STUDIES ON GLYCOSPHINGOLIPIDS: THE ANOMERIC CONFIGURATION OF FABRY TRIHEXOSYL CERAMIDE AND THE RECOGNITION OF AN EXCEPTIONAL CASE OF TAY-SACHS DISEASE WITH VISCERAL INVOLVEMENT

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ABSTRACT

STUDIES ON GLYCOSPHINGOLIPIDS: THE ANOMERIC CONFIGURATION OF FABRY TRIHEXOSYL CERAMIDE AND THE RECOGNITION OF AN EXCEPTIONAL CASE OF TAY-SACHS DISEASE WITH VISCERAL INVOLVEMENT

By

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Two separate research topics are reported in this thesis: (1) the determination of the anomeric configuration of the glycosidic linkages of the carbohydrate moiety of Fabry kidney trihexosyl ceramide and (2) the recognition of an exceptional case of Tay-Sachs disease with visceral involvement and the partial identification of the three accumulating glycosphingolipids.

To establish the anomeric configuration of Fabry trihexosyl ceramide, NMR analyses were carried out on the trimethylsilyl derivatives of the intact lipid, the intact trisaccharide moiety cleaved from this lipid, and glucosyl ceramide derived from Fabry trihexosyl ceramide via mild acid hydrolysis. Comparison of these findings with NMR data from trimethylsilyl derivatives of Gaucher spleen glucosyl ceramide, reference mono- and disaccharides, and other model compounds established an all β

configuration for Fabry trihexosyl ceramide, viz., galactosyl-(β1→4)-galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide.

Todd Thomey was an 18-month old child who died from a lipid storage disorder diagnosed initially as classical Tay-Sachs disease. Hepatosplenomegaly, however, indicated an unusual visceral involvement and fresh-frozen sections of brain, kidney, spleen, and liver from this patient were analyzed for their glycosphingolipid content. Brain, kidney, spleen, and liver from normal juvenile controls were also investigated and the data compared.

Three glycosphingolipids had accumulated in Thomey's organs: (1) normal kidney globoside (galNAc-gal-gal-glc-cer) in the visceral organs, (2) asialo ganglioside G_{M2} (galNAc-gal-glc-cer) in the brain and liver, and (3) ganglioside G_{M2} (galNAc-gal(NANA)-glc-cer) exclusively in the brain. Thus, Thomey was not a classical case of Tay-Sachs disease, but an exceptional case with gross visceral involvement.

Since all three accumulating glycosphingolipids possess a terminal β-N-acetylgalactosamine moiety, it is reasonable to postulate a β-N-acetylhexosaminidase deficiency as the cause of this disease. This possibility

is discussed in relation to the only other reported case of exceptional Tay-Sachs disease in which a deficient β-N-acetylhexosaminidase was demonstrated.

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INTRODUCTION AND LITERATURE REVIEW

Interest in the neutral glycosphingolipids has increased greatly over the past decade due to their implication as haptenic compounds in antigen-antibody reactions and their involvement in several genetic diseases classified as inborn errors of lipid metabolism. By nature, neutral glycosphingolipids are compounds composed of (1) the long-chain base sphingosine and its homologs; (2) the sugars glucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, and, in the case of the blood group substances, fucose; (3) long-chain fatty acids of varying chain length. Though present in most mammalian tissues and organs, as well as in blood, evidence as to the structure and overall function of these compounds has remained scant until recent years. With the development of more sophisticated and efficient techniques of detection and isolation, however, the chemical structures of many glycosphingolipids have now been elucidated.

In one of the earliest structural studies, Nakayama determined via methylation in 1950 that the glycosidic linkage of galactocerebroside was between the anomeric carbon of galactose and the primary hydroxyl group of sphingosine (1). Fujino and Negishi in a

follow-up investigation used enzymatic hydrolysis with β-galactosidase to show that this linkage was of the β-configuration (2). The glycosidic linkage of glucocerebroside isolated from Gaucher spleen was also shown to be of the β-configuration, based on infrared spectroscopy of the glucosyl sphingosine sulfate prepared from this lipid via the addition of methanolic H_2SO_4 to the acyl-free lipid dissolved in anhydrous methanol (3). By careful degradation of kerasin (galactocerebroside) and Gaucher cerebroside and by comparison of the products, Carter, et al. in 1961 confirmed the results of Rosenberg and Chargaff and concluded that kerasin and Gaucher cerebroside were identical compounds except in their sugar content (4).

At about the same time, Rapport, et al. used hapten inhibition in an immunochemical study to show that the disaccharide moiety of cytolipin H (lactosyl ceramide) has the β-configuration (5). Galactosyl-(1→4)-glucosyl-ceramide isolated from human erythrocytes was found by Yamakawa, et al. to also be of the β-configuration (6). Using permethylation and infrared spectroscopy, these investigators determined that the linkage between galactose and glucose in the lactosyl moiety was of the β1→4 type. Continuing their work on human red blood cell glycosphingolipids, Yamakawa, et al. in 1962 carried out a series of studies on globoside (tetrahexosyl ceramide) extracted

from human red cell stroma (7). Combining periodate consumption data with that obtained from permethylation, gas-liquid chromatography, optical rotation, and infrared spectroscopy, these workers proposed the globoside structure to be N- acetyl galactosaminoyl-(β1→6)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl ceramide. Makita, et al. investigated the structure of human kidney globoside, however, using the same methods and postulated N-acetylgalactosaminoyl-(β1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl ceramide as the structure for this compound (8). In the same study, human red cell globoside was reexamined and evidence for a terminal 1→3 linkage was found rather than the 1→6 linkage previously reported (7). In 1965, Yamakawa, et al. settled the problem of human red cell globoside structure (9). Using improved gas-liquid chromatographic techniques, they discovered that the peak previously identified as 2,3,4-trimethylgalactoside was a product of inadequate solid support and stationary phase column conditions and not actually due to the presence of a terminal 1→6 linkage in the globoside carbohydrate moiety. Rather, they found a substantial peak for 2,4,6-trimethylgalactoside and, together with periodate consumption data, these new data indicated a terminal 1→3 linkage. Thus, human red cell globoside was assigned the structure N-acetylgalactosaminoyl-(1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl ceramide and labeled globoside I to distinguish it from

the blood-group active globoside II and III, also isolated from human red cell stroma and found to contain fucose, glucosamine, and sialic acid, in addition to glucose, galactose, and galactosamine. Globoside I was found by Rapport and Graf (10) to be immunochemically identical to cytolipin K, a tetrahexosyl ceramide previously obtained from human kidney containing fatty acid, sphingosine, glucose, galactose, and galactosamine in the molar ratio 1:1:1:2:1 and shown to have haptenic properties (11). Another glycosphingolipid of interest, possessing haptenic activity and having the general globoside structure, is the Forssman hapten isolated from equine kidney and spleen. This compound has the same composition as globoside I and cytolipin K, but was shown by Makita, et al. to differ from these lipids in its mobility on thin-layer chromatography, its optical rotation, and its immunological reactivity (12). Chemical investigation of this glycosphingolipid led to the assignment of the structure N-acetylgalactosaminoyl-(α1→3)-galactosyl-(β1→4)-galactosyl-(1→4)-glucosyl ceramide, thus differing from globoside I and cytolipin K only in the anomeric configuration of the terminal galactosidic linkage. This finding dramatizes how such a seemingly unimportant structural difference can profoundly affect the physicochemical and serological behavior of glycosphingolipids of identical gross chemical composition.

Glycosphingolipids in various disease states have also been studied structurally. In 1963, Sweeley and Klionsky found that trihexosyl ceramide is the lipid that accumulates in the kidney and other organs and tissues of persons afflicted with Fabry's disease, an X-linked lipidosis (13). Further investigations of this glycosphingolipid by these workers revealed the structure to be galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide (14). The determination of the anomeric configuration of the glycosidic linkages of the carbohydrate moiety remained unsolved until the present and will be the topic of part one of this thesis. A study by Makita and Yamakawa of glycosphingolipids isolated from normal human kidney revealed that trihexosyl ceramide from this tissue was structurally identical to that isolated from Fabry kidney (15).

Numerous investigations on glycosphingolipids isolated from mammalian sources other than human have been conducted in recent years. Gray in 1964 isolated and characterized glycosphingolipids from BP8/C3H mouse ascites-sarcoma cells and found lactose and galactosyl-(β1→6)-galactose among the hydrolysis products of dihexosyl ceramide and trihexosyl ceramide, respectively (16). In a later study, Adams and Gray elucidated the oligosaccharide moieties of pig lung glycosphingolipids and found structures similar to glycosphingolipids identified in

human kidney and red blood cells (17). Combining a two-stage methylation-methanolysis with gas-liquid chromatographic and infrared spectroscopic data, they identified the following glycosphingolipids: glucosyl-(β1→1)-ceramide, galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide, a small amount of galactosyl-(β1→4)-galactosyl-(β1→1)-ceramide, galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide, and N-acetylgalactosaminoyl-(1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide. More recently, Miyatake, et al. determined the structure of the main glycosphingolipid of hog erythrocytes to be N-acetylgalactosaminoyl-(β1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide (18). The terminal β-linkage was uncovered via the liberation of the terminal N-acetylgalactosamine with β-N-acetylhexosaminidase obtained from hog epididymis tissue. Structures of the carbohydrate moieties of glycosphingolipids from the kidneys of five different strains of mice were elaborated in 1968 by Adams and Gray (19). In all five strains studied, the major mono-, tri-, and tetrahexosides found were glucosyl-(1→1)-ceramide, galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide, and N-acetylgalactosaminoyl-(1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide, respectively. In one strain, however, galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide was the major dihexoside and in the other strains the less common galactosyl-(β1→4)-

galactosyl-(β1→1)-ceramide was dominant. A similar study of rat kidney glycosphingolipids by Kawanami confirmed the structure of rat kidney globoside to be N-acetyl-galactosaminoyl-(1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl ceramide, with a small amount of N-acetylgalactosaminoyl trigalactosyl ceramide also being present (20). One of the most complex glycosphingolipids to be structurally identified is the pentahexosyl ceramide of rabbit erythrocytes and reticulocytes. Eto, et al. in a chemical and immunochemical study proposed the structure of this lipid to be galactosyl-(1→3)-galactosyl-(1→3)-N-acetyl-glucosaminoyl-(1→3)-galactosyl-(1→4)-glucosyl ceramide (21). It was also shown that this glycosphingolipid inhibits the agglutination of human B-red blood cells with its corresponding antibody, suggesting a terminal α-galactosidic linkage as is found in blood group B carbohydrate.

Another approach to the structural study of glycosphingolipids has been enzymatic hydrolysis. As cited earlier, Fujino and Negishi used α- and β-galactosidase to determine the β-configuration of galactocerebroside in one of the earliest enzymatic analyses (2). Lactosyl ceramide from ox spleen was hydrolyzed by rat brain β-galactosidase in a study carried out by Gatt and Rapport, thus confirming the structure of this glycosphingolipid to be galactosyl-(β1→4)-glucosyl-(1→1)-ceramide (22).

In a subsequent study, Gatt confirmed the β-configuration of Gaucher glucosyl ceramide using enzymatic hydrolysis with ox brain β-glucosidase (23). Frohwein and Gatt employed calf brain β-N-acetylhexosaminidase in the hydrolysis of synthetic N-acetylgalactosaminoyl-(β1→4)-galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide, human red blood cell globoside, and Tay-Sachs ganglioside (24). This work provided further proof for the terminal β-galactosidic linkage in globoside from human red cells. As indicated earlier, Miyatake, et al. in a recent investigation used β-N-acetylhexosaminidase from hog epididymis tissue to establish the terminal β-galactosidic linkage of globoside isolated from hog erythrocytes (18). Thus, enzymatic hydrolysis with the appropriate glycosidases has proven valuable in determining or confirming the anomeric configuration of glycosidic linkages of the carbohydrate moieties of various glycosphingolipids.

Nuclear magnetic resonance spectroscopy (NMR) has been used infrequently to determine anomeric configurations in the glycosphingolipids. In the only published study to date, Kawanami used NMR to establish the structure of trihexosyl ceramide isolated from Nakahara-Fukuoka sarcoma tissue as galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)-ceramide (25). This terminal α-galactosidic linkage is, however, inconsistent with results obtained for the terminal glycosidic linkage in

other glycosphingolipids, with the exception of the Forssman hapten and Gray's BP8/C3H mouse ascites-sarcoma trihexosyl ceramide. In the following study, the complete structure of the carbohydrate moiety of trihexosyl ceramide isolated from the kidneys of two Fabry patients will be discussed. Convincing NMR spectroscopic evidence for exclusive β configurations, viz., galactosyl-(β1→4)-galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide, is presented, thus completing the structural work on this glycosphingolipid begun by Sweeley and Klionsky in 1963 (13).

EXPERIMENTAL

A. Materials

Fabry trihexosyl ceramide (GL-3) was extracted from two portions of fresh-frozen kidney obtained from two deceased male Fabry patients. Glucosyl ceramide (GL-1) was isolated from the spleen of a patient with Gaucher's disease. Glycosphingolipids used as reference standards for thin-layer chromatography (TLC) were isolated and purified from packed human red blood cells by the method of Vance and Sweeley (26). All solvents and chemicals used were analytical or reagent grade unless otherwise indicated.

Heat-activated Unisil (200/325 mesh, Clarkson Chemical Co., Williamsport, Pa.) was used in all silicic acid column chromatography. For all TLC, glass plates were spread-coated to a thickness of 250μ with Silica Gel G (Brinkmann Instruments, Inc., Westbury, New York) using a Desaga TLC spreader. Gas-liquid chromatography (GLC) was carried out on a F & M Model 402 gas chromatograph (Hewlett Packard Co., Avondale, Pa.) equipped with a glass, six-foot 3% SE-30 or OV-1 column (packing from Applied Science Co., State College, Pa.). Hexamethyldisilazane and trimethylchlorosilane used in the silylating

(TMS) reagent were purchased from Applied Science Co., State College, Pa.

B. Methods

Lipid Extraction

Total lipids weighing 3.414 gm were extracted according to the method of Folch, et al. (27) from 32.8 gm (wet weight) of Fabry kidney from two deceased male patients. Prior to column chromatography, the total lipids in chloroform were shaken vigorously with several grams of Celite filter aid and suction-filtered to obtain a clear filtrate. The residue was then washed with chloroform-methanol (CM) 1:1 (v/v) to obtain a second clear filtrate. This filtration removed all non-lipid or CM-insoluble impurities which would interfere with efficient column chromatography.

Column Chromatography and Analytical TLC

A 110-gm Unisil column was prepared and pre-washed with 10 column volumes of CM 9:1 (v/v) and enough $CHCl_3$ to restore column translucency. The $CHCl_3$ filtrate was applied and eluted with CM 9:1 (v/v) until the Lands lipid spot test (28) showed an absence of lipid in the eluate. To remove glycosphingolipids and some polar lipids, CM 1:1 (v/v) was next added, followed by pure methanol to remove remaining lipids. The Lands test was used after these elutions as before.

Analytical TLC was done on 100 µl each of the CM 9:1 (2.399 gm eluted) and 1:1 (452 mg eluted) column eluates and the CM 1:1 (378 mg) Celite filtrate to detect the presence of glycosphingolipids. Upon iodine vapor visualization, spots corresponding to lactosyl ceramide (GL-2) and GL-3 were found in the CM 1:1 column eluate and Celite filtrate, as is shown by Figure 1. Celite 545 in CM 2:1 (v/v) was added to a 1.5-cm i.d. column to give a bed height of 19 cm. The CM 1:1 eluate, redissolved in CM 2:1, was applied and eluted with CM 2:1 to remove colored impurities appearing on TLC. This eluate was then dialyzed against distilled water in the cold overnight with two changes of water. The original Celite filtrate was subjected to mild aklali-catalyzed methanolysis (26) to remove phospholipid impurities and then eluted from a 15-gm Unisil column with CM 19:1, 2:1, and 1:4 (v/v), respectively. Glycosphingolipids were detected via TLC in the CM 2:1 eluate and this fraction was likewise dialyzed against distilled water in the cold. Lipids from the combined dialysates, totaling 528 mg, were then ready for density gradient column chromatography as a final purification step.

