kolodny et al, 1983) when heterozygote levels of amniocyte Hex A activity were assessed by the thermal fractionation procedure with 4MUG.

I assayed cell extracts of two compound heterozygous B^1 TSD patients, one of whom is the same patient whose misdiagnosis was reported by Kolodny et al (1983). A dramatic improvement in the ability to diagnose both patients as severely affected is achieved using the two-substrate assay that employs 4MUGS. In

fibroblast cell extracts both patients have 16% Hex A activity by the thermal fractionation assay procedure. Serum Hex A activity from one of the probands (the misdiagnosed patient) is 29% by thermal fractionation (figure 17). These values are considerably lower by assay with two substrates and application of the formula: % Hex A in fibroblast cell extracts of both patients is 0.7% whereas serum Hex A activity against 4MUGS constitutes 0.9% of total Hex activity. pNPGS has also been used successfully in the diagnosis of B^1 TSD patients (Fuchs et al, 1983; Kytzia et al, 1983; Li et al, 1983). The improvement in diagnosis of the B^1 variants has particularly important implications for prenatal diagnosis. Conzelmann et al (1985) reported the successful prenatal diagnosis of a B^1 variant who would most likely have been misdiagnosed based on the 27% Hex A activity that was measured in amniocytes by the thermal fractionation assay procedure. Cultured amniotic cells were unable to degrade G_{M2} ganglioside and were also significantly deficient in hydrolysis of pNPGS. Thus, the sulfated derivatives of synthetic glucosaminide substrates have already proven valuable in diagnosing B^1 variants of TSD. Does their diagnostic value hold for other TSD variants in whom symptoms are milder and appear at a later age?

A problem often encountered in the diagnosis of many later-onset variants is the lack of correlation between the degree of enzyme deficiency and the severity of the disease. As shown in table 3, a clear distinction does not emerge between the juvenile, chronic, and adult TSD genotypes based solely upon measurement of residual Hex A activity by either the thermal fractionation assay procedure with 4MUG or the two-substrate assay procedure employing both 4MUG and 4MUGS. Different mutant alleles at the α-gene locus yield Hex A with residual activities as measured by synthetic substrates which are nearly identical in patients with vastly

different clinical pictures. These differences in the severity of the disease might be attributable to the effect of the particular mutation on enzyme activity. Thus the mutant Hex A might have altered substrate specificities, or might be more susceptible to proteolytic degradation or inhibition within the lysosomal compartment (Sandhoff and Conzelmann, 1984).

Alternatively, the action of other genes may influence the development of disease. Conzelmann and Sandhoff (1983) proposed a kinetic model that assumes that G_{M2} ganglioside is delivered to the lysosomal compartment of neurons at a constant rate and that the amount of Hex A is sufficient to degrade G_{M2} at a rate at least as fast as that at which it is synthesized. They hypothesize that any reduction of Hex A, whether by a decrease in the amount or turnover of the enzyme, will result in a corresponding increase in the steady state concentration of G_{M2} ganglioside. In most individuals with reduced Hex A (e.g., TS heterozygotes) there is no neuronal accumulation of G_{M2} ganglioside because the enzymatic activity is still sufficient to cope with the substrate influx. However, in others the residual activity falls below a critical threshold value and the enzyme is unable to cope with the influx of G_{M2} ganglioside, which accumulates at a constant rate. Still other variants of TSD are believed to be functioning at a level of Hex A activity that keeps them just above the critical threshold value. Grebner et al (1986) proposed that for these individuals, the balance may be tipped by other physiological factors or even environmental factors. This possibility was first suggested by d'Azzo et al (1984) who demonstrated a defect of α-β subunit association of apparently the same magnitude in two siblings, yet only one of the siblings was affected with the chronic form of TSD; the other sibling was clinically normal. Thus, it is possible that in some individuals the action of other genes might force the level of Hex A activity below the critical threshold value,

resulting in a progressive accumulation of G_{M2} ganglioside and the development of a neurological disorder. Such genes that might tip the balance include those that control the amount of activator protein necessary for the hydrolysis of G_{M2} ganglioside or the amount of a neuraminidase which converts polysialogangliosides into G_{M2} ganglioside. It is also possible that the gene encoding UDP-N-acetylgalactosaminyl transferase, a Golgi enzyme in the biosynthetic pathway of gangliosides which synthesizes G_{M2} ganglioside from G_{M3}, might contribute to the pool of G_{M2} ganglioside provided to the cell and, thus, to different clinical phenotypes.

Besides other genes that might influence the level of Hex A activity and the development of disease, d'Azzo et al (1984) suggested that "environmental factors such as lysosomotropic agents in diet or medications may influence the level of residual enzyme in lysosomes and hence the clinical outcome."

The juvenile TSD cell strains were obtained from patients who are likely to be homozygous for a mutation at the gene locus encoding the α subunit of Hex A (WG306 and WG312, siblings whose parents are first cousins) as well as individuals who are the offspring of unrelated parents and most likely compound heterozygotes for two different mutations at this locus (WG928 and WG1115). The four juvenile TSD probands are virtually identical in residual Hex A activity determined by both thermal fractionation with 4MUG and application of the empirical formula with 4MUGS. In the case of the homozygous siblings, a partial deficiency of Hex A activity by the thermal fractionation procedure is found. These siblings are severely deficient in Hex A activity by the sulfated substrate assay procedure which employs the formula. Hechtman et al (1989; see Appendix B) studied the synthesis and processing of hexosaminidase subunits in cells of the siblings and found that both patients synthesize an α-subunit precursor of normal size but do not produce mature

α subunit. In addition, α-ß subunit association does not occur. d'Azzo et al (1984) found a similar defect of α-ß subunit association in one other juvenile TSD patient. However, they found that the α subunit synthesized by cells of their patient gets phosphorylated, indicating that it reaches the Golgi compartment. In contrast, the α subunit synthesized by cells of the two juvenile TSD siblings studied by Hechtman et al (1989) are not phosphorylated. These mutant α subunits probably fail to exit the RER and are, instead, degraded. Fibroblast cell extracts obtained from the two siblings with juvenile TSD were fractionated by ion-exchange chromatography. Isolated Hex A had an optimum pH identical to that of control Hex A. This indicates that a small amount of Hex A survives the targeting pathway and is probably functional within the lysosomes. Two-dimensional gel electrophoresis of immunoprecipitates would possibly provide evidence of this due to its superior resolution of α and ß chains.

In view of the likelihood that WG928 and WG1115 are compound heterozygotes, the reduced Hex A activity measured by the sulfated substrate assay procedure probably represents a more accurate assessment of the mutant allele that produces a defective α subunit. 4MUG, since it is specific for an active site on the ß subunit, does not accurately assess catalytic activity at the α subunit site. A severe deficiency of Hex A activity has been reported for other juvenile TSD patients based on their ability to degrade G_{M2} ganglioside (O'Brien et al, 1977; Kolodny and Raghavan, 1983; Meek et al, 1984).

Chronic and adult forms of G_{M2} gangliosidosis are indistinguishable from each other when Hex A activity is measured by either assay procedure (table 3).

Neither are these patients clearly distinguishable from patients with an infantile or juvenile presentation based on hydrolysis of 4MUGS by unfractionated cell extracts. G_{M2} ganglioside hydrolysis is also severely affected in these patients (Kolodny and Raghavan, 1983). Chromatographic isolation of Hex isozymes and assay of column fractions with both 4MUG and 4MUGS might provide a useful means of discriminating the different genotypes. A comparison of the chromatographic profiles obtained from the separation of fibroblast Hex isozymes from patients with the infantile (figure 16), B^1 variant (figure 18B), juvenile (Hechtman et al, 1989, figures 1C and 1D, Appendix B) and adult-onset (figure 23B) forms of TSD indicates that these genotypes can be distinguished. Navon et al (1986b) reported a similar finding, comparing chromatographic profiles based on 4MUG hydrolysis by isolated fetal skin fibroblast Hex isozymes to distinguish between a fetus with the adult-onset form of TSD and one with the infantile form of the disease.

Fibroblast cell extracts from two Hex A-deficient healthy adult variants have a partial deficiency of Hex A when measured by the heat inactivation assay with 4MUG (table 3). Hex A specific activities and % Hex A values against 4MUG in these individuals are indistinguishable from those obtained in the four juvenile TSD patients, yet the juvenile patients are affected with a severe neurological disease that invariably leads to death by the age of 15 years, while the Hex A-deficient adults are asymptomatic. The sulfated substrate assay procedure which employs the empirical formula yields Hex A specific activities and % Hex A values that are in the heterozygote-to-normal range. Thus, the sulfated substrate represents a clear improvement in the ability to accurately detect these individuals as unaffected and, once again, yields a diagnosis that is similar to that obtained when G_{M2} ganglioside hydrolysis is assessed in cell extracts and fluids obtained from Hex A-deficient healthy adults. (Tallman et al, 1974; O'Brien et al, 1977 and 1978; Grebner et al,

1986). The 4MUGS assay procedure might prove particularly useful in the prenatal diagnosis of these individuals, providing a clear distinction between a fetus who will develop a severe form of TSD and a fetus who appears to have levels of Hex A activity comparable to those of the severely affected fetus measured by heat inactivation yet who will be clinically normal.

Greenberg and Kaback (1982) have proposed that the rare finding of Hex A deficiency (Hex A^-) in individuals who reach adulthood is most likely due to compound heterozygosity for two mutant alleles, one of which is a 'null' allele of the type that confers infantile TSD when in the homozygous state, and the other of which is a milder mutant allele. This includes the chronic, adult-onset and Hex A-deficient healthy adult variants. Greenberg and Kaback (1982) used three sets of data to estimate the frequency of these milder mutant alleles in the Jewish population of the U.S.: (1) data obtained from TSD carrier screening programs (which includes data only from adult-onset and Hex A-deficient normal adults, since chronic TSD patients develop symptoms in childhood and would not present at a screening clinic for carrier testing), (2) family data on relatives of TSD children, and (3) data on at-risk pregnancies. Based on their calculations they estimated that the frequency of all carriers of milder mutant A^- alleles (i.e., excluding classical TSD alleles) in the American Jewish population is 1:1200 and that the frequency of the adult Hex A^- phenotype is approximately 1:67000. They have determined that about 2-3% of those individuals who are identified as TSD carriers are actually carriers of a milder variant allele and that about 5% of couples identified through screening to be at risk for producing an infantile TSD offspring are actually at risk for producing a child with a Hex A^- phenotype who will survive into adulthood. A major concern thus becomes the likelihood of misdiagnosing a fetus as being affected with a severe form of TSD when, in fact, the fetus may be only mildly affected or unaffected. Clearly assessment of fetal Hex A activity by the assay procedure employing the sulfated synthetic

substrate doesn't resolve all the problems of detection of these milder α-locus alleles. While the most precise diagnostic information is provided by assessment of catabolism of the natural substrate, the procedures of isolating and labelling G_{M2} ganglioside are tedious and enzymatic assays employing the radiolabelled substrate are dependent on the addition of the activator protein which must be prepared in a highly purified state. Many laboratories are not equipped for this kind of analysis.

The use of a synthetic substrate that is preferentially hydrolyzed at the α-subunit active site of Hex A, such as 4MUGS (which is now commercially available), might provide an alternative to natural substrate assays. My results suggest that the accuracy of both pre- and postnatal diagnosis of the later-onset forms of TSD might be improved by performing Hex A assays on unfractionated cell extracts or fluids using the two different assay procedures described earlier (i.e., the thermal fractionation assay procedure with 4MUG and the two-substrate assay procedure that employs both 4MUG and 4MUGS) in combination with chromatographic fractionation of the Hex isozymes and assay of the column fractions with both substrates.

E. Diagnosis of other forms of G_{M2} gangliosidosis using 4MUGS.

Mutations at the ß-gene locus or AP locus also produce forms of G_{M2} gangliosidosis. Mutations at the gene locus encoding the ß subunit of hexosaminidase result in absent or defective Hex A and Hex B since both isozymes contain ß subunits. A number of variant forms of Sandhoff disease have been identified which vary in the amount of residual Hex activity and the age at which clinical symptoms present. Likewise, later-onset forms of G_{M2} gangliosidosis have been identified due to a variant form of activator protein deficiency.

Cell extracts from two patients with ß-locus mutations were assayed with both 4MUG and 4MUGS (table 3). Cells of WG534 were obtained from a patient with the infantile form of SD. Cells of WG1117 were obtained from a Hex A- and Hex B-deficient normal adult. Total Hex activity is severely reduced in both cell strains. The mean total Hex specific activity in 9 normal control cell strains (table 3) is 7557 nmoles 4MUG/ mg protein/ hour. Total Hex specific activity in the infantile SD homozygote (WG534) is 3% of control whereas the clinically normal adult variant (WG1117) has 10% of control total Hex activity. In addition, the great majority of residual Hex activity is thermolabile, constituting 89% and 91% of total activity in WG534 and WG1117 cell extracts, respectively. Cell extracts of both variants hydrolyze a significant amount of 4MUGS. Application of the empirical formula to determine Hex A specific activities against the sulfated substrate yields values that are 2- to 4-fold greater than total Hex (measured with 4MUG). The increased activity is thought to be attributable to Hex S, a dimer of α subunits that accumulates in SD cells in the absence of ß subunits (Beutler et al, 1975; Potier et al, 1979). Because 4MUGS specifically binds to the α-subunit active site, it is hydrolyzed by the Hex S isozyme and accounts for the elevated rate of 4MUGS hydrolysis observed in cell extracts of these two SD variants.

Kytzia et al (1984) reported that pNPGS was unable to differentiate between infantile or juvenile SD patients and normal controls and thus has no diagnostic value for patients with SD. However, by application of the empirical formula that yields a % Hex A value in SD cells, I am able to identify SD variants based on the very high values of '% Hex A' (200-400%) determined with the two-substrate assay procedure that employs 4MUGS. Unfortunately, a clear distinction between the different SD variants or, for that matter, between a SD variant and a SD heterozygote probably will not emerge.

The AB variant form of G_{M2} gangliosidosis hydrolyzes synthetic substrates normally since hydrolysis of these substrates is not dependent upon the action of the AP and since the Hex A synthesized by AB variant cells is normal. A patient with an infantile form of G_{M2} gangliosidosis due to a deficiency of the activator protein (WG802, table 3) hydrolyzed both 4MUG and 4MUGS within the range of the normal controls. Synthetic substrates, therefore, are not suitable for diagnosis of a G_{M2} gangliosidosis that results from a defect at the gene locus that encodes the AP necessary for hydrolysis of G_{M2} ganglioside.

F. Biochemical characterization of the mutation in several infantile TSD probands.

Infantile TSD in individuals of ethnic origins other than Ashkenazi Jewish or French Canadian have been associated with mutations that are CRM-positive for the α subunit of Hex A. Furthermore, the CRM-positive mutations identified include those in which no mature α subunit is produced (due to failure of α-ß subunit association, failure of the α subunit to acquire the mannose-6-phosphate recognition marker that targets the enzyme for the lysosome, or even failure of the α subunit to exit the RER for reasons not yet understood) as well as mutations in which Hex A is synthesized but unable to hydrolyze G_{M2} ganglioside.

1. Infantile TSD in a proband of Italian descent.

Infantile TSD was suspected in a child of Italian descent because of loss of developmental milestones. The diagnosis of TSD was confirmed by Hex A assay of

serum and cultured skin fibroblast cell extracts. The results are presented in table 7. Hex A assays were performed by both the thermal fractionation procedure and the two-substrate procedure that employs 4MUGS. The affected proband has Hex A activity which is in the range of other severely affected probands by the heat inactivation assay with 4MUG. By the two-substrate assay procedure, proband Hex A activity in serum and fibroblasts is also within the range of other infantile TSD probands tested. The much lower values for Hex A activities and % Hex A by the assay procedure that employs 4MUGS exemplifies the improvement in diagnosis that is achieved using this substrate. The parents of the proband test comparably by both assay procedures as heterozygotes. The parents originate from the same small village in Italy and are related through both their fathers and their mothers, indicating that the proband is likely homozygous for an α-locus mutant allele. An older sister of the proband is a normal homozygote. Carrier status was assigned to 6 of eleven other relatives of the proband who requested testing. Their relationship to the proband is indicated in the pedigree shown in figure 15.

The absence of Hex A in fibroblasts of the proband is confirmed by the chromatographic separation of Hex isozymes, shown in figure 16. No enzymatic activity elutes at a position corresponding to normal Hex A (compare to figure 8A) by assay of the column fractions with either 4MUG or 4MUGS. A ß-subunit-containing isozyme of intermediate electronegativity is observed in the chromatographic profile based on 4MUG hydrolysis. This isozyme is not present in normal cells. Its accumulation in cells of the proband further demonstrates the absence of mature α subunits.

Biosynthetic labelling of fibroblast cell proteins of the proband and immunoprecipitation of the Hex subunits were performed by Zokaeem et al (1987,

refer to Appendix B). Precipitation of the subunits with anti-α antiserum indicates that an α-subunit precursor is synthesized by cells of the proband. The proband differs from Ashkenazi Jewish and French Canadian infantile TSD patients in this regard. However, the precursor synthesized is about 2-3 kDa smaller than the precursor synthesized by normal control cells. No mature α subunit is detected (by precipitation with anti-Hex A antiserum) and no enzyme is secreted in response to treatment of cultured cells with NH_4Cl-containing medium. Thus, the mutant α chain fails to undergo further post-translational modifications and is probably degraded within the endoplasmic reticulum. Cells of both parents of the proband are heterozygous for the shortened α subunit providing evidence that the proband is, indeed, homozygous for the truncated α-chain mutation.

The mutation detected in this proband was recently identified by Neufeld (1988, personal communication) as a deletion of cytosine at nucleotide position 1510 of the α-chain gene. The deletion of this single nucleotide produces a frameshift mutation which, in turn, results in a premature termination codon at nucleotides 1522-1524.

Infantile TSD was identified in another patient of Italian origin (Proia and Neufeld, 1982) unrelated to the patient described here. While the mutation has yet to be characterized, cells of the patient were found to produce an insoluble α-chain precursor that fails to leave the endoplasmic reticulum for further processing and is, instead, degraded. However, the α precursor polypeptide synthesized by cells of this patient is of normal 67 kDa size. It is, therefore, interesting to note that there is

heterogeneity not only in the α-locus mutations that give rise to the infantile TSD phenotype between different groups, but also that heterogeneity is evident within ethnic groups. This has been demonstrated in Ashkenazi Jewish and French Canadian populations, and now in probands of Italian descent.

2. Infantile TSD in two B^1 variant probands.

The mutant Hex A synthesized by two B^1 TSD variants was characterized and is discussed here. The two probands are unrelated and presented at 6 months of age with the typical signs of classical TSD. As mentioned previously, both probands have significant Hex A activity against the synthetic substrate, 4MUG, yet their Hex A is unable to hydrolyze G_{M2} ganglioside or 4MUGS; thus, the designation of these probands as B^1 variants. Both patients are the offspring of unrelated parents from different ethnic backgrounds.

Sera obtained from the parents of the two probands were assayed by both thermal fractionation with 4MUG and the two-substrate assay procedure that employs 4MUGS. All four parents are unambiguously identified as carriers by the assay procedure that employs 4MUGS. However, by thermal fractionation two parents (the father of patient 1 and the mother of patient 2) fall slightly outside the range for other obligate heterozygotes, into an inconclusive zone for classification. Thus, the mothers and fathers of both of the B^1 variants I tested are likely carriers for different mutant alleles. Assuming this is an accurate assessment, both probands would be compound heterozygotes, which is suggested as well by the different ethnic backgrounds of their parents and the absence of consanguinity. The majority of the B^1 variants described in the literature to date are thought to be genetic compounds (Goldman et al, 1980; Charrow et al, 1985; Conzelmann et al, 1985; Besley et al, 1987; Gordon et al,

1988). Most of the probands have an infantile-onset form of TSD and Hex A activities that approach the heterozygote range when assessed by the heat inactivation procedure with 4MUG. B^1 homozygotes, born to related parents, have also been reported (Goldman et al, 1980; Li et al, 1981; Inui et al, 1983) with a juvenile presentation and Hex A activities in the normal range determined by thermal fractionation.

The parents of the two probands I studied would most likely have been advised of their risk for having an affected fetus. However, both Charrow et al (1985) and Besley et al (1987b) reported on B^1 TSD probands who had one parent testing well within the range of homozygous normals by the thermal fractionation assay with 4MUG. By assay with the sulfated substrate, however, they were clearly identified as heterozygotes. The two sets of parents reported by Charrow et al (1985) and Besley et al (1987b) would not be considered at risk for having an affected child since only one parent would be identified as a carrier based solely on 4MUG hydrolysis. The frequency of the B^1 allele(s) is not known and would be impossible to determine by screening programs using 4MUG only.

Normal control and patient fibroblast Hex isozymes were separated by ion-exchange chromatography. Based on 4MUG hydrolysis, an intermediate isozyme is present in the patients' profiles that is not seen in the profile of the control. This isozyme, not present in normal cells, accumulates in TSD cells. Its presence has been noted in the chromatographic profiles of Hex isozymes isolated from fibroblasts obtained from classical TSD patients (Hechtman et al, 1983) as well as from patients with later-onset forms of the disease in whom α subunits are produced yet defective in their ability to complex with β subunits (Argov and Navon, 1984).

Comparable values of 60% Hex A are obtained for the normal control when

Hex A activity is measured either by the thermal fractionation assay with 4MUG or by chromatographic fractionation of control Hex isozymes and assay with 4MUG. In contrast, proband 1 Hex A activity against 4MUG, measured after chromatographic separation of Hex isozymes, accounts for 30% (29% for proband 2) of the total recovered Hex activity, or approximately twice the amount obtained by the heat inactivation assay on unfractionated cell extract. If it is assumed that the probands are compound heterozygotes for a mutant allele that does not encode an α subunit polypeptide (or encodes an α subunit incapable of complexing with ß subunits) and a mutant allele that encodes an α polypeptide that does associate with ß subunits, then the Hex A activity that is measured is attributable only to the latter. The 30% Hex A activity measured after chromatography is 1/2 that of the normal control. Thus, it is likely that this allele synthesizes a normal amount of α subunit.

It was necessary to rule out the possibility that the patients, in addition to having mutations at the gene locus encoding the α subunit of Hex, might also have a ß-locus mutation producing a heat-labile form of Hex B. Mutations of this sort have been been associated with G_{M2} gangliosidosis (Momoi et al, 1978; Lane and Jenkins, 1978; Dlott et al, 1988) as well as occurring as a benign condition (Dreyfus et al, 1975 and 1977; Hechtman and Rowlands, 1979; Navon et al, 1985; Dlott et al, 1988). While it is unlikely that the patients would possess mutations at both α- and ß-gene loci, a heat-labile form of Hex B could account for the thermolabile Hex activity measured in the heat inactivation assay with 4MUG. As expected, Hex B isolated from fibroblasts of both patients is as thermostable as normal control Hex B. However, the patients' Hex A differs from control Hex A in its thermal characteristics. It is more stable to heating than normal Hex A. The increased thermal

stability of both probands' Hex A is consistent with the finding of increased Hex A activity observed by chromatographic fractionation of patient fibroblast cell extracts (29-30%) over that which is measured in the assay dependent upon thermal fractionation (16%). Patient Hex A also differs significantly from control Hex A in the optimum pH of its activity against 4MUGS. The pH optimum of control Hex A is 3.9. The Hex A of the two B^1 probands has a much lower pH optimum of 2.3, indicating that these enzymes may be nonfunctional within the lysosome.

