Enzymatic Hydrolysis of Sphingolipids. VI. Hydrolysis of Ceramide Glycosides by Calf Brain β-*N*-Acetylhexosaminidase*

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ABSTRACT: Trihexosylceramide (GalNAc-β-1,4-Gal-β-1,4-Glc-β-1,1-(2-*N*-acyl)sphingosine) was prepared and used as substrate for calf brain β-*N*-acetylhexosaminidase. Two other glycosphingolipids, "globoside" from human erythrocyte stroma and the ganglioside which accumulates in brain tissue of patients with Tay-Sachs' disease, were also hydrolyzed. The rate of hydrolysis of the latter compound was much slower than those of the other two substrates. The β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase from calf brain did not hydrolyze these glycosphingolipids. The hydrolysis of trihexosylceramide had a pH optimum of 3.8, a K_m of 0.6 mM, and a V_{max} of 0.5 μmole/mg of protein per hr. It was inhibited by several lipids, by two "SH inhibitors," by free and acetylated hexosamines, and by mono- or divalent ions. The hydrolysis of globoside was similar to that of trihexosylceramide in most respects. "Tay Sachs' ganglioside" strongly inhibited the hydrolysis of trihexosylceramide. The possibility that this might be a cause for the accumulation of the latter compound in brain tissue of patients with Tay-Sachs' disease is discussed.

N-Acetylgalactosamine is a component of the carbohydrate moiety of brain gangliosides (Wiegandt, 1966). In most gangliosides it is linked, through both the reducing group and through a nonreducing hydroxyl group, to two galactose units. In one ganglioside, which accumulates in brain tissue of patients with infantile amaurotic familial idiocy (Tay-Sachs' disease), *N*-acetylgalactosamine occurs in the terminal position of the carbohydrate chain. A second glycosphingolipid also accumulates in this pathological tissue (Gatt and Berman, 1963a; Makita and Yamakawa, 1963). It is a trihexosylceramide[1] having the following composition: *N*-acetylgalactosaminylgalactosylglucosylceramide. Removal of the terminal *N*-acetylgalactosamine from this compound yields lactosylceramide. Enzymes which catalyze the stepwise degradation of the latter compound have been isolated from brain (Gatt and Rapport, 1966a,b; Gatt, 1963, 1966a,b).

This paper reports the removal of the terminal *N*-acetylgalactosamine of trihexosylceramide using the β-*N*-acetylhexosaminidase of calf brain (Frohwein and Gatt, 1967). The hydrolyses of two other glycosphingolipids, "globoside" (*N*-acetylgalactosaminylgalactosylgalactosylglucosylceramide, Makita *et al.*, 1964) and "Tay-Sachs' ganglioside" (*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide, Ledeen and Salsman, 1965) have also been studied. A preliminary communication has appeared (Frohwein and Gatt, 1966a).

Experimental Procedure

Substrates. Trihexosylceramide (*N*-acetylgalactosaminyl-β-1,4-galactosyl-β-1,4-glucosyl-β-1,1-(2-*N*-acyl)-di[^{3}H]sphingosine) was prepared as follows. ^{3}H-labeled tetrahexosylceramide (70 mg) (Gal-β-1,3-GalNAc-β-1,4-Gal-β-1,4-Glc-β-1,1-(*N*-acyl)di[^{3}H]sphingosine, Gatt and Rapport, 1966b) was dissolved in 5 ml of chloroform–methanol (2:1). Chloroform–methanol solutions containing 20 mg of Triton X-100 and 20 ml of sodium taurocholate were added. The solvent was evaporated and the residue was suspended in 19 ml of water. Sodium acetate buffer (1 ml of 1 M, pH 5) and 10 ml of rat brain β-galactosidase (Gatt and Rapport, 1966a), which had been prepared from 15 g of brain tissue, were added. The mixture was incubated for 17 hr at 37° to split off the terminal galactose. The reaction was terminated by the addition of 120 ml of chloroform–methanol (2:1), the phases were separated, the lower phase was evaporated, and the trihexosylceramide was purified by chromatography on a column (10 × 1 cm) of 4 g of Unisil. It was eluted using a discontinuous gradient of methanol in chloroform. The fraction eluted with 15–25% methanol was collected. It was identified by thin layer chromatography on silica gel plates in three solvent systems: (a) chloroform–methanol–water (75:25:4), (b) chloroform–methanol–water (60:35:8) (Müldner *et al.*, 1962), and (c) propanol–water (7:3) (Kuhn *et al.*, 1961). The quantity of the glycolipid was determined by the anthrone-phosphoric acid method of Radin *et al.* (1955), using an equimolar mixture of glucose and galactose as standard. The compound had a radiochemical purity of 95%. The impurities consisted of about 1% lacto-

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[1] Ceramide is the trivial name for *N*-acylsphingosine.

sylceramide and 2%, each, of glucosylceramide and tetrahexosylceramide.

