The Metabolism of Tay-Sachs Ganglioside: Catabolic Studies with Lysosomal Enzymes from Normal and Tay-Sachs Brain Tissue

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ABSTRACT The catabolism of Tay-Sachs ganglioside, *N*-acetylgalactosaminyl-(*N*-acetylneuraminosyl)-galactosylglucosylceramide, has been studied in lysosomal preparations from normal human brain and brain obtained at biopsy from Tay-Sachs patients. Utilizing Tay-Sachs ganglioside labeled with ^{14}C in the *N*-acetylgalactosaminyl portion or ^{3}H in the *N*-acetylneuraminosyl portion, the catabolism of Tay-Sachs ganglioside may be initiated by either the removal of the molecule of *N*-acetylgalactosamine or *N*-acetylneuraminic acid. The activity of the *N*-acetylgalactosamine-cleaving enzyme (hexosaminidase) is drastically diminished in such preparations from Tay-Sachs brain whereas the activity of the *N*-acetylneuraminic acid-cleaving enzyme (neuraminidase) is at a normal level. Total hexosaminidase activity as measured with an artificial fluorogenic substrate is increased in tissues obtained from patients with the B variant form of Tay-Sachs disease and it is virtually absent in the O-variant patients. The addition of purified neuraminidase and various purified hexosaminidases exerted only a minimal synergistic effect on the hydrolysis of Tay-Sachs ganglioside in the lysosomal preparations from the control or patient with the O variant of Tay-Sachs disease.

INTRODUCTION

Infantile Tay-Sachs disease, the most common of the lipid storage diseases, is a fatal inborn error of ganglioside metabolism, inherited as an autosomal recessive trait. The earliest clinical descriptions of the disease date from the 19th century and over 599 cases have been reported (1). Onset of the disease is in the first 6 months of life and is characterized by apathy, hyperacusis, motor weakness, and appearance of a macular cherry-red spot in the retina. Seizures and progressive mental deterioration follow with blindness, deafness, and spasticity, leading to a state of decerebrate rigidity. These infants usually die by 3 yr of age (2).

A change in the chemical composition of the brain of such patients was first detected by Klenk who showed that there was an increase in the ganglioside content compared with normal human brain tissue (3). The particular ganglioside which accumulated to the greatest extent was shown by Svennerholm (4) to be a minor component of normal cerebral gangliosides, called Tay-Sachs ganglioside or ganglioside G_{M2}, *N*-acetylgalactosaminyl (*N*-acetylneuraminosyl-) galactosyl glucosyl ceramide. The corresponding asialo-derivative of G_{M2} called G_{A2} also accumulates (20% w:w compared with G_{M2}) in the brain of Tay-Sachs patients. In the "O"-variant form of Tay-Sachs disease, the levels of G_{A2} in brain are five times higher and the accumulation of G_{M2} is somewhat more rapid than in patients with the classic type of this disease (5, 6). These patients also stored globoside. Most recently, an "AB"-variant has been described which is clinically and chemically indistinguishable from classical Tay-Sachs disease (called variant B) (7).

On the basis of previous information about the nature of the enzymatic defects in related lipid storage diseases (8), it is logical to look for a defect in the catabolism of the accumulated ganglioside and the corresponding asialo compound. Since the enzymatic degradation of G_{M2} could conceivably proceed either through the initial removal

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of *N*-acetylneuraminic acid to yield G_{A2} or *N*-acetylgalactosamine to yield *N*-acetylneuraminosylgalactosyl-glucosylceramide, a deficiency of either of the respective enzymes might lead to an accumulation of the ganglioside. Assays for hexosaminidase with artificial substrates showed that there was a higher total hexosaminidase activity in brain tissue from patients with Tay-Sachs disease than that in controls (5). The possibility had to be considered that a neuraminidase deficiency was responsible for the ganglioside accumulation. To examine this alternative, G_{M2} was biosynthetically labeled in the *N*-acetylneuraminosyl portion of the molecule (9) and the activity of this neuraminidase was determined. The specific activity of this enzyme was similar in tissue specimens obtained from normal humans and patients with Tay-Sachs disease (10). The question was then partially resolved by the demonstration that there were two hexosaminidase isozymes in normal human tissues (11) and one of these, hexosaminidase A, was diminished in tissue from patients with Tay-Sachs disease (12). Both isozymes are virtually absent in patients with the O-variant form of Tay-Sachs disease (13). In the AB-variant, the activity of each isozyme is increased when chromogenic or fluorogenic substrates are utilized for the assay of hexosaminidase activity (7).