Density Gradient Column Chromatography

In this procedure, a density gradient is established between two solvents of unequal density. The level

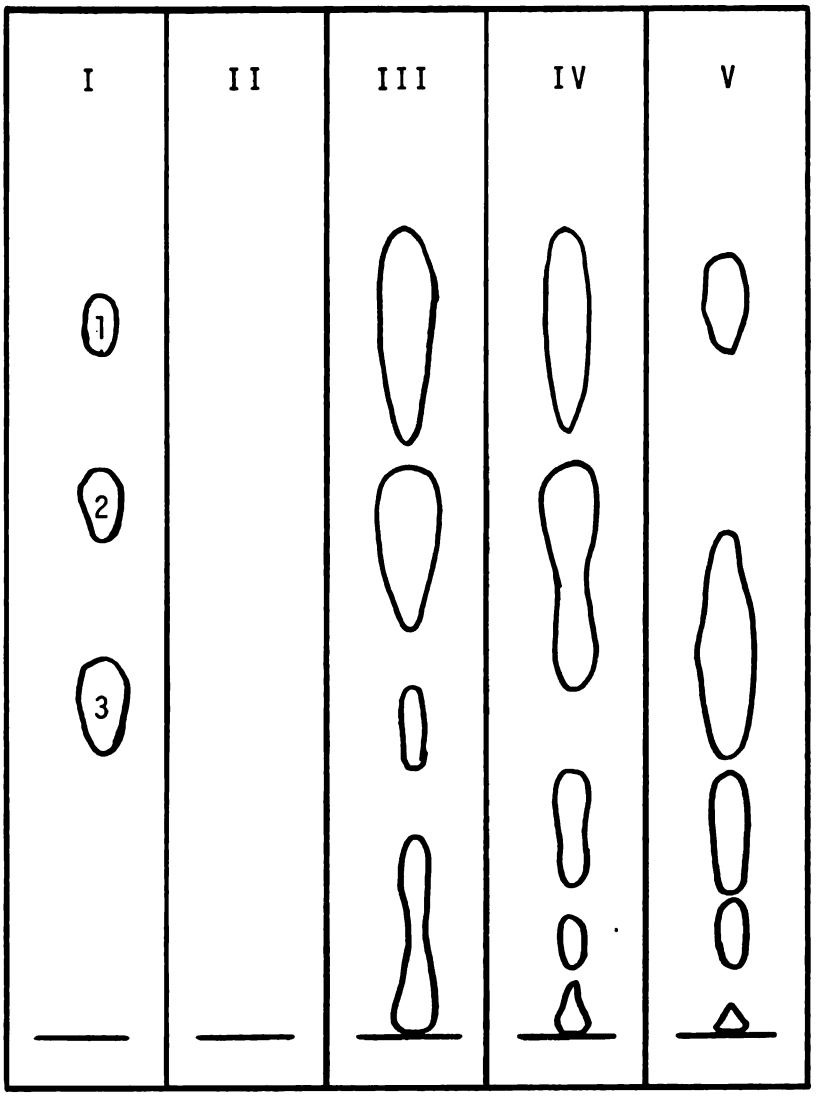

Figure 1. Thin-layer chromatography of Fabry kidney glycosphingolipids. I. Human red blood cell glycosphingolipid reference standards: (1) lactosyl ceramide (GL-2), (2) trihexosyl ceramide (GL-3), and (3) globoside (GL-4). II. CM 9:1 fraction. III. CM 1:1 fraction. IV. Celite filtrate. V. Human plasma phospholipids.

of the less dense solvent must be higher than that of the denser solvent to prevent backward flow due to the force of gravity. The required height difference is given by the following relationship:

$$\text{height ratio} = \frac{\text{sp. gravity more dense solvent}}{\text{sp. gravity less dense solvent}}.$$

For this study, the mixing bottle contained 1700 ml of CM 94:6 (v/v, sp. gravity = 1.447) and to another bottle was added enough CM 65:35 (v/v, sp. gravity = 1.225) to give a solvent height 1.16 greater than that in the mixing bottle. A 150-gm Unisil column with 2.5-cm i.d. was prepared for this elution. After fitting a Gilson automatic fraction collector with 225 30-ml test tubes, the elution was started and allowed to run overnight. The Lands spot test showed lipid present in tubes 70 through 190. The contents of every three tubes were pooled and, beginning with tubes 70-72 (fraction 24), were taken to dryness and weighed. Fractions 28-30, 32-35, and 41-54 contained the bulk of the weight as is shown by the graph in Figure 2. Analytical TLC of these fractions showed GL-2 to be present in 28-30 and 32-35 and GL-3 in 41-54. Phospholipid impurities persisted, however, and the fractions were subjected to mild alkali-catalyzed methanolysis. After repeated hexane extraction of the methanolysates, analytical TLC showed the fractions to contain pure GL-2 and GL-3. Final weights of pure GL-2 and GL-3 were 63 mg and 272 mg, respectively.

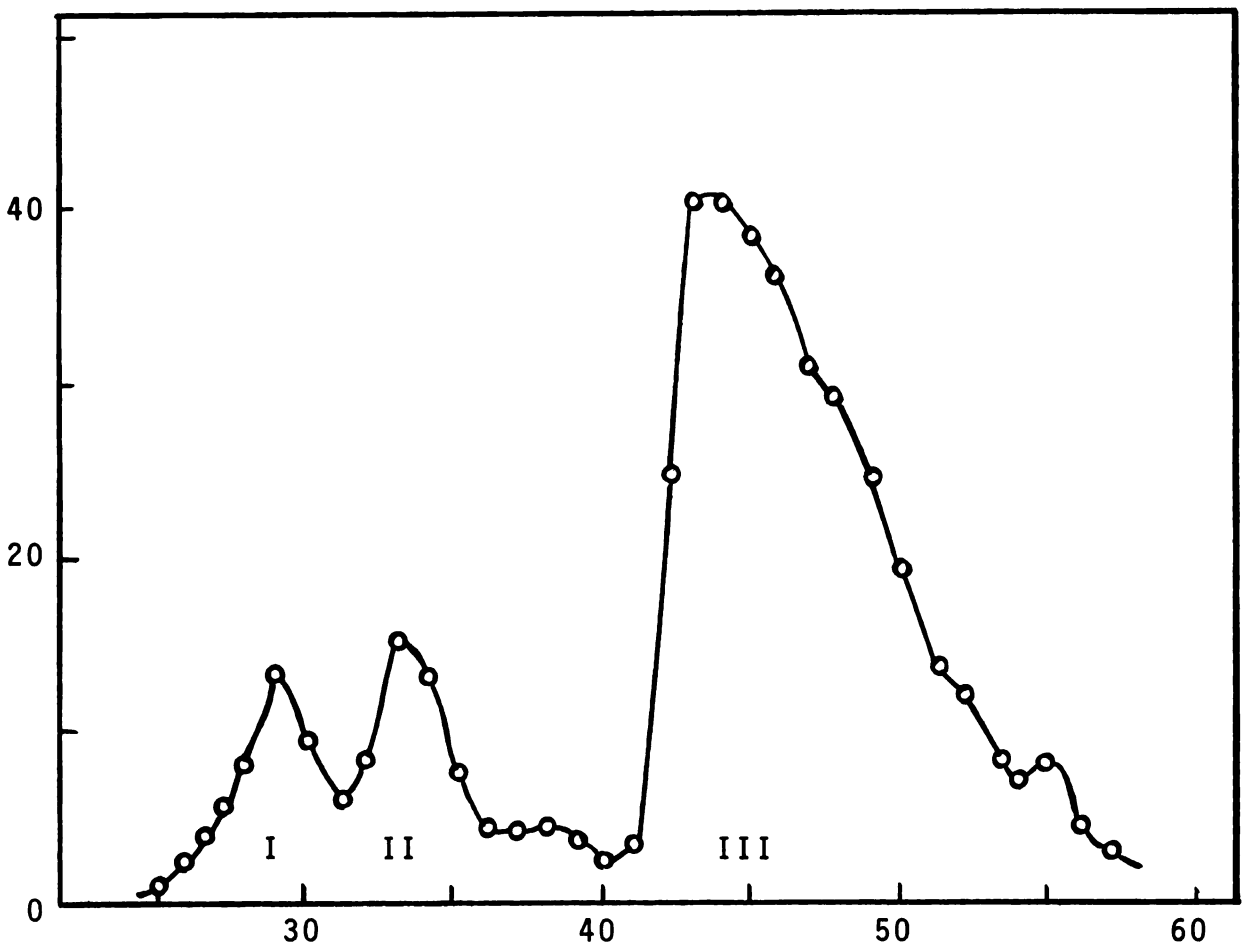

Figure 2. Density gradient elution curve of Fabry kidney glycosphingolipids. Ordinate: weight of lipid eluted in mg; abcissa: fraction number (3 tubes = 1 fraction). I & II = dihexosyl ceramide; III = trihexosyl ceramide.

Degradation of GL-3

To obtain the intact oligosaccharide moiety for structural analysis, 100 mg of pure GL-3 was subjected to the method of Hakomori (29). This procedure involves an osmium tetroxide-catalyzed periodate oxidation of the sphingosine moiety of the glycosphingolipid. The following is a stepwise account of the degradation:

(1) One hundred mg of Fabry GL-3 was dissolved in 25 ml of pyridine-acetic anhydride 3:2 (v/v) and allowed to stand at room temperature in a stoppered flask overnight.

(2) To remove the acetylating agent, 25 ml of water was added to the flask and the contents lyophilized.

(3) The yellowish residue was dissolved in a small volume of CM 1:1, transferred to a screw-cap test tube, and evaporated to dryness under N_2. To insure complete removal of water, first absolute ethanol and then acetone were successively added, evaporating to dryness under N_2 each time.

(4) The residue was next dissolved in 25 ml of dry dioxane and 3.75 ml of freshly-prepared 0.2M $NaIO_4$ plus 0.50 ml of 1% OsO_4 in dry ethyl ether added with thorough mixing. The mixture was allowed to stand in the cold overnight.

(5) A few drops of ethylene glycol were then added to react with excess $NaIO_4$. When no additional

precipitation was observed, the mixture was divided between two 40-ml centrifuge tubes, twice the volume of $CHCl_3$ was added to each with mixing, and both tubes were spun in a table centrifuge for about five minutes. The $CHCl_3$ lower phases were removed, divided among four 40-ml centrifuge tubes, equal volumes of distilled water added, and the tubes centrifuged again for five minutes. This water wash was repeated ten times, the aqueous upper phases being discarded each time. The four $CHCl_3$ extracts were then combined and evaporated to dryness under N_2.

(6) Vacuum desiccation over KOH pellets for 24 hours was employed to remove all harmful OsO_4 vapors.

(7) The dry residue was dissolved in 10 ml of methanol, 2.50 ml of freshly-prepared 0.5% NaOMe in methanol added with mixing, and the solution allowed to stand at room temperature for one hour. Next was added enough aqueous 0.5% HOAc to bring the solution to neutrality. After the subsequent addition of twice the volume of distilled water, the solution was centrifuged for ten minutes. The clear, yellowish supernatant was removed, taken to dryness on a flash evaporator, and the residue dissolved in 7.0 ml of methanol.

GLC of Oligosaccharide

Two μmoles of a mannitol standard solution (2.0 μmole/ml) were added to 0.14 ml (1/50 of the original

material) of the Fabry oligosaccharide in MeOH, the solution evaporated to dryness, and 3.0 ml of 0.75 N HCL in dry MeOH added for overnight methanolysis at 82°C. Subsequent isothermal GLC on a 3% OV-1 column at 170°C of the TMS derivative of the dry methanolysate indicated a trisaccharide with a galactose:glucose ratio of 2.06:1.00. The total amount of GL-3 oligosaccharide obtained was calculated to be 20.5 μmoles.

To prove the intactness of the trisaccharide, another 1/50 of the original material in solution was evaporated to dryness and TMS reagent added directly and allowed to stand overnight. Likewise, an aqueous solution of 5 mg each of D-glucose, D-lactose, and raffinose was allowed to equilibrate for one hour and then evaporated to dryness under N_2. One ml of TMS reagent was next added to this mixture and allowed to stand overnight. A programmed GLC run on a 3% OV-1 column, beginning at 140°C and rising at 2°/min, of the TMS sugar mixture gave the α- and β-anomers of the three sugars at the retention times listed in Table 1. The TMS Fabry trisaccharide programmed under identical conditions gave α- and β-anomers only in the region of the trisaccharide raffinose.

NMR of TMS Oligosaccharide

The TMS derivative of the oligosaccharide was prepared and purified as a pale yellow oil upon two

Table 1.--Programmed GLC of TMS Reference Sugars and TMS Fabry Trisaccharide.

Sugar	Anomer	Retention Time (Minutes)	
		Sugar Mixture	Fabry Trisaccharide
Glucose	α	20.5	-
	β	25.5	-
Lactose	α	53.5	-
	β	58.5	-
Raffinose	α	100.5	87.7
	β	105.5	111.7

washings with dry ethyl acetate. The oil was then taken up in a small volume of dry ethyl acetate and sent to Dr. C. E. Griffin at the University of Pittsburgh for NMR spectrometry. A Varian Associates HA-100 spectrometer was used in this analysis. External $CHCl_3$ was used as a lock signal with the TMS oligosaccharide being dissolved in $CDCl_3$. Sweep widths of 50-200 Hz were used in scanning the anomeric proton region. All signals were accumulated by a Northern Scientific computer and 100-200 sweeps were taken to obtain chemical shift and coupling constant values.

The data collected in this analysis were coupled with NMR data previously obtained from intact TMS Fabry GL-3 and compared with spectra of TMS lactose and other established NMR values for anomeric configurations of glycosidic linkages cited in the literature.

To establish the anomeric configuration of the remaining glycosidic bond in the glucosyl-(1→1)-ceramide portion of Fabry GL-3, mild acid hydrolysis (0.3N HCl in CM 2:1, v/v, at 60°C for three hrs.) was used on 50 mg of GL-3 to cleave off the two galactose units, leaving the GL-1 moiety intact. Purity of the derived GL-1 (yield, 5 mg) was established by TLC and the pure TMS derivative was prepared for NMR as before. The TMS derivative of known glucosyl-(β1→1)-ceramide from Gaucher spleen (3,4) was likewise prepared and, together with the TMS

Fabry GL-1, was sent to Dr. Griffin for NMR analysis. The data were compared with the NMR spectra of TMS α- and β-D-methyl glucoside and galactoside and of intact TMS Fabry GL-3.

Optical Rotation of GL-3

Optical rotation data were obtained using a Zeiss polarimeter fitted with a 1.0-cm cell. Pure Fabry GL-3 and pure GL-3 isolated from human red blood cells were dissolved in dry pyridine and their rotations versus the mercury 578 mμ and 546 mμ wavelengths compared.