Tay-Sachs' ganglioside (GalNAc-β-1,4-(NANA[2]-β-2,3)-Gal-β-1,4-Glc-β-1,1-(2-*N*-acyl)sphingosine (Ledeen and Salsman, 1965) was isolated from brain tissue of patients with Tay-Sachs' disease (Gatt and Berman, 1963b).

Globoside (GalNAc-β-1,3-Gal-β-1,4-Gal-β-1,4-Glc-β-1,1-(2-*N*-acyl)sphingosine (Makita *et al.*, 1964; Yamakawa *et al.*, 1965) was prepared as follows. Human blood was centrifuged and the plasma was siphoned. The packed erythrocytes were hemolyzed with five volumes of 0.5% acetic acid and the stroma were collected by centrifugation for 10 min at 20,000*g*. The paste was stirred with four volumes of acetone for 1 hr; the mixture was filtered and the res due was again treated with acetone. The powder obtained after filtration was air dried.

Dried stroma (160 g) was refluxed for 1 hr with 1 l. of chloroform–methanol–water (64:32:4). After filtration, the residue was again refluxed with 1 l. of the above solvent mixture. The two extracts were combined, evaporated *in vacuo*, and subjected to mild alkaline hydrolysis (for 2 hr, at 37°, with 200 ml of 0.4 N KOH in 90% methanol). Chloroform (360 ml) and water (115 ml) were added, the upper phase was removed, and the lower phase was washed with 500 ml of a mixture of methanol–water–chloroform (96:94:6) (Folch *et al.*, 1957). It was chromatographed on 100 g of silicic acid (Mallinckrodt) using 500 ml each of the following chloroform–methanol mixtures: 95:10, 85:15, 80:20, and 70:30. The fraction eluted with 30% methanol in chloroform was again chromatographed on 30 g of Florisil. This column was eluted with 300 ml of chloroform–methanol (2:1) and then with 1 l. of chloroform–methanol (1:1). This latter fraction was evaporated to dryness and the globoside was crystallized two times from pyridine–acetone (1:4).

Marker Lactosylceramide. Bovine brain gangliosides were hydrolyzed with 0.015 N HCl for 1 hr at 100°. Four volumes of chloroform–methanol (2:1) was added, the phases were separated, and the lower phase was chromatographed on a column of Unisil. The column was washed with chloroform–methanol (90:10) and lactosylceramide was then eluted with chloroform–methanol (87:13).

Enzymes. *N*-Acetylhexosaminidase, *N*-acetylglucosaminidase, and *N*-acetylgalactosaminidase of calf brain were prepared as described by Frohwein and Gatt (1966b, 1967).

Chemicals. Triton X-100 was obtained from Rohm and Haas; Unisil (activated silicic acid, 200–325 mesh) from Clarkson Chemical Co., Williamsport, Pa.; silicic acid from Mallinckrodt; silica gel G from Desaga; and Florisil from Floridin Co., Tallahassee, Fla. Commercial sodium taurocholate was purified by dissolution in ethanol and fractional precipitation from this solution with ether. This procedure was repeated until the taurocholate precipitate was white. It was then washed with ether, dried, and dissolved in chloroform–methanol (2:1).

Preparation of Reaction Mixtures. Chloroform–methanol solutions of substrates and detergents were mixed and the solvent was evaporated at 70° under a stream of nitrogen. Buffer (0.01–0.02 ml) and water (0.02 ml) were added and tubes were gently shaken to disperse the mixed micelle of lipid and detergent. Enzyme and water were then added to a final volume of 0.1 ml and the tubes were placed in a water bath at 37°.