The Hex isozyme profile of fibroblasts obtained from the two probands revealed severely reduced, though not completely absent, Hex A activity against 4MUGS. Kinetic analyses were performed to determine whether the residual Hex A activity is attributable to hydrolysis occurring at the mutant α-subunit active site or at the ß-subunit active site of Hex A. Km values of the probands' Hex A activity against 4MUGS are virtually identical to the Km of normal control Hex A, suggestive of 4MUGS hydrolysis occurring at a site with an identical binding affinity on both control and patient Hex A. However, Vmax values for patient Hex A differ significantly from that of control Hex A. Patient 1 Hex A has a maximum velocity that is about 1/3 that of control, whereas the maximum velocity of patient 2 Hex A is similar to that of control Hex B against 4MUGS. Vmax values are expressed as nanomoles of 4MUGS hydrolyzed per unit of enzyme (as measured by 4MUG hydrolysis) and, therefore, they reflect reduced catalytic activity at the α-subunit active site. If the Vmax had been expressed instead as nmoles of 4MUGS hydrolyzed per mg of protein, it would be difficult to determine whether the reduced value for patient Hex A was indicative of a decreased amount of enzyme or a normal amount of enzyme with reduced catalytic activity.

The results of inhibition studies also point to the existence of an α subunit

active site on patient Hex A capable of binding 4MUGS. The inhibition of 4MUG and 4MUGS hydrolysis by control Hex A and Hex B and both probands' Hex A was studied in the presence of an inhibitor specific for the ß-subunit active site (NG) as well as one specific for the α-subunit active site (NGP). The significance of these inhibition studies as evidence for two distinct active sites on Hex A has been discussed previously (page 170). The discussion here will, therefore, be limited to the results as they pertain to the mutant Hex A.

4MUG hydrolysis by both patients' Hex A is inhibited to the same extent by NG as is normal control Hex A and Hex B, indicating that hydrolysis of this substrate occurs at an active site common to normal and mutant Hex A and normal Hex B, i.e., at the active site on the ß subunit. Normal and proband Hex A-catalyzed hydrolysis of 4MUGS is inhibited only slightly in the presence of NG, thus establishing that the catalytic site on the mutant Hex A has characteristics associated with an α-subunit active site.

4MUGS hydrolysis by control and proband Hex A is strongly inhibited in the presence of low concentrations of NGP, indicating that the inhibitor and the sulfated substrate are competing for the same active site on both enzymes, i.e., for an active site on the α subunit of normal and mutant Hex A. While patient 1 Hex A-catalyzed hydrolysis of 4MUGS is as sensitive to inhibition by NGP as is 4MUGS hydrolysis by normal control Hex A, the inhibition profile for patient 2 Hex A is intermediate between that of control Hex A and control Hex B. Thus, both the α- and ß-subunit active sites of patient 2 Hex A may contribute to 4MUGS hydrolysis. This finding is consistent with the much lower Vmax of this proband's Hex A.

While B^1 variants have been described by others, some of whom have reported a similar dramatic improvement in the ability to diagnose these patients using

sulfated synthetic substrates, few have attempted to characterize the mutant Hex A synthesized by cells of the B^1 TSD patients. The few who have performed such analyses (Kytzia et al, 1983 and Charrow et al, 1985) have found the Hex A synthesized by their respective patients to be essentially identical to normal control Hex A in apparent Km, optimum pH, and thermostability characteristics. However, these parameters were assessed using the unsulfated substrate, 4MUG. Since this substrate binds to the cataltyic site on the ß subunit of Hex A which is unaffected by the mutation, it does not accurately assess the product of the mutant α gene.

The eluciation of the mutation in one B^1 TSD variant, recently presented by Ohno and Suzuki (1988a), may provide some insight into the possible mutation mechanism operating in the two B^1 variants I have studied. Ohno and Suzuki (1988a) cloned and characterized mutant cDNA from fibroblasts of the B^1 variant reported previously by Goldman et al (1980) and Sonderfeld et al (1985). Northern analysis of mRNA preparations from the patient's cells gave a band of normal size for the α chain of Hex A, yet the amount was approximately half that of normal control message. Their findings were consistent with those of Sonderfeld et al (1985) who reported reduced biosynthesis of a normal-sized α subunit precursor polypeptide that was processed normally by cells of this patient. Ohno and Suzuki (1988a) have suggested that since there is no record of consanguinity in the parents of their B^1 TSD patient, it is likely that the child is a compound heterozygote for a mRNA-negative α gene and a B^1-type α gene. Comparison of the mutant B^1 cDNA with normal cDNA revealed a single base substitution that produces a change of one amino acid at the

NH_2-terminus of the mature α chain. The changed amino acid presumably results in different folding of the protein. A mutation of this type might be expected to affect such characteristics of the mutant protein as its thermostability, optimum pH, and catalytic activity, particularly if the mutation affects the protein at or near the active site on the α subunit.

Ohno and Suzuki (1988a) have proposed that it is likely that other B^1 TSD patients possess mutant α alleles that affect the same region of the protein since the mutant Hex As synthesized by cells of these patients appear to have the same unique substrate-specificity characteristics. While it is tempting to speculate that the mutation in the two B^1 variants I have studied is similar to that found by Ohno and Suzuki (1988a), this remains to be determined. Indeed, it is possible that the two probands I have studied possess slightly different mutations. This is suggested by the observed differences in thermal stability, Vmax, and inhibition of 4MUGS hydrolysis by NGP. It is most likely that the molecular heterogeneity that has been discovered in other TSD variants will also hold for the B^1 variants of TSD.

G. Prognostic value of 4MUGS.

A problem that has been encountered in carrier screening is the detection of healthy adults, usually in their late-teens, who have very low levels of Hex A activity measured by the standard thermal inactivation assay with 4MUG. The significance of such findings is not known since 4MUG has no prognostic value in determining whether an individual with low Hex A activity is awaiting a neurological disorder or whether that individual can expect to remain symptom-free. The prognostic value of the sulfated substrate assay in differentiating between these individuals was

investigated.

The thermal fractionation assay procedure is not a discriminant of genotypes. Adult TSD (WG1116) and Hex A-deficient healthy adult (WG1107 and WG1111) variants have Hex A specific activities that are significantly lower than the normal controls or obligate heterozygotes tested (table 9). Such individuals ascertained through routine screening would probably be diagnosed as likely to develop a disease. While the two Hex A-deficient healthy adults included in this study have a partial Hex A deficiency measured by thermal fractionation, others have been reported with a severe deficiency, indistinguishable from infantile TSD probands (Vidgoff et al, 1973; Kelly et al, 1976; Grebner et al, 1986).

The two-substrate assay procedure that employs 4MUGS and the formula does distinguish between neurologically affected and healthy adults. Hex A specific activity is very low in the adult TSD cell extract yet is in (or approaches) the heterozygote range in the two Hex A-deficient healthy adult cell strains. However, while the sulfated substrate assay procedure is able to differentiate between affected and unaffected adults, a clear distinction does not emerge between patients affected with an early-onset form of TSD and those in whom the onset of symptoms occurs in adulthood. The implications of this are most serious for prenatal diagnosis.

Hexosaminidase isozymes were fractionated by ion-exchange chromatography from fibroblast cell extracts of a normal control, the adult-onset TSD patient (WG1116) and one of the Hex A-deficient healthy adults (WG1107). The appearance of an intermediate isozyme in the chromatographic profiles obtained by 4MUG hydrolysis in the two α-locus variants is not unexpected. Cells from both adult-onset TSD patients (d'Azzo et al, 1984; Frisch et al, 1984; Navon et al, 1986) and Hex A-deficient healthy adults (Grebner et al, 1986; Navon et al, 1986) have been shown to synthesize reduced amounts of α-subunit precursor polypeptide. The

presence of Hex I in cells of adult-onset TSD patients (Argov and Navon, 1984) and Hex A-deficient healthy adults (Grebner et al, 1986) has been previously reported. Nor is the significant peak co-chromatographing with Hex A in the 4MUGS profile of the Hex A-deficient healthy adult unexpected, since Hex A of these individuals is capable of hydrolyzing G_{M2} ganglioside (Tallman et al, 1974; O'Brien et al, 1977 and 1978; Grebner et al, 1986). A small, though measureable, amount of Hex A activity against 4MUGS is detected in the adult TSD chromatographic profile. This contrasts with the relative absence of a Hex A peak in the chromatographic profiles of infantile TSD probands (figures 16 and 18) and might provide a useful means of discriminating these genotypes in a prenatal diagnosis. Navon et al (1986b) reported on the clear differentiation between an adult-onset TSD fetus and one with infantile TSD based on the chromatographic profiles of fetal fibroblast Hex isozyme activity against 4MUG.

In the normal control cell extract, chromatographic fractionation and assay with 4MUG yields a peak of Hex A activity that represents 70% of total recovered activity, a value that is in agreement with that obtained by the heat inactivation assay. Significantly higher Hex A activity is measured chromatographically by 4MUG hydrolysis in the adult TSD and Hex A-deficient healthy adult cell extracts. As shown in figure 23, the adult-onset TSD Hex A peak represents 22% of the total recovered Hex activity. This is about 1/3 that of the normal control cell extract after chromatographic separation of the Hex isozymes and is consistent with the reduced α subunit synthesis observed in adult-onset TSD cells. However, the 22% Hex A by chromatography contrasts sharply with the 8% Hex A measured by thermal fractionation in the adult-onset TSD cell extract. The discrepancy is accounted for by a species of Hex A synthesized by cells of the adult TSD patient that is more stable to heating than normal control Hex A (figure 24). Navon et al (1986b) also reported a

discrepancy between Hex A activity obtained by the heat inactivation assay and by ion-exchange chromatography of Hex isozymes obtained from a fetus with the adult-onset form of TSD. The Hex A of the fetus was indistinguishable from that of an infantile TSD fetus by the heat inactivation assay with 4MUG. However, by chromatographic fractionation of fetal Hex isozymes and assay of the column fractions with 4MUG, the fetus had Hex A activity that accounted for 17% of the total recovered Hex activity.

Chromatographic isolation of the Hex A-deficient healthy adult isozymes and assay with 4MUG yields a Hex A-associated peak that represents 34% of total recovered Hex activity (figure 23). This value is approximately 1/2 that obtained by chromatography of the normal control cell extract, which is consistent with Grebner et al's (1986) report that cells of this Hex A-deficient normal individual synthesize a reduced amount of α subunit. This is not unexpected if such individuals are, as suggested by O'Brien (1978), compound heterozygotes for a 'classical' TSD allele and another mutant allele that produces an α subunit capable of associating with β subunits to form Hex A with altered substrate specificity. Compound heterozygosity was demonstrated in one such woman classified as a Hex A-deficient healthy adult who gave birth to a TSD infant (Vidgoff et al, 1973). However, it is possible that the Hex A-deficient healthy adult phenotype could also be attributable to homozygosity for the milder mutant allele or, alternatively, that other factors affecting Hex A could explain the apparent severe deficiency of this enzyme against unsulfated synthetic substrates, particularly in those individuals whose Hex A deficiency is not uniform in cells and fluids.

The profile of Hex isozymes fractionated from cells of the clinically normal adult (WG1107) by ion-exchange chromatography differs significantly from the profile obtained by Grebner et al (1986) for this same individual. They found very

low Hex A activity that constitutes only 13% of the total recovered Hex activity. It is tempting to speculate that this individual might have a nonuniform deficiency of Hex A, since Grebner et al (1986) isolated plasma Hex isozymes whereas I fractionated Hex isozymes obtained from his fibroblasts. However, Grebner et al (1986) reported that this individual appeared to have uniformly low levels of Hex A activity in plasma, leukocytes, and fibroblasts. Thus, the higher % Hex A obtained by chromatography is difficult to reconcile with Grebner's findings. In addition, the 34% Hex A recovered by chromatography (figure 23) is very different from the 15% Hex A measured in the thermal fractionation assay with 4MUG (table 9). This is also difficult to reconcile in light of the finding that Hex A isolated from cells of this individual is identical to normal control Hex A in its thermolability (figure 24).

The inexplicable findings obtained for the Hex A-deficient healthy adult described here should not detract from the prognostic value of the sulfated substrate in distinguishing between individuals who have an adult-onset form of TSD and normal individuals who can expect to remain symptom-free. These genotypes are clearly indistinguishable based on the thermal fractionation assay with 4MUG. However, the healthy adults with low Hex A activity measured by thermal fractionation are clearly able to hydrolyze 4MUGS. They have also been shown to hydrolyze the natural substrate (Kolodny and Raghavan, 1983; Grebner et al, 1986) and thus their cells do not accumulate G_{M2} ganglioside (Grebner et al, 1986). These findings argue for the conversion of the automated assay procedure to use with the sulfated substrate for carrier screening.

H. The TSD gene in French Canadians of Quebec.

French Canadians of eastern Quebec represent the only other ethnic group in whom TSD is prevalent and in whom a high heterozygote frequency has been noted.

The clinical presentation of TSD in individuals of French Canadian descent as well as the absence of detectable Hex A in the probands' tissues and fluids are findings identical to those in infantile TSD probands of Ashkenazi Jewish ancestry. Cells of Ashkenazi Jewish infantile TSD patients synthesize a primary RNA transcript whereas the deletion mutation in infantile TSD probands of French Canadian ancestry is inconsistent with transcription (Proia and Neufeld, 1982; Myerowitz and Proia, 1984; Paw and Neufeld, 1988). The elucidation of the intron-exon organization of the α-chain gene (Myerowitz et al, 1985; Proia and Soravia, 1987) has led to the identification of a number of TSD mutations, one of which is the 5' end gene deletion characterized by Myerowitz and Hogikyan (1986, 1987). This deletion explains the absence of mRNA in cells from patients of French Canadian descent. It is believed to have arisen from unequal crossover between Alu sequences. Unequal crossover between Alu sequences during meiosis has also been implicated in the generation of deletion mutations of the low density lipoprotein receptor (Lehrman et al, 1985, 1986, 1987; Hobbs et al, 1986) and globin (Vanin et al, 1983; Henthorn et al, 1986) genes.

The majority of TSD families in Quebec are situated in communities along the north and south shores of the St. Lawrence River in the eastern portion of the province. Andermann (unpublished) has extensively studied the genealogies of five TSD families, four of whom originate from communities along the south shore in Quebec (numbers 1, 2, 4, and 5 on the map, figure 25). The fifth family is from northern New Brunswick (number 3, figure 25). While Dr. Andermann has found common surnames in the pedigrees of these families, a single ancestor common to all of the families has not been identified. In spite of this, the similarity of these communities to other geographic isolates, such as the Pennsylvania Dutch in whom TSD occurs (Kelly et al, 1975), is suggestive of the founder effect as an explanation

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of the increased frequency of the TSD gene in this region (Andermann et al, 1977).

Myerowitz and Hogikyan (1986) found TSD probands from two of these five families to be homozygous for a 5' end deletion which they identified using several probes, one of which is the 300-bp intronic probe that I employed in my study. Recently, the infantile form of TSD has been found in French Canadian families whose ancestors originated from regions of Quebec that are geographically remote from the communities along the south shore in the eastern portion of the province. These families correspond to numbers 6, 7, and 10-13 in figure 25.

The TSD gene in the French Canadian population of Quebec was assessed with respect to the homogeneity of the 5' end deletion. Southern analysis of genomic DNA isolated from leukocytes of 63 individuals and fibroblasts of 6 infantile TSD probands (figure 26) indicates that normal homozygotes typically possess one fragment of 9.5-kb size, generated from digestion of the DNA with EcoRI, eletrophoresis of the fragments, and hybridization with the 300-bp intronic probe. A single band of 9.5 kb is also obtained by Southern analysis of genomic DNA isolated from fibroblasts of an Ashkenazi Jewish infantile TSD proband (WG93 in figure 26). Three probands from southeastern Quebec and one from northern New Brunswick (WG107, WG884, WG1065, and WG733, respectively) are homozygous for a fragment of 23 kb, the much larger fragment generated as a consequence of the deletion which abolishes three EcoRI restriction sites (figure 2). Thus, the size of fragments generated by digestion and Southern analysis of genomic DNAs isolated from a normal control, an Ashkenazi Jewish TSD proband, and the two French Canadian TSD DNAs, WG107 and WG733, analyzed by Myerowitz and Hogikyan (1986) are in complete agreement with their findings.

All of the individuals from the south shore who have Hex A activities in the heterozygote range by serum assay, including the obligates (such as WG885 and WG886 in figure 26), are also heterozygous for the 5' end deletion, possessing both

9.5- and 23-kb fragments by Southern blotting. This is indicative of the homogeneity of this mutation in the south shore communities. The obligate heterozygotes (D.P. and R.R.) who originate from Quebec City are first cousins and both are homozygous for the 9.5 kb fragment. Their child (WG 1499), affected with infantile TSD, likewise has a single band of 9.5 kb. Clearly a mutant allele is segregating in this family that is distinctly different from the mutant allele segregating in the other French Canadian TSD families studied.

Genomic DNA isolated from a sixth infantile TSD proband who is a B^1 compound heterozygote (WG1108) was also analyzed because his mother is of French Canadian descent. Her family originated from St. Germain, Quebec (near Drummondville). Assuming the mother of this patient is a carrier of the deletion-type mutant allele observed in the other obligate heterozygotes, Southern analysis of the proband's DNA should reveal both 9.5- and 23-kb fragments inherited from his father and mother, respectively. However, only a 9.5 kb fragment is observed. Thus, the mutant α-locus allele for which the mother is heterozygous is not of the type observed in the obligate heterozygotes who originate from southeastern Quebec. The results obtained in the families not possessing the 5' end deletion are surprising, considering the commonly held notions about inbreeding among the French Canadian population. The molecular defect, whether the same or different, in the families originating from Quebec City and St. Germain remains to be determined. Additional studies eliminating one of the Ashkenazi Jewish mutations in these families are discussed below.

Cells of the B^1 TSD patient synthesize Hex A. The chromatographic profile of the proband's fibroblast Hex isozymes (figure 18B) indicates that the CRM-positive mutant allele inherited from the patient's father produces an apparently normal amount

of α subunit that associates with ß subunits. In contrast, the mutant allele inherited from the proband's mother either produces no α subunit or produces an α subunit that is incapable of complexing with ß subunits. It is very difficult to determine the contribution of maternally- and paternally-inherited alleles to the synthesis and maturation of the α subunit in cells of an individual who is heterozygous for two different mutant α-locus alleles. Thus, as I was unable to obtain cells directly from the mother of the proband it was not possible to determine whether the mutant α -locus allele for which she is heterozygous is a CRM-negative or CRM-positive mutation. However, since the parents of the infantile TSD proband, WG 1499, are related it is likely that they are carriers of an identical α-locus mutant allele. Fibroblasts of the proband were found to produce ß subunit of normal size which associates normally with other ß subunits to form Hex B and is proteolytically processed to mature size in the lysosome. In contrast, neither precursor nor mature α subunit is detected in cells of this proband. The proband is thus homozygous for a CRM-negative mutation at the α-locus. Biosynthetic labelling and immunoprecipitation of fibroblast cell proteins of two other French Canadian TSD probands (corresponding to numbers 10 and 12 on the map, figure 25), performed recently by Bernard Boulay, indicate that they are also homozygous for a mutant allele that fails to synthesize an α precursor polypeptide. Southern analysis has not yet been performed on DNA isolated from these two probands. However, it will be interesting to discover if the CRM-negative mutation for which they are homozygous is of the deletion-type characterized by Myerowitz and Hogikyan (1986).

Southern analysis of genomic DNA obtained from fiboblasts of WG1108 and

WG1499 clearly indicates that the α-locus mutation inherited from their French Canadian parents differs from the 5' end gene deletion found in other French Canadian probands originating from southeastern Quebec and northern New Brunswick. McKusick (1986) has suggested that TSD in the French Canadian population might represent an infusion of the TSD gene from Jewish fur traders at the time when various settlements were being established throughout Quebec. It has become feasible only recently to rule out this possibility. Only now are the defects in these two ethnic groups being investigated at the molecular level. Two mutations responsible for infantile TSD in the Ashkenazi Jewish population have been characterized. As well, one mutation associated with infantile TSD in the French Canadian population has been identified. While it is not beyond possibility that the 5' end deletion mutation detected in French Canadians of eastern Quebec will also be found by genomic DNA analysis of Ashkenazi Jewish TSD probands, this has not yet been seen. DNAs isolated from WG1108 and WG1499 fibroblasts were amplified by the PCR technique (performed by E. Arpaia, Hospital for Sick Children, Toronto, Ontario, Canada) to determine if the mutation in these two patients is similar to the intron 12 splice junction mutation found in the α-chain gene of some Ashkenazi Jewish TSD probands (Arpaia et al, 1988; Myerowitz, 1988; Ohno and Suzuki, 1988b). The two fragments of 85- and 35-bp generated by Dde I digestion of PCR-amplified Ashkenazi Jewish TSD DNA were not found in similarly treated DNAs isolated from the two French Canadian probands who do not possess the 5' end gene deletion. Instead, only one fragment of 120 bp was generated from DNA amplified from these patients as well as from normal controls.

Thus, the mutation in these two probands is not the 5' end deletion mutation found in French Canadians of southeastern Quebec nor is it the splice junction mutation of intron 12 found in some Ashkenazi Jewish probands. It is likely that

multiple mutant alleles will be identified in the Ashkenazi Jewish population that produce an infantile form of TSD. It is not yet known whether WG1108 and WG1499 possess different mutant alleles inherited from their French Canadian parents or even if the mutations they possess will be found to differ from those likely to be discovered in the Ashkenazi Jewish population. Likewise, DNA has not yet been analyzed from TSD probands and their parents who reside in communities along the north shore of the St. Lawrence River. The finding of an identical mutation in all individuals tested would have been strongly in favor of the founder effect as an explanation of the high heterozygote frequency in the French Canadian population. However, this was not the case. It is possible that the founder effect may explain the incidence of TSD in the French Canadian population of southeastern Quebec, since these communities constitute a 'genetic isolate'. Yet it clearly does not account for all cases of infantile TSD in the French Canadian population. The theory of selective advantage is somewhat strengthened by the discovery of multiple mutant alleles that contribute to the same phenotype. However, just what the selective factor could be and how heterozygosity for the TSD allele would result in a "fitter" genotype is unknown.

V. SUMMARY.

I have attempted to deal with some of the problems inherent in TSD diagnosis and carrier detection by developing a 'new' substrate that measures the amount and catalytic activity of Hex A with increased accuracy and reliability. The improvement that assay with this substrate represents over the standard thermal fractionation assay procedure is attributable to the specificity of 4MUGS for the α-subunit active site of Hex A. 4MUGS represents a significant improvement in the ability to detect TS heterozygotes, particularly carriers of variant α-locus alleles, by both manual and automated assay procedures. The ability to identify patients with variant TSD phenotypes is also greatly improved by use of the assay procedure that employs the sulfated substrate. Biochemical characterization of mutant Hex As synthesized by TSD variants leads to an appreciation of the heterogeneity that exists not only between different ethnic groups but even between different kindreds within the same ethnic group.

A multiplicity of mutant alleles at the α-locus produce biochemical and clinical heterogeneity. Thus, the TSD mutations have come to resemble the ß-thalassemias in the wide variations in severity of disease that are produced by numerous mutations. Mutations that are biochemically distinct can produce clinically indistinguishable effects. On the other hand, there are essentially benign mutations that do not affect the health of the individual in any discernible manner. The identification of these mutations is a new and exciting area of TSD research that has been made possible by the elucidation of the structures of the normal α- and ß-chain genes and by the development of techniques that amplify segments of DNA for analysis.

There is currently no treatment for TSD. It is possible that sometime in the future enzyme replacement therapy or even gene therapy might be feasible. But until there is a treatment for TSD efforts must be aimed at preventing the births of infants affected with this lethal disease as well as improving the ability to detect milder forms of the disease which have no significant effect on longevity. Therefore, the effect that a particular mutation has on the amount and catalytic activity of Hex A will remain of prime importance for the diagnosis of TSD and the detection of carriers.