RESULTS

A. NMR Analyses

The NMR spectrum of TMS GL-1 derived from Fabry GL-3 via acid hydrolysis exhibited an anomeric proton resonance at τ = 6.03 ppm, J = 7.0 Hz. These data compare very favorably with those obtained for TMS Gaucher spleen GL-1, glucosyl-(β1→1)-ceramide (3,4): τ = 6.02 ppm, J = 7.0 Hz; TMS lactose: H^{β}_{G}, τ = 5.86 ppm, J = 7.6 Hz; H^{α}, τ = 5.11 ppm, J = 3.0 Hz; TMS methyl β-galactoside: τ = 6.06 ppm, J = 7.0 Hz; and methyl β-glucoside: τ = 5.95 ppm, J = 7.0 Hz, in DMSO-d_6 (30). Values for the corresponding α-anomers are quite different, indicating a β-linkage for Fabry GL-1. For TMS maltose, O-α-D-glucopyranosyl-(1→4)-β-D-glucopyranose, these parameters are: H^{α}_{G}, τ = 4.69 ppm, J = 3.2 Hz; H^{β}, τ = 5.34 ppm, J = 7.1 Hz, in acetone-d_6 (31); TMS methyl α-galactoside: τ = 5.53 ppm, J = 3.0 Hz; and methyl α-glucoside: τ = 5.45 ppm, J = 3.0 Hz, in DMSO-d_6 (30).

Two anomeric proton resonances were identified for TMS Fabry GL-3 trisaccharide: a doublet at τ = 5.98 ppm, J = 7.3-7.5 Hz, with relative intensity of 2.5, and a doublet at τ = 5.00 ppm, J = 3.0 Hz, with relative intensity of 0.5. Broadened upfield resonances indicated

protons with slightly differing chemical shifts or coupling constants. The H_G^β of the two galactosidic linkages plus the anomeric proton of β-glucose adequately account for the 2.5 proton doublet at τ = 5.98 ppm, while the anomeric proton of α-glucose is assumed to contribute the 0.5 proton doublet at τ = 5.00 ppm. In addition, the spectrum of intact TMS Fabry GL-3 exhibited broadened doublets at τ = 6.01 ppm, J = 7.0 Hz, and τ = 5.81 ppm, J = 7.0 Hz, in the H_G^β chemical shift region and showed no doublets in the H_G^α chemical shift region (τ = 4.5-5.5 ppm), as is illustrated by Figure 3. The two smaller resonances present in this region will be discussed in the following section. These data, therefore, coupled with NMR parameters cited in the preceding paragraph strongly suggest an all-β anomeric configuration for Fabry GL-3.

B. Optical Rotation of Fabry and Red Cell GL-3

For Fabry GL-3, it was found that $[\alpha]_{546}^{23} = +30^o$ and $[\alpha]_{578}^{23} = +26^o$ (c = 2.01). For human red blood cell GL-3, it was found that $[\alpha]_{546}^{23} = +18^o$ and $[\alpha]_{578}^{23} = +15^o$ (c = 2.00). These data cannot readily be compared to those determined by Kawanami for Nakahara-Fukuoka sarcoma GL-3 (25): $[\alpha]_{589}^{23} = +23.9^o$ (c = 1.02), and by Makita for normal human kidney GL-3 (32): $[\alpha]_{589}^{23} = +26.1^o$ (c = 2.13), since the standard sodium D-line (589 mμ) was not used. An earlier rotation, however, taken by Sweeley and Klionsky (14) gave $[\alpha]_{589}^{25} = +34.2^o$ (c = 2.02) for Fabry GL-3.

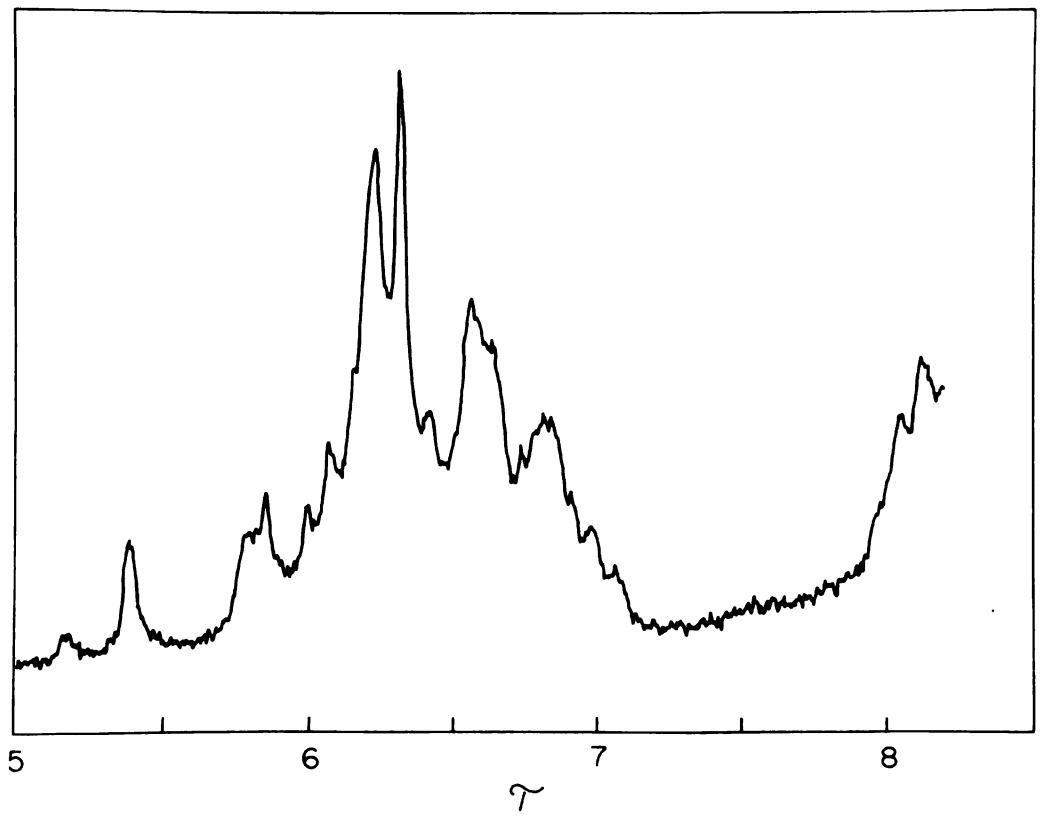

Figure 3. NMR spectrum of Fabry trihexosyl ceramide (GL-3) dissolved in $CDCl_3$.

DISCUSSION

The method of Hakomori (29) used in this work to obtain the intact trisaccharide unit from Fabry GL-3 provides a convenient means of obtaining the oligosaccharide moieties from both simple and complex glycosphingolipids. Free from the long-chain base (LCB) and acyl portions of the original molecule, the anomeric configuration of the oligosaccharide moiety can then be examined via NMR spectrometry. Thus, possible interference from protons in the LCB and acyl moieties is eliminated and anomeric configurations can be unambiguously assigned.

In order to assign anomeric configurations to the glycosidic linkages of Fabry GL-3, it was necessary to compare the NMR data obtained with values for reference sugars analyzed under identical conditions and with known NMR parameters for various sugars cited in the literature (30,31,33-35). Typical chemical shift values for glycosidic linkages are those established by van der Veen (in D_2O): H_G^{α}, τ = 4.88 ± 0.33 ppm; H_G^{β}, τ = 5.56 ± 0.06 ppm (34). This difference is further emphasized when the C-1, C-2 proton-proton spin-spin coupling constants are considered. The diaxial relationship of the protons in β-linkages yields a coupling constant (J_{12} = 7.2 ± 0.2 Hz)

more than twice that found for the axial-equatorial arrangement in α-linkages (J_{12} = 3.2 ± 0.6 Hz) (34). This is a result of the fact that C-1 protons of β-glycosidic linkages are more highly shielded than those of α-linkages. From NMR data previously cited for intact TMS Fabry GL-3 and TMS Fabry GL-1 derived from GL-3, unambiguous anomeric assignment of the three glycosidic linkages of Fabry GL-3 as all-β has been established. The resonance at τ = 5.38 ppm was due to the presence of some $CHCl_3$ as an impurity in the $CDCl_3$. The slight resonance at τ = 5.18 ppm remains unassigned, since it is downfield from the H_G^{α} region in TMS methyl α-galactoside (τ = 5.53 ppm) and is displaced upfield from the expected anomeric region (τ = 4.6-4.9 ppm) for a galactosyl-(α1→4)-galactosyl linkage. It can thus be assumed that this resonance is not due to an anomeric proton. Figure 4 illustrates the structure of the intact Fabry GL-3 molecule.

The findings contrast with those of Kawanami (25) and Gray (16) in their studies of glycosphingolipids of cancer tissues. It may be possible, however, that the GL-3 configurations found by these investigators are peculiar to glycosphingolipids of cancerous tissues and, therefore, cannot be accurately compared with the data presented here.

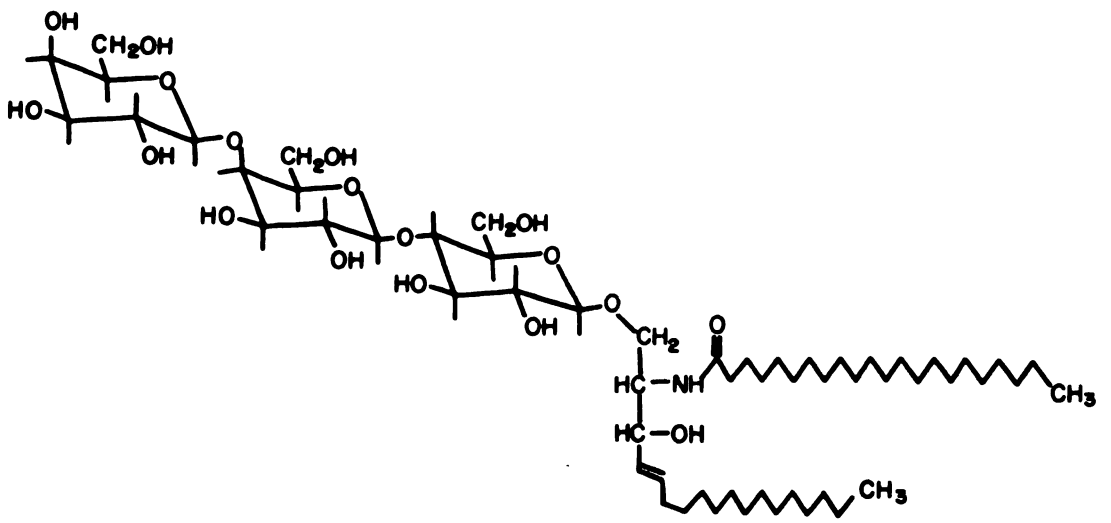

Figure 4. Structure of Fabry GL-3, galactosyl-(β1→4)-galactosyl-(β1→4)-glucosyl-(β1→1)-ceramide.

A very recent paper by Kint (36) shows the complete absence in leukocytes of the enzyme α-galactosidase from males with Fabry's disease and a reduced level of this enzyme in female carriers. Kint uses this evidence, without structural study of Fabry GL-3 itself, to suggest that the accumulating GL-3 possesses a terminal α-galactosidic linkage. Since the NMR analyses of highly purified Fabry trisaccharide and intact GL-3 have unequivocally shown the glycosidic linkages of this compound to be all β, Kint's hypothesis seems untenable. In addition, his enzyme assays were carried out using artificial substrates; nowhere does he show that his nonspecific α-galactosidase preparation cleaves the terminal galactose moiety of Fabry GL-3. Until this is demonstrated, coupled with a careful structural analysis of purified GL-3 from his patients, his proposal of a GL-3 with a terminal α-galactose moiety is indeed questionable.

SUMMARY

From the data presented, it has been concluded that the glycosidic linkages in Fabry GL-3 are unmistakably in the β anomeric configuration. Thus it now seems certain that Fabry's disease is strictly an enzyme deficiency disease and not a combination of a deficient enzyme and defective substrate.

NMR analysis of the TMS derivative of the intact glycosphingolipid proved invaluable in this study, indicating that the determination of anomeric configurations of other glycosphingolipids via this method is quite feasible. Despite the high proton content of the intact compound, the NMR absorptions of interest occur in a region of the spectrum relatively free from conflicting absorptions. An assignment of anomeric configuration could therefore be made without ambiguity.

A CASE OF TAY-SACHS DISEASE WITH VISCERAL INVOLVEMENT: EVIDENCE FOR THE ACCUMULATION OF THREE GLYCOSPHINGOLIPIDS IN THE BRAIN AND VISCERAL ORGANS

INTRODUCTION AND LITERATURE REVIEW

Classical Tay-Sachs disease is a biochemical malady affecting the central nervous system (CNS), resulting in death before the age of four. Onset generally begins by six months of age and involves progressive developmental retardation, dementia, paralysis, and blindness, characterized by a cherry-red spot in the retina. These clinical symptoms are a direct result of the steady accumulation of ganglioside G_{M2}, N-acetylgalactosaminoyl-(β1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide (37,24),

$$\left(\begin{matrix}3\\ \uparrow\\ 2\end{matrix}\right)$$

N-acetylneuraminyl

and, to a lesser extent, its asialo derivative, N-acetylgalactosaminoyl-(β1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide (37,24), in the ganglion and glial cells of the CNS. An excellent review (38) covers the medical and biochemical history of classical Tay-Sachs disease and, therefore, those details will not be discussed here. Information is not so abundant, however, concerning aberrations of Tay-Sachs disease involving the visceral organs. Only a few such instances have been recorded and some of them have not been clearly differentiated from other ganglioside storage disorders.

Perhaps the earliest recorded definitive case of Tay-Sachs disease with visceral involvement was described by Turban in 1944 (39). A non-Jewish child died at age two from a progressive neurological illness. Histologically, the brain was typical of Tay-Sachs disease and the cerebral cortex exhibited a fivefold increase in gangliosides. Vacuolation of the liver and renal tubules was noted and foam cells were found in the pulmonary alveoli.

Similarly, Norman, et al. (40) in 1959 reported a case of a non-Jewish child who died at age 17 months from Tay-Sachs disease and who exhibited gross visceral involvement. These workers found that, in addition to a large excess of gangliosides in the brain, the liver, spleen, lymph glands, thymus, bone marrow, adrenals, lung, and intestine exhibited lipid storage. A large excess of hexosamine was discovered upon chemical analysis of the liver and spleen. This case had originally been diagnosed as a Niemann-Pick, but absence of sphingomyelin accumulation in the brain and viscera plus the excessive ganglioside accumulation confirmed that this was a Tay-Sachs patient. No attempt was made in this study to characterize the accumulating ganglioside or hexosamine material.

In still another case reported in 1964, Norman et al. (41) investigated the brain and viscera of a non-Jewish child who died at age 16 months from Tay-Sachs

disease with visceral involvement. Gangliosides, as estimated from the neuraminic acid content, were elevated 3-1/2 times in cerebral cortex and 10 times in white matter. Foam cells were found in the alveolar septa and vacuolation of liver and renal tubular cells was evident. The ganglioside accumulation in this case also resembled that of gargoylism, especially the levels noted in the cortex. But a final diagnosis of Tay-Sachs disease was made on the basis of the tenfold increase in white matter gangliosides. Confusion existed at this time because ganglioside separation techniques were still not entirely reliable and the nature of the stored ganglioside in Tay-Sachs disease remained uncertain. Svennerholm and Raal (42) had shown in 1961 that more than 90% of the total Tay-Sachs gangliosides were of an "abnormal" type with higher R_f-values on TLC than normal brain gangliosides. But the final structure of Tay-Sachs ganglioside remained somewhat ambiguous until Ledeen's work in 1965 (37) finally established its structure to be N-acetylgalactosaminoyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide, verifying

$$\left(\begin{matrix}3\\ \uparrow\\ 2\end{matrix}\right)$$

N-acetylneuraminyl

the structure proposed by Makita and Yamakawa in 1963 (43).