Determination of the Reaction Products. (a) The tritium-labeled lipid product was determined as follows. Chloroform–methanol (2:1) (0.4 ml) was added to 0.1 ml of reaction mixture. The tubes were thoroughly shaken and centrifuged, and the upper phase was removed. The lower (chloroform) phase was decanted quantitatively into a small test tube containing marker lactosylceramide. The solvent was evaporated and the residue was chromatographed on a thin layer silica gel plate in chloroform–methanol–water (75:25:4). The lactosylceramide spot was visualized in iodine vapor, scraped off, and eluted, once with boiling chloroform–methanol–water (64:32:4) and twice with boiling methanol. The extracts were decanted into counting vials containing 1 mg of egg lecithin. The solvents were evaporated under an infrared lamp and 1 ml of hot toluene was added followed by 10 ml of scintillation fluid (3 g of PPO and 100 mg of dimethyl-POPOP in 1 l. of toluene). The vials were counted in a liquid scintillation counter.

(b) The *N*-acetylgalactosamine released was determined by the method of Reissig *et al.* (1955), as modified by Frohwein and Gatt (1967). The assay was performed either directly on the reaction mixture or on the upper phase obtained in method a. In the latter case, this upper phase was evaporated at 100° under a stream of air or nitrogen.

Results

Effect of Detergents. Hydrolysis of ceramide glycosides by the β-*N*-acetylhexosaminidase required the addition of a detergent; without it, only very little activity was obtained. Addition of sodium taurocholate stimulated the reaction 30-fold or more. The optimal concentrations of taurocholate were 1.2 mg/ml of reaction mixture for the hydrolysis of trihexosylceramide and about 3 mg/ml for the hydrolyses of globoside or Tay-Sachs' ganglioside. It should be noted that similar concentrations of taurocholate were inhibitory when added to reaction mixtures in which *p*-nitrophenyl *N*-acetylgalactosaminide was used as substrate. No activity was obtained using the following detergents: Triton X-100, Cutscum, Cetavlon, and Tween 20. When these compounds were added to reaction mixtures having optimal concentration of taurocholate, inhibition ensued.

[2] Abbreviations used: NANA, *N*-acetylneuraminic acid; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)].

Hydrolysis of Trihexosylceramide. This hydrolysis was directly proportional to the reaction time (up to at least 2.5 hr) and to the concentration of purified *N*-acetylhexosaminidase (up to at least 30 μg of protein in 0.1 ml of reaction mixture). The hydrolysis by crude enzyme preparations showed very poor proportionality to enzyme concentration: the specific activity rapidly decreased with increasing amounts of protein. The optimal pH of the reaction was 3.8 with citrate or phosphate–citrate buffers (Figure 1, curve A). The range in which the enzyme acted on this substrate was limited to less than 2 pH units. In contrast, when using *p*-nitrophenyl *N*-acetylgalactosaminide and the same buffers, hydrolysis was obtained over a range of 5 or more pH units (Figure 1, curve C). The optimal pH of hydrolysis of trihexosylceramide changed to 4.4 if acetate was used instead of phosphate–citrate buffers (Figure 1, curve B). As seen in Figure 1, acetate also inhibited the reaction. The maximal hydrolysis in 0.017 M acetate buffer (pH 4.4) was only 60% of that obtained in phosphate citrate buffer (pH 3.8). Higher concentrations of acetate inhibited even more. Thus in 0.025 M acetate, the maximal activity was only 35% of that obtained with phosphate citrate. The K_m of hydrolysis of trihexosylceramide in phosphate citrate buffer was 6×10^{-4} M; the V_{max} was 0.5 μmole/mg of protein per hr.

Identification of the Products of Hydrolysis. A. *N*-ACETYLGALACTOSAMINE released was determined by the method of Reissig *et al.* (1955) as described in Experimental Procedure. It was also identified by electrophoresis on borate-impregnated paper (Crumpton, 1959), using *N*-acetylgalactosamine as reference compound.