Claims to originality

To the best of my knowledge the following work is original:

1. I performed the first synthesis of 4-methylumbelliferyl-ß-D-N-acetylglucosamine-6-sulfate (4MUGS) and demonstrated its specificity for the α subunit of Hex A. The following parameters of normal control Hex A and Hex B activities were assessed using this substrate:

   i) kinetics

   ii) pH optimum

   iii) inhibition at α- and ß-subunit active sites

   iv) thermal characteristics

2. I undertook a comparison of manual screening methods (the standard thermal inactivation assay procedure employing 4MUG and an assay procedure employing both 4MUG and 4MUGS) for serum and compared the methods by the following criteria:

   i) the separation of means for normal control and heterozygote groups

   ii) the dispersion of Hex A and Hex B values within each group around the means

   iii) the correlation of % Hex A values obtained from each of the two forms of bivariate analyses

   iv) the ability to distinguish heterozygotes from controls by a single measurement

3. I developed an empirical equation that transforms 4MUGS assay values into equivalent 4MUG enzyme units in order to facilitate comparison of the two assay

procedures. This equation incorporates the relative rates of hydrolysis of 4MUGS/ 4MUG by isolated Hex A and Hex B (i.e, the r_A and r_B constants). The r_B constant has been utilized as a measure of the purity of 4MUGS preparations.

4. I evaluated the usefulness of the two-substrate assay procedure for special problems encountered in screening:

i) the testing of pregnant women for carrier status using serum as the enzyme source

ii) the detection of carriers of variant α-locus alleles such as the B^1 heterozygotes

iii) the differentiation between adults with low Hex A activity who are likely to develop a neurological disease and those who can expect to remain symptom-free

5. I demonstrated the feasibility of adapting the automated assay procedure employed for mass carrier screening to use with both 4MUG and 4MUGS. This adaptation can be achieved with an absolute minimum of changes to the apparatus.

6. I evaluated the usefulness of the sulfated substrate for the diagnosis of TSD variants, particularly with regard to the ability of the assay procedure to distinguish between the variants. I demonstrated that:

i) residual Hex A activities in infantile TSD probands more closely approximate zero

ii) later-onset TSD variants (such as juvenile, chronic, and adult) are more accurately distinguished from infantile forms of TSD

7. I undertook a detailed kinetic characterization of the B^1 mutation using the 4MUGS substrate and in so doing provided evidence for heterogeneity.

8. I demonstrated that TSD in the French Canadian population of Quebec is attributable to more than one mutant allele, leading to an appreciation of heterogeneity within populations.

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Appendix A

Appendix A: Derivation of empirical formula for the calculation of Hex A units using two-substrate assays.

TERM	SUBSTRATE	ENZYME SOURCE	DIRECT MEASUREMENT or ATTRIBUTED TO	ENZYME(S)
G	4MUG	mixture	direct	A + B
S	4MUGS	mixture	direct	A + B
g_A	4MUG	mixture	attributed	A
g'_A	4MUG	isolated A	direct	A
s_A	4MUGS	mixture	attributed	A
s'_A	4MUGS	isolated A	direct	A
g_B	4MUG	mixture	attributed	B
g'_B	4MUG	isolated B	direct	B
s_B	4MUGS	mixture	attributed	B
s'_B	4MUGS	isolated B	direct	B

Each term represents a measurement of fluorometric units with one of the two substrates, using either an unfractionated mixture of Hex A and Hex B or one of the two enzymes resolved by ion-exchange chromatography. The equation applied in the text determines the activity of Hex A in an unfractionated mixture of the two isozymes in terms of the rate of hydrolysis of 4MUG by Hex A. This calculation facilitates comparison of the number of Hex A units determined by thermolability with the number of Hex A units determined using both 4MUG and 4MUGS in an assay without thermal fractionation. The prime (') symbol has been employed to denote the rate of hydrolysis of a substrate by pure enzyme. The derivation of the equation depends upon the assumption that in an isozyme mixture Hex A and Hex B behave independently. Thus, isolated enzyme activity should be equivalent to activity attributable to the same isozyme in an unfractionated enzyme mixture.

Derivation

(1) $r_A = \frac{s'_A}{g'_A}$ = ratio of the rate of hydrolysis of the two substrates by pure Hex A.

(2) $r_B = \frac{s'_B}{g'_B}$ = ratio of the rate of hydrolysis of the two substrates by pure Hex B.

(3) $S = s_A + s_B$ by definition

(4) $G = g_A + g_B$ by definition

(5) $g'_B = \frac{s'_B}{r_B}$ by rearrangement of (2)

(6) $G = g_A + \frac{s'_B}{r_B}$ by substitution into (4)

(7) $s_B = S - s_A$ by rearrangement of (3)

(8) $G = g_A + \frac{S - s_A}{r_B}$ by substitution into (6)

(9) $s'_A = r_A \times g'_A$ by rearrangement of (1)

(10) $G = g_A + \frac{S - (r_A \times g'_A)}{r_B}$ by substitution into (8)

(11) $(r_B \times G) = (r_B \times g_A) + S - (r_A \times g'_A)$ multiplying (10) by r_B

(12) $(r_A \times g'_A) - (r_B \times g_A) = S - (r_B \times G)$ by rearrangement of (11)

(13) $g_A (r_A - r_B) = S - (r_B \times G)$ by rearrangement of (12)

(14) $g_A = \frac{S - (G \times r_B)}{r_A - r_B}$ dividing (13) by $(r_A - r_B)$

Appendix B

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Synthesis of 4-methylumbelliferyl-β-D-*N*-acetylglucosamine-6-sulfate and its use in classification of GM_2 gangliosidosis genotypes

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Summary

Measurement of hexosaminidase A (Hex A) is an important clinical chemical procedure in the classification of GM_2 gangliosidosis genotypes. We have synthesized a new substrate which may be useful in both the biochemical diagnosis of GM_2 gangliosidosis and the detection of heterozygotes for the Tay–Sachs disease (TSD) allele.

4-Methylumbelliferyl-β-D-*N*-acetylglucosamine-6-sulfate (4MUGS) was synthesized by sulfation of 4MU-β-D-*N*-acetylglucosamine (4MUG) with chlorosulfonic acid and purified through gel filtration and ion-exchange chromatography. The structure of 4MUGS was verified by elemental analysis and NMR.

Hex A is approximately 100 times more active toward 4MUGS than Hex B. The advantage of this increased specificity is that Hex A can be determined in a one-step procedure which allows separation of normal control serum values from those of obligate heterozygotes.

Alternatively, assay values obtained using both substrates can be transformed by application of an empirical equation that allows the calculation of both Hex A and Hex B without the requirement of thermal fractionation. Lower values for % Hex A in serum have been obtained for Tay–Sachs homozygotes using the 4MUGS assay procedure.

The results of Hex A assays on fibroblast cell strains obtained from Tay–Sachs

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homozygotes, variant forms of GM_2 gangliosidosis and normal controls are also discussed.

Introduction

The GM_2 gangliosidoses are a group of neurodegenerative diseases characterized by autosomal recessive inheritance. Gangliosides are acidic glycolipids containing *N*-acetylneuraminic acid. The enzyme which catalyzes the hydrolysis of GM_2 ganglioside is hexosaminidase A which cleaves the *N*-acetylgalactosaminyl linkage to yield GM_3 ganglioside. It is this pathway that is blocked in the GM_2 gangliosidoses resulting in accumulation of GM_2 ganglioside primarily in nerve tissue.

There are two major hexosaminidase isozymes: hexosaminidase B (Hex B), a homopolymer of 4 β subunits and the more anionic form, hexosaminidase A (Hex A), with a subunit structure of $\alpha\beta_2$. A third isozyme, hexosaminidase I (Hex I), is the major form of heat-stable hexosaminidase activity in serum with an isoelectric point intermediate between Hex A and Hex B [1].

Present methods of Hex A determination rely primarily on the use of the fluorogenic substrate 4-methylumbelliferyl-β-D-*N*-acetylglucosamine (4MUG) or the less sensitive colorimetric substrate para-nitrophenyl-β-D-*N*-acetylglucosamine (PNP-β-glcNac).

There are, however, disadvantages associated with 4MUG as a substrate for the determination of Hex A. Since 4MUG is cleaved by both Hex A and Hex B, the two isozymes must be distinguished by their differential thermolabilities. Hex A is inactivated at 50 °C, whereas Hex B is stable at this temperature [2].

Sulfation of PNP-β-glcNac [3,4] produces a substrate specific for Hex A, thus offering the potential for an assay method for Hex A which does not rely on differential heat lability. Recent evidence [5] suggests that assays of Hex A with this substrate may improve the classification of GM_2 gangliosidosis genotypes. However, fluorescent substrates will continue to be more useful in clinical chemistry because of their greater sensitivity.

In this paper we report on the synthesis of 4-methylumbelliferyl-β-D-*N*-acetylglucosamine-6-sulfate (4MUGS), a substrate specific for Hex A. 4MUGS can be prepared in high yield by sulfation of 4MUG with chlorosulfonic acid [6] and purified as a sodium salt using three chromatographic steps.

We have compared the results of assays using sera obtained from obligate Tay–Sachs (TS) heterozygotes, infantile TS homozygotes and normal controls by thermal fractionation with 4MUG and by the 4MUGS substrate. Both procedures give identical classification of all individuals tested. There is a slight statistical improvement in classification using the sulfated substrate assay.

Fibroblast cell extracts obtained from individuals diagnosed with several of the variant forms of GM_2 gangliosidosis were assayed for Hex A. The classification of the infantile and juvenile TS forms is identical with both synthetic substrates. However, quite different results for the chronic GM_2 gangliosidosis and Sandhoff homozygote cell strains were obtained when assayed with 4MUGS. The potential of

the sulfated substrate for the classification of the variant forms of GM_2 gangliosidosis is discussed.

Materials and methods

Preparation of 4-methylumbelliferyl-β-D-N-acetylglucosamine-6-sulfate

Reagents 4MUG was purchased from Koch-Light (Edmonton, Alberta, Canada). Traces of free 4MU were removed by stirring 1 g of 4MUG overnight in 450 ml of acetone at 4°C, followed by filtration and storage in a vacuum dessicator. Pyridine (technical grade) was distilled and stored over KOH pellets. Dichloromethane was purchased from Eastman (Rochester, NY, USA) and dried over Davison molecular sieves, 4 Å pore size, purchased from Fisher (Montreal, Quebec, Canada). 4-Dimethylaminopyridine was purchased from Sigma (St. Louis, MO, USA). Chlorosulfonic acid was purchased from Fisher. Diethylaminoethyl (DEAE) cellulose, purchased from Bio-Rad (Richmond, CA, USA) was washed with 1 liter 1 N HCl followed by distilled water. It was then washed with 1 liter 0.5 N NaOH and washed again with distilled water to neutrality. This was followed by equilibration in 10 mM NaH_2PO_4–NaOH, pH 6.0. The cation exchange resin, AG50W-X12, 200–400 mesh, hydrogen form, was purchased from Bio-Rad. It was washed with 0.5 l 1 N NaOH and then with distilled water to neutrality. Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden).

Synthesis One gram (2.67 mmol) of washed 4MUG was dissolved in 35 ml distilled pyridine. Fifty milligrams of 4-dimethylaminopyridine were added. The mixture was stirred in a sealed vial for 30 min at 0°C, 266.5 μl (4.01 mmol) chlorosulfonic acid were added to 3.5 ml dry dichloromethane. The chlorosulfonic acid solution was injected dropwise by syringe into the reaction mixture, which was allowed to stir at 0°C for 3–4 h and then at 25°C for 2 h. The reaction mixture was chilled and 10 ml cold distilled water were added.

Purification The reaction mixture was taken to dryness in a vacuum at 25°C. Residual pyridine was removed by taking the residue up in distilled water and lyophilizing. This was repeated twice. The sulfated material was separated from unreacted starting material by ion exchange chromatography on DEAE cellulose. The redissolved residue was applied to a DEAE cellulose column (10.2 × 2.5 cm) equilibrated in 10 mM NaH_2PO_4–NaOH, pH 6.0. The column was washed with distilled water and the eluate was monitored for absorption at 320 nm (A_{320}). After the ultraviolet absorption of the eluate decreased to zero, the column was washed with 0.15 M ammonium acetate. Column fractions eluting with the salt solution with high A_{320} were pooled and lyophilized, leaving a wet salt residue. The ammonium salt of 4MUGS was dissolved in distilled water and NH_4^+ exchanged for Na^+ by application of the redissolved residue to a column of AG50W-X12 (7.9 × 1.8 cm). Fractions with high A_{320} in the distilled water eluate were pooled and lyophilized. Inorganic salts were removed from the redissolved residue by gel filtration on a

Sephadex G-25 column (61.2 × 2.5 cm) swollen in and eluted with distilled water. Fractions with high A_{320} were pooled, lyophilized and stored in a vacuum dessicator at 4°C.

Analysis of 4MUGS Sulfur analysis was performed by Dr. R.N. Pandey, Guelph Chemical Laboratories, Ltd. (Guelph, Ontario, Canada). Nuclear magnetic resonance (NMR) was performed in D_2O/pyridine at 200 and 400 mHz. UV spectra of 4MUG and 4MUGS were determined using 0.25 mM solutions of both 4MUG and 4MUGS in 0.1 M sodium citrate–sodium phosphate, pH 4.4.

Measurement of hexosaminidases Hexosaminidase activity was determined fluorometrically according to the method of Leaback and Walker [7]. Reaction mixtures contained sodium citrate–sodium phosphate buffer, 0.05 M, pH 4.4, human serum albumin (HSA), 2.5 mg/ml (Connaught Labs., Toronto, Ontario, Canada), 4MUG, 0.5 mM and enzyme in a volume of 200 μl. Enzyme was omitted in blank tubes. Reaction mixtures were incubated for 30 min at 37°C and terminated with 5 ml of 0.2 M 2-amino-2-methyl-1-propanol (AMP), pH 10.2. Fluorometric units were recorded on a Turner fluorometer using 4-methylumbelliferone (4MU) standards which were purchased from Koch-Light. One unit of enzyme activity is that amount of enzyme which hydrolyzes 1 nmol 4MUG/min at 37°C. Thermolability studies were performed by heating enzyme preparations for 2 h at 50°C for fibroblasts (30 min at 52°C for sera) in a volume of 100 μl containing 0.1 M sodium citrate–sodium phosphate buffer, pH 4.4 and HSA 5 mg/ml.

For Hex A determinations with the 4MUGS substrate, reaction mixtures contained sodium citrate buffer, 0.04 M, pH 4.2, HSA 2.5 mg/ml, 4MUGS, 0.5 mM and enzyme in a volume of 200 μl. The substrate concentration used resulted in a maximum ratio of Hex A/Hex B activities. Reactions were incubated at 37°C for 30 min and were terminated with 5 ml of 0.2 M AMP, pH 10.2.

For purposes of classification of genotypes it is desirable to obtain estimates of both Hex A and Hex B from assay values. When thermal fractionation is used the percentage of Hex A and Hex B can be directly determined from assay values since the turnover numbers for 4MUG hydrolysis for the two isozymes are equal [8]. Since the V_{max} for the hydrolysis of the 4MUGS substrate is very different for the two isozymes, we have developed an empirical formula which allows the transformation of assay values obtained using mixtures of the two enzymes with each of the two substrates. The formula incorporates relative rates of hydrolysis of the two substrates by each of the two partially purified enzymes and allows the calculation of Hex A and Hex B enzyme units in terms of nanomoles of 4MUG hydrolyzed. The purpose of this transformation is to facilitate comparison with the quantity of '% Hex A' obtained by thermal fractionation. The conversion of fluorometric measurements obtained with 4MUGS to enzyme units based on 4MUG hydrolysis is achieved by employing the following formula:

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

For the definition of terms and derivation of the formula, refer to the Appendix, pages 86–88.

Separation of Hex A and Hex B Resolution of the β-hexosaminidases by ion exchange chromatography was performed according to the procedure of Nakagawa et al [9].

Fibroblasts Human fibroblasts were grown from skin biopsies in roller bottles (480 cm^2) using Eagle's MEM plus 10% FCS. Confluent cultures were harvested by trypsinization. Cell pellets were washed three times with normal saline and stored at −20 °C. Cells were disrupted by three cycles of freezing and thawing and the supernatants were obtained by centrifuging at 10000 × *g* for 10 min at 4 °C. Cell strains WG103, WG93 (obtained from patients of Ashkenazic Jewish ancestry), WG107 and WG884 (from French–Canadian patients) were classified as having the classical infantile form of Tay–Sachs disease (TSD). In these cases GM_2 gangliosidosis was confirmed by thin layer chromatography of brain gangliosides. Cell strain WG534 was obtained from a patient with Sandhoff's disease, also confirmed by thin layer chromatography of brain lipids. WG312 came from an individual with juvenile TSD. Diagnosis was made on the basis of clinical criteria, reduced Hex A activity in skin fibroblasts and age of onset. The patient corresponds to IV-6 in pedigree N reported by Andermann et al [10]. Cell strain WG928 was obtained from an individual with chronic GM_2 gangliosidosis exhibiting dystonic features, as reported by Meek et al [11]. These patients, initially diagnosed at the Montreal Neurological Institute, were biopsied at our facility.

Serum Serum was prepared from clotted whole blood by centrifugation at 25 °C. Sera was obtained from children with infantile TS disease, obligate TS heterozygotes (i.e. parents of children with infantile TS disease) and normal controls.

Protein was determined by the method of Lowry et al [12] using crystalline bovine serum albumin, purchased from Sigma, as the standard.

Results

Synthesis of 4-methylumbelliferyl-β-D-N-acetylglucosamine-6-sulfate

Ion exchange chromatography on DEAE cellulose resolves the negatively-charged product, 4MUGS, from unreacted 4MUG. The starting material, which is uncharged, was eluted with distilled water. 4MUGS, which eluted with 0.15 M ammonium acetate, represented 83% of the UV-absorbing material applied to the column.

The UV absorption spectra of 4MUG and the sodium salt of the 4MUGS preparation obtained after gel filtration on Sephadex G-25 were nearly identical, with the absorption maximum for both compounds occurring at 320 nm. The identity of the two maxima indicates that the umbelliferone ring was not modified by the sulfation reaction. 4MUGS had a slightly greater optical density at the absorption minimum (λ = 254 nm). Since this wavelength corresponds to the λ_{max} of

pyridine a second cycle of gel filtration was undertaken to separate possible contaminating pyridinium salts. No change in the absorption spectrum of the product occurred following rechromatography on Sephadex G-25.

The yield of lyophilized material following purification on Sephadex G-25 was 76% of expected by weight and 77% by recovery of A_{320} units.

NMR spectra of 4MUG and 4MUGS, dissolved in D_2O/pyridine, are shown in Figs. 1A and B. Comparing the original glycoside and the sulfated product, the only significant change is in the position of the two 6-hydrogens which are displaced downfield, as expected on 0-sulfation at the 6-position. Not only are the positions of the two 6-hydrogens characteristic, but their splitting patterns are characteristic indicating sulfation at position 6. The pattern of hydrogens 3 and 4 in the 4MUG and 4MUGS spectra indicates there is no substitution at these positions, thus disulfation has not occurred. All the protons are accounted for in the 4MUGS spectrum, indicating the presence of only one compound, and, therefore, the absence of starting material.

The theoretical percent sulfur was calculated to be 10.97 for a disulfated product and 6.65% for a monosulfated compound. The value found by elemental analysis, 6.41%, is thus more consistent with a monosulfated product.

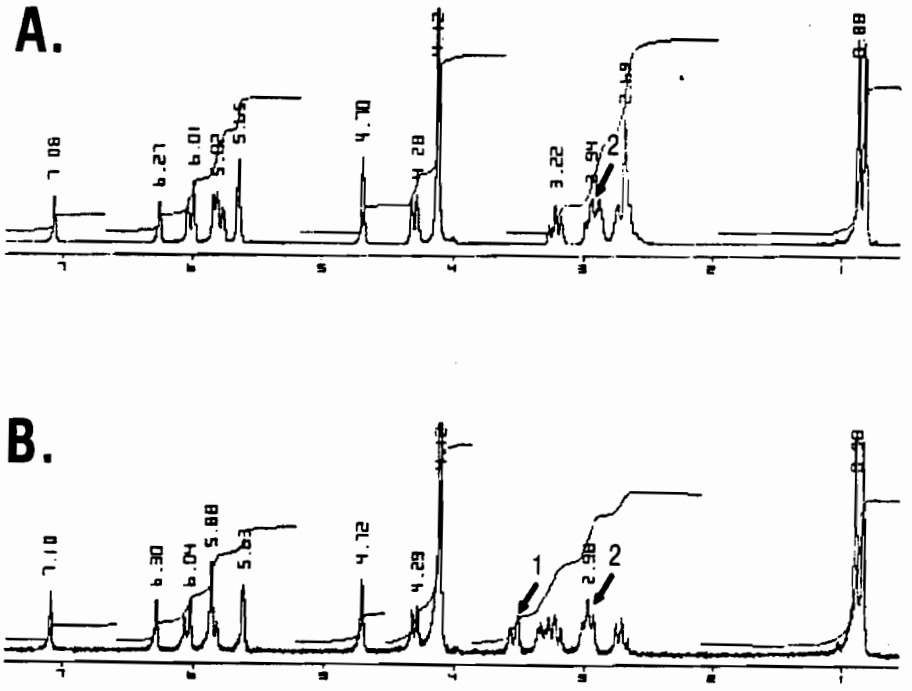


Fig. 1. NMR spectra of 4MUG (A) and 4MUGS (B). Compounds were dissolved in D_2O/pyridine and NMR spectra performed at 200 mHz. Arrow 1 indicates downfield displacement of C6 hydrogen atoms in 4MUGS. Arrow 2 indicates the identity of C3 and C4 hydrogen atoms in both 4MUG and 4MUGS.

Activity of Hex A and Hex B toward 4-methylumbelliferyl-β-D-N-acetylglucosamine-6-sulfate

Hex A and Hex B from fibroblasts and Hex A, Hex B and Hex I from normal human serum were separated by ion exchange chromatography on DEAE cellulose (Fig. 2). The human liver isozymes were enriched 1100-fold according to a previously published procedure [13] before resolution by ion exchange chromatography. Column fractions were assayed for hexosaminidase activity with both 4MUG and 4MUGS substrates. All three serum enzymes have activity toward 4MUG while Hex B and Hex I have barely detectable activity toward 4MUGS. Comparable results are obtained upon fractionation of normal liver and fibroblast hexosaminidases except that the intermediate species, Hex I, is absent in these tissues.

Enzyme-containing fractions were dialyzed against column buffer and concentrated by aquacide II (Calbiochem-Behring, LaJolla, CA, USA). The relative rates of hydrolysis of 4MUGS/4MUG by Hex A and Hex B separated from normal liver, fibroblast and serum (and Hex I obtained from serum only) were as follows: for Hex A, 0.10 (liver), 0.066 (fibroblast) and 0.10 (serum); for Hex B, 0.0034 (liver), 0.0015 (fibroblast) and 0.0014 (serum); for Hex I, 0.012 (serum). The empirical formula (stated in 'Materials and Methods') incorporates these relative rates of hydrolysis for the determination of Hex A specific activity and % Hex A using the sulfated synthetic substrate.

pH-activity curves of Hex A and Hex B for 4MUGS are shown in Fig. 3. The pH optima of Hex A and Hex B, respectively, 3.9 and 3.2, are lower than the pH optima for the 4MUG substrate which is 4.4 for both isozymes (data not shown). For maximum selectivity of Hex A determination using the 4MUGS substrate, a buffer at pH 4.2 was used.