The activity of these hexosaminidases with regard to their ability to catalyze the catabolism of the natural substrate G_{M2} has not been investigated thoroughly. This information is extremely important since widely divergent results have been obtained through the use of the chromogenic or fluorogenic substrates. The use of such "artificial" substrates does not necessarily imply a defect in the natural substrate cleavage. The catabolism of G_{M2} labeled in the *N*-acetylneuraminosyl and *N*-acetylgalactosaminyl moieties had been reported in preparations of skeletal muscle and the release of *N*-acetylgalactosamine was diminished in such preparations from patients with Tay-Sachs disease (10). However, the possibility of a two-step catabolic scheme in which *N*-acetylneuraminic acid was first cleaved could not be ruled out using such preparations. We have resolved this uncertainty by the present investigations in which G_{M2} labeled either in the *N*-acetylneuraminosyl *or* the *N*-acetylgalactosaminyl moiety is used to study the catabolism occurring in normal and Tay-Sachs tissue.

Additionally, because of the impending potential use of highly purified enzyme preparations in enzyme replacement therapy in Tay-Sachs and other related diseases, we have studied the synergistic effect of added purified enzymes on the levels of hydrolyses in normal and Tay-Sachs lysosomal preparations.

METHODS

Materials. *N*-acetylmannosamine-[^{3}H] (U) (SA 5.06 Ci/mmole) and uridinediphosphate *N*-acetylgalactosamine-1-^{14}C (SA 43 mCi/mmole) were obtained from New England Nuclear Corp. (Boston, Mass.). Authentic G_{M2} was prepared from frozen postmortem brain of Tay-Sachs patients. 4-Methylumbelliferyl-β-*N*-acetyl-D-glucosamide was purchased from Pierce Chemicals (Rockford, Ill.).

Radioactive G_{M2}. *N*-acetylneuraminic acid-labeled G_{M2} was prepared biosynthetically as previously described from precursor *N*-acetyl-D-mannosamine-[^{3}H] (9). The specific radioactivity of the compound was 5.04×10^5 cpm/μmole. Hydrolysis indicated that radioactivity was associated only with the *N*-acetylneuraminic acid portion of the molecule. *N*-acetylgalactosamine-labeled G_{M2} was prepared from G_{M3} and UDP-*N*-acetylgalactosamine-1-^{14}C as described (14). The specific radioactivity of this compound was 6.7×10^6 cpm/μmole. Hydrolysis indicated that all of the radioactivity was associated with *N*-acetylgalactosamine. Both compounds were chromatographically pure in three solvent systems.

For incubations with normal brain tissue preparations, the *N*-acetylgalactosamine-labeled G_{M2} was diluted to 1.0×10^6 cpm/μmole. For Tay-Sachs brain preparations, this substrate was used without dilution because of the endogenous dilution (*vide infra*).

Preparation of cortical lysosomes. Human cortical gray matter was obtained at biopsy from "normal" and Tay-Sachs patients and was kept at 0° in Elliot's solution B (15) until use. The cortical gray matter was homogenized by hand utilizing a TenBroeck glass homogenizer in 10 vol (w:v) of 0.25 M sucrose solution containing 1 mM EDTA at pH 7.4. Lysosomes were prepared by the procedure of Sellinger and Nordrum (16) with minor modifications. Evaluation of the activities of nine lysosomal marker enzymes (including acid phosphatase, β-galactosidase, and sphingomyelinase) showed that the pellet obtained in this procedure was enriched three to fivefold over homogenate in the content of these hydrolases (17). This lysosomal pellet was resuspended in the original sucrose-EDTA solution so that the final protein concentration was between 2 and 3 mg/ml.