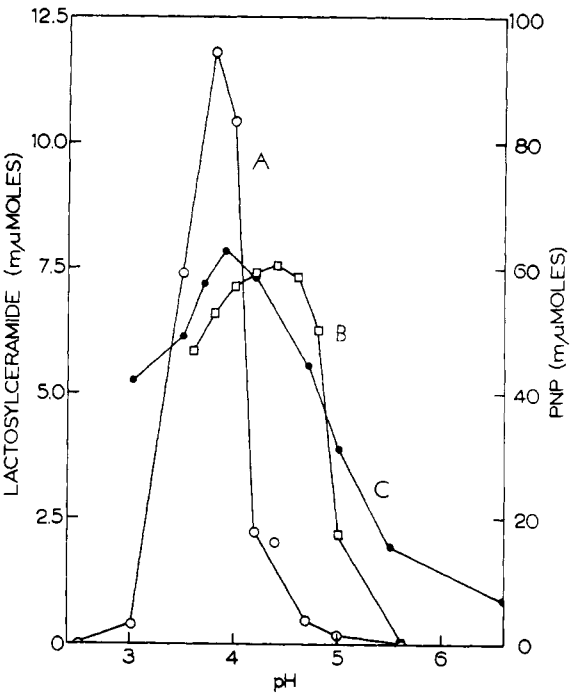

FIGURE 1: Effect of pH on the hydrolysis of trihexosylceramide and *p*-nitrophenyl *N*-acetylgalactosaminide by β-*N*-acetylhexosaminidase. Curves A and B: Incubation mixtures in volumes of 0.1 ml each contained 30 mμmoles of [^{3}H]trihexosylceramide, 0.6 mg of sodium taurocholate, 30 μg of enzyme, and 1.25 μmoles each of phosphate and citrate buffers (curve A) or 1.7 μmoles of potassium acetate buffers (curve B). After 2 hr at 37°, [^{3}H]lactosylceramide was isolated and its radioactivity was determined as described in Experimental Procedure. Curve C: Incubation mixtures, in volumes of 0.2 ml each contained 0.2 μmole of *p*-nitrophenyl *N*-acetylgalactosaminide, 8 μg of enzyme, and 10 μmoles each of sodium and citrate buffers. After 90 min at 37°, 0.8 ml of 0.125 M sodium borate was added and the color was read at 420 mμ.

TABLE I: Stoichiometry of the Hydrolysis of Trihexosylceramide.[c]

Compound	Amt (mμmoles)
Trihexosylceramide hydrolyzed[a]	26.0
Lipid hydrolysis products[b]	24.2
N-Acetylgalactosamine formed	27.5

[a] Calculated from the difference in radioactivity of trihexosylceramide incubated with and without enzyme. [b] Sum of lactosylceramide, glucosylceramide, and ceramide. [c] The reaction mixture, in a volume of 0.1 ml, contained 64 mμmoles of [^{3}H]trihexosylceramide, 120 μg of sodium taurocholate, 1.25 μmoles each of sodium phosphate and citrate (pH 3.8), and 37 μg of enzyme. After incubation for 20 hr at 37°, 0.4 ml of chloroform–methanol (2:1) was added and the phases were separated. The upper phase was removed and the *N*-acetylgalactosamine content was determined. The lower phase was chromatographed on thin layer plates of silica gel. The spots corresponding to trihexosylceramide, lactosylceramide, glucosylceramide, and ceramide were scraped off, extracted, and counted as described in the Experimental Procedure.

B. LACTOSYLCERAMIDE, the product of the hydrolysis of trihexosylceramide, was identified by chromatography on thin layer plates of silica gel in the following three solvent systems: (1) chloroform–methanol–water (75:25:4), (2) chloroform–methanol–2 N NH_4OH (60:35:8) (Müldner *et al.*, 1962), and (3) 1-propanol–water (7:3) (Kuhn *et al.*, 1961). Authentic lactosylceramide ("Cytolipin H," obtained from Dr. Maurice M. Rapport) was used as reference compound.

Under the hydrolysis conditions of trihexosylceramide described in Figure 1, lactosylceramide was the only lipid product formed. If the reaction was run for longer periods (up to 18 hr, when about 75% of the substrate was hydrolyzed), some glucosylceramide and ceramide were obtained as well. This is due to some contamination with β-galactosidase and β-

glucosidase (Gatt and Rapport, 1966a,b; Gatt, 1966a). The stoichiometry of the hydrolysis of trihexosylceramide is shown in Table I. The amount of substrate hydrolyzed was approximately equimolar to amounts of each of the products formed.

Hydrolysis of Globoside and Tay-Sachs' Ganglioside. The hydrolysis of globoside and Tay-Sachs' ganglioside by the enzyme was also tested. Both substrates were not available with radioactive label and were assayed by determining the *N*-acetylgalactosamine released.