Lineweaver–Burke plots for normal liver and fibroblast Hex A and Hex B, determined with 4MUGS, are shown in Fig. 4. Liver Hex A (Fig. 4, top) has a Km

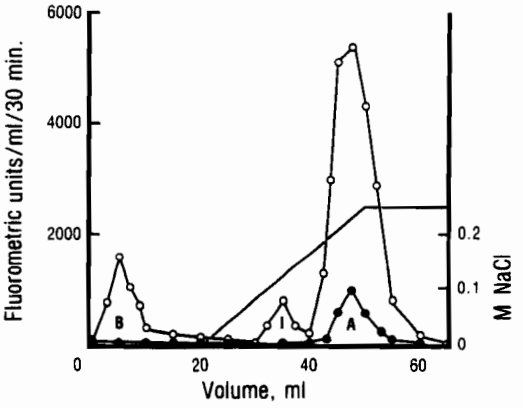
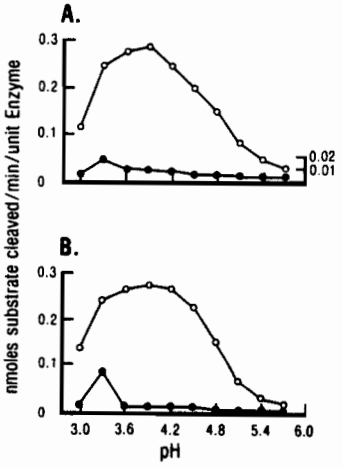


Fig. 2. DEAE cellulose chromatography of serum hexosaminidases. 1.3 ml control serum were dialyzed against 300 ml column buffer and hexosaminidases resolved by ion-exchange chromatography according to the procedure of Nakagawa et al. Fractions were assayed with 4MUG (○——○) and 4MUGS (●——●).

of 0.94 mM and a V_{max} of 0.31 nmol/min per Unit of enzyme, while liver Hex B has a Km of 5.6 mM and a V_{max} of 0.03 nmol/min per Unit of enzyme. The Km of normal fibroblast Hex A (Fig. 4, bottom) is 0.65 mM with a V_{max} of 0.40 nmol/min per Unit of enzyme. Fibroblast Hex B has a Km of 5.9 mM while its V_{max} is 0.004 nmol/min per unit of enzyme. The V_{max} for liver Hex A is 10-fold greater than the V_{max} for liver Hex B, whereas a 100-fold difference is observed for the V_{max} of the fibroblast isozymes. A possible explanation is the presence of a sulfatase in the partially purified liver Hex B preparation. A sulfatase which acts on 4MUGS could convert this substrate to 4MUG, which is easily hydrolyzed by Hex B.

Phosphate is a powerful inhibitor of most sulfatases. The presence of phosphate in reaction mixtures containing a Hex B preparation and 4MUGS should inhibit the release of 4MU if the hydrolysis of the glycoside linkage is dependent on the action of a sulfatase. In the presence of sodium citrate–sodium phosphate buffer (100 mM NaH_2PO_4) the release of 4MU was inhibited 20% compared to the reaction mixture containing no phosphate ion when a partially purified liver Hex B preparation was used. When either fibroblast Hex B or an unfractionated extract of a homozygous Tay–Sachs cell strain was used phosphate inhibition of 4MU release from 4MUGS was not observed. Thus, in the liver Hex B preparation some sulfatase activity contributes to the hydrolysis of the glycoside linkage of 4MUGS although the phosphate inhibitable component does not appear to be large enough to account for the higher V_{max} determined for liver Hex B compared to the fibroblast Hex B preparation.



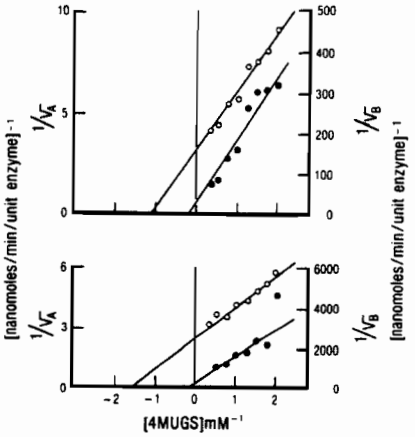


Fig. 3. pH optima of hexosaminidase-catalyzed hydrolysis of 4MUGS. Liver (A) and fibroblast (B) Hex A (○———○) and Hex B (●———●) were assayed in sodium citrate buffer at the indicated pH's. One unit of enzyme activity is that amount of enzyme which hydrolyzes 1 nmol of 4MUG/min.

Fig. 4. Lineweaver–Burke plots of liver (top) and fibroblast (bottom) Hex A and Hex B. ○———○, Hex A; ●———●, Hex B.

Hydrolysis of 4MUGS by fibroblast extracts

The results of fibroblast Hex A assays using both synthetic substrates are shown in Table I. The range for % Hex A in the normal controls ($n = 7$) was 56–79% when 4MUG was used and 46–73% with 4MUGS. For the homozygous infantile Tay–Sachs cell strains ($n = 4$), the range for % Hex A was < 1–5% with 4MUG and < 1–2% with 4MUGS. Percent Hex A values for the juvenile Tay–Sachs cell strain were comparable by both procedures — 19% Hex A was found by thermolability; 20% Hex A by the 4MUGS assay. Quite striking differences, however, were observed in the % Hex A determined by the two procedures for the chronic GM_2 gangliosidosis and Sandhoff cell strains.

Determination of Hex A in human serum

Serum hexosaminidase assays using both 4MUGS and thermal fractionation of activity toward 4MUG were undertaken in order to compare the usefulness of the two procedures for classification of Tay–Sachs carriers. The reliability of the two procedures was assessed by comparing the coefficients of variation (SD/mean) for

TABLE I

Determination of Hex A activity in human fibroblast cell extracts

Fibroblast cell strain	Hex A by thermolability using 4MUG		Hex A by application of formula using 4MUGS	
	% Hex A	Spec. act. [a]	% Hex A	Spec. act. [a]
Normal Controls				
1	76	10300	50	6,680
2	58	3,900	46	3,080
3	70	9,280	63	8,290
4	56	3,660	67	4,360
5	69	3,340	73	3,530
6	61	3,260	63	3,380
7	79	6,940	56	4,920
Infantile TSD				
WG 103	<1	< 32	1.4	45
WG 107	5	120	1.4	33
WG 884	<1	< 82	<1	87
WG 93	4	140	2	69
Juvenile TSD				
WG 312	19	768	20	803
Chronic GM_2 gangliosidosis				
WG 928	11	422	3	108
Sandhoff				
WG 534	89	242	–	995 [b]

[a] Spec. act. is expressed in nmol substrate hydrolyzed/mg protein per h and represents the mean of four determinations.

[b] Application of the formula may not be valid, possibly due to the presence of another enzyme in Sandhoff cells.

eight determinations performed on single serum samples obtained from a normal control and an obligate heterozygote. The coefficients of variation for the control were 0.010 for total hexosaminidase (4MUG), 0.020 for heat labile hexosaminidase (4MUG) and 0.0084 for hexosaminidase activity against 4MUGS. For the heterozygote the coefficients of variation were 0.0073 for total Hex, 0.032 for heat labile Hex and 0.012 for 4MUGS Hex activity. Thus, the reliability of Hex A measurements was improved approximately 3-fold by use of 4MUGS for the measurement of Hex A.

Figure 5 presents the results of Hex A and Hex B determinations using sera obtained from 37 normal controls, 19 obligate Tay–Sachs heterozygotes and 5 infantile Tay–Sachs homozygotes. Each point represents the mean of four determinations on a single serum sample. The values plotted on the ordinates of Figs. 5A and B represent the sum of Hex B and Hex I. However, since these isozymes behave identically in the thermal fractionation procedure and since both species have identical ratios of activity toward the two substrates, the ordinates have been labelled 'Hex B' for convenience.

Figure 5A shows the values of Hex A and Hex B, obtained from measurements of heat labile and heat stable hexosaminidases, plotted as a two-discriminant test. In

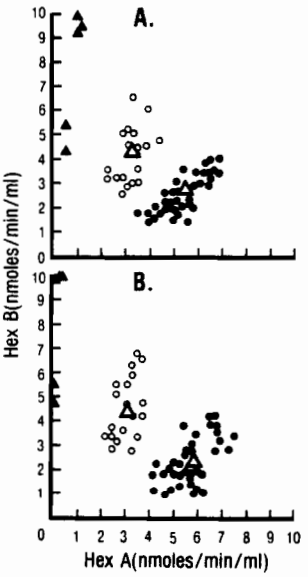


Fig. 5. Determination of Hex A in human serum. Hex A was determined by thermolability using 4MUG (A) and by application of the equation using 4MUGS (B). (A) Thermal fractionation with 4MUG: Hex A, mean ± SD for controls 5.38 ± 0.91, for heterozygotes 3.29 ± 0.59. Hex B, mean ± SD 2.61 ± 0.77 for controls, 4.11 ± 1.19 for heterozygotes. (B) 4MUGS procedure: Hex A, mean ± SD 5.66 ± 0.82 for controls, 3.12 ± 0.54 for heterozygotes. Hex B, mean ± SD 2.32 ± 0.98 for controls, 4.33 ± 1.27 for heterozygotes. ● = normal controls, ○ = obligate Tay–Sachs heterozygotes, ▲ = infantile Tay–Sachs homozygotes, △ = mean values for carrier and normal control groups.

Fig. 5B, Hex A and Hex B values were obtained by measurement of total Hex activity with 4MUG and transformation of 4MUGS assay results using the equation derived in the Appendix. Using this transformation, the values for Hex A and Hex B can be plotted as a two-discriminant test, comparable to that of Fig. 5A.

While the two graphs look essentially similar, closer inspection of the distribution of data points indicates that greater separation of means for each group as well as smaller SD values are obtained with 4MUGS (Fig. 5B) than when heat-labile hexosaminidase activity is measured with 4MUG (Fig. 5A).

The classification of genotypes based on two discriminants has been shown to allow greater resolution of test values for the two groups than can be obtained with a single test [14]. However, we have provided a comparison of serum Hex A values for the three genotypes plotted as a single discriminant and without reference to total Hex activity. The data, plotted in Fig. 6, indicate that the range of Hex A values (measured as 4MUGS-cleaving activity) for control sera does not overlap with the range of heterozygote values, whereas considerable overlap is observed between the control and heterozygote groups when serum Hex A activity is based on thermal fractionation.

In Fig. 7, % Hex A values determined by both procedures are plotted for each subject. The values of % Hex A plotted on the ordinate were obtained by measuring total activity against both 4MUG and 4MUGS and transforming these values by the equation. The % Hex A values plotted on the abscissa were obtained by measurement of heat labile Hex activity with 4MUG. The graph indicates a high degree of correlation ($r = 0.78$) between the two sets of values. The control group had a range

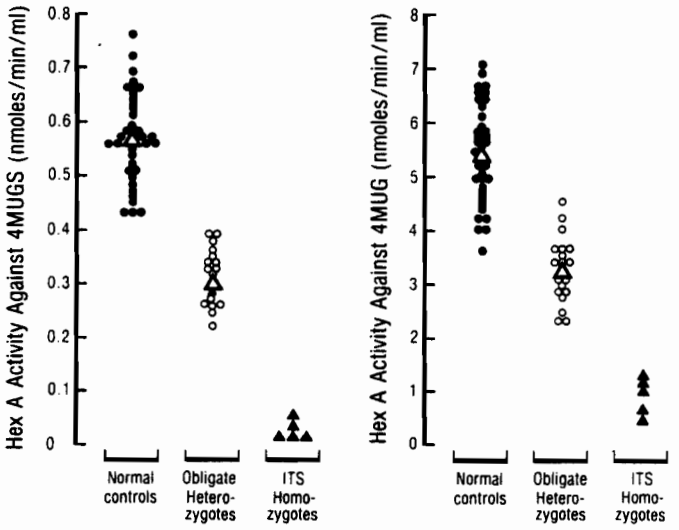


Fig. 6. Activity of sera toward 4MUGS (left) compared to heat labile activity toward 4MUG (right). Hexosaminidase A measurement using 4MUGS: mean ± SD 0.57 ± 0.08 for controls, 0.31 ± 0.05 for heterozygotes. ● = normal controls, ○ = obligate Tay–Sachs heterozygotes, ▲ = infantile Tay–Sachs homozygotes, △ = mean values for carrier and normal control groups.

of 61–81% Hex A measured as heat-labile hexosaminidase. The range for heterozygotes measured by thermal fractionation was 34–54% Hex A. Using the 4MUGS procedure, the range for the controls was 60–88% Hex A and for heterozygotes 35–55% Hex A.

Discussion

The usefulness of an enzyme assay for carrier screening and diagnosis of affected homozygotes depends upon the ability of the procedure to yield values which divide the population at risk into distinct clusters.

The synthetic substrate for Hex A assay employed by most laboratories, 4MUG, depends upon the differential thermolabilities of the isozymes. While satisfactory for most diagnostic purposes, the requirement for thermal fractionation introduces a tedious procedure as well as associated inaccuracies due to variability in the amount of Hex A remaining or the amount of Hex B inactivated.

A procedure is required which detects enzyme activity unique to the Hex A isozyme. Other procedures currently in use employ the radiolabelled physiological substrate, GM_2 ganglioside. This has been used for diagnostic purposes with intact fibroblasts [15] and with extracts of fibroblasts in the presence of a Hex A activator protein prepared from human tissues [16]. While the use of the natural substrate yields more precise diagnostic information, the difficulties inherent in isolating and labelling the substrate make it unlikely that it would come into routine laboratory use.

The two procedures used in this study for determination of Hex A were: (i) thermal fractionation of hexosaminidase activity toward 4MUG and (ii) a new assay procedure based on the differential activities of Hex A and Hex B toward 4MUGS.

Although a detailed kinetic analysis of the interaction of 4MUGS with the hexosaminidase isozymes has yet to be undertaken, the analysis depicted in Fig. 4

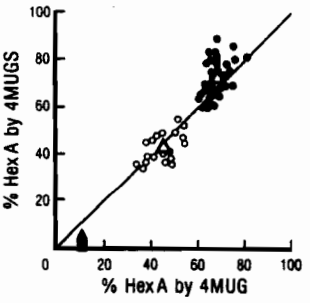


Fig. 7. Correlation of % Hex A determined with 4MUGS and by thermal fractionation with 4MUG. Percent Hex A mean by thermolability 68% for controls and 45% for heterozygotes. Percent Hex A mean by the 4MUGS procedure 72% for controls, 42% for heterozygotes. ● = normal controls, ○ = obligate Tay–Sachs heterozygotes, ▲ = infantile Tay–Sachs homozygotes, △ = means for carrier and normal control groups. The line represents the expected agreement between pairs of assay values. Correlation coefficient = 0.78.

strongly suggests that an active site located on the α subunit of Hex A has a much greater affinity for the sulfated substrate than does the active site located on the β subunit common to both isozymes. Kresse et al [17] and Fuchs et al [5] have reported that PNP-β-glcNac-6-SO_4 behaves as a substrate specific for Hex A. The α subunit also possesses a site for GM_2 ganglioside and is the subunit encoded by the locus at which the TSD mutation occurs. Li et al [18] have shown that one of the forms of the so-called AB variant of TSD possesses normal levels of Hex A and Hex B when measured with PNP-β-glcNac, but is completely deficient in activity toward the 6-O sulfated derivative of this substrate.

The results of both assay procedures, using extracts of infantile Tay–Sachs fibroblast cell strains (Table I), indicate that detectable activity is present in the cells of three of the four patients studied. Infantile TSD in the Ashkenazic Jewish and French–Canadian populations is associated with a mutation that results in complete absence of the α subunit of Hex A ([19] and E. Neufeld, pers. commun.). It should, in theory, be possible to obtain zero activity for Hex A measurements using fluids and tissues obtained from affected individuals from these populations by using the constant r_B to determine the portion of 4MUGS activity attributable to Hex B.

Although the source of the observed residual Hex A activity in homozygous TSD fibroblasts was not determined, two possibilities may account for it. The activity of Hex B toward the sulfated substrate is much lower than Hex B activity toward 4MUG. Consequently, greater amounts of this enzyme are required to obtain accurate measurements of 4MUGS hydrolysis. This may introduce inaccuracies into the calculation of r_B.

Alternatively, the apparent Hex A activity observed in TSD fibroblasts against 4MUGS may be due to the action of a sulfatase. If present, a sulfatase would hydrolyze the 6-O sulfate linkage of 4MUGS to yield 4MUG which can be rapidly hydrolyzed by Hex B. Although phosphate, a potent inhibitor of most sulfatases, did not inhibit the hydrolysis of 4MUGS to 4MU using extracts of TSD cells, it is impossible to rule out completely that the low levels of 4MUGS hydrolysis in TSD cell extracts are due to the action of a sulfatase not inhibited by phosphate.

The juvenile TSD cell strain (WG312) shows moderately reduced Hex A activity by both assays. The % Hex A determined for this patient is comparable to results published in the initial report describing this variant [10]. The agreement between Hex A values obtained by both assay procedures, together with the fact that the child is the offspring of consanguinous parents, indicates that this patient is most likely a homozygote. It should be noted, however, that the juvenile TSD phenotype has been associated with residual Hex A values ranging from 0–35% and that in some families evidence supports the hypothesis that the patients are compound heterozygotes [20].

The chronic GM_2 gangliosidosis cell strain (WG928) was obtained from a patient with dystonic features whose clinical and biochemical phenotype have been reported elsewhere [11]. The striking differences between heat labile hexosaminidase and 4MUGS activity suggest that this genotype may represent a genetic compound of one null allele and another allele with severely reduced, but not completely deficient, activity of the α subunit active site.

Sandhoff disease involves a mutation at the gene locus encoding the β subunit that is common to both Hex A and Hex B. The finding of severely reduced total hexosaminidase in the Sandhoff cell strain (Hex S; 3.2% of the mean of seven normal controls) together with the observation that 89% of the hexosaminidase activity is thermolabile is not unexpected. What is surprising, however, is that specific activity measured by the 4MUGS procedure is 4-fold greater than specific activity determined by thermal fractionation. It has been noted that Hex S, an isozyme more thermolabile and more anodal than Hex A, accumulates in cells which are homozygous for the Sandhoff mutation [21,22]. If this isozyme is composed entirely of α subunits [23] then Hex S may be the source of this elevated rate of 4MUGS hydrolysis.

Serum was analyzed using both assay procedures for the discrimination of Tay-Sachs homozygotes, heterozygotes and normal controls. While both methods are satisfactory for classifying genotypes using a two-discriminant plot (Figs. 5A and 5B), the 4MUGS procedure does offer a slight improvement in classification as shown by the greater difference in the means for enzyme activity and the smaller standard deviations.

In the single-discriminant plot (Fig. 6), however, the control and heterozygote ranges of assay values for 4MUGS hydrolysis do not overlap whereas there is considerable overlap in the values for heat-labile hexosaminidase. In theory, therefore, a one-step assay procedure could be used for the detection of Tay–Sachs heterozygotes by measurement of hydrolysis of 4MUGS. We do not suggest, however, that the superior resolution offered by the use of two discriminants be abandoned in favor of the increased speed of the one-step 4MUGS procedure.

Among the five infantile TSD homozygotes (two Jewish, two French–Canadian and one Italian) whose serum Hex A was determined, the range for % Hex A (9–11%) by thermolability was much higher than the range (0–2%) measured with 4MUGS. The measurement of thermolabile hexosaminidase in fluids obtained from TSD homozygotes frequently gives values as high as 15% Hex A [24]. This artefact is presumably due to loss of variable amounts of the Hex B during the thermal fractionation procedure.

The inaccuracies associated with the thermal fractionation procedure make it difficult to reliably differentiate infantile, juvenile and chronic cases of GM_2 gangliosidosis, or to differentiate any of these phenotypes from the Hex A deficient healthy adult [25] based upon differences in residual Hex A activity. We suggest that the 4MUGS assay procedure, used in conjunction with the thermal fractionation procedure, may have the benefit of biochemically differentiating some of the clinically diverse cases of GM_2 gangliosidosis which are associated with deficient or defective Hex A.

Appendix

Derivation of empirical formula for calculation of Hex A units using two substrate assays

Term	Substrate	Enzyme source	Direct measurement or attributed to	Enzyme(s)
G	4MUG	mixture	direct	A + B
S	4MUGS	mixture	direct	A + B
g_A	4MUG	mixture	attributed	A
g'_A	4MUG	isolated A	direct	A
s_A	4MUGS	mixture	attributed	A
s'_A	4MUGS	isolated A	direct	A
g_B	4MUG	mixture	attributed	B
g'_B	4MUG	isolated B	direct	B
s_B	4MUGS	mixture	attributed	B
s'_B	4MUGS	isolated B	direct	B

Each term represents a measurement of fluorometric units with one of the two substrates, using either an unfractionated mixture of Hex A and Hex B or one of the two enzymes resolved by ion exchange chromatography. The equation applied in the text determines the activity of Hex A in an unfractionated mixture of the two isozymes in terms of the rate of hydrolysis of 4MUG by Hex A. This calculation facilitates comparison of the number of Hex A units determined by thermolability with the number of Hex A units determined using both 4MUG and 4MUGS in an assay without thermal fractionation. We have employed the prime symbol (′) to denote the rate of hydrolysis of a substrate by pure enzyme. The derivation of the equation depends upon the assumption that in an isozyme mixture Hex A and Hex B behave independently. Thus, isolated enzyme activity should be equivalent to activity attributable to the same isozyme in an unfractionated enzyme mixture.

Derivation

(1) $r_A = s'_A/g'_A$ = ratio of the rate of hydrolysis of the two substrates by pure Hex A.

(2) $r_B = s'_B/g'_B$ = ratio of the rate of hydrolysis of the two substrates by pure Hex B.

(3) $S = s_A + s_B$, by definition.

(4) $G = g_A + g_B$, by definition.

(5) $g'_B = s'_B/r_B$, by rearrangement of (2).

(6) $G = g_A + s'_B/r_B$, by substitution into (4).

(7) $s_B = S - s_A$, by rearrangement of (3).

(8) $G = g_A + \dfrac{S - s_A}{r_B}$, by substitution into (6).

(9) $s'_A = r_A \cdot g'_A$, by rearrangement of (1).

(10) $G = g_A + \dfrac{S - (r_A \cdot g'_A)}{r_B}$, by substitution into (8).

(11) $(r_B \cdot G) = (r_B \cdot g_A) + S - (r_A \cdot g'_A)$, multiplying (10) by r_B.

(12) $(r_A \cdot g'_A) - (r_B \cdot g_A) = S - (r_B \cdot G)$, by rearrangement of (11).

(13) $g_A(r_A - r_B) = S - (r_B \cdot G)$, by rearrangement of (12).

(14) $g_A = \dfrac{S - (G \cdot r_B)}{r_A - r_B}$, dividing (13) by $(r_A - r_B)$.

Example of application of formula

(1) Fluorometric unit values obtained per 30 min incubation, normal control no. 1 (Table I):

4MUGS = 96.7 μl of undiluted extract;
4MUG = 28.75 μl of extract diluted 100 : 1.
S = FU/ml/30 min = 96 700 for the 4MUGS substrate;
G = FU/ml/30 min = 2.875×10^6 for the 4MUG substrate.

(2) $$g_A = \frac{S - (G \cdot r_B)}{r_A - r_B},$$
$$= \frac{96\,700 - (2.875 \times 10^6)\,(0.0015)}{0.0643},$$
= 1 436 820 equivalent number of 4MUG FU/ml per 30 min attributed to Hex A.