Experimental conditions. Utilizing the respective labeled ganglioside, we have shown elsewhere[1] that the catabolism of G_{M2} can be initiated in similar lysosomal preparations either by the sialidase reaction to yield reaction products which we have identified as *N*-acetylneuraminic acid and G_{A2} or by the hexosaminidase reaction to yield reaction products which were identified as *N*-acetylgalactosamine and G_{M3}. Studies on the kinetic parameters of these reactions have indicated that the optimal pH for the sialidase is 4.4 in a citrate-phosphate buffer, that the reaction is proportional to protein content up to 400 μg protein per incubation and with time up to 5 hr. Similarly, the optimal pH for the hexosaminidase was 5.1 in a potassium acetate buffer, and this reaction displayed similar proportionality to protein and time. Since the effect of all detergents was inhibitory, both of these reactions were carried out without additional detergent. The procedure for the determination of hexosaminidase activity utilizing the fluorogenic substrate has been described previously (17). Incubation conditions are summarized in Table I.

Correction for dilution with endogenous ganglioside. A 0.2 ml portion of the resuspended lysosomes (400–600 μg of protein) from control and Tay-Sachs patients was added

[1] Tallman, J., and R. O. Brady. The catabolism of Tay-Sachs ganglioside in rat brain lysosome. *J. Biol. Chem.* In press.

TABLE I

Conditions of Incubation for Lysosomal Neuraminidase and Hexosaminidase Determinations

Enzyme	Substrate	Concentration of substrate	Concentration of buffer/pH	Amount of protein	Time
		mM		μg	hr
Neuraminidase	AcneuG$_{M2}$-^{3}H	0.075	0.1 M Citrate-phosphate/4.4	200–300	4
G$_{M2}$-hexosaminidase	NAcgalG$_{M2}$-^{14}C	0.100	0.1 M Potassium acetate/5.1	300–400	5
Total hexosaminidase	4MeUmb-Gluc NH_2	0.25	0.15 M Citrate-phosphate/4.4	10–20	0.25

to 4 ml of a solution of chloroform-methanol, 2:1 (v:v). The suspension was filtered and the residue on the filter paper was washed with 2 ml of chloroform-methanol 1:1. The filtrates were combined and the volume was reduced under a stream of N_2. The amount of *N*-acetylneuraminic acid was determined on a portion of the residual solution (18) and the remainder was subjected to thin-layer chromatography to isolate the individual gangliosides (19). More than 95% of the gangliosides in the lysosomal preparations from the patients with Tay-Sachs disease was shown