Eeg-Olofsson, et al. in 1966 (44) and Suzuki, et al. in 1969 (45) reported cases of Tay-Sachs disease in which ganglioside G_{M2} was elevated in the livers and

spleens of their patients. These were the first instances in which Tay-Sachs ganglioside was shown to accumulate in organs other than the CNS. Eeg-Olofsson, et al. found that the liver G_{M2} of their patient contained equal amounts of C_{18} and C_{22} + C_{24} fatty acids. On this basis, they concluded that Tay-Sachs disease could be considered a generalized disorder of ganglioside metabolism, since the fatty acid pattern indicated both a neural and visceral synthesis of ganglioside G_{M2}.

Sandhoff, et al. (46,47) recorded still another variation of Tay-Sachs disease in 1968 when they reported a case of a non-Jewish male who died at age 2-1/2 in which normal kidney globoside, N-acetylgalactosaminoyl-(β1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1)-ceramide (8,9,24), accumulated in the visceral organs in addition to the excessive presence of ganglioside G_{M2} and its asialo derivative in the brain. Three cases of classical Tay-Sachs disease examined by these investigators showed no increased globoside levels in the visceral organs. Enzyme assays of brain and visceral tissue homogenates from seven normals, three classical Tay-Sachs cases, and the exceptional case of Tay-Sachs, showed normal to somewhat above normal activities for neuraminidase and nonspecific β-N-acetylhexosaminidase in the controls and classical Tay-Sachs patients. The exceptional case of Tay-Sachs, however, exhibited an almost complete

absence of hexosaminidase in all tissues assayed; neuraminidase showed normal activity. These investigators therefore postulated the missing hexosaminidase as the cause of glycosphingolipid accumulation, since all three excessive compounds possess a terminal β-N-acetylgalactosamine moiety. This same case was also reported by Pilz, et al. (48).

In 1969 Taketomi and Kawamura (49) reported a thorough investigation of cerebral and visceral glycosphingolipids in a case of Tay-Sachs disease. They found the usual excessive accumulation of ganglioside G_{M2} in the brain, but failed to detect any ganglioside storage in the kidney, spleen, or liver. In addition, they found only a trace of glucosyl ceramide and a small amount of lactosyl ceramide in the brain. On the other hand, lactosyl ceramide was the major glycosphingolipid of Tay-Sachs spleen and trihexosyl ceramide and hematoside (ganglioside G_{M3}) dominated the liver glycosphingolipids. Asialo G_{M2} was limited to the Tay-Sachs brain. Kidney globoside was present in the kidney, spleen, and to a lesser extent in the liver of this patient. Amounts of this glycosphingolipid in these organs were not quantitated, but from the discussion presented, they did not seem exceptional. In a similar case of Tay-Sachs disease with visceral involvement, Grégoire, et al. (50) detected the storage of asialo G_{M2} in both cerebral grey and white

matter and the liver, in addition to the usual ganglioside G_{M2} accumulation in the brain.

This study presents the case of Todd Thomey, a child who died at age 18 months from a lipid storage disorder diagnosed as classical Tay-Sachs disease. The cherry-red spot was present in the retina and hepatosplenomegaly was observed, strongly suggesting visceral involvement. Neutral glycosphingolipids and gangliosides were extracted from Thomey's cerebral grey and white matter, kidney, spleen, and liver and compared with those from normal controls identically extracted. The data obtained indicate a type of Tay-Sachs disease with visceral involvement very similar to the case of Sandhoff, et al. (46,47).

EXPERIMENTAL

A. Materials

Portions of Thomey brain, kidney, liver, and spleen (fresh-frozen at autopsy) were shipped to this laboratory over dry ice via air express, courtesy of Dr. William Krivit, the University of Minnesota Medical School, Minneapolis, Minnesota. Identical organ sections from three different normal controls were also obtained from Dr. Krivit. Glycosphingolipids used as reference standards for TLC were extracted and purified from packed human red blood cells by the method of Vance and Sweeley (26). All chemicals and solvents used in this work were analytical or reagent grade unless otherwise noted.

Heat-activated Unisil (200/325 mesh, Clarkson Chemical Co., Inc., Williamsport, Pa.) was used in all silicic acid column chromatography. With the exception of the first Thomey kidney, the Thomey spleen, and the second Thomey liver glycolipid separations, all analytical and preparative TLC was carried out on Quantum precoated TLC plates (Quantum Industries, Chicago, Ill.). The other TLC separations were done on glass plates coated to a thickness of 250μ with Silica Gel G (Brinkmann Instruments, Inc., Westbury, New York) using a

Desaga TLC spreader. A F & M Model 402 gas chromatograph (Hewlett Packard Co., Avondale, Pa.) fitted with a glass, six-foot 3% SE-30 or OV-1 column (packing from Applied Science Co., State College, Pa.) was used in all GLC analyses. Hexamethyldisilazane and trimethylchlorosilane used in the silylating (TMS) reagent and bis-trimethylsilyltrifluoroacetamide (BSTFA) used in direct probe mass spectrometry (MS) analyses were purchased from Applied Science Co., State College, Pa.

B. Methods

Lipid Extraction and Purification

Total lipids were extracted from all organs according to the method of Folch, et al. (27). Glycosphingolipids were prepared from the total lipid extract by the method of Vance and Sweeley (26). In the majority of analyses, a modification of the Folch extraction was used. This procedure involved taking up the total lipids in 30-40 ml of $CHCl_3$, pouring the solution into a suitable length of dialysis tubing, and dialyzing against a large volume of distilled water for two days with several changes of water. Ideally, this method minimizes retention of low molecular weight gangliosides in the organic phase, which often occurs in the conventional Folch procedure (51).

TLC of Glycosphingolipids

Two TLC systems were utilized in this work. For separation of neutral glycosphingolipids, the $CHCl_3$:MeOH:H_2O 100:42:6 (v/v) system was used with single development (26). Ganglioside separation was accomplished using the $CHCl_3$:MeOH:2.5N NH_3(aq) 60:40:9 (v/v) system with double development (52). Pre-coated TLC plates were used in the majority of separations after sharper bands and minimal overlapping were observed with their use.

Preparation of TMS Glycosphingolipids

The silylating agent consisted of dry pyridine, hexamethyldisilazane, and trimethylchlorosilane mixed in the ratio 5:2:1 (v/v) in that order. Generally, 50-100 µl of TMS reagent was added to 100-300 µg of glycosphingolipid and allowed to stand about 10 minutes before GLC injection.

GLC of TMS Glycosphingolipids and Fatty Acid Methyl Esters

Thomey and normal liver, kidney, and spleen TMS glycosphingolipids and fatty acid methyl esters (FAME) were run on a six-foot 3% OV-1 column. A six-foot 3% SE-30 column was used in Thomey and normal brain analyses. All neutral TMS glycosphingolipids were isothermally eluted at 170°C; all TMS gangliosides were programmed from

150°C to 220°C at 2°/min. All FAME were programmed from 180°C to 240°C at 2°/min.

Direct Probe Mass Spectrometry of TMS Glycosphingolipids

The BSTFA derivatives of whole Thomey and normal glycosphingolipids were prepared and analyzed according to the method of Sweeley and Dawson (53). Only those glycosphingolipids which accumulated in Thomey's viscera and their normal counterparts were examined. An LKB 9000 mass spectrometer (LKB Produktor, Stockholm, Sweden) consisting of a gas chromatograph and a single-focusing 60° magnetic sector mass spectrometer directly coupled to Becker-Ryhage type molecular separators was employed for these analyses. The GC column used was a six-foot, coiled glass column packed with 3% OV-1 on 100-200 mesh, acid-washed, silanized Gas Chrom S. For direct probe analysis, the instrument was operated at 3500 volts and 70 eV electron energy with a 60 μamp electron current and ion source temperature of 290°C.

RESULTS

A. TLC of Neutral Glycosphingolipids and Gangliosides

Figures 5 through 13 illustrate the preparative TLC separations of neutral glycosphingolipids and gangliosides from each type of tissue extracted. From 6 to 12 mg of material was applied to each plate. The shaded bands in the chromatograms of both Thomey and normal lipids are the bands of interest to be compared. For all neutral glycosphingolipid separations, the solvent system was $CHCl_3$:MeOH:H_2O 100:42:6 (v/v); for all ganglioside separations, the $CHCl_3$:MeOH:2.5N NH_3(aq) 60:40:9 (v/v) system was used. Human red blood cell glycosphingolipids were used as reference standards for all neutral glycosphingolipid TLC separations. The identification of the major gangliosides on TLC, however, rested solely on the literature citations for the solvent system used, as suitable ganglioside reference standards were not available. Verification of these assignments was accomplished via GLC analyses after acid-catalyzed methanolysis.

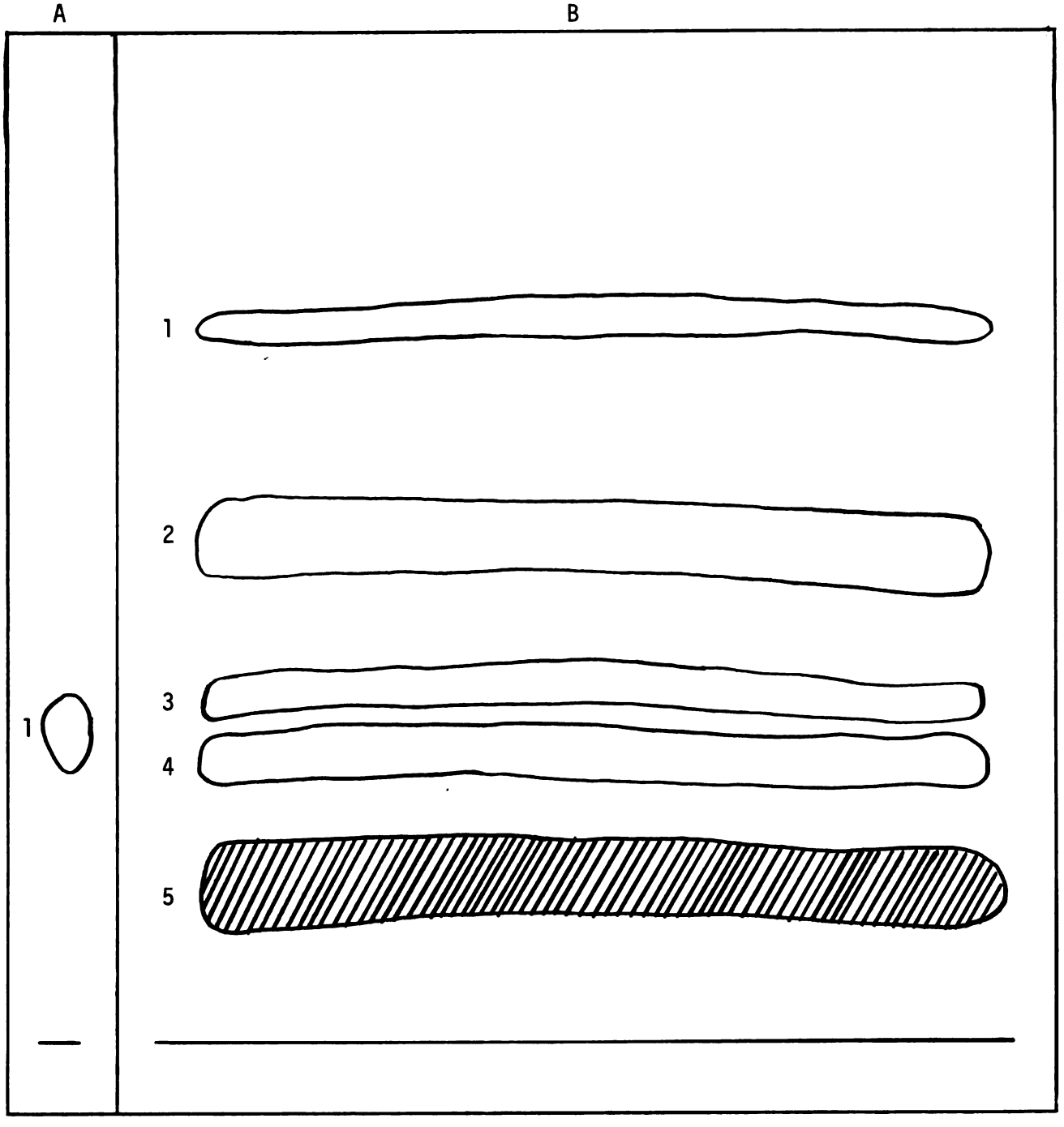

Figure 5. TLC of Thomey kidney neutral glycosphingolipids. A. (1) Normal red blood cell gal-gal-glc-cer. B. (1) Glc-cer and gal-cer, (2) gal-gal-cer and gal-glc-cer, (3) SO_3gal-glc-cer, (4) gal-gal-glc-cer, and (5) galNAc-gal-gal-glc-cer. In Figures 5 through 11, the solvent system was $CHCl_3$:MeOH:H_2O 100:42:6 (v/v).

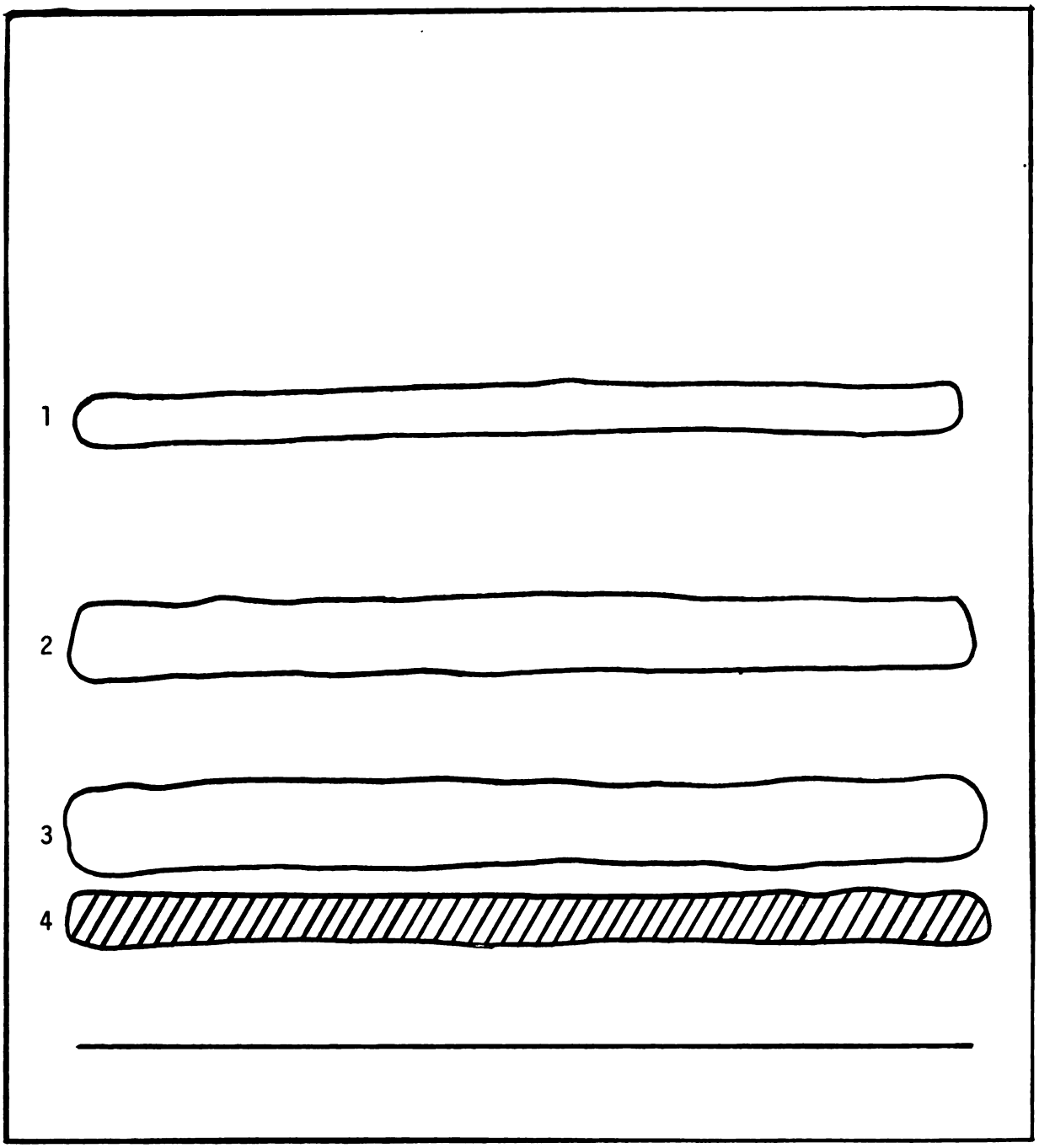

Figure 6. TLC of normal human juvenile kidney neutral glycosphingolipids: (1) glc-cer and gal-cer, (2) gal-gal-cer and gal-glc-cer, (3) gal-gal-glc-cer, and (4) galNAc-gal-gal-glc-cer.