(3) % Hex A $= g_A/G,$
$$= \frac{1\,436\,820}{2.875 \times 10^6},$$
= 50%.

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A Shortened β-Hexosaminidase α-Chain in an Italian Patient with Infantile Tay-Sachs Disease

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SUMMARY

Fibroblasts derived from a β-hexosaminidase A (HexA)–deficient infant with clinically classic Tay-Sachs disease synthesized a precursor α-chain that was smaller than its normal counterpart. Fibroblasts from the infant's parents, who were consanguinous, produced both normal and mutant α-chains. The size difference, estimated to be 2–3 kilodaltons on the basis of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis, persisted after removal of oligosaccharides with endo-H and is therefore attributable to a shortened polypeptide. The mutant α-chain did not undergo the further posttranslational modifications characteristic of its normal counterpart—i.e., synthesis of the mannose phosphate recognition marker, association with the β-chain to give HexA, and proteolytic conversion to the mature form. Nor was it secreted, even in the presence of NH_4Cl. Instead, it disappeared in the course of a 20-h chase. These results suggest that the mutant α-chain was trapped in an early biosynthetic compartment, either the endoplasmic reticulum or the *cis*-Golgi. The mutation appears to be different from all those previously described in patients with clinically classic Tay-Sachs disease.

INTRODUCTION

Tay-Sachs disease is a neurologic disease caused by mutation of the locus encoding the α-chain of β-hexosaminidase; this results in absence of the A

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isozyme (HexA) and neuronal accumulation of G_{M2} ganglioside (Sandhoff and Christomanou 1979; O'Brien 1983). There is clinical heterogeneity in the disease, which ranges from the infantile (classic) form to juvenile, chronic, and adult forms with progressively later onset and milder clinical manifestations (Kolodny and Raghavan 1983; O'Brien 1983); asymptomatic adults have also been reported, though some of them later developed neurologic problems (Navon et al. 1986). The milder forms are associated with detectable ability to hydrolyze G_{M2} ganglioside, whereas such residual activity is lacking in the classic disease (Conzelmann et al. 1983).

Recent studies have revealed molecular heterogeneity within clinically classic Tay-Sachs disease. Fibroblasts derived from a number of Tay-Sachs patients of Ashkenazi-Jewish origin were found to synthesize no α-chain protein (Proia and Neufeld 1982) and to contain no detectable mRNA (Myerowitz and Proia 1984; Myerowitz et al. 1985; Korneluk et al. 1986). This was also found for fibroblasts from non-Jewish Tay-Sachs patients of French-Canadian origin, but the mutations were shown to differ. A 5–8-kb deletion has been found at the 5′ end of the α-chain gene in the French-Canadian patients, whereas the genomic DNA of Ashkenazi patients was found to be generally intact (Myerowitz and Hogikyan 1986). Ashkenazi Tay-Sachs fibroblasts appear able to initiate mRNA transcripts, suggesting a defect in mRNA processing or transport (B. H. Paw and E. F. Neufeld, unpublished results).

Cells from several classic Tay-Sachs patients belonging to neither of the above groups synthesize an altered α-chain. The altered α-chain may give rise to a defective HexA either with low catalytic activity and/or stability (Kolodny et al. 1983) or with different substrate specificity (Kytzia et al. 1983). Fibroblasts of one patient of Italian descent were found to produce an α-chain that appeared to be trapped in the endoplasmic reticulum (Proia and Neufeld 1982).

Fibroblasts from a number of patients with milder forms of HexA deficiency were found to synthesize an α-chain precursor that failed both to associate with the β-chain and to be converted to the mature form (d'Azzo et al. 1984). It is likely that in several of these cases there was compound heterozygosity with the Ashkenazi classic Tay-Sachs (null) allele.

We here describe a defect of the α-chain that differs from all those previously described, one that was found in cells of an Italian non-Jewish patient with clinically classic Tay-Sachs disease.

CASE HISTORY

The proband was 12 mo old when she was brought to the Montreal Children's Hospital for evaluation because of loss of milestones. Her parents came from the same town of 3,500 inhabitants in Italy. Her grandmothers were second cousins, and her grandfathers were third cousins. She had an unaffected sister. At 12 mo she appeared to be unaware of her environment; she did not babble and could not sit unsupported. She startled easily. Cherry-red spots were seen in the fundi. Diagnosis of Tay-Sachs disease was confirmed by the finding of

profound HexA deficiency in serum and fibroblasts. The family returned to Italy where the infant died at 2½ years of age.

MATERIAL AND METHODS

Fibroblasts from the patient and her parents were provided by the Repository for Mutant Cell Strains, Montreal, Canada, under the numbers WG1051, WG1053, and WG1054, respectively. The normal cell strain IMR90, used as a control, was obtained from the Institute for Medical Research, Camden, NJ.

All procedures for maintenance and labeling of cells; for preparation of cell extracts and medium concentrates; for immunoprecipitation of HexA, HexB, and their subunits; and for electrophoretic separation and fluorographic visualization of the β-hexosaminidase subunits were as described elsewhere (Proia et al. 1984), except for the following differences in culture media: the growth medium used in the present study was Eagle's Minimal Essential Medium in Earle salts (Gibco) supplemented with nonessential amino acids, penicillin, streptomycin, 1 mM pyruvate, and 15% fetal bovine serum (Tissue Culture Biologicals or Irvine Scientific). Labeling medium was Eagle's Minimal Essential Medium in Earle salts prepared from the Gibco Selectamine Kit to be free of leucine or phosphate for labeling with [^{3}H]leucine or [^{32}P]phosphate, respectively; it was supplemented with antibiotics, 1 mM pyruvate, nonessential amino acids, and 5% dialyzed fetal calf serum (Gibco).

Activity of HexA was determined fluorometrically using 4-methyl-umbelliferyl-β-*N*-acetylglucosaminide 6-sulfate (4MUGS), as well as the unsulfated 4-methylumbelliferyl-β-*N*-acetylglucosaminide (4MUG), as substrates (Bayleran et al. 1984). Endo-β-*N*-acetylglucosaminidase H (endo H) was purchased from Miles Scientific; it was used as described elsewhere (Proia and Neufeld 1982) but with the concentration of endo-H increased to 0.03 units.

RESULTS

Absence of HexA Activity in Proband's Serum and Fibroblasts

The HexA activity in the sera of the proband and members of her family was determined by the conventional measurement of heat-labile β-hexosaminidase activity toward 4MUG substrate, as well as by hydrolysis of the more specific 4MUGS substrate. Table 1 shows (1) the proband's HexA activity, as measured by both procedures, to be in the range characteristic of Tay-Sachs homozygotes, (2) that of her parents to be in the range characteristic of Tay-Sachs heterozygotes, and (3) that of her sister to be in the range characteristic of controls. A profound deficiency of HexA was also found in extracts of her fibroblasts. The apparent "HexA" activity observed in the serum and cell extracts of the proband and of other Tay-Sachs patients by means of the thermolability assay is an artifact due to variable loss of HexB during heating rather than true residual activity of HexA; use of the sulfated substrate, toward which HexB is essentially inactive, gives a more reliable estimate of HexA activity. On DEAE-cellulose chromatography of an extract of the proband's fibroblasts,

TABLE 1

DETERMINATION OF HexA ACTIVITY

TISSUE AND SUBJECT (*N*)	HexA SPECIFIC ACTIVITY AS			
	Thermolability (%)		4MUG Equivalents[a] (%)	
Serum:				
Proband	64	(10)	0.6	(0)
Mother	147	(45)	131	(40)
Father	242	(39)	230	(38)
Sister	389	(65)	410	(68)
Controls (45)	218–474	(60–81)	243–457	(60–88)
Heterozygotes (28)	130–276	(34–57)	79–228	(35–55)
Tay-Sachs patients (5)	32–74	(9–11)	0.6–17	(0–2)
Fibroblasts:				
Proband	120	(5)	33	(1.4)
Controls (8)	3,260–9,280	(47–79)	3,080–8,294	(46–89)
Tay-Sachs patients (7)	32–140	(1–5)	9–87	(0.4–2)

NOTE.—For serum, specific activity is expressed as nmol of 4MUG hydrolyzed/h/ml; for fibroblast extract, serum activity is expressed as nmol of 4MUG hydrolyzed/h/mg protein.

[a] Measured using 4MUGS as substrate and converted to 4MUG equivalents by means of the formula of Bayleran et al. (1984).

no activity eluting in the position of HexA was detected with either the 4MUG or the 4MUGS substrate (not shown).

Synthesis of an α-Chain Precursor with Abnormal Electrophoretic Mobility

Figure 1 shows the results of a 3-h pulse of [^{3}H]leucine administered to normal cells and to cells of the proband, 1051. Three goat antisera were used in this and subsequent experiments: anti-HexA, which precipitates all α- and β-chains, whether free or associated into HexA, HexB, or HexS; anti-HexB, which precipitates all β-chains but only those α-chains that are associated with β into HexA; and anti-α-chain, which reacts only with α-chains that are monomeric (Proia et al. 1984). After a 3-h pulse, the two chains of β-hexosaminidase were found to be in the precursor form, a finding in agreement with previous studies (Hasilik and Neufeld 1980*a*). The normal precursor α-chain (α_p), which previously had been assigned an apparent molecular weight of 67,000, and the normal precursor β-chains (two bands that differ in oligosaccharide content), which previously had been assigned apparent molecular weights of 63,000 and 61,000, respectively (Hasilik and Neufeld 1980*a*; Proia and Neufeld 1982), are clearly seen in the normal cell extract immunoprecipitated with anti-HexA (fig. 1). By contrast, the labeled material precipitated from extracts of 1051 by anti-HexA is seen as a broad band in the region of the normal β-chain. Use of anti-HexB and of anti-α-chain showed that this broad band was a composite of the normal β-chain doublet and of an altered α-chain monomer that migrated more rapidly than its normal counterpart.

Similar labeling of fibroblasts derived from the patient's mother (1053) and father (1054) revealed two precursor α-chains, one of normal and one of abnormal mobility, the normal chain being more intensely labeled (fig. 2). After the 5-h

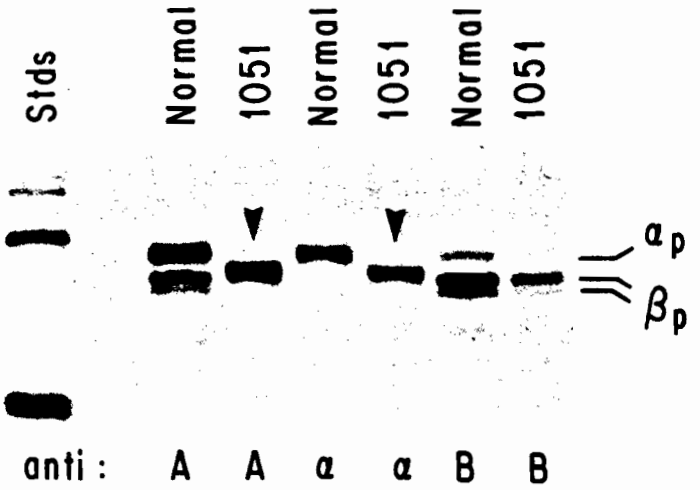

FIG. 1.—Synthesis of abnormal precursor α-chain by 1051 fibroblasts. Confluent cultures of normal (IMR90) and the proband's (1051) fibroblasts in 100-mm Petri dishes were labeled with [^{3}H]leucine (0.25 mCi/dish) for 3 h. Cell extracts from each plate were treated with one of the indicated antisera—anti-HexA, anti-α-chain, or anti-HexB—according to the method described elsewhere (d'Azzo et al. 1984; Proia et al. 1984). The immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions, and the radioactive bands were visualized by means of fluorographic exposure for 2 days. α_p = Precursor α-chain; and β_p = precursor β-chain. Arrowheads point to the α-chain of 1051.

labeling period, some of the normal precursor α-chain was precipitated by anti-HexB, a result showing that it had associated with the β-chain. But the abnormal α-chain was not seen in the anti-HexB immunoprecipitates, even after much longer (21-day) fluorographic exposure (not shown), a result indicating that it did not associate with the β-chain.

Persistence of Abnormal Electrophoretic Mobility after Treatment with Endo-H

Both α- and β-precursor chains have *N*-linked "high mannose" oligosaccharides that can be removed by the bacterial enzyme endo-H (Proia and Neufeld 1982). Figure 3 shows that the difference in mobility between the normal and mutant α-chains persisted after the treatment with endo-H. For this experiment, the [^{3}H]leucine-labeled cell extracts were subjected to sequential immunoprecipitation with anti-α-chain and anti-HexB. Because of the short (1-h) labeling time, none of the normal α-chain would have been associated with β (Proia et al. 1984), and thus the sequential immunoprecipitation gave a complete separation of α- and β-chains.

Apparent molecular weights were determined on the basis of the electrophoretic mobilities. For normal cells the weights were as follows: precursor α-chain, 67,000; endo-H treated, 63,000; precursor β-chain, 63,000 and 61,000; and endo-H treated, 57,000. For cells of 1051 the weights were as follows:

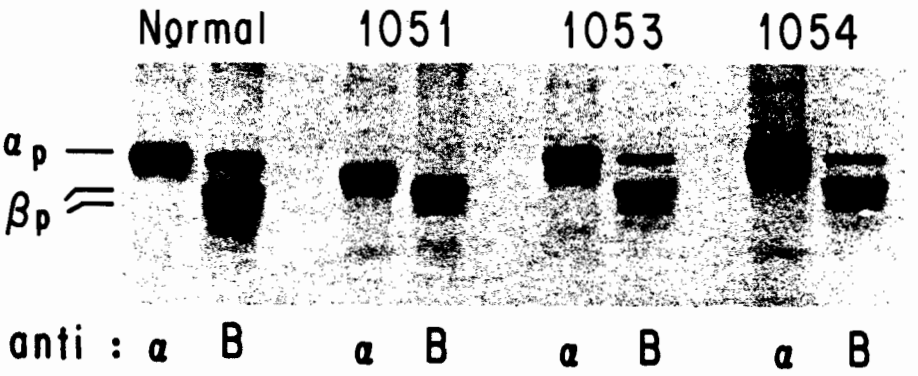

FIG. 2.—Synthesis of both normal and abnormal precursor α-chains by the parents' fibroblasts. Confluent cultures of the mother's (1053), the father's (1054), the proband's (1051), and normal (IMR90) fibroblasts in 100-mm petri dishes were exposed for 5 h to [^{3}H]leucine (0.25 mCi/dish). Cell extracts were treated sequentially with anti-α-chain and anti-HexB. The immunoprecipitates were subjected to SDS-PAGE as in fig. 1; fluorographic exposure was for 3 days.

precursor α-chain, 65,000; endo-H treated, 61,000; precursor β-chain, 63,000 and 61,000; and endo-H treated, 57,000.

Lack of Phosphorylation of the Abnormal α-Chain

Enzymes destined for lysosomes normally acquire the mannose 6-phosphate recognition marker by way of a two-step enzymatic pathway that takes place in the Golgi (reviewed in Kornfeld 1986; von Figura and Hasilik 1986). Both chains of β-hexosaminidase acquire this marker, as can be readily demonstrated by exposure of intact fibroblasts to [^{32}P]phosphate (Hasilik and Neufeld 1980*b*).

Figure 4 shows that although the 1051 fibroblasts synthesized a considerable amount of α-chain precursor (seen in the ^{3}H lane), they did not phosphorylate it, as evidenced by the absence of a detectable band of comparable mobility in

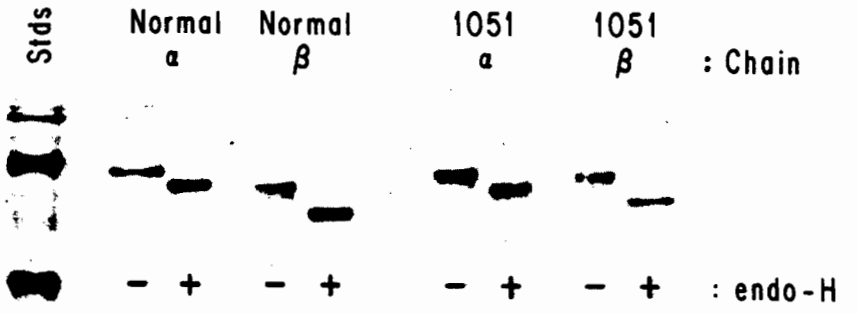

FIG. 3.—Effect of endo-H on the electrophoretic mobility of the normal and 1051 precursor α- and β-chains. Confluent cultures of normal (IMR90) and the proband's (1051) fibroblasts in 150-cm^2 flasks were labeled with [^{3}H]leucine (1.5 mCi/flask) for 1 h. Cell extracts were subjected to sequential precipitation with anti-α-chain and anti-HexB to bring down α- and β-chains, respectively. The immunoprecipitates were treated with endo-H, concentrated by means of lyophilization, and subjected to SDS-PAGE. Fluorographic exposure was for 11 days.

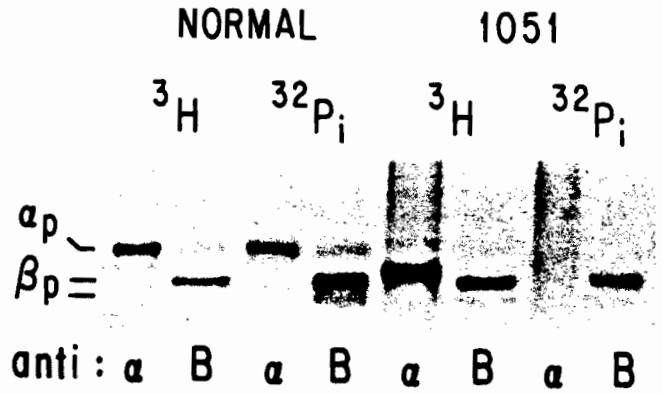

FIG. 4.—Lack of incorporation of [^{32}P] into the 1051 α-chain. Parallel cultures of normal (IMR90) and the proband's (1051) fibroblasts were labeled in 100-mm petri dishes with [^{3}H]leucine (0.25 mCi) or [^{32}P]phosphate (1 mCi) for 3 h. Cell extracts were treated by means of sequential immunoprecipitation with anti-α-chain and anti-HexB. The immunoprecipitates were subjected to SDS-PAGE. Fluorographic exposure was for 2 days; a Dupont Cronex Lightning Plus screen was used to intensify the ^{32}P signal.

the ^{32}P lane. The β-chain precursor of 1051, which served as internal control, was phosphorylated. ^{32}P/^{3}H ratios, determined after excision and solubilization of appropriate areas of the gel, were found to be (per 1,000 cpm ^{3}H) as follows: 1051 α-chain, 0; 1051 β-chain, 9; normal α-chain, 17; and normal β-chain, 42. The quantitative measurement confirms the impression given by visual examination of figure 4—i.e., that the 1051 cells were less efficient than normal cells in phosphorylating their β-chain precursor. The reason for this is not known. However, a greater amount of material was loaded onto the gel to compensate for this reduced phosphorylation, so that phosphorylation of the 1051 α-chain, had it occurred at one-third the level of the β-chain, would have been detected very easily on the film.

Lack of Maturation and Disappearance of the Abnormal α-Chain

Normal cells are known to process the precursor α-chain to a smaller mature form with an apparent molecular weight of 54,000; the proteolytic processing, which is thought to occur in lysosomes, takes ~10 h for completion (Proia et al. 1984). Figure 5 shows that there was no band corresponding to mature α-chain in the 1051 cells after a 20-h chase. The shortened precursor α-chain that had been present in the 3-h pulse was no longer seen on precipitation with any of the three antisera; nor had it become converted to any other species during the chase period. The precursor β-chain, an internal control, was converted in normal fashion to the major chain of 29,000 plus several smaller fragments that comprise the mature β (Hasilik and Neufeld 1980*a*).

Lack of Secretion of the Abnormal α-Chain

The α-chain that disappeared from the 1051 fibroblasts during the chase was not found in the medium (fig. 6, right-hand lanes). For this determination, the medium concentrate was treated sequentially with anti-α-chain, anti-HexB,

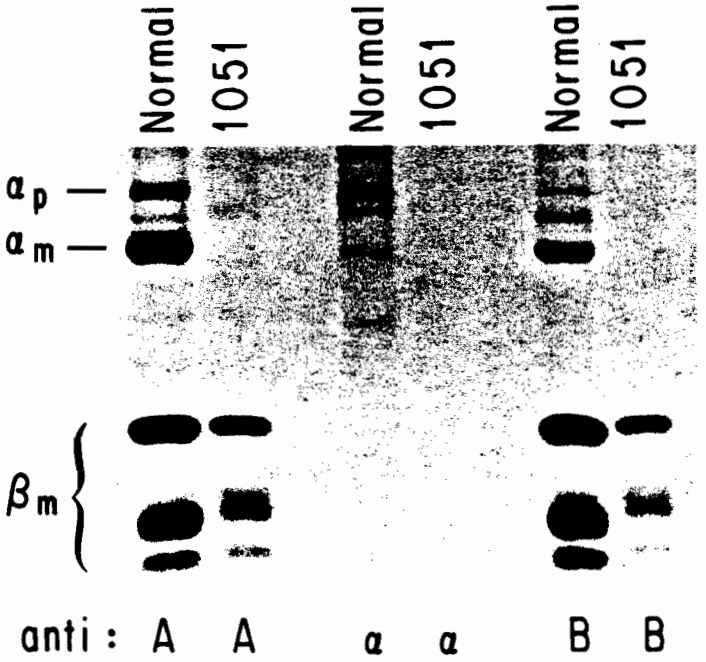

FIG. 5.—Loss of the 1051 α-chain during chase. Confluent cultures of normal (IMR90) and the proband's (1051) fibroblasts were labeled by means of a 3-h exposure to [^{3}H]leucine conducted in parallel with the experiment described in fig. 1 (pulse). Unlabeled leucine was then added (1 mg/plate), and the incubation was continued for 2 h (chase). The cell extract from each plate was treated with one of the three designated antisera: anti-HexA, anti-α-chain, or anti-HexB. Immunoprecipitates were subjected to SDS-PAGE. Fluorographic exposure was for 16 days. α_m = Mature α-chain; and β_m = mature β-chain fragments. Other abbreviations are as in fig. 1.

and anti-HexA to recover, respectively, any monomeric α-chain, HexA plus Hex$_{S}$, and HexS that might have been secreted. It can be seen that whereas a small amount of both α- and β-chains appear in the lane corresponding to normal secretions treated with anti-HexB, only a trace of β-chain is present in the 1051 immunoprecipitates (fig. 6, arrow).

Figure 6 (left-hand lanes) also shows that the amount of HexA and HexB secreted by normal fibroblasts was greatly enhanced if the pulse-chase was carried out in the presence of 10 mM NH_4Cl, as has been demonstrated elsewhere (Hasilik and Neufeld 1980*a*). Even a small amount of monomeric α-chain could be seen in the NH_4Cl-induced secretions of normal cells (fig. 6, arrowhead). However, the 1051 fibroblasts failed to secrete any α-chain, free or associated, in the presence of NH_4Cl, even though their secretion of β-chain (presumably as HexB) was much enhanced.