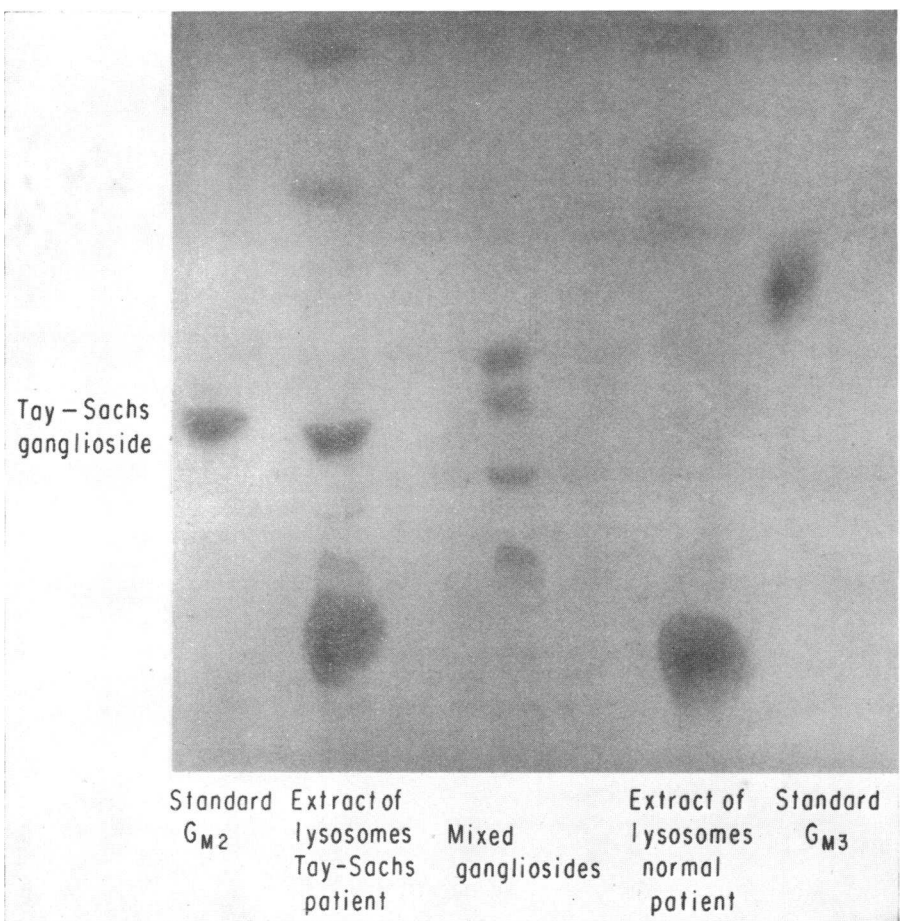

FIGURE 1 Thin-layer chromatogram showing the accumulation of ganglioside G$_{M2}$ in lysosomes of a patient with Tay-Sachs disease. The gangliosides became bluish purple when sprayed with resorcinol reagent (24). The two large slow-moving spots in the second and fourth lanes do not show this color reaction and are most likely some residual sucrose from the density gradient centrifugation used for isolation of lysosomes. Also present in the second and fourth lanes are various of the neutral lipids which show very high Rf. These also do not contain sialic acid.

TABLE II

Ganglioside Catabolism by Human Brain Lysosomes

Source of tissue	Age	Enzyme: Hexosaminidase — G_{M2}-^{14}C ganglioside	Enzyme: Hexosaminidase — Substrate 4-methylumbelliferyl-β-*N*-acetyl-D-glucosaminide	Enzyme: Sialidase — G_{M2}-^{3}H ganglioside
		pmoles/mg protein/hr	*nmoles/mg protein/hr*	*pmoles/mg protein/hr*
Control series				
Exploratory frontal lobotomy	21 yr	163	148	271
Epileptigenic focus	25 yr	104	121	193
Schilder's disease	7 yr	226	132	–*
Abortus	13 wk	53	–	–
Lipidosis "unknown"	–	107	–	187
Degenerative disease	43 yr	186		216
Accident victim or "trauma"	22 yr	143	–	–
Mean ±SD		140±53	134±11	217±33
Tay-Sachs patients				
V. D. (classic)	28 months	0	513	225
J. K. (classic)	20 months	0	1416	–
D. T. (variant)	13 months	6	3	232
Mixed experiment				
Degenerative disease control and patient D.T.	–	138 (theory = 93)	–	210 (theory = 224)

* –, not determined.

to be G_{M2} and appropriate corrections were made to correct for the dilution of the labeled substrate. No G_{M2} was detected in the lysosomal preparations from the control brain specimens (Fig. 1).