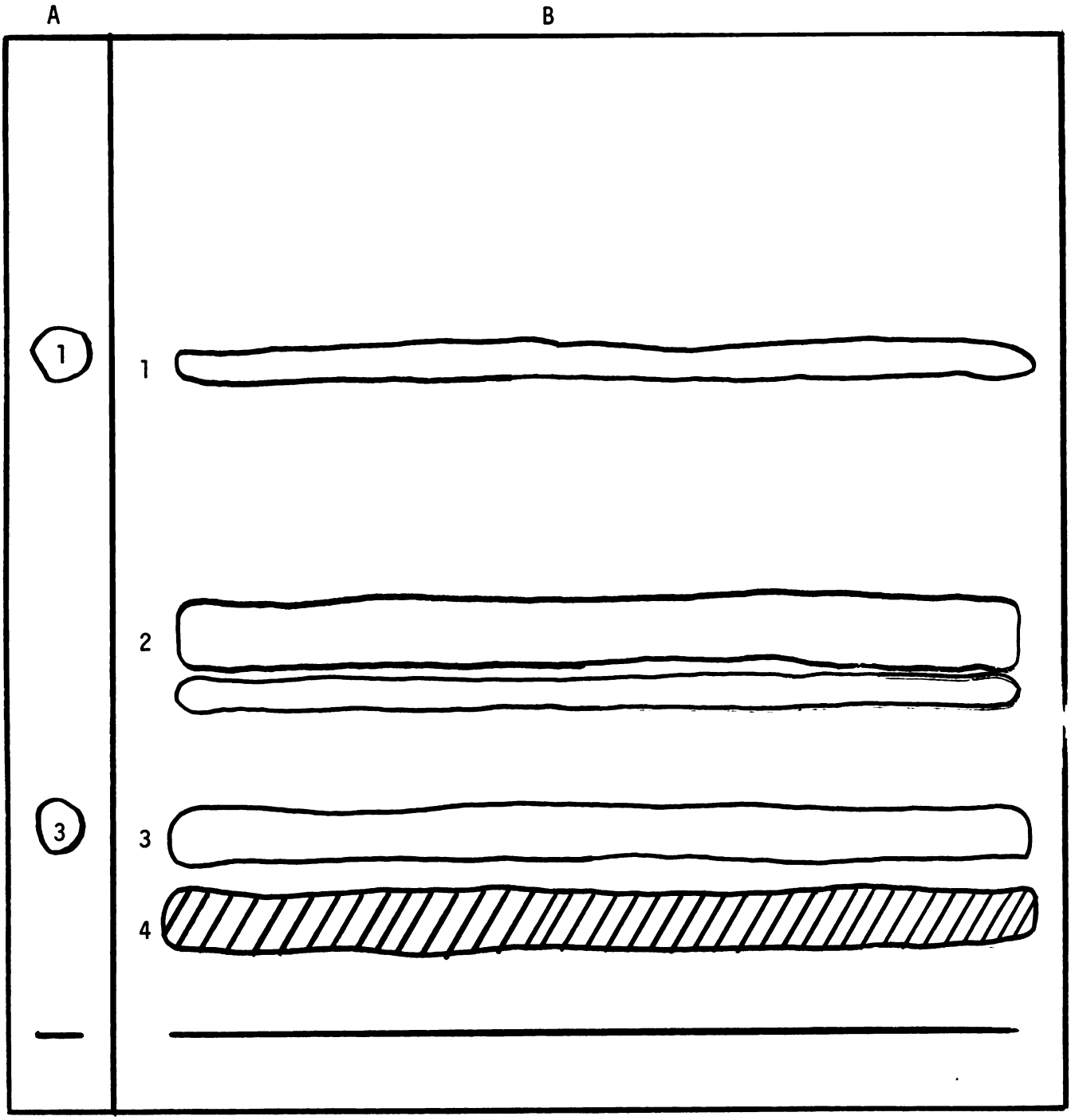

Figure 7. TLC of normal human juvenile spleen neutral glycosphingolipids. A. (1) Gaucher spleen glc-cer and (2) normal red blood cell gal-gal-glc-cer. B. (1) Glc-cer and gal-cer, (2) gal-glc-cer, (3) gal-gal-glc-cer, and (4) galNAc-gal-gal-glc-cer.

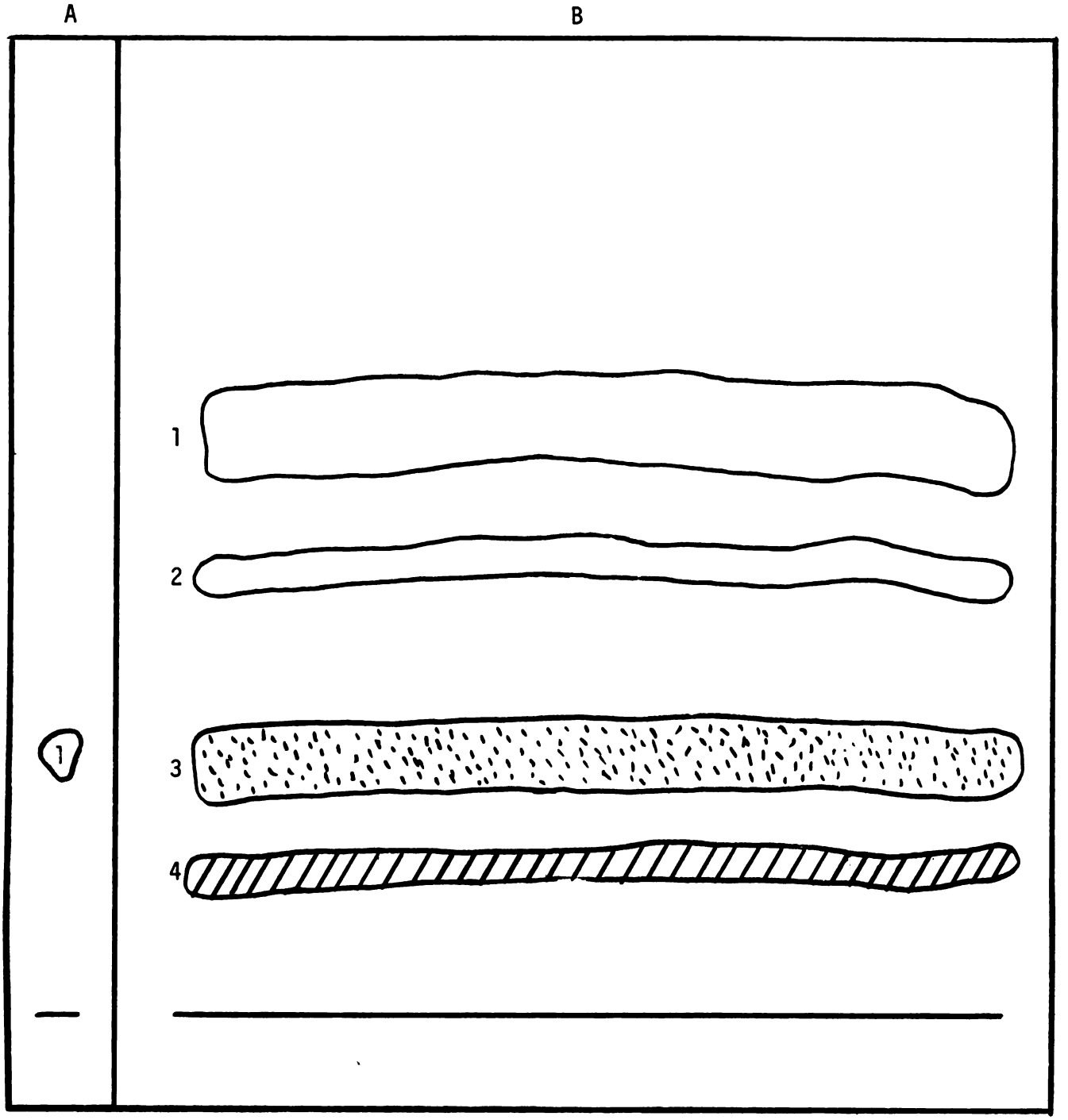

Figure 8. TLC of Thomey liver neutral glycosphingolipids. A. (1) Normal red blood cell gal-gal-glc-cer. B. (1) Glc-cer and gal-cer, (2) gal-glc-cer, (3) galNAc-gal-glc-cer, and (4) galNAc-gal-gal-glc-cer.

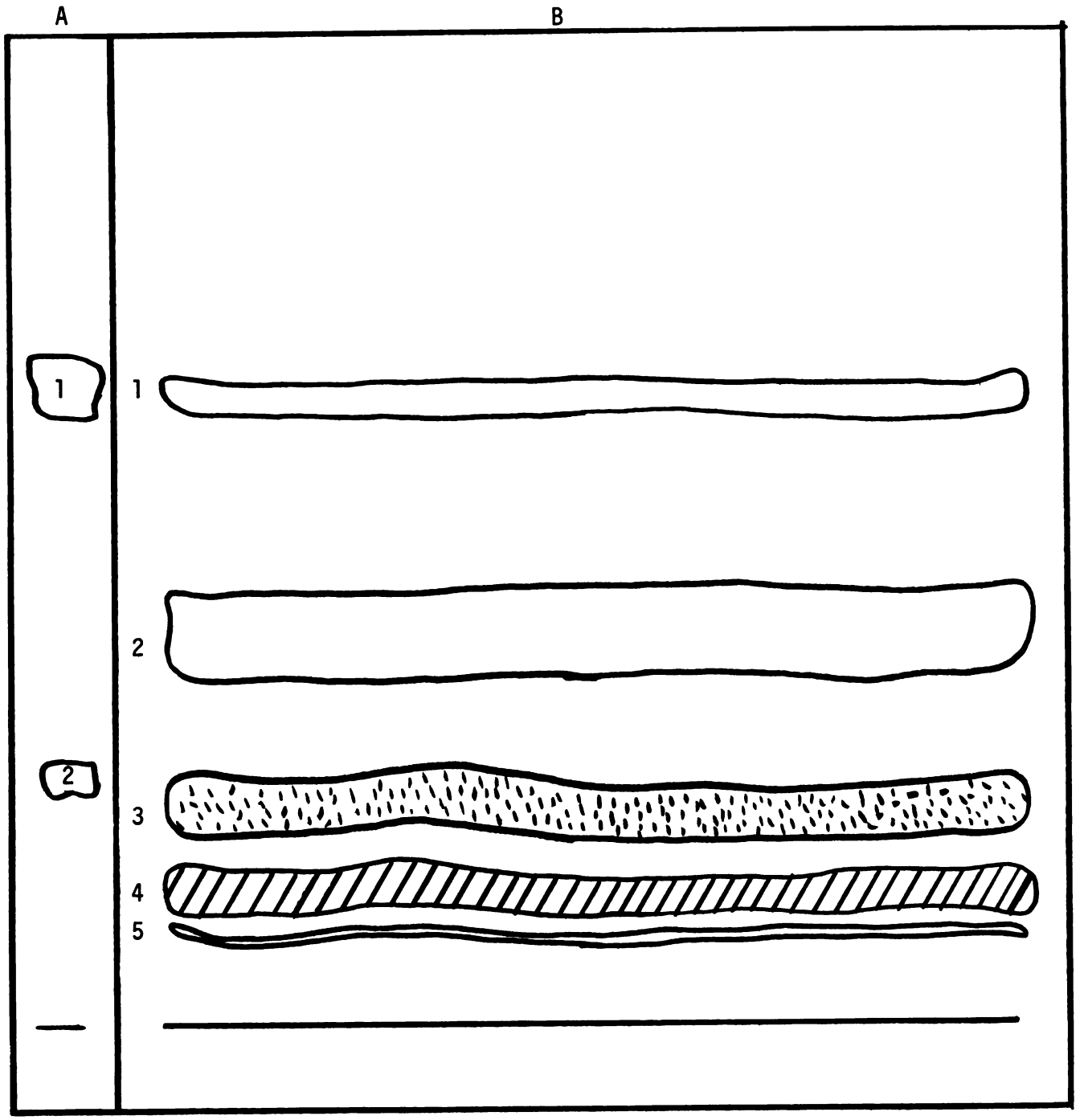

Figure 9. TLC of normal human juvenile liver neutral glycosphingolipids. A. (1) Gaucher spleen glc-cer and (2) normal red blood cell gal-gal-glc-cer. B. (1) Glc-cer and gal-cer, (2) gal-glc-cer, (3) gal-gal-glc-cer, (4) galNAc-gal-gal-glc-cer, and (5) G_{M3}, NANA-gal-glc-cer.