The hydrolysis of globoside was directly proportional to enzyme concentration up to at least 0.1 mg. The K_m was 2×10^{-4} M and the V_{max} 0.35 μmole/mg × hr. The product (galactosylgalactosylglucosylceramide) was identified by thin layer chromatography, using the three solvent systems employed for the identification of lactosylceramide. The reference compound (digalactosylglucosylceramide, isolated from tissue of patients with Fabry's disease) was a generous gift of Dr. Charles C. Sweeley.

The rate of hydrolysis of Tay-Sachs' ganglioside was much lower than the corresponding rates of the other two substrates. Larger amounts of enzyme (about 0.4 mg of protein/reaction mixture) and longer incubation periods (18 hr) were employed. Under these conditions (at a substrate concentration of 0.1 μmole/0.1 ml) about 40 mμmoles of *N*-acetylgalactosamine was released. In control experiments, which were similarly incubated in the absence of enzyme, *N*-acetylgalactosamine was not formed.

Effect of Inhibitors. Several compounds were tested and found to inhibit the hydrolysis of trihexosylceramide. Attempts were made to determine the type of inhibition and K_i values of the various inhibitors by plotting $1/V$ against $1/S$, according to Lineweaver and Burk. The resulting curves were not typical, either for the competitive or for the noncompetitive types of inhibition. In many cases, the results obtained in the presence and the absence of inhibitor yielded two curves which intersected at positive values of $1/S$, *i.e.*, at high substrate concentration an activation, rather than an inhibition, ensued. Effects of this nature have been ascribed to inhibitors acting on the substrate rather than on the enzyme (Webb, 1963). Because of this, the K_i values could not be determined. Instead, that concentration of each inhibitor which reduced the reaction rates by 50% was calculated from plots of velocity against concentration of inhibitor.

The inhibitors tested fell into two categories. In the first group, five- to tenfold excess of inhibitor over substrate was required for 50% inhibition. This group included long-chain fatty acids; glucosyl-, galactosyl-, and lactosylceramide; lecithin, sphingomyelin, and monosialoganglioside; *p*-nitrophenyl *N*-acetylgalactosaminide; free and *N*-acetylated glucosamine or galactosamine; and calcium ions. Sodium and potassium ion concentrations of 20–30 mM gave optimum hydrolysis and at higher concentrations inhibited; thus 0.15 M solutions gave about 50% inhibition. In the second category were *p*-hydroxymercuribenzoate and silver nitrate; 50% inhibition by these two compounds was obtained at 2×10^{-5} M inhibitor concentration. Two other SH inhibitors, *N*-ethylmaleimide and iodoacetate, did not inhibit the reaction even at over a hundred times higher concentrations.

One compound which inhibited the reaction was of special interest. "Tay-Sachs' ganglioside" (GalNAc-β-1,4-(NANA-β-2,3)-Gal-β-1,4-Glc-β-1,1-(2-*N*-acyl)-sphingosine) has a terminal *N*-acetylgalactosamine and was therefore hydrolyzed by the enzyme. The rate of hydrolysis of this compound was, however, very low. When this compound was added to incubation mixtures in which trihexosylceramide ("asialo-Tay-Sachs' ganglioside") was used as substrate, it acted as a potent inhibitor: 50% inhibition was obtained at 2×10^{-4} M ganglioside and by plotting $1/V$ against concentration of the inhibitor K_i of approximately 8×10^{-5} M was calculated.

Discussion

Three separate enzymes which hydrolyzed β-*N*-acetylhexosaminides were purified from calf brain (Frohwein and Gatt, 1966b, 1967). One ("*N*-acetylhexosaminidase"), of particulate origin, was effective with both *N*-acetylglucosaminides and -galactosaminides. The two others were obtained from the 100,000*g* supernatant; one of these ("*N*-acetylglucosaminidase") hydrolyzed glucosaminides and the other ("*N*-acetylgalactosaminidase") hydrolyzed galactosaminides preferentially. Several nonlipid *N*-acetylhexosaminides were used as substrates for these three enzymes (Frohwein and Gatt, 1967). In this communication, three sphingolipids, having a terminal *N*-acetylgalactosamine unit, were employed.