Preparation of supplemental enzymes. The details of the purification of heart muscle neuraminidase (20), urinary hexosaminidases A and B, and placental hexosaminidase A[2] will be published. Briefly, the neuraminidase is purified by gel filtration on Sephadex G-150 (Pharmacia, Uppsala, Sweden), chromatography on carboxymethyl Sephadex (CM-50) followed by isoelectric focusing. The purified enzyme is enriched 3000-fold over the starting extract and this enzyme catalyzes the hydrolysis of the molecule of *N*-acetylneuraminic acid from G_{M2}. The urinary hexosaminidase A was purified by ammonium sulfate precipitation, gel filtration on Sephadex G-200, DEAE-Sephadex, and carboxymethyl Sephadex. The purified hexosaminidase is 6000-fold purified over the initial activity. Hexosaminidase B from a similar protein extract was completely separated from hexosaminidase A and purified 100-fold by ammonium sulfate precipitation, Sephadex G-200, and DEAE Sephadex. Placental hexosaminidases were purified using much the same techniques. The activity of these hexosaminidases during purification was assayed with the artificial substrate, 4-methylumbelliferyl-β-*N*-acetyl-D-glucosaminide.

[2] Johnson, W. G., and R. O. Brady. Manuscripts in preparation.

RESULTS

Lysosomes from fresh normal human brain contain enzymes which catalyze the cleavage of both *N*-acetylgalactosamine and *N*-acetylneuraminic acid moieties of G_{M2} (Table II). The mean catalytic activity of G_{M2} hexosaminidase in lysosomes obtained from the seven control brain samples was 140 pmoles/mg of protein per hr. Approximately 62% of the total activity present in crude tissue could be recovered in this lysosome fraction. This enzyme was drastically diminished in similar preparations from patients with Tay-Sachs disease in which the mean G_{M2} hexosaminidase activity was 2 pmoles/mg of protein per hr. These values are corrected for dilution by endogenous G_{M2}. G_{M2} neuraminidase activity in the control lysosomes averaged 217 pmoles/mg of protein per hr and for two of the Tay-Sachs specimens, the mean was 228 pmoles/mg of protein per hr. The recovery of total sialidase activity present in the crude tissue in the lysosomal pellet was 52% for control and 44% for Tay-Sachs. Mixing Tay-Sachs brain lysosomes with normal brain lysosomes led to G_{M2} hexosaminidase activity of 138 pmoles/mg per hr and G_{M2} neuraminidase activity of 210 pmoles/mg per hr. The lysosomes obtained from the con-

TABLE III

Effect of the Addition of Purified Enzymes to Various Lysosomal Fractions

Addition	1	2	3	4	5	6	7	8	9	10	11
Neuraminidase	−	−	−	+	−	−	−	+	+	+	+
Urinary hexosaminidase A	+	−	−	+	−	−	−	+	+	−	−
Placental hexosaminidase A	−	+	−	−	−	−	−	−	−	+	+
Urinary hexosaminidase B	−	−	+	+	−	−	−	+	+	+	+
Lysosomal extract*											
O variant	−	−	−	−	−	+	+	−	½	−	½
Lysosomal extract‡											
Normal	−	−	−	−	+	−	+	½	−	½	−
*p*moles GalNAc incubation/hr	0	14	0	38	49	1.2	63	23	0	21	0
*p*moles GalNAc/total mg lysosomal protein/hr	—	—	—	—	186	6	138	180	0	164	0

* 2.4 mg protein/ml lysosomal extract. 80-μl portions were used except where indicated by ½.

‡ 3.3 mg protein/ml lysosomal extract. 80-μl portions were used except where indicated by ½.

trol brain specimens contained negligible quantities of G_{M2} whereas similar preparations from the Tay-Sachs patients showed clear evidence of the accumulation of this ganglioside (Fig. 1). In the lysosomes obtained from patients with the classic form of Tay-Sachs disease, total hexosaminidase activity measured with the artificial fluorogenic substrate was increased 4 and 10 times, respectively, over the mean of hexosaminidase activity in the control preparations. As expected, total hexosaminidase activity in the brain lysosome preparation from the patient with the O-variant form of Tay-Sachs disease contained only 2% of that in the controls.