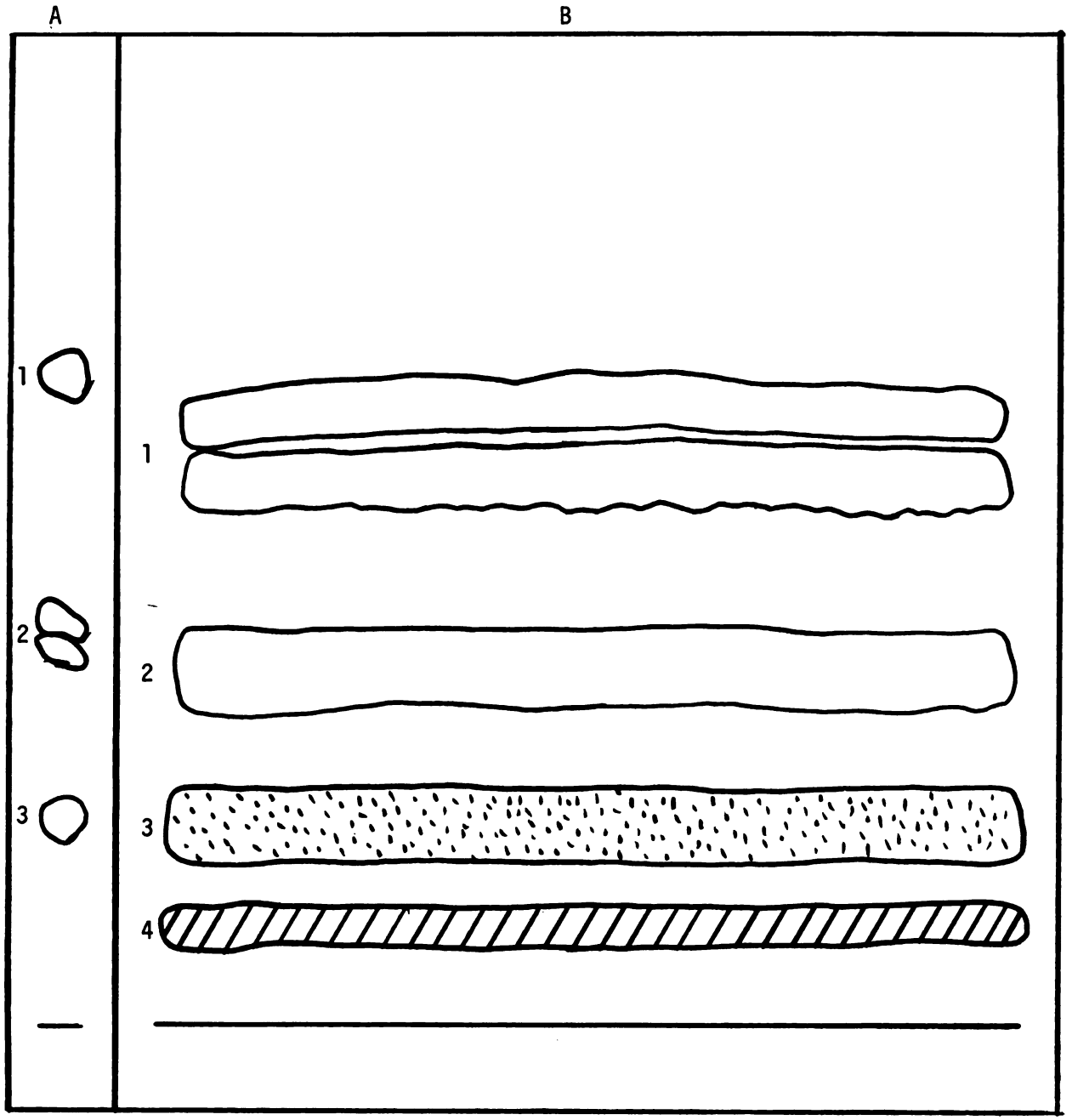

Figure 10. TLC of Thomey cerebral white matter neutral glycosphingolipids. A. (1) Gaucher spleen glc-cer, (2) normal red blood cell gal-glc-cer, and (3) normal red blood cell gal-gal-glc-cer. B. (1) Glc-cer and gal-cer, (2) sulfatide, SO_3gal-cer, and gal-glc-cer, (3) galNAc-gal-glc-cer, and (4) galNAc-gal-gal-glc-cer.

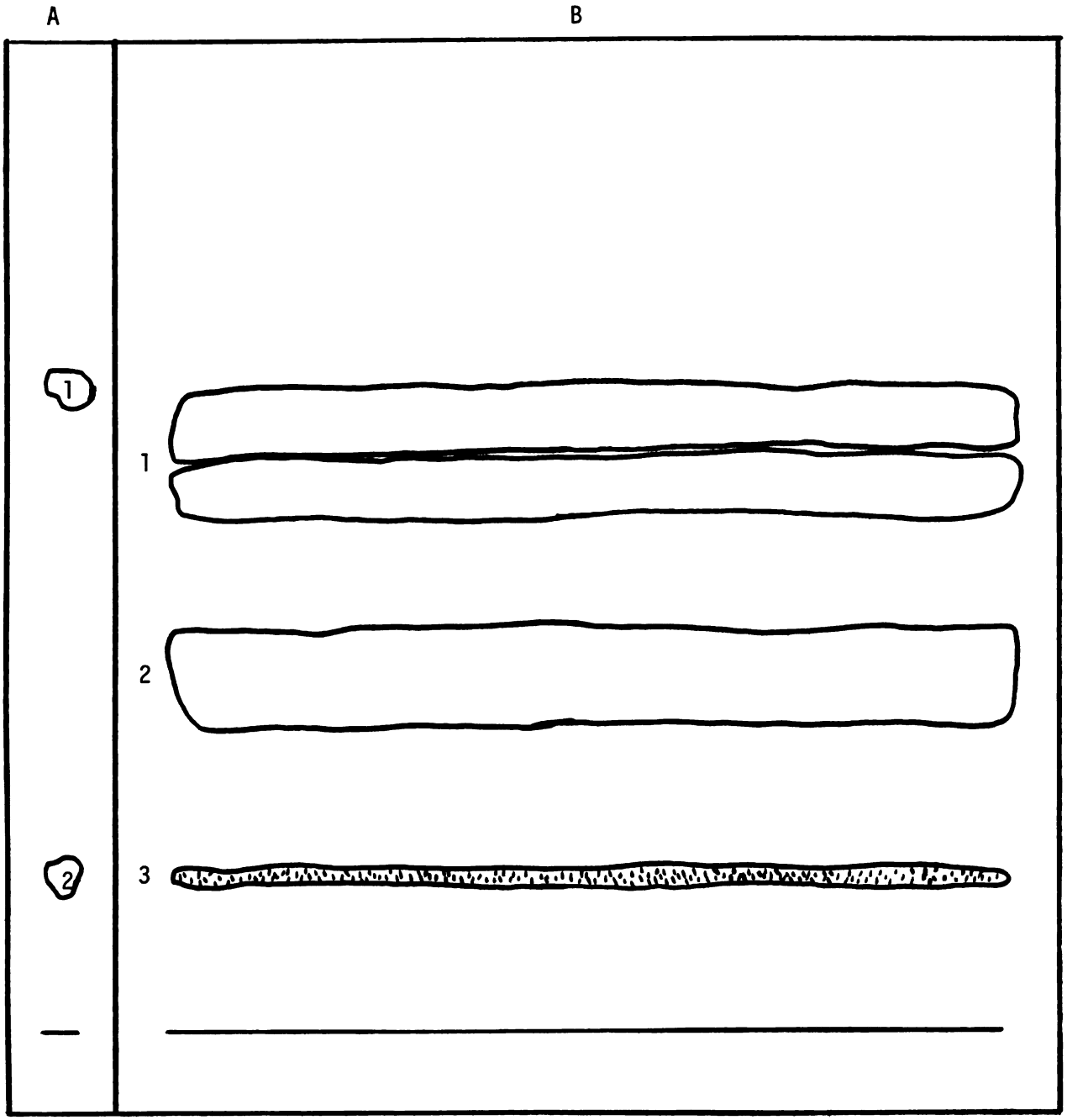

Figure 11. TLC of normal human juvenile cerebral white matter neutral glycosphingolipids. A. (1) Gaucher spleen glc-cer and (2) normal red blood cell gal-gal-glc-cer. B. (1) Gal-cer, (2) sulfatide, SO_3gal-cer, and gal-glc-cer, and (3) gal-gal-glc-cer (tentative).

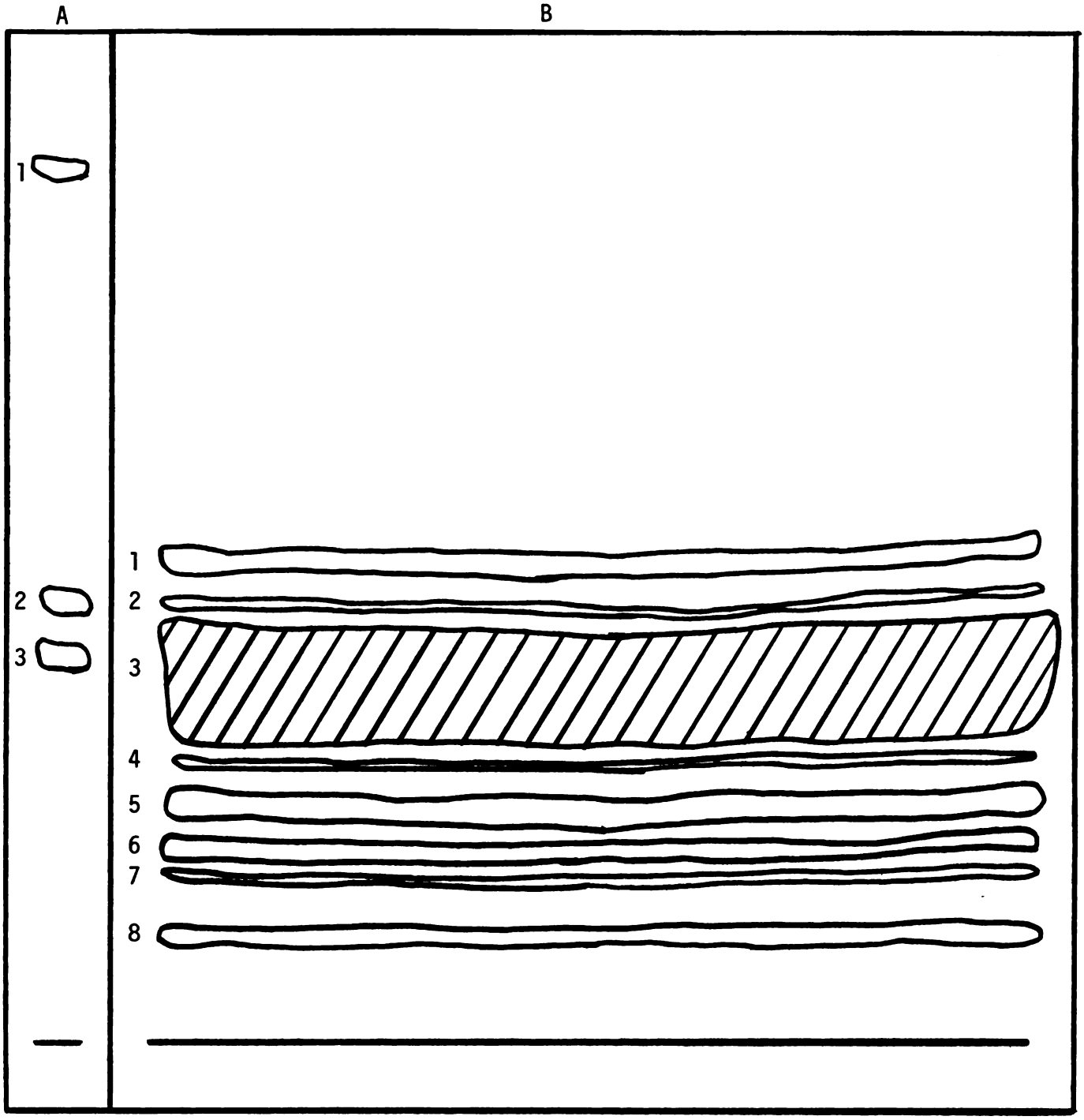

Figure 12. TLC of Thomey cerebral white matter gangliosides. A. (1) Gaucher spleen glc-cer, (2) normal red blood cell gal-gal-glc-cer, and (3) normal red blood cell galNAc-gal-gal-glc-cer. B. (1) Unclassified, (2) G_{M3}, (3) G_{M2}, (4) unclassified, (5) G_{M1}, (6) G_{D1a}, (7) G_{D1b}, and (8) G_{T1}. Solvent system: $CHCl_3$:MeOH:2.5N NH_3 (aq) 60:40:9 (v/v).

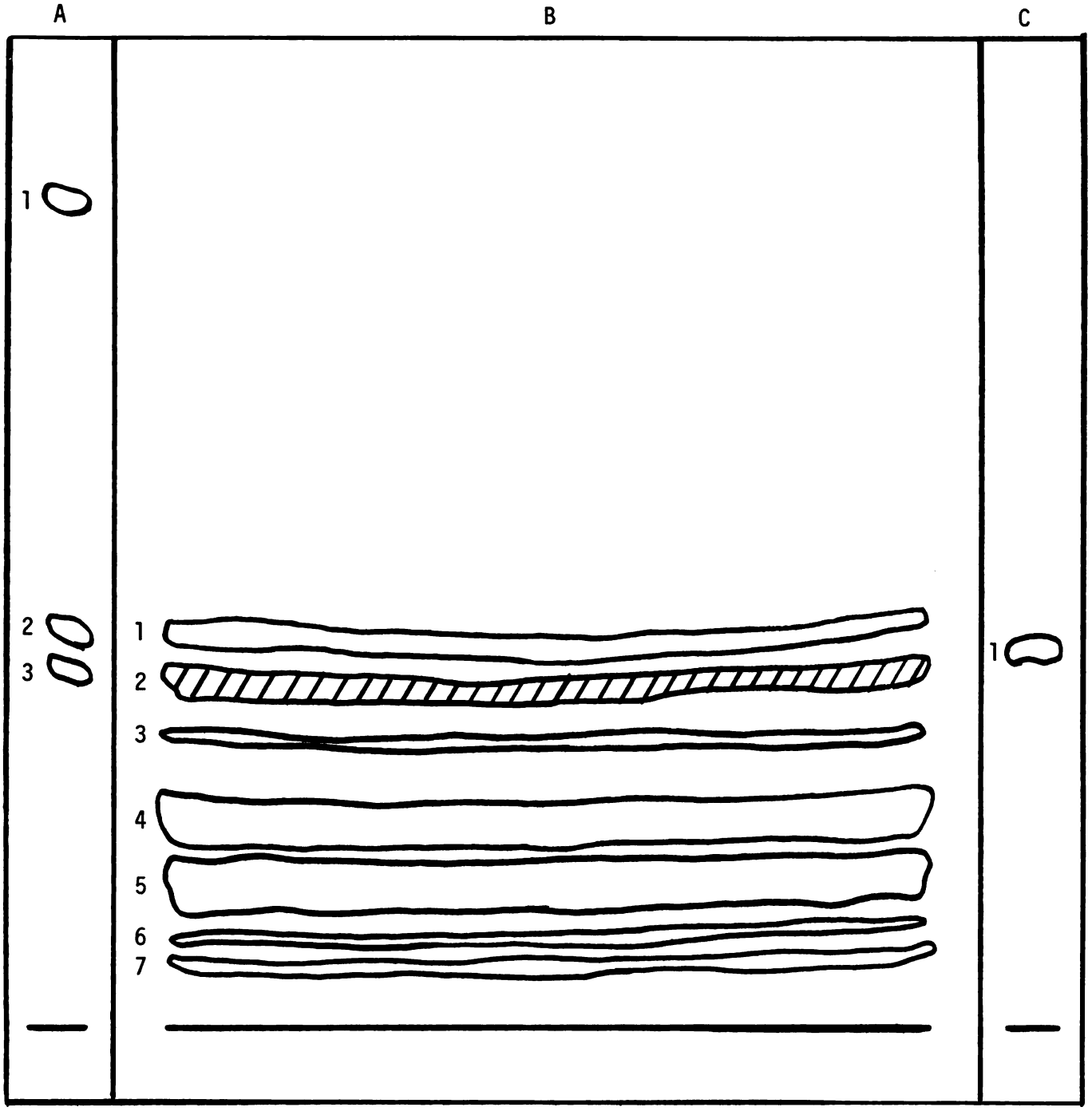

Figure 13. TLC of normal juvenile cerebral white matter gangliosides. A. (1) Gaucher spleen glc-cer, (2) normal red blood cell gal-gal-glc-cer, and (3) normal red blood cell galNAc-gal-gal-glc-cer. B. (1) Unclassified, (2) G_{M2}, (3) unclassified, (4) G_{M1}, (5) G_{D1a}, (6) G_{D1b}, and (7) G_{T1}. C. (1) Thomey G_{M2}. Solvent system: $CHCl_3$:MeOH:2.5N NH_3 (aq) 60:40:9 (v/v).