It was expected that the *N*-acetylglucosaminidase would not, but that the *N*-acetylhexosaminidase and *N*-acetylgalactosaminidase would hydrolyze the lipid substrates. However, the two enzymes obtained from the 100,000*g* supernatant were ineffective when tested at pH values between 3.8 and 8.0. The sphingolipids were hydrolyzed only by the "particulate" *N*-acetylhexosaminidase.

The optimal pH of hydrolysis by the *N*-acetylhexosaminidase was 3.8, both for the lipid substrates and for *p*-nitrophenyl *N*-acetylgalactosaminide. The hydrolysis of the lipid substrates was however confined to a rather narrow pH range, as opposed to that of *p*-nitrophenyl *N*-acetylgalactosaminide (Figure 1, curves A and C). Thus, at pH 5, the latter substrate was still hydrolyzed at about half the rate obtained at pH 3.8, while the trihexosylceramide was not hydrolyzed at all. As a consequence, the ratio of the hydrolysis rates of the nitrophenyl galactosaminide and trihexosylceramide varied with the pH of the reaction. This raises the possibility that this *N*-acetylhexosaminidase preparation contains two separate enzymes, one acting on lipid and the other on nonlipid substrates.

The hydrolysis of trihexosylceramide occurred only when the substrate was in a suitable physical form. To obtain this, it required the anionic detergent taurocholate. Excess of cations or the presence of any of

numerous lipids inhibited the reaction. Plots of $1/V$ against $1/S$ indicate that the inhibition is an effect on the substrate, presumably a change in micellary structure. In contrast, the inhibitory effect of silver nitrate and *p*-hydroxymercuribenzoate are probably due to their binding of SH groups in the enzyme, and similar inhibitions ensue with nonlipid substrates (Frohwein and Gatt, 1967).

Of the three lipid substrates tested, globoside and trihexosylceramide were hydrolyzed at similar rates. However, for hydrolysis of Tay-Sachs' ganglioside (trihexosylceramide to which an *N*-acetylneuraminic acid moiety is attached), much higher enzyme concentrations and longer periods of incubation were required. Another feature of the hydrolysis of Tay-Sachs' ganglioside was a lack of linearity with time. Only little hydrolysis occurred in the first few hours of incubations, so that incubations of 18–24 hr were required. Hydrolysis due to bacterial contamination was ruled out by running suitable controls from which enzyme was omitted.

It is possible that the *N*-acetylgalactosamine group of the Tay-Sachs' ganglioside is sterically hindered by the *N*-acetylneuraminic acid. A reciprocal hindrance of the *N*-acetylneuraminic acid by the *N*-acetylgalactosamine has also been observed. Thus Tay-Sachs' ganglioside is not attacked by neuraminidase from bacteria (Wiegandt, 1966) or brain (Z. Leibovitz and S. Gatt, unpublished experiments). However, the sialic acid residue of "hematoside" (*N*-glycolylneuraminylgalactosylglucosylceramide) is removed by the bacterial sialidase (Wiegandt, 1966). Similarly, the hematoside-like product obtained by the action of *N*-acetylhexosaminidase on Tay-Sachs' ganglioside (NANA-Gal-Glc-ceramide) could be split to yield NANA and lactosylceramide by the action of brain sialidase (Z. Leibovitz and S. Gatt, unpublished experiments).

Tay-Sachs' ganglioside accumulates in excessive amounts in brain tissue of patients with infantile amaurotic familial idiocy (Tay-Sachs' disease) (Fredrickson and Trams, 1966). The biochemical aberration leading to this accumulation is not known. The possibility that this might be due to a deficiency in a degradative reaction is being investigated.

Gatt and Berman (1961, 1963a) and Makita and Yamakawa (1963) have demonstrated the accumulation of relatively large quantities of trihexosylceramide in brain tissue of Tay-Sachs' disease; this compound is not found in nonpathological brain (Suzuki and Chen, 1967). In the present communication it has been shown that the hydrolysis of this trihexosylceramide by β-*N*-acetylhexosaminidase is strongly inhibited by Tay-Sachs' ganglioside. This observation might explain the accumulation of the trihexosylceramide in brain tissue of Tay-Sachs' disease. The large amounts of gangliosides present in the pathological tissue might inhibit the normally rapid hydrolysis of trihexosylceramide, resulting in its accumulation in the diseased tissue.

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