DISCUSSION

The α-chain synthesized by 1051 cells was abnormal in many ways. It had an increased electrophoretic mobility in SDS-PAGE, corresponding to a reduction

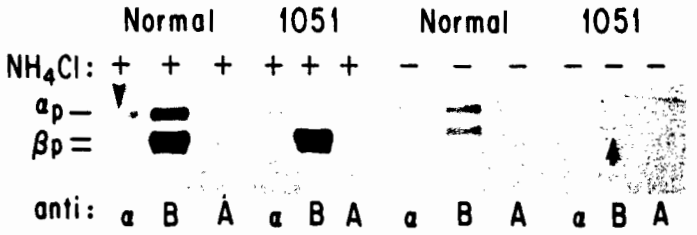

FIG. 6.—Absence of α-chain from the secretions of 1051 fibroblasts. Confluent cultures of normal (IMR90) and the proband's (1051) fibroblasts in 100-mm petri dishes were labeled for 3 h with [^{3}H]leucine (0.25 mCi/dish) after which unlabeled leucine (1 mg/dish) was added and incubation continued for an additional 18 h (chase). Where indicated, 10 mM NH_4Cl was included in the labeling medium. Medium concentrates were treated by means of sequential immunoprecipitations with the three indicated antisera and subjected to SDS-PAGE. Fluorographic exposure was for 17 days.

in molecular size of 2–3 kilodaltons. Though it was *N*-glycosylated, as shown by susceptibility to digestion with endo-H, it did not undergo the further post-translational modifications characteristic of the normal α-chain—namely, acquisition of the mannose 6-phosphate recognition marker, association with the β-chain to give HexA, and processing to the mature form. This means that the mutant α-chain entered the endoplasmic reticulum, the site of *N*-glycosylation (Kornfeld and Kornfeld 1985) but failed to reach lysosomes, where proteolytic conversion to the mature form is believed to take place (Frisch and Neufeld 1981; Gieselman et al. 1983; Skudlarek et al. 1984). It may be that the α-chain was not transported from the endoplasmic reticulum to the *cis*-Golgi, the probable site of the first step leading to the synthesis of the mannose 6-phosphate recognition marker (Kornfeld 1986; von Figura and Hasilik 1986). An alternative hypothesis would have the α-chain reach the *cis*-Golgi but not be recognized as a substrate by phospho-*N*-acetylglucosamine transferase, the first of two enzymes needed to synthesize the marker. In contrast to a recently described mutant β-galactosidase that, lacking the recognition marker, was secreted into the medium (Hoogeveen et al. 1986), the 1051 α-chain was not secreted even in the presence of 10 mM NH_4Cl, an agent that causes newly made lysosomal enzymes to be secreted rather than packaged into lysosomes (Gonzalez-Noriega et al. 1980; Neufeld and Hasilik 1980*a*). Instead, the abnormal α-chain decayed within 20 h, presumably owing to proteolytic digestion within the endoplasmic reticulum or early Golgi.

The difference in molecular size persisted after removal of oligosaccharide chains by means of digestion with endo-H. This indicates that the size difference was due to a shortened polypeptide rather than to a reduction in carbohydrate content in the mutant α-chain. We do not yet know whether the shortening is the result of an internal deletion or of premature termination at the carboxyl end. The missing stretch of polypeptide may contain specific signals for posttranslational modifications or intracellular transport; it is possible, however, that the shortened polypeptide does not fold correctly and for that reason is not recognized by enzymes or receptors along the pathway to lysosomes.

The consanguinity of the parents suggested that the proband would be homozygous for an α-chain mutation, rather than be a compound heterozygote with two different mutant alleles. This was borne out by biochemical evidence; cells of both parents synthesized the shortened α-chain. We currently have no explanation for the reduced amount of abnormal α-chain, relative to its normal counterpart, in the cells from the parents. Cells of the proband did not appear to synthesize a reduced amount of α-chain, as gauged by comparison with β-chain synthesis.

Several of the abnormalities of the 1051 α-chain are reminiscent of those described for GM1110, which were derived from another classic Tay-Sachs patient of Italian origin (Proia and Neufeld 1982). The α-chain of GM1110 was likewise *N*-glycosylated but not phosphorylated, secreted, or processed to the mature form. It was also thought to be trapped in the endoplasmic reticulum. However, the GM1110 α-chain was of normal size and of reduced solubility. The structural difference between the α-chains of 1051 and GM1110 underscores the heterogeneity of mutations of the α-chain gene that can give rise to clinically classic Tay-Sachs disease; these may differ not only between major ethnic or demographic groups but even between different kindreds within a group.

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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

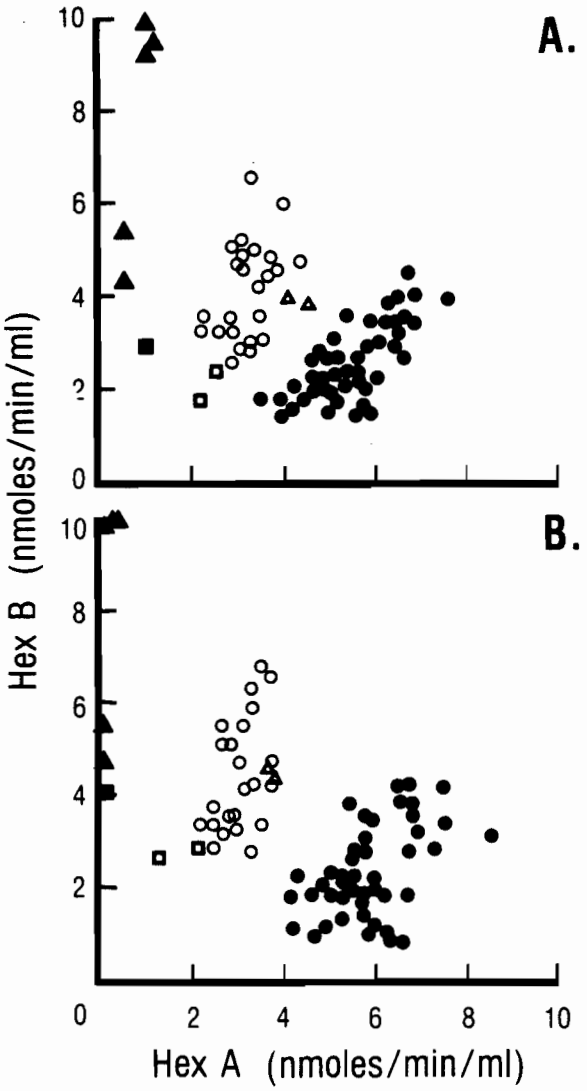

FIG. 1.—Determination of hexosaminidase isozymes in human serum. Hex A and B were determined (*A*) by thermolability, with use of 4MUG, and (*B*) by application of the equation, with use of 4MUGS. *A*, Thermal fractionation with 4MUG: mean ± SD Hex A = 5.53 ± 0.96 for controls and 3.28 ± 0.64 for heterozygotes; 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

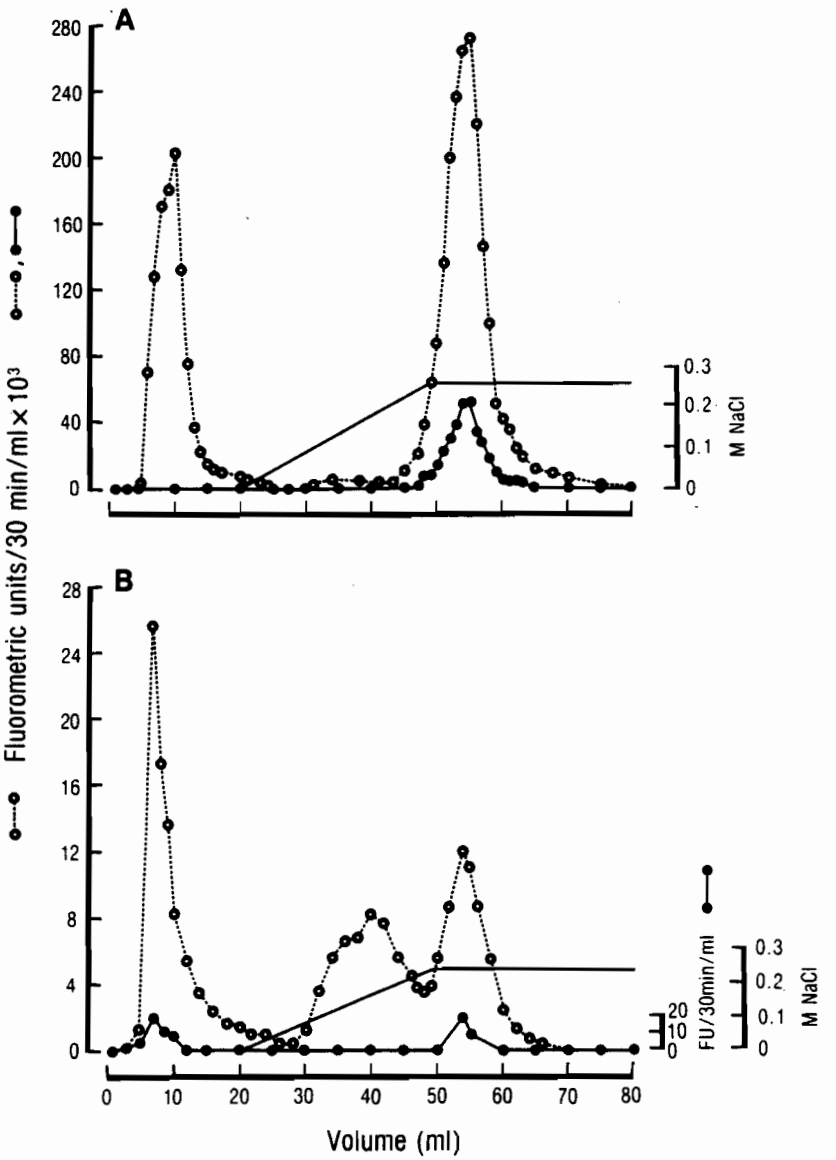

FIG. 2.—DEAE cellulose chromatography of fibroblast hexosaminidases in (*A*) normal control and (*B*) patient 1. Hexosaminidases were resolved by salt-gradient elution according to the procedure of Nakagawa et al. (1977) Fractions were assayed with 4MUG (○---○) and 4MUGS (●—●).

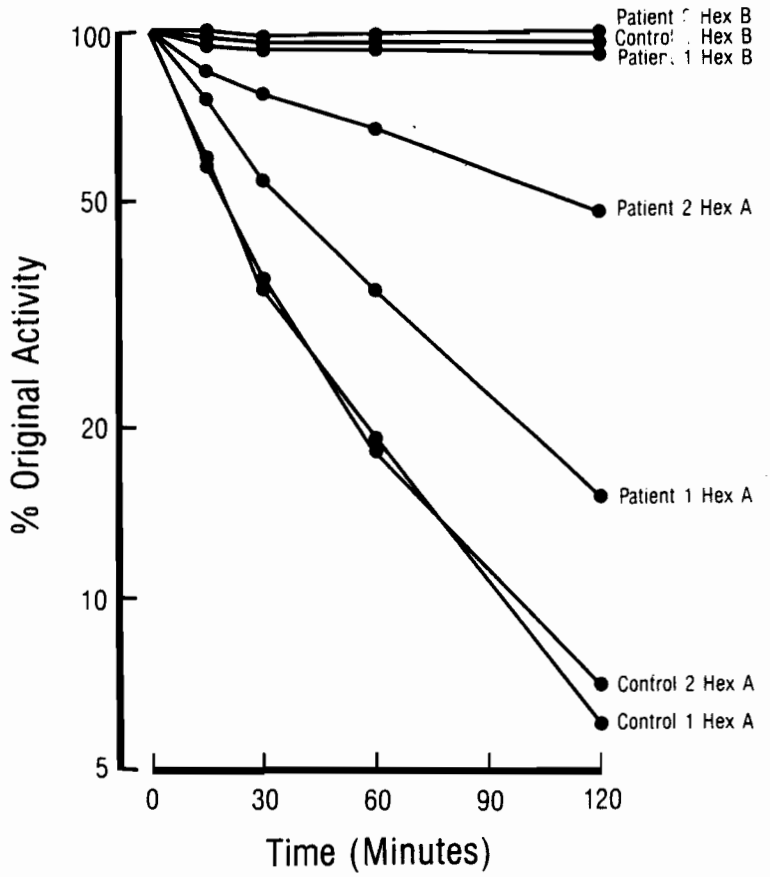

FIG. 3.—Thermal inactivation of control and patient Hex A and Hex B. Hex A was denatured at 42 C and assayed with 4MUGS at 37 C. Hex B was denatured at 47 C and assayed at 37 C with 4MUG.

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,

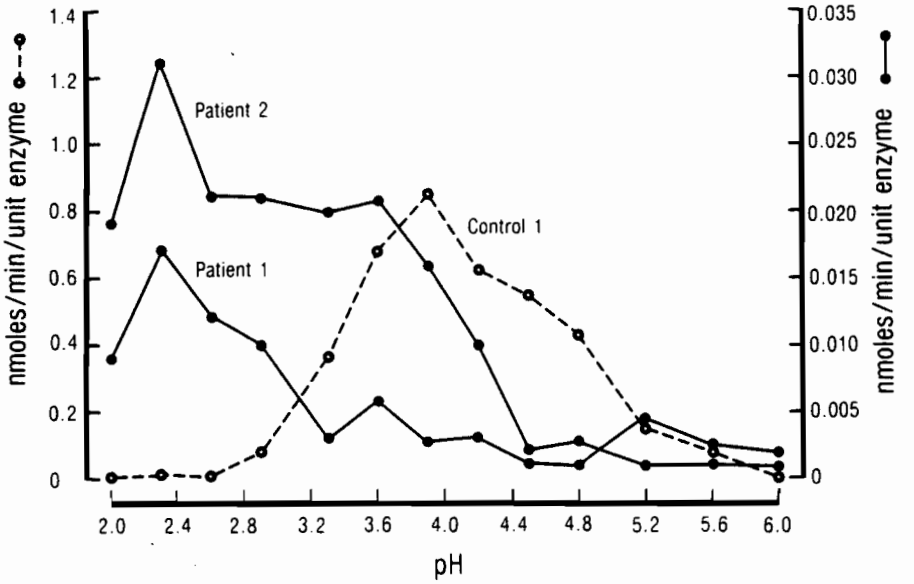

FIG. 4.—pH activity curve of Hex A–catalyzed hydrolysis of 4MUGS. Normal control and patient fibroblast Hex A were assayed in 0.04 M sodium citrate buffer at the indicated pH's with 1.5 mM substrate. One unit of enzyme activity is that amount of enzyme which catalyzes the hydrolysis of 1 nmol 4MUG/min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

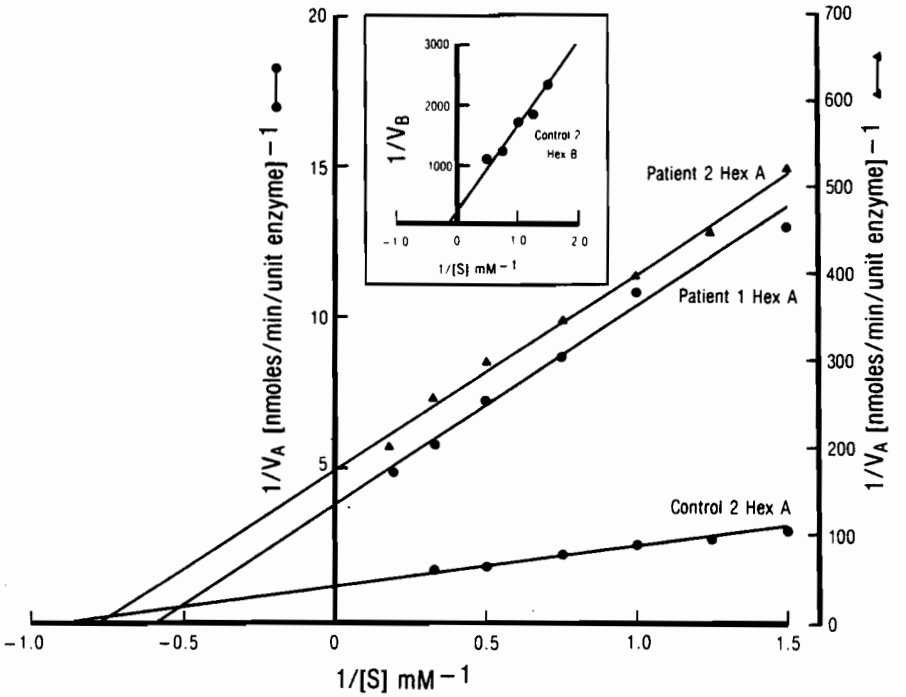

FIG. 5.—Lineweaver-Burke plots of control and patient fibroblast Hex A and control fibroblast Hex B (*insert*) for hydrolysis of 4MUGS. K_m for control Hex A = 1.3 mM; V_{max} = 0.91 nmol/min/unit enzyme. K_m for patient 1 Hex A = 1.7 mM; V_{max} = 0.26 nmol/min/unit enzyme. K_m for patient 2 Hex A =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

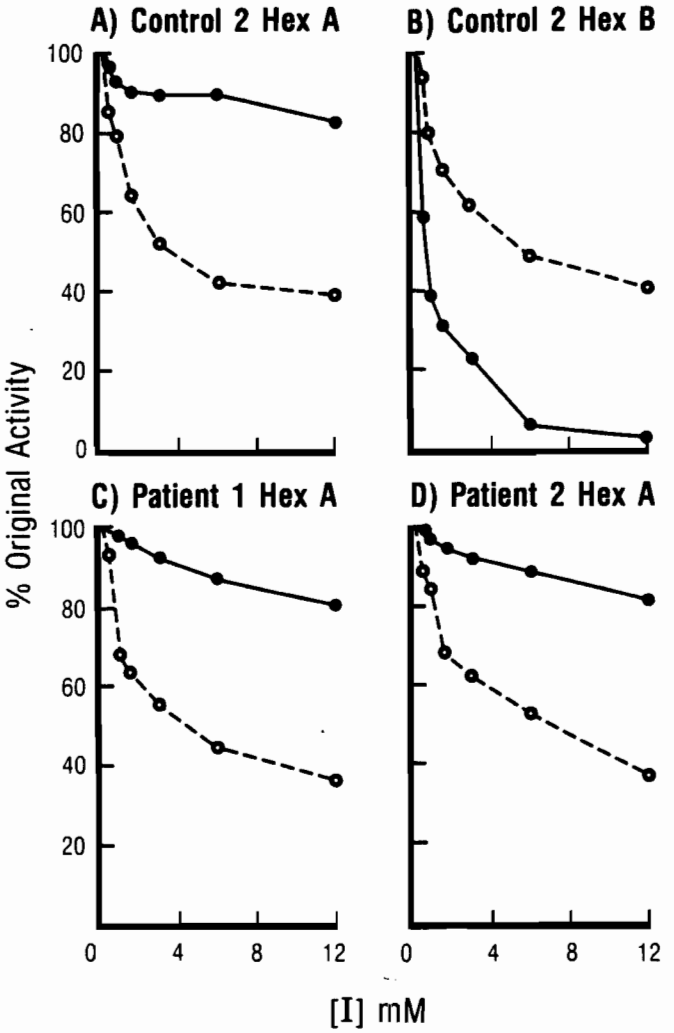

FIG. 6.—Inhibition of 4MUG (○---○) and 4MUGS (●——●) hydrolysis by *N*-acetylglucosamine. Concentration of both substrates was 0.5 mM.

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

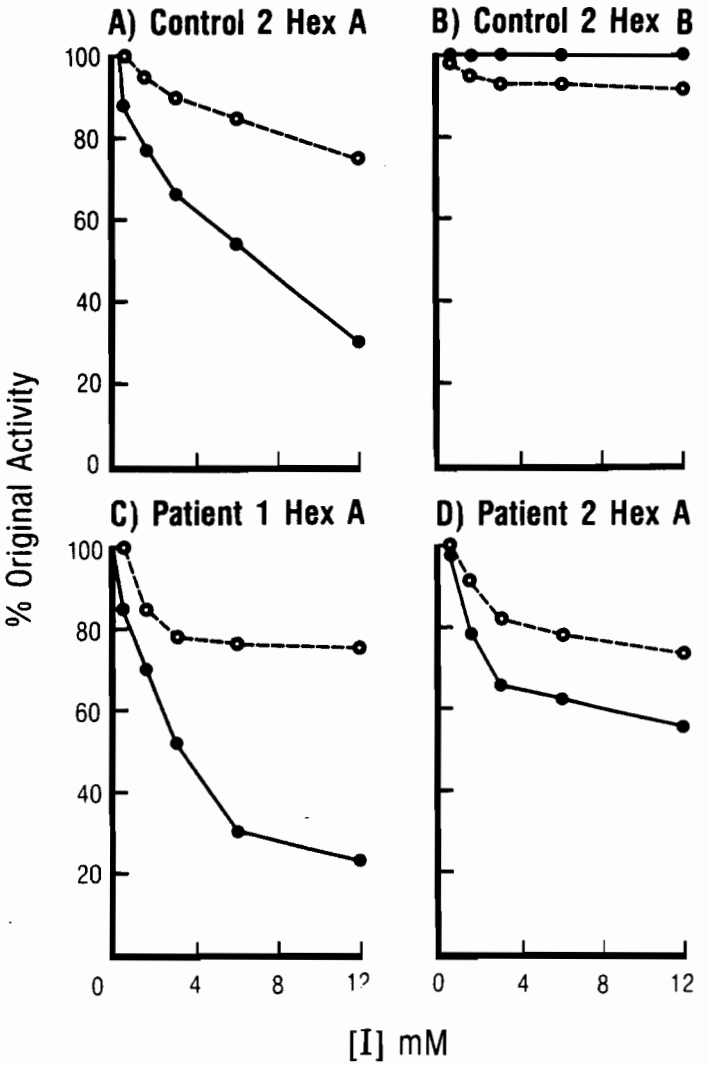

FIG. 7.—Inhibition of 4MUG (○---○) and 4MUGS (●——●) hydrolysis by *N*-acetylglucosamine-6-PO_4. Concentration of both substrates was 0.5 mM.

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.

ACKNOWLEDGMENTS

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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.

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Tay-Sachs Disease: B^1 Variant

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This first child of non-Jewish parents had nystagmus at 4 months of age, bilateral cherry-red macular spots at 7 months of age, and hyperacusis at 8 months of age; the patient has deteriorated progressively following a clinical course typical of Tay-Sachs disease B variant. Total β-*N*-acetylhexosaminidase assayed with 4-methylumbelliferyl-β-glucosamine (4 MU GlcNAc) as substrate was within the normal range in plasma and cultured dermal fibroblasts and 2/3 the normal mean in leukocytes. The hexosaminidase A activity, assayed with the same substrate in plasma and cultured fibroblasts, approximated Tay-Sachs disease heterozygote levels; however, the activity of hexosaminidase A assayed with 4 MU Glc NAc-6-sulfate in the plasma, leukocytes, and cultured fibroblasts was < 8, 2, and 1%, respectively of the control mean. This female infant with the B^1 variant of Tay-Sachs disease demonstrated an earlier onset and more rapidly progressive course than was observed in 4 of the 5 previously reported patients with this Tay-Sachs disease variant.

Gordon BA, Gordon KE, Hinton GG, Cadera W, Feleki V, Bayleran J, Hechtman P. Tay-Sachs disease: B^1 variant. Pediatr Neurol 1988;4:54-7.

Introduction

Four variant forms of infantile G_{M2} gangliosidosis have been described, each with a unique enzymopathy. In two of these variants, the classic Tay-Sachs disease (i.e., the B variant) and Sandhoff (O) variant, the enzyme defect is readily identified by hexosaminidase assays employing the synthetic substrate, 4-methylumbelliferyl-α-*N*-acetylglucosamide (4 MU GlcNAc) or *p*-nitrophenyl-α-*N*-acetylglucosamide (pNpGlcNAc) [1]. For the *in vivo* hydrolysis of ganglioside G_{M2}, a protein activator is required in addition to hexosaminidase A [2- 4]. The activator deficiency in tissues of the AB variant only became apparent when an immunoassay for the activator was performed or an attempt was made to use the patient tissues as a source of activator.