B. GLC of TMS Neutral Glycosphingolipids, Gangliosides, and FAME

Tables 2A through 10 show the levels of neutral glycosphingolipids and gangliosides in the tissues examined expressed as µmole hexose/gm wet weight of tissue. Prior to overnight acid-catalyzed methanolysis at 82°C, 0.1 or 0.2 µmole of a mannitol standard solution (2.0 µmole/ml, in MeOH) was added to each fraction to insure quantitation of material. Areas of GLC peaks were calculated via planimetry and the amounts of glucose (glc), galactose (gal), N-acetylgalactosamine (galNAc), and N-acetylneuraminic acid (NANA) were calculated from the following formulas:

(1) $\frac{\text{area glc or gal}}{\text{area mannitol}} \times 1.25 \times \mu\text{moles mannitol added} = \mu\text{moles glc or gal}$

(2) $\frac{\text{area galNAc}}{\text{area mannitol}} \times 1.36 \times \mu\text{moles mannitol added} = \mu\text{moles galNAc}$

(3) $\frac{\text{area NANA}}{\text{area mannitol}} \times 0.98 \times \mu\text{moles mannitol added} = \mu\text{moles NANA}$

To carry out the re-N-acetylation of galNAc and NANA, about 0.2 ml of acetic anhydride was added to each FAME-extracted, neutralized methanolysate suspected of containing galNAc or NANA and allowed to stand in a closed culture tube a minimum of six hours at room temperature. Silver carbonate used in the neutralization was allowed to remain in the methanolysate during re-N-acetylation,

Table 2A.--Neutral Glycosphingolipids of Thomey Kidney.[a]

Det.	No.	MHC[b]	Gal/ Glc	DHC[c]	Gal/ Glc	SULF[d]	Gal/ Glc	TRHC[e]	Gal/ Glc	GLOB[f]	Gal/ Glc	GalNAc/ Glc
1	I	0.10	0.29	0.20	9.82	-	-	0.12	1.98	0.78	2.20	-[g]
	II	0.26	0.81	0.11	10.22	-	-	0.11	1.93	0.69	2.26	-[g]
	III	0.08	0.42	0.15	8.35	-[g]	-	-[g]	-	-[g]	-	-
	IV	0.09	0.37	0.12	7.97	0.03	1.39	0.11	2.00	0.82	2.07	0.74
4	I	0.12	0.58	0.17	7.28	0.06	1.36	0.16	1.91	0.58	1.88	0.67
	II	0.10	0.83	0.15	6.80	0.05	1.14	0.13	1.85	0.49	1.98	0.66
Averages		0.12±0.04	0.55	0.15±0.02	8.41	0.05±0.01	1.30	0.13±0.01	1.93	0.67±0.11	2.08	0.69
Martensson[h]		0.12		0.16		0.05		0.32		0.48		

[a]In this and all other tables, values are given as μmole hexose (glc or gal)/gm wet weight of tissue.

[b]MHC = monohexosyl ceramide (glc-cer and gal-cer).

[c]DHC = dihexosyl ceramide (gal-glc-cer and gal-gal-cer).

[d]SULF = dihexosyl sulfatide (SO_3gal-glc-cer), tentative identification.

[e]TRHC = trihexosyl ceramide (gal-gal-glc-cer).

[f]GLOB = tetrahexosyl ceramide (galNAc-gal-gal-glc-cer).

[g]Not determined.

[h]These values are μmoles/gm dry weight of normal kidney tissues x 0.25 (55,57); conversion has given equivalent μmoles/gm wet weight of tissue.

Table 2B.--Neutral Glycosphingolipids of Thomey Kidney.

Det. No.		MHC[a]	Gal/ Glc	DHC	Gal/ Glc	SULF	Gal/ Glc	TRHC	Gal/ Glc	GLOB	Gal/ Glc	GalNAc/ Glc
2	I	0.44	0.38	0.61	2.55	-	-	0.92	1.50	4.05	1.96	0.79
	II	0.26	0.50	0.39	7.77	0.18	1.38	0.39	1.94	3.98	1.98	0.64
3	I	0.44	0.61	0.49	3.28	-	-	0.77	1.52	3.53	2.05	0.83
	II	0.22	0.46	0.33	5.52	0.12	1.54	0.32	2.10	3.20	2.09	0.86
Averages		0.34±0.10	0.49	0.46±0.09	4.78	0.15±0.03	1.46	0.60±0.24	1.77	3.69±0.33	2.04	0.77

[a]MHC, DHC, etc. have the same meaning as in Table 2A.

Table 3.--Neutral Glycosphingolipids of Thomey Spleen.

Det.	MHC[a]	Gal/Glc	DHC	Gal/Glc	TRHC	Gal/Glc	GLOB	Gal/Glc	GalNAc/Glc
1	0.28	-[b]	0.14	0.80	0.11	1.48[c]	1.82	1.95	0.89
2	0.32	-[b]	0.11	1.06	0.14	1.34[c]	2.25	2.04	0.91
Averages	0.30±0.02	-	0.13±0.01	0.93	0.12±0.02	1.41	2.04±0.22	2.00	0.89

[a]MHC, DHC, etc. have the same meaning as in Table 2A.

[b]Only glc was present.

[c]TLC separation was done on a homemade plate and TRHC and GLOB separated poorly. But from the galNAc present in TRHC, the amount of GLOB in TRHC was calculated, subtracted out, and added to GLOB to give the values presented in the table.

Table 4.--Neutral Glycosphingolipids of Thomey Liver.

Det.	No.	MHC	Gal/ Glc	DHC	Gal/ Glc	TRHC[a]	Gal/ Glc	GalNAc/ Glc	GLOB	Gal/ Glc	GalNAc/ Glc
1	I	-[b]	-	0.06	1.04	0.73	1.07	0.95	0.22	1.76	0.89
2	I	0.10	0.20	0.07	1.10	-[c]	-	-	-[c]	-	-
3	I	0.13	0.49	0.09	0.97	1.33	1.04	0.96	0.24	2.07	0.81
4	I	0.15	0.53	0.12	1.11	1.16	1.09	0.81	0.32	1.88	0.81
	II	0.10	0.67	0.10	0.97	1.32	1.06	0.61	0.42	1.91	0.72
Averages		0.12±0.02	0.47	0.09±0.02	1.04	1.13±0.21	1.07	0.80	0.30±0.07	1.91	0.80

[a]TRHC = galNAc-gal-glc-cer only.

[b]Lost.

[c]This separation was done on a homemade TLC plate and TRHC and GLOB ascended as one band; yield of material was 1.19 µmole glc/gm wet weight of tissue.

Table 5.--Neutral Glycosphingolipids of Normal Human Juvenile Kidney.

Control No.	MHC	Gal/Glc	DHC	Gal/Glc	TRHC	Gal/Glc	GLOB	Gal/Glc	GalNAc/Glc
1	0.09	0.80	0.06	2.74	0.08	2.81	0.09	2.18	-[a]
2	0.15	0.40	0.22	3.80	0.50	1.88	0.35	1.98	0.75
3	0.09	0.41	0.21	4.70	0.45	1.92	0.42	1.74	0.79
4	0.08	0.45	0.09	4.34	0.21	1.84	0.19	2.00	0.72
Averages	0.10±0.02	0.52	0.14±0.07	3.90	0.31±0.17	2.11	0.26±0.12	1.98	0.75
Martensson[b]	0.12		0.16		0.32		0.48		

[a]Not determined.

[b]See Table 2A.

Table 6.--Neutral Glycosphingolipids of Normal Human Juvenile Spleen.

Control No.	MHC	Gal/Glc	DHC	Gal/Glc	TRHC	Gal/Glc	GLOB	Gal/Glc	GalNAc/Glc
1	0.22	-[a]	0.29	1.00	0.03	1.61	0.05	1.87	0.57
2	0.05	0.17	0.09	1.06	0.05	1.85	0.08	1.65	0.70
3	0.08	0.06	0.10	1.19	0.10	1.94	0.11	1.80	0.54
Averages	0.12±0.07	0.08	0.16±0.09	1.08	0.06±0.03	1.80	0.08±0.02	1.77	0.60

[a]Only glc was present.

Table 7.--Neutral Glycosphingolipids of Normal Human Juvenile Liver.

Control No.	MHC	Gal/ Glc	DHC	Gal/ Glc	TRHC[a]	Gal/ Glc	GLOB	Gal/ Glc	GalNAc/ Glc	G_{M3}	Gal/ Glc	NANA/ Glc
1	0.05	-[b]	0.11	1.12	0.03	1.85	0.02	1.84	0.55	0.01	1.25	0.85
2	0.07	-[b]	0.12	1.06	0.03	1.84	0.04	2.00	0.50	<0.01	0.47	0.96
3	0.04	0.18	0.08	1.15	0.05	2.16	0.02	1.92	0.61	<0.01	1.26	0.93
Averages	0.05±0.01	0.06	0.10±0.02	1.11	0.04±0.01	1.95	0.03±0.01	1.92	0.55	0.01±<0.01	0.99	0.91

[a]TRHC = gal-gal-glc-cer only.

[b]Only glc was present.

Table 8.--Neutral Glycosphingolipids of Thomey and Normal Juvenile Cerebral Grey and White Matter.

	MHC											
	Band A	Gal/ Glc	Band B	Gal/ Glc	DHC[a]	Gal/ Glc	TRHC[b]	Gal/ Glc	GalNAc/ Glc	GLOB	Gal/ Glc	GalNAc/ Glc
T. Grey Matter	0.39	0.51	0.39	18.00	0.29	5.09	4.61	1.06	0.80	0.11	2.07	0.71
T. White Matter	2.16	12.80	4.00	-[c]	2.84	16.10	3.20	1.08	0.77	0.30	2.14	0.76
N. Grey Matter	5.00[d]		-[c]		1.66	11.60	<0.01	2.00	-[e]	<0.01	1.59	-[e]
N. White Matter	135.70		-[c]		57.10	14.95	0.11	1.55	-[e]	-[f]	-	-

[a]DHC = dihexosyl ceramide (gal-glc-cer, minor) and sulfatide (SO_3gal-cer, major).

[b]TRHC = galNAc-gal-glc-cer only.

[c]Only gal was present.

[d]For normal controls MHC bands were unresolvable on TLC.

[e]GalNAc was not detected.

[f]GLOB was not detected.

Table 9.--Thomey and Normal Juvenile Cerebral Grey Matter Gangliosides.

Thomey Grey Matter					Normal Grey Matter				
Compound[a]	Amount	Gal/Glc	GalNAc/Glc	NANA/Glc	Compound[a]	Amount	Gal/Glc	GalNAc/Glc	NANA/Glc
					1	0.01	0.93	-	-
					2	0.01	-[b]	-	-
					3	0.01	-[b]	-	-
					4	0.03	0.86	trace	0.35
1 (G_{M3})	0.07	0.71	-	0.33	5 (G_{M3})	0.01	0.71	trace	0.71
2 (G_{M2})	5.43	1.10	0.77	0.64	6 (G_{M2})	0.04	1.14	trace	0.80
3 (G_{M1})	0.30	1.93	0.64	0.88	7 (G_{M1})	0.32	2.03	0.73	0.78
4 (G_{D1a})	0.33	1.98	0.69	1.17	8 (G_{D1a})	0.46	2.05	0.80	1.63
5 (G_{D1b})	0.14	1.74	0.76	1.20	9 (G_{D1b})	0.20	1.85	0.68	1.41
6 (G_{T1})	0.11	1.93	0.56	1.05					

[a]Numbers represent sequence of bands on TLC, beginning near solvent front and extending down to near origin. Designations under numbers in parentheses were made on the basis of comparison with published photos of TLC of cerebral gangliosides in the same solvent system (45).

[b]Only glc was present.

Table 10.--Thomey and Normal Juvenile Cerebral White Matter Gangliosides.

Thomey White Matter					Normal White Matter				
Compound[a]	Amount	Gal/Glc	GalNAc/Glc	NANA/Glc	Compound[a]	Amount	Gal/Glc	GalNAc/Glc	NANA/Glc
1	0.01	0.59	-	trace	1	<0.01	1.61	-	trace
2 (G_{M3})	0.02	0.72	trace	0.50	2 (G_{M2})	0.01	0.86	0.43	0.39
3 (G_{M2})	0.92	1.03	0.70	0.60	3	0.01	1.12	trace	0.71
4	0.04	1.12	0.33	0.98					
5 (G_{M1})	0.09	1.77	0.62	0.55	4 (G_{M1})	0.09	2.05	0.72	0.71
6 (G_{D1a})	0.09	1.89	0.68	1.06	5 (G_{D1a})	0.13	2.03	0.79	1.41
7 (G_{D1b})	0.04	1.44	0.82	1.09	6 (G_{D1b})	0.02	1.63	0.63	1.64
8 (G_{T1})	0.02	1.44	0.57	0.78	7 (G_{T1})	0.04	1.82	0.67	1.48

[a]Numbering and designations are the same as in Table 9.

since it has been observed to catalytically promote re-N-acetylation by an unknown mechanism (54). Sugar ratios given in the tables are all relative to glc and were found by taking ratios of the μmolar amounts of the moieties present. In the neutral glycosphingolipid determinations, bands appearing on TLC closer to the origin than globoside sometimes contained NANA, in addition to glc and/or gal. Only in the normal liver did NANA consistently appear and, considering the gal/glc ratios, this substance was probably hematoside. No attempt was made to characterize other bands appearing lower than globoside in separations where the compound could not be identified as hematoside from the calculated ratios. Where available, values from the literature for visceral and cerebral glycosphingolipids are included in the tables.

The Thomey kidney data deserve special mention. First, the discrepancy between extractions 1 and 4, and 2 and 3, is most likely due to an error in weighing the wet tissue, since the glycosphingolipid amounts in both sets of data are proportional. These extractions were all done in the same manner and a weighing error therefore appears to be the best explanation for this discrepancy. Second, the gal/glc values for trihexosyl ceramide indicated the presence of two glycosphingolipids. A separation was achieved after the total glycosphingolipids

had been standing in CM 2:1 for several days or more and then applied to a TLC plate. When the glycosphingolipids were run immediately after mild alkali-catalyzed methanolysis, however, only one band was observed for trihexosyl ceramide. The gal/glc ratio for this late-separating glycosphingolipid indicates that it is probably the dihexosyl sulfatide isolated from human kidney by Martensson (55). However, due to some overlap of trihexosyl ceramide, the gal/glc ratio does not show equimolar amounts of gal and glc. Similar separation problems were not encountered with other visceral and cerebral glycosphingolipids.

Tables 11 and 12 illustrate the kinds of FAME present in the visceral and cerebral glycosphingolipids isolated. Values are given as percent of total FAME present (total area of all FAME GLC peaks). Where available, values from the literature are presented for comparison. Identifications of FAME were made on the basis of retention times of a standard mixture of 16:0, 18:0, 20:0, 22:0, and 24:0 FAME and by "carbon-number" (56).

C. Mass Spectrometry of TMS Glycosphingolipids

The mass spectra shown in Figures 14 and 15 verify that Thomey kidney globoside is the same compound as that of normal kidney, due to the presence of a terminal galNAc moiety. The presence of galNAc is indicated by

Table 11.--FAME from Thomey Abnormal and Corresponding Normal Juvenile Visceral Glycosphingolipids[a]

FAME	T. Kidney Globoside (3 det.)	N. Kidney Globoside (2 det.)	N. Kidney Globoside[b]	T. Spleen Globoside	N. Spleen Globoside	T. Liver TRHC[c]	N. Liver TRHC[d]	N. Liver Globoside
16:0	5.8	7.1	5.5	5.3	6.8	6.2	12.6	7.0
16:1	-	-	-	-	-	-	-	0.5
18:0	3.0	3.4	3.2	4.6	2.7	55.0	6.1	9.4
18:1	0.5	1.1	0.3	1.0	1.0	4.7	2.2	2.8
20:0	6.4	6.2	6.8	3.1	4.1	7.2	5.2	3.7
22:0	23.4	22.7	20.6	13.3	21.4	5.8	25.7	11.4
22:1	-	0.9	1.9	1.7	-	-	-	3.7
23:0	3.7	3.1	2.8	5.2	8.0	1.6	13.0	3.1
24:0	29.6	31.5	24.8	25.0	36.2	7.4	29.2	20.2
24:1	23.7	22.4	32.0	38.0	19.5	11.1	5.2	37.8

[a]Values represent percent of total FAME present in a given sample.