The effect of adding purified heart muscle neuraminidase and human urinary and placental hexosaminidases to the lysosome preparation obtained from the variant form of Tay-Sachs disease was examined (Table III). Neither of these purified hexosaminidase preparations significantly catalyed the hydrolysis of G_{M2} by themselves in vitro. This result confirms the previous studies of Sandhoff, Harzer, Wassle, and Jatzkewitz (7) and Frohwein and Gatt (21). However, when purified heart muscle neuraminidase was included in the incubation mixture with these enzymes, a small amount of *N*-acetylgalactosamine was released from the lipid substrate. Conversely, the addition of purified hexosaminidase did not enhance the G_{M2} neuraminidase reaction. In spite of the lack of G_{M2} hexosamine-cleaving activity of purified hexosaminidases, the possibility existed that the addition of these enzymes to lysosomes from brain tissue from Tay-Sachs patients might bring about the catabolism of the ganglioside either because of the presence in lysosomes of a necessary cofactor or because of an altered steric orientation of the G_{M2} *in situ*. There was no evidence of release of *N*-acetylgalactosamine-^{14}C when purified hexosaminidases were added to the lysosomes from the Tay-Sachs patient or when the very active sialidase was added to such preparations.

DISCUSSION

Studies on the metabolism of gangliosides in brain of patients with Tay-Sachs disease were required for the explicit identification of the enzymatic lesion in this condition. These investigations are hampered by the fact that brain tissue normally exhibits only very low activity with regard to the catabolism of the accumulating ganglioside (G_{M2}). Steric hindrance of the terminal sugar has been proposed as an explanation for this phenomenon. No one has yet been able to demonstrate the production of either *N*-acetylgalactosamine or *N*-acetylneuraminic acid by colorimetric methods. Our radioactive products were identified as the authentic sugar in each case and represented assay systems 500 times more sensitive than the colorimetric determination. The present experiments were performed with G_{M2} specifically labeled with ^{14}C in the *N*-acetylgalactosaminyl moiety or in the *N*-acetylneuraminosyl portion with ^{3}H in order to provide sensitive probes for delineating the pathway(s) of catabolism of this ganglioside. We have found that brain lysosomal preparations contain enzymes which catalyze the cleavage of the *N*-acetylgalactosaminyl portion of G_{M2} and the *N*-acetylneuraminosyl moiety by this method. The activity of the hexosamine-cleaving enzyme is virtually nonexistent in preparations of brain tissue obtained from patients with Tay-Sachs disease. The activity of the neuraminic acid hydrolytic enzyme in preparations from Tay-Sachs patients was similar to that in the control brain tissue specimens. Thus, the present experiments extend and confirm the investigations with skeletal muscle biopsies that the enzymatic defect in Tay-Sachs disease studies is a deficiency of the hexosamine

cleaving enzyme which normally is one of the pathways available for ganglioside catabolism in brain (10). The other route via the neuraminidase is unaffected in Tay-Sachs disease. Although the values reported for these enzymes are low, we feel that they reflect quite accurately the *in situ* activities for these enzymes. All catabolic schemes which have been proposed show G_{M2} as an intermediate (22–24). This compound must be catabolized in normal tissue even at these low levels. It is significant to note that these levels are sufficiently high to account for all G_{M2} turnover (24).