In the fourth variant, B^1 [3,5-9], tissues from the patient have appreciable hexosaminidase A activity when assayed with either of the above synthetic substrates, as well as adequate tissue activator levels, but *in vitro* assays fail to hydrolyze the natural substrate, ganglioside G_{M2}. It has recently been established that the synthetic substrates that are sulfated in the 6 position will identify the enzyme defect in the hexosaminidase A of tissues from B^1 variant patients [5,6,8].

Case Report

The patient was born after an uncomplicated pregnancy, labor, and delivery to a 26-year-old primigravida mother. Birth weight was 3,230 gm. Her parents were nonconsanguineous; the mother was of German/Romanian origin and the father was of English/Irish origin. She was referred at age 7 months for assessment of nystagmus which had been recognized 4 months earlier. Examination revealed bilateral, rapid pendular horizontal nystagmus of fine amplitude with associated head nod. Gray macular areas with cherry-red spots were observed bilaterally.

On examination at 8 months of age her weight was 9.6 kg (90th percentile), height 74 cm (97th percentile), and head circumference 45.5 cm (75th percentile). She was a placid infant. Cardiovascular examination was normal. There was no visceromegaly. Nystagmus had become intermittent, while visual pursuit was inconsistent. Her hands were held in a "clawed" position. She reached for large objects with a palmar grasp. There was moderate head lag. She did not sit with or without support or attempt to roll over. She was mildly hypotonic. Deep tendon reflexes were normoactive and symmetric; plantar responses were extensor. She demonstrated an exaggerated but fatigable startle. Developmental age was at a 4 month level.

When examined at 10 months of age the nystagmus was no longer present. Head lag was less evident and she rolled over. Deep tendon reflexes were increased with clonus at the ankles and hyperextension of the legs in suspension. The startle response had become more pronounced.

On examination at 21 months of age her weight was 14.4 kg, length 94 cm, and head circumference 51 cm, all of which were above the 90th percentile. She was almost unaware of her environment; her optic discs were pale. She had no speech and made few noises. During examination she moved her upper limbs to push the examiner away. She had just begun having numerous seizures with head and eyes turned to the left and secondary generalization. These seizures occurred in paroxysms every 5-10 min for as long as 2-3 hours and to date have been resistant to antiepileptic drug treatment. At 25 months of age the head circumference had increased dramatically to 54 cm. She was totally dependent on others for her care.

Methods

Total β-*N*-acetylhexosaminidase activity was assayed with 4 MU-GlcNAc (Sigma Chemical Co., St. Louis, MO) as substrate [10]. The contribution of isoenzyme A to total hexosaminidase activity was assessed after inactivating this isoenzyme by incubation at 50°C for 4 hours. The same enzyme activity was assayed with 4 MU GlcNAc-6-S [11]. This latter substrate was synthesized by Bayleran et al. [11].

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Table 2. Tay-Sachs disease B^1 variant

Patient Number, Reference	1, [3,5]	2, [6]	3, [7,8]	4, [7,8]	5, [9]	6, (Present Patient)	B/B^1 Compound, [16]
Ethnic background	Puerto Rican	French	Puerto Rican	Puerto Rican	—	English/ Irish/ German/ Romanian	Irish/ American
Sex	M	?	F	M	?	F	F
Symptoms							
Early development	N	N	N	N	Abn	Abn	N
Developmental arrest (months)	24	—	30	—	7	10	18
Dementia	Yes	—	Yes	Yes	Yes	Yes	Yes
Dysphagia	Yes	—	Yes	Yes	—	—	Yes
Seizures	Yes	—	Yes	No	Yes	Yes	Yes
Signs							
Head size	Micro	—	—	—	—	Macro	Macro
Hyperacusis	Yes	—	—	—	Yes	Yes	Yes
Ophthalmologic:							
Nystagmus	Yes	—	Yes	—	—	Yes	Yes
Cherry-red spot	Yes	—	—	—	—	Yes	Yes
Macular deposits	—	—	Yes	Yes	—	—	—
Early hypotonia	Yes	—	—	—	—	Yes	Yes
Late:							
Spasticity	Yes	Yes	Yes	Yes	Yes	Yes	—
Ataxic gait	Yes	—	—	Yes	—	—	Yes
Paraplegia, quadriplegia	Yes	Yes	Yes	—	Yes	Yes	Yes
Muscle atrophy	Yes	—	Yes	Yes	—	—	—
Death	56 mos	—	8 yrs	7 yrs	23 mos	—	—
Biochemistry							
Hex A:							
4 MU/pNP-GlcNAc	N	N	N	—	Hz	Hz	Hz
4 MU/pNP-GlcNA-6-S	↓	↓	—	↓	↓	↓	↓
G_{M2} + activator	↓	—	—	—	↓	—	↓
Brain G_{M2}	↑	—	↑	↑	—	—	—

5 patients, as well as our patient and an additional one identified as B/B^1 compound heterozygote are summarized in Table 2. The initial patient reported by Goldman et al. [3,5] and the third and fourth patients reported by Inui et al. [7,8] were Puerto Rican, while parents of the others were of European origin. The 4 previously reported patients as well as the B/B^1 compound patient did not show conspicuous developmental problems for the first $1^1/_2$ to 2 years of life, although 1 patient was described as developmentally delayed. Initial problems in this group were ataxic gait, dysphagia and/or increasing spasticity, and ophthalmologic findings including nystagmus, cherry-red macular spots, or brownish macular deposits. If early hypotonia were evident, progressively increasing spasticity, hyperreflexia, and paraplegia or quadraplegia would ensue. Muscle atrophy was frequently encountered later. Epilepsy was observed in most patients: they manifested either myoclonus, absence, or clonic seizures as they deteriorated. Our patient, as well as one described by Conzelmann et al. [9], experienced a plateau in development within the first year of life, then had a more precipitous decline than reported for the others (i.e., a course more typical of the B variant of Tay-Sachs disease). In the 4 patients with a later onset of clinical manifestations and slower progression, death occurred at $4^1/_2$ to 8 years of age in 3; however, the patient whose course most closely paralleled our patient, died at 23 months of age. The B/B^1 compound patient reported by Charrow et al. [15] had many similarities to the first four B^1 variant patients reported. Studies of brain gangliosides at autopsy revealed that G_{M2}, which normally constitutes < 5% of the total brain

Table 1. β-*N*-acetylhexosaminidase activity in normal controls

	Plasma			Leukocytes			Fibroblasts		
	Total* (nM/ml/hr)	%A†	Total‡ (nM/ml/hr)	Total* (nM/ml/hr)	%A†	Total‡ (nM/mg/hr)	Total* (nM/mg/hr)	%A†	Total‡ (nM/mg/hr)
Mean	1,157	70	29.6	925	65	180.8	18,616	55	800
(Range)	(637 – 1,947)	(64 – 79)	(24.0 – 36.0)	(766 – 1,091)	(63 – 68)	(127.6 – 270.9)	(7,564 – 33,970)	(45 – 66)	(687 – 891)
Patient	2,406	42	2.4	620	57	3.3	11,551	38	3.7
Father	1,046	44	15.8	1,240	76	64.6	13,670	41	183
Mother	1,294	52	14.2	1,078	65	66.6	12,974	38	105

* Assayed with 4 MU GlcNAc as substrate [10].
† Percent of isoenzyme assayed with 4 MU GlcNAc as substrate in a thermal inactivation assay.
‡ Assayed with 4 MU GlcNAc-6-S as substrate [11].

Results

Activities of a series of acid hydrolases including β-galactosidase, α-*N*-acetylneuraminidase, acid-β-glucosidase, and arylsulfatase A were within normal limits. The total β-*N*-acetylhexosaminidase assayed in the patient's plasma, leukocytes, and cultured fibroblasts with 4 MU GlcNAc as substrate was below normal only in leukocytes (Table 1). The percentage of isoenzyme A in plasma and fibroblasts (i.e., hexosaminidase thermolabile at 50°C after 4 hours) was reduced approximately to levels observed in the B variant Tay-Sachs disease heterozygotes. With the sulfated substrate 4 MU GlcNAc-6-S, levels of hexosaminidase activity in the patient's plasma, leukocytes, and cultured fibroblasts were < 8, 2, and 1% of the control mean, similar to activities observed in tissues from a Tay-Sachs disease B variant homozygote.

With the 4 MU GlcNAc as substrate and plasma, leukocytes, or cultured fibroblasts from the parents: total hexosaminidase activities as well as the proportion of isoenzyme A in leukocytes were within the control range. The percentage of the A isoenzyme in the parents' plasma or fibroblasts was within the range previously observed in Tay-Sachs disease B variant heterozygotes. With the 4 MU GlcNAc-6-S as substrate, the hexosaminidase activities in either parent's plasma, leukocytes, and cultured fibroblasts were < 53, 37, or 22% of the respective control mean.

Discussion

The total β-*N*-acetylhexosaminidase and proportion of isoenzyme A assayed with the 4 MU-GlcNAc as substrate suggested that neither B or O variants of Tay-Sachs disease were responsible for her neurodegenerative features. The marked lack of activity when 4 MU GlcNAc-6-S was utilized as substrate indicated a severe dysfunction of β-*N*-acetylhexosaminidase A isoenzyme similar to that reported by other authors in B^1 variant Tay-Sachs disease [5,6,8,9,11], (*vide infra*).

The β-*N*-acetylglycosaminidic linkages have a widespread distribution in glycolipids, glycoproteins, or glycosaminoglycans. They differ in susceptibility to hexosaminidase isoenzymes. Hexosaminidase A is the only isoenzyme capable of hydrolyzing this linkage in ganglioside G_{M2} (in the presence of the activator protein) [1], in the 6-sulfated oligosaccharide derived from such glycosaminoglycans as keratan sulfate [12], or in the 6-sulfated synthetic substrate 4 MU GlcNAc-6-S [5,6,8,11,12]. Other hexosamine-containing oligomers, including the commonly used synthetic substrate 4 MU GlcNAc, are also susceptible to hydrolysis by hexosaminidase B.

Hexosaminidase A consists of two different polypeptide subunits, α and β, whereas hexosaminidase B consists of only β subunits [1]. From competition experiments performed with the natural substrate ganglioside G_{M2} (in the presence of activator) or the synthetic substrates, 4 MU GlcNAc and 4 MU GlcNAc-6-S, and hexosaminidases A, B, or a homopolymer of α subunits, Kytzia and Sandhoff [13] reported that ganglioside G_{M2} and the 4 MU GlcNAc-6-S interact with the same active site on the α-subunit. However, 4 MU GlcNAc interacts with an active site on the β-subunit. The activator that binds G_{M2} also probably binds to the α-subunit.

The B^1 variant of Tay-Sachs disease like the B variant, is a consequence of a mutation involving the α-gene locus. By fusion experiments the B and B^1 variant mutations have been demonstrated to be allelic [14]. The B^1 variant patients synthesize a hexosaminidase A which has lost its activity against sulfated synthetic substrates (or the G_{M2} ganglioside in the presence of activators) but has retained its activity against the nonsulfated synthetic substrate. It appears that the mutation has inactivated the reactive site on the α-subunit without interfering with other functions of the enzyme.

Five patients with the B^1 variant of Tay-Sachs disease have been reported in the literature. Characteristics of these

oblasts

A[+]	Total[2] (nM/mg/hr)
55	800
45 – 66)	(687 – 891)
38	3.7
41	183
38	105

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Table 2. Tay-Sachs disease B^1 variant

Patient Number, Reference	1, [3,5]	2, [6]	3, [7,8]	4, [7,8]	5, [9]	6, (Present Patient)	B/B^1 Compound, [16]
Ethnic background	Puerto Rican	French	Puerto Rican	Puerto Rican	—	English/ Irish/ German/ Romanian	Irish/ American
Sex	M	?	F	M	?	F	F
Symptoms							
Early development	N	N	N	N	Abn	Abn	N
Developmental arrest (months)	24	—	30	—	7	10	18
Dementia	Yes	—	Yes	Yes	Yes	Yes	Yes
Dysphagia	Yes	—	Yes	Yes	—	—	Yes
Seizures	Yes	—	Yes	No	Yes	Yes	Yes
Signs							
Head size	Micro	—	—	—	—	Macro	Macro
Hyperacusis	Yes	—	—	—	Yes	Yes	Yes
Ophthalmologic:							
Nystagmus	Yes	—	Yes	—	—	Yes	Yes
Cherry-red spot	Yes	—	—	—	—	Yes	Yes
Macular deposits	—	—	Yes	Yes	—	—	—
Early hypotonia	Yes	—	—	—	—	Yes	Yes
Late:							
Spasticity	Yes	Yes	Yes	Yes	Yes	Yes	—
Ataxic gait	Yes	—	—	Yes	—	—	Yes
Paraplegia, quadriplegia	Yes	Yes	Yes	—	Yes	Yes	Yes
Muscle atrophy	Yes	—	Yes	Yes	—	—	—
Death	56 mos	—	8 yrs	7 yrs	23 mos	—	—
Biochemistry							
Hex A:							
4 MU/pNP-GlcNAc	N	N	N	—	Hz	Hz	Hz
4 MU/pNP-GlcNA-6-S	-	-	—	↓	↓	↓	↓
G_{M2} + activator	-	—	—	—	↓	—	-
Brain G_{M2}	*	—	↑	↑	—	—	—

5 patients, as well as our patient and an additional one identified as B/B^1 compound heterozygote are summarized in Table 2. The initial patient reported by Goldman et al. [3,5] and the third and fourth patients reported by Inui et al. [7,8] were Puerto Rican, while parents of the others were of European origin. The 4 previously reported patients as well as the B/B^1 compound patient did not show conspicuous developmental problems for the first 1½ to 2 years of life, although 1 patient was described as developmentally delayed. Initial problems in this group were ataxic gait, dysphagia and/or increasing spasticity, and ophthalmologic findings including nystagmus, cherry-red macular spots, or brownish macular deposits. If early hypotonia were evident, progressively increasing spasticity, hyperreflexia, and paraplegia or quadraplegia would ensue. Muscle atrophy was frequently encountered later. Epilepsy was observed in most patients; they manifested either myoclonus, absence, or clonic seizures as they deteriorated. Our patient, as well as one described by Conzelmann et al. [9], experienced a plateau in development within the first year of life, then had a more precipitous decline than reported for the others (i.e., a course more typical of the B variant of Tay-Sachs disease). In the 4 patients with a later onset of clinical manifestations and slower progression, death occurred at 4 ½ to 8 years of age in 3; however, the patient whose course most closely paralleled our patient, died at 23 months of age. The B/B^1 compound patient reported by Charrow et al. [15] had many similarities to the first four B^1 variant patients reported. Studies of brain gangliosides at autopsy revealed that G_{M2}, which normally constitutes < 5% of the total brain

ganglioside in these patients, constituted more than 55% of the total. The activity of total hexosaminidase or isoenzyme A assayed with 4 MU or pNP-GlcNAc was either normal or at heterozygote levels but was severely depressed when assayed with the sulfated synthetic substrate.

Our patient, diagnosed at 8 months of age, appears to have a clinical course similar to that observed in the classic B patients rather than the later onset and somewhat more protracted course reported in the previously described B^1 variant patients.

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The mutation mechanism causing juvenile-onset Tay-Sachs disease among Lebanese

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Expression of the hexosaminidase isozymes was evaluated in fibroblast cell lines obtained from two sibs of Lebanese-Christian origin who presented with juvenile-onset Tay-Sachs disease. In the normal control fibroblasts the *a* subunit of hexosaminidase A (hex A) is synthesized as a 67 KD precursor which is cleaved in lysosomes to a mature 54 KD peptide. The patients' fibroblasts were capable of synthesizing the 67 KD precursor but failed to convert it to the mature subunit. The *a* subunit precursor synthesized by patients' cells could not be phosphorylated, nor was the patients' *a* subunit precursor secreted into the medium in response to NH_4Cl, which caused accumulation of both *a* and β subunit precursor in the medium of the normal control fibroblasts. The measurement of residual enzyme activity in the fibroblasts of patients which best correlated with the onset of the illness was the ion exchange chromatographic separation of Hex A-associated hydrolysis of the synthetic substrate 4-methylumbelliferyl N-acetyl-β-D-glucosamine-6-sulfate (4MUGS). The patients had 0.32% and 0.36% of Hex A-associated 4MUGS cleaving activity compared to normal control fibroblasts as compared to $<0.016\%$ for infantile Tay-Sachs disease fibroblasts. The residual Hex A activity in patients' cells had a pH optimum identical with normal enzyme (pH 3.9–4.0), a reduced specific activity for 4MUGS (relative to hydrolysis of unsulfated synthetic substrate), and a greatly enhanced thermal stability. The occurrence of this form of Tay-Sachs disease in Lebanon, the fact that the condition has been described in three unrelated Lebanese immigrant families in Canada, together with the fact that the grandparents of the unrelated probands come from villages in both th northern and southern regions of Lebanon, leads us to speculate that a gene causing juvenile-onselt Tay-Sachs disease may not be infrequent in Lebanon.

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Mutations at three gene loci result in the occurrence of variants of the neurodegenerative lipid storage diseases known as the G_{M2} gangliosidoses. Biochemically, the common feature of all forms of G_{M2} gangliosidoses is the accumulation in neurones of the acidic glycolipid G_{M2} ganglioside. The genetic defect occurs in the locus encoding the *a* subunit of the enzyme hexosaminidase A (Hex A) in Tay-Sachs disease (TSD) or in the locus encoding the β subunit common to both Hex A and Hex B in Sandhoff disease. A rare form of G_{M2} gangliosidosis has also been described in which the mu-

tation occurs at a locus encoding an activator protein required for the action of Hex A on glycolipid substrates.

The existence of a variety of mutant alleles at the α subunit locus has been reported. Their biochemical characterization contributes much information to the understanding of genetic control of protein processing, maturation and targeting. In some cases the same clinical phenotype is compatible with different mutant alleles. Thus, the classical or infantile onset form of TSD occurs in the Ashkenzai Jewish population as a result of at least two mutant alleles, one of which is an exon 12 splicing defect (Arpaia et al. 1988, Myerowitz 1988). An identical phenotype in French Canadians occurs as a result of at least two mutant alleles (Bayleran et al. 1988), one of which is a 7 KB deletion which includes exon 1 (Myerowitz & Hogikyan 1986). In contrast to these mutations which result in deficiency of Hex A, other allelic mutations, known as B1 mutations, produce normal amounts of catalytically defective enzyme (reviewed in Gordon et al. 1988).

Mutations occurring at the α locus are also associated with later-onset forms of TSD (reviewed in Johnson et al. 1980) or with variants of G_{M2} gangliosidosis with no cortical involvement. Examples of such mutations include juvenile- (Brett et al. 1973, Meek et al. 1984, Parnes et al. 1985) and adult-onset TSD as well as chronic forms of the disease which present without intellectual impairment (Rapin et al. 1976, Willner et al. 1981, Argov & Navon 1984). Many of these variant forms of G_{M2} gangliosidoses are likely to be the result of compound heterozygosity at the α locus. The existence of two different mutant α locus alleles in the same cell presents difficulties in evaluating the contributions of each mutant allele to the cellular or enzymatic phenotype.

We present here the enzymatic analysis of a juvenile-onset form of Tay-Sachs disease which has been identified in Lebanese immigrant families in Canada. The clinical defect was initially described by Andermann et al. (1977).

The relationship of this mutant allele to forms of Tay-Sachs disease occurring in Lebanon is difficult to assess. A survey of 15 cases of G_{M2} gangliosidoses in Lebanon revealed only two likely cases of infantile-onset TSD with both Muslim and Christian ethnic background (Der Kaloustian et al. 1981). The mutation identified in Canada occurs in three Christian Lebanese families and is clearly a later-onset form of the disease. No common ancestor was found linking any of these families. Grandparents came from villages in both northern and southern Lebanon, but the majority of the gradparents originated from adjacent small villages in the vicinity of Mt. Hermon in southern Lebanon and in what is now the Golan Heights in Israel (Andermann et al. 1977). These preliminary findings suggest a relatively high frequency of this mutant allele which is consistent with the occurrence of a number of cases of juvenile-onset TSD in the Maronite community of Lebanon itselg (V. M. Der Kaloustian, personal communication).

Material and Methods

Patients

The two patients whose cultured fibroblasts are the subject of this investigation are sibs of family N reported by Andermann et al. (1977). The parents are first cousins. The probands had normal developmental milestones until the age of 18 months to 2 years, followed by progressive deterioration of gait and mental functioning with gradual development of ataxia and spasticity. Unlike infantile TSD patients, these patients had no seizures. However, the EEGs showed active generalized epileptic dis-

charges with severe generalized disturbance of cerebral activity, suggesting diffuse cortical and subcortical grey matter encephalopathy. Preliminary diagnosis of juvenile Tay Sachs disease was made by brain biopsy on the older sibling and subsequently confirmed by serum hexosaminidase assays performed on both patients and the parents.

By the age of 5 years, the older boy had no comprehensible speech and no manifestation of interest in his surroundings. He had a markedly ataxic gait, with spasticity in all four limbs, particularly the lower extremities. Grasping and sucking reflexes and a very active reflex were present. The younger sibling, who talked well by the age of 2 years, had lost the ability to speak by 3½ years. He was also markedly ataxic and had an unusual gait with abduction of the arms. Both boys had generalized hyperreflexia. The patients died at ages 8 and 7, respectively.

Cultured fibroblasts

Conditions for culturing human skin fibroblasts were described previously (Bayleran et al. 1984). Fibroblasts obtained from the older of the two patients are referred to as WG 306 and the younger as WG 312.

Biosynthetic Labelling Studies

Biosynthetic labelling of proteins with $^{3[H]}$ Leucine or $^{32}PO_4$, immunoprecipitation, electrophoresis and radioautography of protein subunits were performed according to Proia et al. (1984). Goat antisera directed against purified Hex A, Hex B and *a* chain were a generous gift of Dr. Elizabeth Neufeld. The following modifications were used: fibroblast lysates were prepared in a buffer containing 1.4 M NaCl and prior to the preclearing step, lysates were centrifuged at 145 000 × g for 45 min at 4°C.

Assay of Hexosaminidases

Hexosaminidases were assayed fluorimetrically using the unsulfated synthetic substrate 4 methylumbelliferyl N-acetyl-β-D-glucosamine (4MUG) (St. Louis, Mo, USA) and using the sulfated substrate 4 methylumbelliferyl N-acetyl-β-D-glucosamine-6-SO_4 (4MUGS). The sulfated substrate was purchased from the Sick Children's Hospital Development Corp. (Toronto, Ontario, Canada) and was purified by chromatographic procedures reported earlier (Bayleran et al. 1984). Fibroblast Hex A and B were separated by ion exchange chromatography according to Nakagawa et al. (1977).

Protein

Protein was determined by the method of Lowry et al. (1951) using crystalline BSA as standard.

Results

Hexosaminidase Activity in Patients' Fibroblasts

Hexosaminidases A and B were measured in normal control and mutant fibroblasts by four procedures: a) thermal fractionation of fibroblast lysate hexosaminidase activity using 4MUG; b) measurement of enzyme activity toward both 4MUG and 4MUGS with application of an empirical formula (Bayleran et al. 1984); c) hydrolysis of 4MUGS by fibroblast lysates; and d) ion exchange chromatographic separation of isozymes.

The first two procedures gave values for Hex A activity which were greatly reduced compared to values for Hex A in normal control fibroblasts, but were significantly greater than assay values obtained using fibroblasts obtained from patients with classical infantile Tay-Sachs disease (of Ashkenazic Jewish origin). The results are shown in Table 1.