In order to obtain a full understanding of the pathological biochemistry and physiology of Tay-Sachs disease, the following observation must be also taken into account. Hexosaminidase B which is present in the tissues of patients with the AB- and B-variant forms of Tay-Sachs disease can catalyze the hydrolysis of the asialo-G_{M2} produced via the neuraminidase pathway (7). Since G_{M2} neuraminidase is normal and the hexosaminidase B isozyme is markedly increased in brain tissue of these patients and presumably able to function at least initially in Tay-Sachs patients, why does G_{M2} accumulate at all? One reasonable explanation for this accumulation is the following. The activity of G_{M2} neuraminidase is quite low in brain preparations compared with that in other tissues (25). Since cerebral ganglioside turnover is very rapid in the neonatal period of life, it may be assumed that a major portion of ganglioside catabolism must also occur via the hexosaminidase pathway. Both these enzymes catalyze rate-limiting reactions in ganglioside metabolism.[1] Because the hexosaminidase is deficient in patients with Tay-Sachs disease, all G_{M2} catabolism must proceed exclusively via the neuraminidase route which, under these conditions, is insufficient to prevent the accumulation of G_{M2}. Along with this accumulation, there are notable changes occurring in the lysosomal structure (17). The smaller amount of pathological involvement of systemic tissues (except for neurons in the myenteric plexus) in the classic form of Tay-Sachs disease my be due to the fact that the rapidity and quantity of ganglioside turnover in peripheral tissues is much less than that in brain. Also, catabolism of G_{M2}, peripherally, can also occur through the neuraminidase pathway and the combined activity of G_{M2} neuraminidase which is functioning and hexosaminidase B which is augmented in Tay-Sachs disease is sufficient to prevent the accumulation of a significant quantity of Tay-Sachs ganglioside in non-neural tissues. The absence of even hexosaminidase B in patients with the O-variant form of Tay-Sachs disease may be responsible for the even greater accumulation of G_{M2} in the brain of these infants compared with that in conventional cases of Tay-Sachs disease (26).

A number of other observations which were made in the course of these investigations deserve brief comment. The higher-than-expected rate of catabolism of G_{M2} by the mixture of lysosomes from the normal and Tay-Sachs variant may be related to the higher substrate concentration of G_{M2} available for hydrolysis by the hexosaminidase in the control lysosome preparation. Our choice of G_{M2} concentration for use in the bulk of the assays performed (0.1 mM) was based on the known critical micellar concentration of ganglioside. We wished to stay just below this concentration in our experiments in order to prevent aggregation of G_{M2}. Thus we did not work at the absolute V_{max} of either the G_{M2} hexosaminidase or neuraminidase, although the reactions were carried out at optimal pH in a proportional range. Additionally, the correction applied to substrate specific radioactivity for the presence of G_{M2} in Tay-Sachs presumes that both the added ganglioside and that *in situ* are in the same pool (or mix). The possibility exists that the stored G_{M2} in the Tay-Sachs lysosomes does not mix with the G_{M2}-^{14}C and therefore a low value is assumed for the radioactivity of G_{M2} which is used to calculate the enzymic activity resulting in the higher value. Another possibility is that there is a cooperating factor in the Tay-Sachs lysosomes which when added to the hexosaminidase from normal lysosomes increases its activity (27).

The inability of added purified enzymes to enhance significantly the enzymatic activities of the lysosomes may be related to structural ordering of these enzymes within the lysosome. This postulated steric restriction might also contribute to the accumulation of G_{M2} in Tay-Sachs disease. The sialidase pathway may not be arranged in the brain lysosomal membrane in an appropriate way to degrade the G_{M2} with facility. Furthermore, the presence of an altered hexosaminidase A protein which binds, but cannot hydrolyze G_{M2}, might prevent some of the G_{M2} from being catabolized by the sialidase. The pathological sequela of either of these constrictions is formation of storage inclusion bodies and the deleterious changes in the neurons which are characteristic of Tay-Sachs disease. This finding also makes the possibility of enzyme replacement therapy less appealing than might otherwise be assumed. The inability of the enzymes to work in synergism will remain a problem in all attempts at this therapy in Tay-Sachs patients.

A complete understanding of the catabolism of G_{M2} demands a quantitative assessment of the relative contributions of the neuraminidase and hexosaminidase pathways. The optimal pH for the neuraminidase in brain lysosomes is 4.4; the hexosaminidase has a pH optimum of 5.1.[1] The pH of the lysosomal environment is not known, and the possibility that the pH is not optimal for one or the other of these enzymes in normal or Tay-Sachs brain cannot be overlooked. We are at present evaluating the contribution of each pathway under varying conditions in control human brain preparations.

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