A more definitive measurement of Hex A activity which correlated with the differences in age of onset is obtained by chromatographic separation of enzymatic activity

Table 1

Hexosaminidase A determinations in control and mutant fibroblast cell lines

Cell line	Thermolabile hexosaminidase	4MUG and 4MUGS hydrolysis by formula	nmol/h/mg protein	
			4MUGS hydrolysis	Hex A associated-4MUGS hydrolysis
Normal control	57	76	1094	1094
WG 306 JTSD	14	0.92	7.14	3.5
WG 312 JTSD	13	2.15	10.1	3.9
WG 93 ITSD	5.2	0.29	4.2	<0.175

catalyzing hydrolysis of the 4MUGS substrate. The results of these separations are shown in Fig. 1. Fig. 1C and D show separation of hexosaminidase isozyme in lysates of fibroblasts WG 306 and WG 312 respectively. When hydrolysis of 4MUGS is monitored in each column fraction, respectively 49% and 39% of the recovered enzyme activity cochromatographs with Hex A. The remainder of 4MUGS hydrolyzing activity cochromatographs with Hex B. By contrast, in infantile TSD fibroblasts (Fig. B), all detectable 4MUGS hydrolyzing activity cochromatographs with Hex B. In normal control fibroblasts a numerically larger scale is required for the representation of 4MUGS hydrolysis by chromatographic column fractions. Therefore the small Hex B-associated hydrolysis of 4MUGS, though present, does not appear on this chromatographic profile. Table 1 also records Hex A associated 4MUGS hydrolysis for the juvenile and infantile onset TSD fibroblasts.

Fig. 1C and D also show that chromatographic recovery of Hex A-associated 4MUG hydrolysis is greatly reduced in the juvenile TSD cells compared to the normal control cells. This finding is similar to the results obtained for infantile TSD cells but strikingly different from results obtained with fibroblasts from infantile patients who are α locus compound heterozygotes for the B1 allele (Bayleran et al. 1987). The significance of this difference is described in the Discussion.

α Chain Maturation in Juvenile TSD Fibroblasts

Fig. 2 shows the biosynthetic labelling and immunoprecipitation of hexosaminidase subunits. Fig. 2 (top) shows the expected pattern of α subunit labelling in normal control cells and Ashkenazi Jewish infantile TSD cells following a 2-h pulse with ^{3}[H]-leucine and a 1-h chase with unlabelled leucine. A 67 KD α subunit precursor is seen in normal cells (lane 1) and is absent in classical TSD cells (lane 6). In the juvenile TSD cells WG 306 α subunit precursor of normal size is also synthesized (lane 3). After a chase period of 17 h, no α subunit precursor can be immunoprecipitated with antiserum directed against free α subunit from either control (lane 2) or juvenile TSD cells (lane 4).

Fig. 2 (bottom) shows the results of immunoprecipitation, electrophoresis and fluorography using antiserum directed against Hex B. This procedure detects both β subunits as well as α subunits associated with them but not free α subunits. In normal control cells the β subunit precursor is detected after pulse-chase (1 hour) (lane 2), whereas the overnight chase results in the appearance of the mature β subunits (28 and 29 KD) as well as the mature β-bound α

subunits (54 KD) (lane 3). In both classical infantile TSD cells (WG 93) and WG 306 (lane 5) only the mature β subunits are precipitated. WG 312, the cell line obtained from the affected sib of WG 306 has a labelling pattern identical to WG 306 (data not shown).

It would appear that an α subunit precursor is synthesized in the cells of the Lebanese JTSD patient but the precursor fails to mature and is degraded.

Fig. 3 shows the biosynthetic labelling of α and β subunits with $^{32}PO_4$. After the pulse and 1-h chase period, labelled α and β subunit precursor are detected in the normal control cells (lanes 2 and 6), whereas in the JTSD cells only the β subunit precursor is labelled. These results support the hypothesis that α subunit precursor is synthesized normally in the JTSD cells but not phosphorylated. Since the site of phosphorylation of enzymes destined for the lysosome is the Golgi apparatus, the defect in the JTSD fibroblasts may be due to failure of the mutant α precursor to reach the Golgi or to failure of the subunit to be recognized as an acceptor substrate for the Golgi apparatus enzyme UDP-N-acetylglucosaminyl phosphate transferase.

In Fig. 4 nexosaminidase subunits were labelled with $^{3[H]}$-leucine and following a 17-h chase period were immunoprecipitated from the culture medium with antiserum directed against Hex A. This antiserum detects free α subunits as well as α and β subunits bound in Hex A or Hex B. The

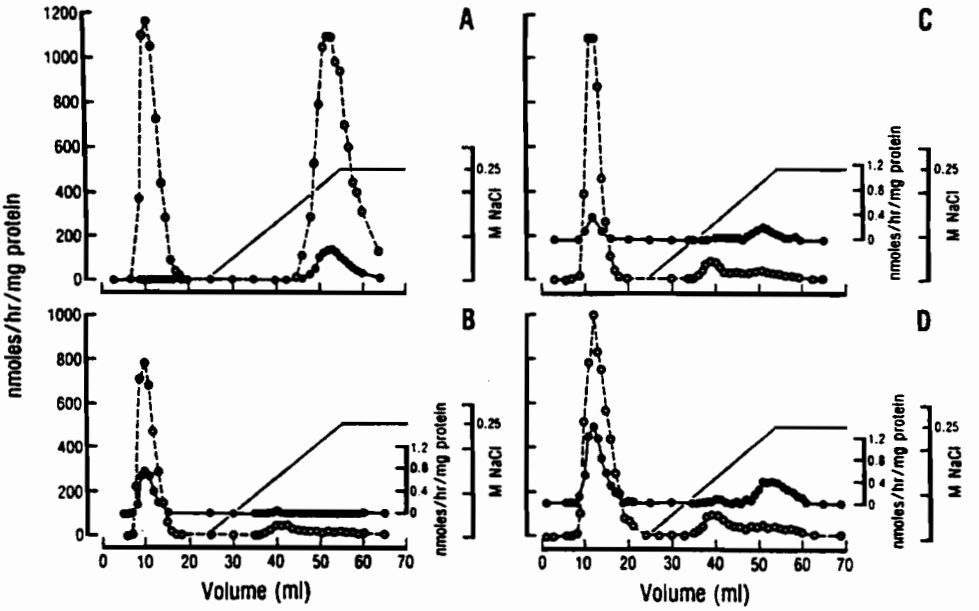

Fig. 1. Separation of hexosaminidase isozymes from normal and Tay-Sachs disease fibroblast cell lines. Fibroblasts were disrupted by sonication in water followed by centrifugation at 10000 × g at 4°. Soluble extracts were chromatographed on DEAE Cellulose according to Nakagawa et al. (1977). Column fractions were assayed for hexosaminidase activity with 4MUG (open circles, dashed lines). Enzyme units for 4MUG hydrolysis are represented on left scale. Column fractions were also assayed for 4MUGS (closed circles, solid lines). Enzyme units for 4MUGS hydrolysis are represented on the left scale in Fig. 1A and on the right hand scale in Fig. 1B, C and D. Enzyme units recovered are normalized to mg soluble fibroblast protein applied. A) MCH24, normal control cell line, B) WG 93 Ashkenazi-Jewish infantile Tay-Sachs disease cell line. C) WG 306 Lebanese-Juvenile Tay-Sachs disease cell line; D) WG 312 Lebanese-Juvenile Tay-Sachs disease cell line (sib of WG 306).

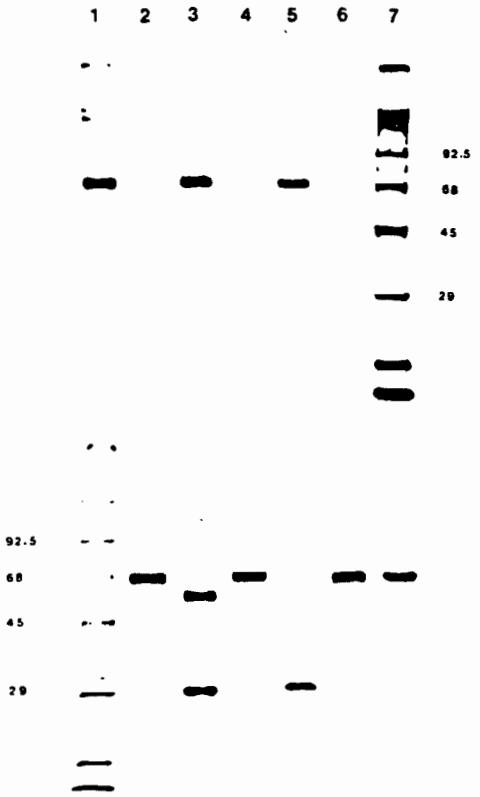

Fig. 2. Immunoprecipitation and electrophoresis of hexosaminidase subunits biosynthetically labelled with ^{3}[H] leucine from cell lysates. Top. Radiolabelled antigens precipitated with anti α-antiserum. Pulse period for all lanes is 2 h. *Lane 1* normal control fibroblasts, chase period 1 h. *Lane 2* normal control fibroblasts, chase period 17 h. *Lane 3* Juvenile Tay-Sachs disease fibroblasts (WG 306). Chase period 1 h. *Lane 4* Juvenile Tay-Sachs disease fibroblasts (WG 306). Chase period 17 h. *Lane 5* Normal control fibroblasts. Chase period 1 h. Chase period 17 h. *Lane 6* Infantile Tay-Sachs disease fibroblasts (WG 93). Chase period 1 h. *Lane 7.* Molecular weight standards. Bottom. Radiolabelled antigens precipitated with antiHex B antiserum. Pulse period for all lanes is 1 h. *Lane 1.* Molecular weight standards. *Lane 2.* Normal control fibroblasts. Chase period 1 h. *Lane 3.* Normal control fibroblasts. Chase period 17 h. *Lane 4.* Juvenile Tay-Sachs disease fibroblast (WG 306). Chase period 1 h. *Lane 5.* Juvenile Tay-Sachs disease fibroblasts (WG 306). Chase period 17 h. *Lane 6.* Normal control fibroblasts. Chase period 1 h. *Lane 7.* Infantile Tay-Sachs disease fibroblasts. Chase period 1 h. Numbers at side are molecular weights in kilodaltons.

medium in which normal cells are grown shows the presence of both α and β Hex subunits secreted in response to the presence of NH_4Cl in the medium (lane 1). Under these conditions the mature α and β subunits are not secreted into the medium. In the JTSD cells only β subunit secretion occurs in the presence of NH_4Cl (lane 2).

Ammonium chloride is understood to act by preventing dissociation of the mannose-6-phosphate receptor from lysosomal enzymes within the lumen of the lysosome or a prelysosomal compartment. Since the MRP functions in the transport of lysosomal enzymes from the Golgi apparatus and since the MRP is present in limiting amounts, a block in recycling of the MRP results in bulk movement of phosphorylated lysosomal enzymes outside the cell. Alternatively, lysosomal enzyme which cannot be phosphorylated (for example, in cells with Mucolipidoses II or III) will be secreted into the medium in the absence of weak bases. The fact that α chains are synthesized but not secreted by the JTSD fibroblasts we interpret as evidence that the α subunit precursor is degraded before it reaches the Golgi apparatus, and that this event most likely occurs in the rough endoplasmic reticulum of the cell.

Characteristics of Residual Hex A Activity in Mutant Fibroblasts

Hexosaminidase A activity obtained by ion exchange chromatography of lysates of normal control and JTSD fibroblasts were compared with respect to thermolability, pH optima and substrate specificity. The results of these comparisons are presented in Table 2. Hexosaminidase A obtained from chromatography of JTSD patient cells is strikingly more stable to heating at 47°C than is normal fibroblast Hex A. In fact, its thermostability is comparable to that of Hex B. This surprising finding makes it difficult to explain why any thermolabile

hexosaminidase activity should be detectable in the unfractionated fibroblast lysate. Hexosaminidase A, which was prepared chromatographically from secretions induced by culturing normal control fibroblasts for 17 h in the presence of 10 mM NH_4Cl-containing medium, was thermostable (Table 2), in contrast to cellular Hex A from normal control cells. The extracellular form of Hex A is known to consist of unprocessed α and β subunits (Hasilik et al. 1982).

Hex A prepared from JTSD fibroblasts hydrolyzes 4MUGS at a rate relative to 4MUG hydrolysis that is intermediate between normal fibroblast Hex A and Hex B. The pH optima of Hex A from both normal and JTSD fibroblasts for 4MUGS hydrolysis is 3.9–4.0.

Discussion

The detailed study of the effect of different α and β locus mutations on the enzymatic phenotype of cultured cells obtained from patients with variant forms of G_{M2} gangliosidoses serves two important purposes: (1) it may provide new insights into basic eucaryotic cellular mechanisms of expressing genetic information and targeting gene products; and (2) it may offer explanations in

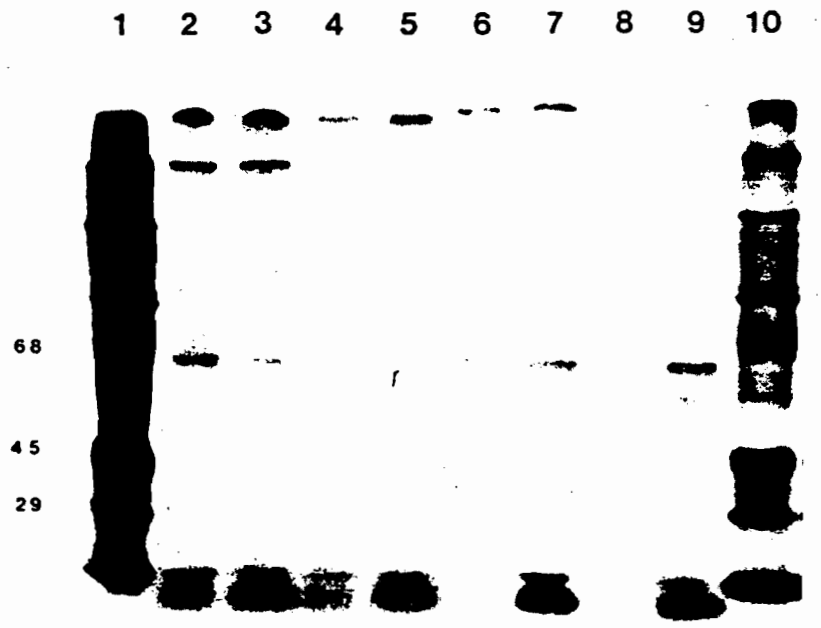

Fig. 3. Immunoprecipitation and electrophoresis of hexosaminidase subunits biosynthetically labelled with $^{32}PO_4$ from cell lysates. *Lanes 1 and 10.* Molecular weight standards. *Lane 2.* Normal control 1 h chase, precipitation with anti-α-antiserum. *Lane 3.* Normal control, 17 h chase precipitation with anti-α-serum. *Lane 4.* Juvenile Tay-Sachs disease fibroblasts, 1 h chase, precipitation with anti-α-antiserum. *Lane 5.* Juvenile Tay-Sachs disease fibroblasts, 17 h chase, precipitation with anti-α-antiserum. *Lane 6.* Normal control fibroblasts, 1 h chase, precipitated with anti-Hex B antiserum. *Lane 7.* Normal control fibroblasts, 17 h chase period, precipitation with anti-hex B antiserum. *Lane 8.* Juvenile Tay-Sachs disease fibroblasts, 1 h chase period, precipitation with anti-Hex B antiserum. *Lane 9.* Juvenile Tay-Sachs disease fibroblasts, 17 h chase period, precipitation with anti-Hex B antiserum. Numbers at left are molecular weights in kilodaltons.

biochemical or enzymatic terms for phenotypic (i.e. clinical) differences between patients carrying different mutant alleles. In this discussion we compare findings in the Lebanese JTSD patients with observations made on previous patients investigated both at our centre and elsewhere.

We believe that the mutation observed in this family (and by inference, in the other Lebanese-Canadian families reported by Andermann et al. (1977)) is one which affects primarily the quantity of mature Hex A delivered to the lysosome by degradation of the α subunit precursor in an early compartment of the protein targeting pathway. This class of α locus mutation is quite distinct from the B1 mutations in which a normal amount of Hex A is produced but the α subunit, although capable of associating with the β subunit, is catalytically defective and unable to degrade G_{M2} ganglioside (Kytzia & Sandhoff 1985) and 4MUGS (Bayleran et al. 1984). A third class of α locus mutations suggested to account for a number of cases of so-called "chronic" or adult-onset forms of TSD was also documented by d'Azzo et al. (1984). In cells with these mutant alleles the defective α chains are unable to associate with β subunits. The α subunit produced by these "association defective" patients is, however, phosphorylated and does accumulate in the culture medium in response to NH_4Cl. Thus, the block in maturation of Hex A in these patients with milder clinical abnormalities does occur at a later stage in the targeting pathway than is the case for the patients described here.

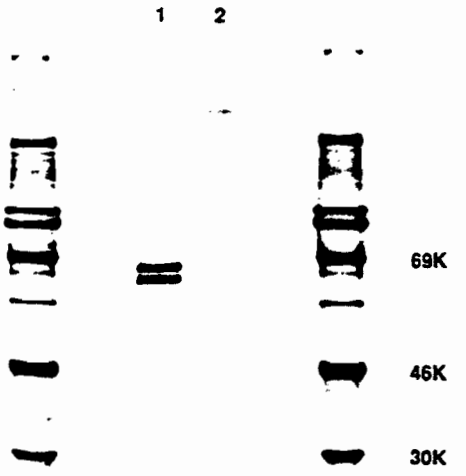

Fig. 4. Immunoprecipitation and electrophoresis of culture medium. Hexosaminidases biosynthetically labelled with ^{35}S-leucine. Chase period 17 h in the presence of 10 mM NH_4Cl. Antigens precipitated with anti Hex A antiserum. *Lane 1* normal control cells. *Lane 2.* Juvenile Tay-Sachs Disease fibroblasts.

The mutation mechanism observed in the Lebanese JTSD patients is evidently not an uncommon mutation mechanism at a number of human gene loci. Sequestration of mutant gene product within the endoplasmic reticulum accounts for a frequent form of α_1 antitrypsin deficiency (Hercz & Harpaz 1980).

An ITSD patient of Italian origin reported by Zoakeem et al. (1987) also had a mutant Hex A α subunit which also undergoes degradation in an early processing compartment. Although it is tempting to conclude that the same type of mutation mechanism is compatible with both ITSD and JTSD phenotypes, there are also some striking differences in enzymatic phenotype between the Italian and the Lebanese patients which are relevant to their clinical differences. Thus, the Italian patient produces an α chain truncated by 3–4 KD at the C-terminal end (Zoakeem et al. 1987). The cells of this patient produce no Hex A-associated 4MUGS cleaving activity (unpublished) in common with cells obtained from null mutant patients of French Canad-

Table 2

Properties of fibroblast and medium hexosaminidases from control and mutant fibroblast cell lines

Enzyme source[4]	4MUGS/4MUG[1]	$T_{1/2}$[2]	pH optimum[3]
Normal control Hex A	0.143	35 min	3.9–4.0
Normal control Hex B	0.001	810 min	–
WG 306 Hex A	0.011	810 min	3.9–4.0
WG 312 Hex A	0.0099	810 min	3.9–4.0
NH_4Cl-culture medium Hex A[5]	0.129	240 min	–

[1] Ratio of rate of hydrolysis of substrates. Concentration = 1 mM.
[2] Time required for inactivation of 50% of enzyme at 47°C.
[3] 0.08 Citric Acid-NaOH.
[4] Isozymes separated by the procedure of Nakagawa et al. (1977).
[5] Culture medium plus NH_4Cl (10 mM) exposed to confluent normal cells 24 h.

ian and Ashkenazi Jewish origins. By contrast, the cells of the Lebanese TSD patients do produce some Hex A-associated 4MUGS cleaving activity (0.32% and 0.36% relative to normal control). Nevertheless, the possibility that the many mutations which prevent proteins from exiting the RER all alter a common recognition signal remains an intriguing hypothesis.

A number of methods for measurement of hexosaminidase A activity in patients' cells and fluids are in use. Many of these methods will discriminate between the general categories of "affected" and "unaffected" (e.g. Raghaven et al. 1985) but not between the variant phenotypes of G_{M2} gangliosidosis which differ in severity, age of onset, cortical involvement and speed of progression. Thus, the common sense suggestion that the clinical severity of an enzyme deficiency disease will be inversely related to the amount of residual enzymatic activity in the patient is frequently negated in practice by limitations on the accuracy or sensitivity of available methods of enzymatic assays.

A survey of 25 cases of juvenile TSD revealed a range of leucocyte % Hex A (measured as thermolabile hexosaminidase activity toward 4MUG) among patients that varied between 0–35 (Johnson et al. 1980). This range is partly accounted for by the fact that many different alleles are associated with the JTSD phenotype (Greenberg & Kaback 1982). However, it also reflects differences between laboratories in the application of the thermal fractionation procedure for differential determination of hexosaminidases.

Conzelmann et al. (1983) have demonstrated that measurement of fibroblast Hex A catalyzed hydrolysis of G_{M2} ganglioside in the presence of human liver Hex A activator protein detects residual enzyme activities that correlate well with the neurological severity of the variant phenotype. It would be desirable to have an assay for residual Hex A activity which correlated as well with phenotypic severity but which is accessible to laboratories that are not equipped for the isolation and purification of both G_{M2} ganglioside and activator protein. We propose that the combination of ion exchange chromatographic separation of Hex A with the use of the synthetic fluorescent substrate 4MUGS may serve this purpose.

It is instructive to compare the enzymatic phenotype of the Lebanese JTSD patient with those of patients carrying the B1 mutations. Two B1 compound heterozygous patients investigated by us had the infantile-onset form of TSD. Other patients classified

as B1 have juvenile onset forms of the disease (Charrow et al. 1985). It is likely that the phenotypic differences between JTSD and ITSD B1 patients may be due to differences in the catalytic site of the enzyme, which may reflect the ability of the enzyme to function under physiological conditions.

Thus the two unrelated B1 ITSD patients we recently investigated have half of the normal amount of Hex A, but this enzyme has a pH optima of 2.4 when 4MUGS is used as the substrate. This species of enzyme is likely to be completely nonfunctional in lysosomes (Bayleran et al. 1987). The pH optima of fibroblast Hex A obtained from the JTSD patients who are the subjects of the present study is pH 3.9–4.0, which is identical to that of Hex A from normal control cells. On the basis of this property, we expect the small amount of Hex A that survives the targeting pathway in JTSD cells to be functional in lysosomes.

A property of Hex A is strikingly different in the normal control and JTSD genotype is the greater thermostability of the mutant Hex A. The physiological significance of this property is not known. It is possible that the greater thermostability of the mutant Hex A is a direct consequence of the effect of the amino acid substitution on protein folding, as has been suggested by Ohno & Suzuki (1988) in the case of the B1 mutation.

Alternatively, we suggest that the greater thermostability of the mutant Hex A may be a consequence of failure of the α subunit precursor to undergo the final proteolytic cleavage step that occurs in lysosomes (Frisch & Neufeld 1981). This suggestion is made because normal fibroblast Hex A, obtained by chromatographic separation of NH_4Cl-induced secretions, is also a considerably more thermostable enzyme than normal cellular Hex A (Table 2). This enzyme, which is secreted from the cell without entering the lysosome, is an unprocessed form of the Hex A with a 67 KD α subunit. The thermolability of the normal cellular enzyme may therefore be acquired following proteolytic cleavage of the precursor of the α subunit in the lysosomes.

Few, in any, cases of juvenile TSD have been the subject of detailed biochemical analysis. It remains to be seen therefore, whether a biochemical defect similar to the type demonstrated here underlies the phenotype of other cases of juvenile Tay-Sachs disease.

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