[b]Martensson, E., Biochim. Biophys. Acta, 116, 296 (1966).

[c]TRHC = trihexosyl ceramide (galNAc-gal-glc-cer).

[d]TRHC = trihexosyl ceramide (gal-gal-glc-cer).

Table 12.--FAME from Thomey and Tay-Sachs Cerebral Glycosphingolipids.[a]

FAME	T. Grey Matter TRHC[b]	T. White Matter TRHC[b]	Tay-Sachs[c] TRHC[b]	T. Grey Matter G_{M2}	T. White Matter G_{M2}	Tay-Sachs[c] G_{M2}	T. White Matter Globoside
16:0	2.0	3.8	3.4	2.3	2.5	6.6	11.8
16:1	-	-	-	1.1	0.6	-	1.5
18:0	91.4	87.6	84.1	89.8	90.6	87.0	50.0
18:1	2.0	3.8	2.5	1.7	1.3	2.8	10.3
20:0	4.6	4.8	2.2	5.1	5.0	3.6	3.0
22:0	-	-	7.8	-	-	-	3.0
22:1	-	-	-	-	-	-	7.4
23:0	-	-	-	-	-	-	-
24:0	-	-	-	-	-	-	6.6
24:1	-	-	-	-	-	-	4.4

[a]Values represent percent of total FAME in a given sample.

[b]TRHC = trihexosyl ceramide (galNAc-gal-glc-cer).

[c]Taketomi, T., and Kawamura, N., J. Biochem., 66, 165 (1969).

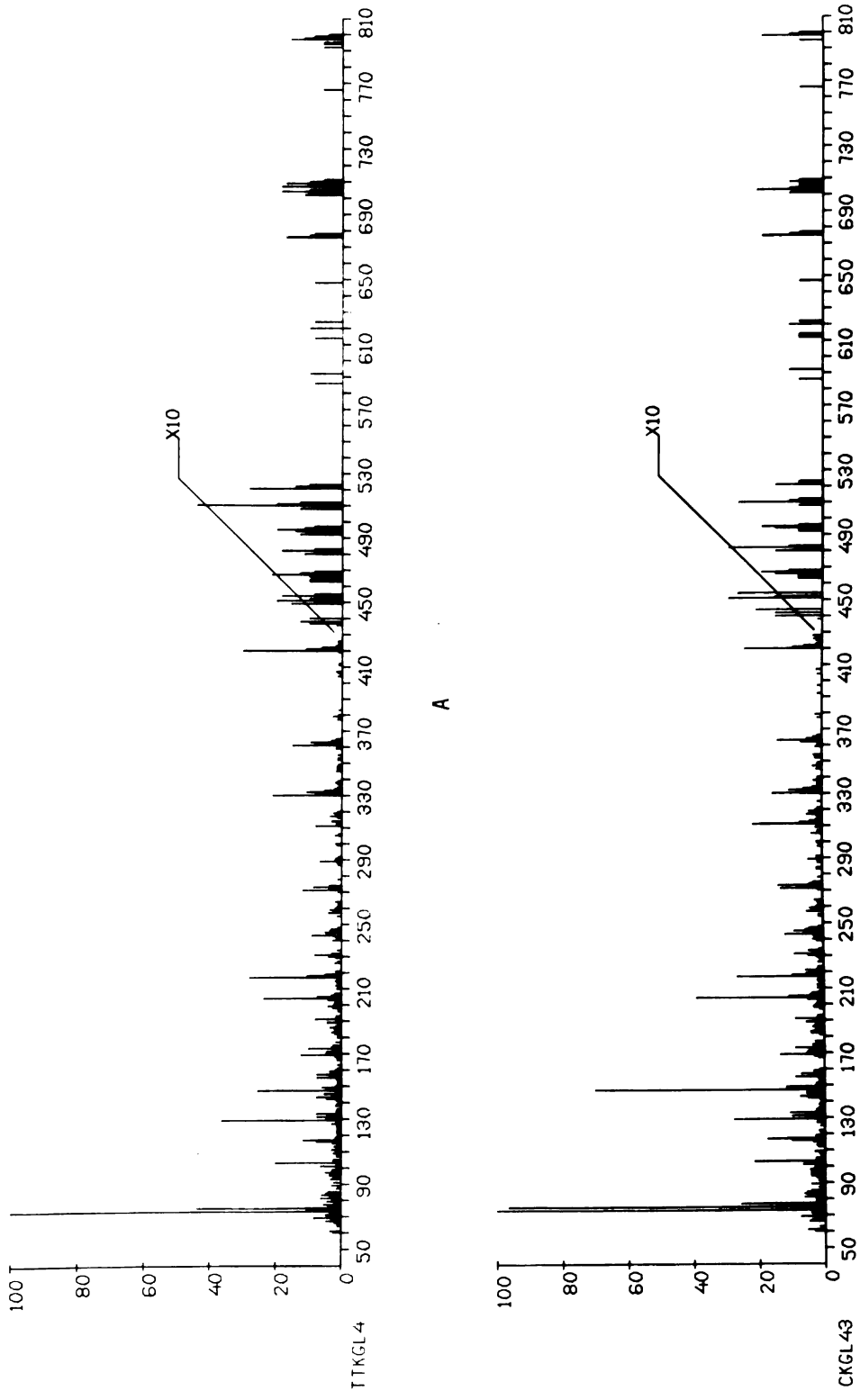

Figure 14. Mass spectra of TMS glycosphingolipids. A. Thomey kidney globoside, gal-NAc-gal-gal-glc-cer. B. Normal kidney globoside, galNAc-gal-gal-glc-cer.

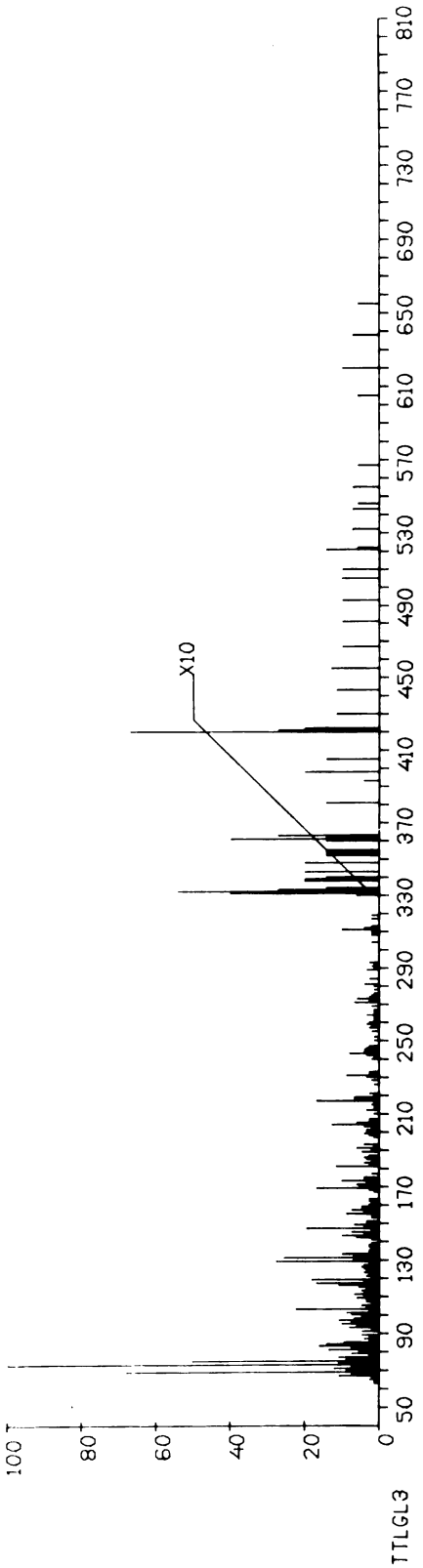

Figure 15. Mass spectrum of Thomey liver TMS trihexosyl ceramide, galNAc-gal-glc-cer.

an ion at m/e 173 and a terminal galNAc exhibits a strong ion at m/e 420 (53). Both of these ions are present in the spectra of Thomey and normal globoside. The mass spectrum of Thomey liver trihexosyl ceramide confirms this lipid to be asialo ganglioside G_{M2}, since the ions at m/e 173 and 420 indicate a terminal galNAc.

DISCUSSION

Quantitative determinations of visceral and cerebral glycosphingolipids in either disease or normal states are scarce in the literature and therefore few comparisons can be made with the data obtained in this study. Martensson's investigation of normal kidney glycosphingolipids (57), however, was very thorough and the data for Thomey kidney and the control kidneys are in close agreement with his values. The glycosphingolipid levels in Martensson's study were originally given as μmole/gm dry weight of tissue. Assuming visceral tissues to be composed of 75% water on the average (45), multiplication of Martensson's values by 0.25 transforms the data to μmole/gm wet weight of tissue. The Thomey data showed an elevation in globoside and a depression in trihexosyl ceramide (gal-gal-glc-cer) as compared with normal levels. This finding is consistent with a metabolic block at the site of globoside catabolism to gal-gal-glc-cer, assuming that gal-gal-glc-cer is derived from globoside via enzymatic cleavage of the terminal β-N-acetylgalactosamine moiety.

A similar result was obtained with spleen from Thomey. The depression in the amount of gal-gal-glc-cer was not observed here, but there was an enormous

twenty-fivefold increase in the amount of globoside present. Svennerholm and Svennerholm (58) reported that normal spleen consisted chiefly of lactosyl ceramide (gal-glc-cer, 50-60%); globoside was present only to the extent of 12-18% of the total glycosphingolipids. The three normal controls corresponded closely to the Svennerholms' criteria, but the Thomey spleen contained an overwhelming proportion of globoside.

Results with liver were somewhat different. In the same study, Svennerholm and Svennerholm (58) reported that the dominant glycosphingolipid of normal liver was gal-glc-cer (65-75%), while gal-gal-glc-cer and globoside were present in far lesser amounts, 7-10% and 8-12%, respectively. Levels for the normal controls corroborated these findings, but Thomey liver exhibited a thirtyfold increase in galNAc-gal-glc-cer and an elevenfold increase in globoside. Normal gal-gal-glc-cer was not found in Thomey's liver. More significant, however, is the fact that Thomey galNAc-gal-glc-cer was found via GLC and MS analysis to be asialo ganglioside G_{M2}. This finding is in accord with a diagnosis of Tay-Sachs disease with visceral involvement. Grégoire, et al. (50) had previously reported the storage of asialo G_{M2} in the liver in a case of Tay-Sachs disease with visceral involvement.

In all normal and Thomey visceral tissues studied, no gangliosides were detected in the aqueous phases of the

Folch washes or the dialysis-modified Folch procedure. In the Thomey kidney and spleen there was a trace of globoside observed on TLC of the lyophilized material from the Folch upper phase. Due to the excess of globoside in these tissues, traces of this compound partitioned into the aqueous methanolic Folch upper phase. Eeg-Olofsson, et al. (44) and Suzuki, et al. (45), however, reported ganglioside G_{M2} accumulation in the livers and spleens of their Tay-Sachs patients. Taketomi and Kawamura (49), on the other hand, did not detect ganglioside G_{M2} storage in the viscera of their Tay-Sachs patient. These differences might be attributed to variations in methodology, since it seems inconsistent that asialo G_{M2} would accumulate to such an extent in the liver, for example, without at least some storage of ganglioside G_{M2} occurring as well. More careful ganglioside extraction and assay procedures would perhaps reveal the presence of ganglioside G_{M2} in the visceral tissues. Alternatively, these storage differences might be due to genetic variations that are not yet understood.

Analysis of Thomey cerebral grey and white matter revealed the classical storage pattern for Tay-Sachs ganglioside. Asialo G_{M2} dominated the mixture of neutral glycosphingolipids in grey matter and was also present to a significant extent in the glycosphingolipids of white matter. The ganglioside G_{M2} was present in ten- to

twentyfold excess over the other grey and white matter gangliosides found in the aqueous phase of the modified Folch extracts. In addition, glucocerebroside (glc-cer) was detected to a significant extent in Thomey grey matter and the levels of gal-cer and sulfatide (SO_3gal-cer) were severely depressed in Thomey white matter. Gal-glc-cer was present to a minor extent in both Thomey and normal grey and white matter. These observations are consistent with previous studies on neutral glycosphingolipids of Tay-Sachs brain (44,49,59) and of normal brain (45,49).

The galNAc/glc and NANA/glc ratios found in these studies deserve special comment. All of these ratios were consistently low and were especially so in the case of the brain polysialo gangliosides. The consistency of these low ratios probably indicates an inadequate re-N-acetylation procedure. It has been the practice in our laboratory to add some (ca. 0.2 ml) acetic anhydride to the neutralized methanolysate in the presence of Ag_2CO_3 and let the re-N-acetylation mixture stand for a minimum of 6 hours up to a maximum of 24 hours. From the variability of the data, it is obvious that this procedure will have to be made more rigorous if reliable results are to be obtained. Although the mechanism of Ag_2CO_3 catalysis is not known, optimal acetic anhydride concentration and reaction time could be achieved via re-N-acetylation experiments with known micromolar amounts of

N-acetylneuraminic acid. The standardization of this procedure would greatly facilitate the accurate GLC analysis and identification of hexosamine- and NANA-containing glycosphingolipids.

The investigations of the glycosphingolipid and ganglioside content of Thomey brain and visceral organs, coupled with identical studies on normal controls, have led to an accurate identification of Thomey's lipid storage disorder. An examination of the data accumulated conclusively establishes Todd Thomey as a case of Tay-Sachs disease with gross visceral involvement. Three glycosphingolipids were found to accumulate in excessive amounts: (1) normal kidney globoside (galNAc-gal-gal-glc-cer) in the visceral organs, (2) asialo ganglioside G_{M2} (galNAc-gal-glc-cer) in the brain and liver, and (3) ganglioside G_{M2} (galNAc-gal(NANA)-glc-cer) in the brain. These three compounds share a common structural feature: all possess a terminal β-N-acetylgalactosamine moiety (8,24). (The presence of a terminal β linkage was not proven, however, for the compounds isolated in this study.) Thus, it appears that Todd Thomey is a case of Tay-Sachs disease with visceral involvement very similar, if not identical, to the case described by Sandhoff, et al. (46,47). In that study, an absence of the enzyme β-N-acetylhexosaminidase was postulated as the cause of the excessive kidney

globoside accumulation in the visceral organs and of the accumulation of ganglioside G_{M2} and its asialo derivative in the CNS. Classical Tay-Sachs patients exhibited normal β-N-acetylhexosaminidase activity; normal neuraminidase activity was observed in both the exceptional and classical cases. In a later publication, Sandhoff (60) investigated this enzyme deficiency more closely and found that β-N-acetylhexosaminidase consisted of two main fractions with isoelectric points at pH 7.3 and 5.0. In classical cases of Tay-Sachs disease, patients exhibited a lack of the fraction with isoelectric point at pH 5.0 in brain and liver, but the activity of the pH 7.3 fraction was elevated three- to fourfold in the brains of these patients. The exceptional case of Tay-Sachs disease, however, exhibited a complete absence of both enzyme components. These preliminary data would suggest a correlation of kidney globoside storage with the absence of the pH 7.3 fraction of β-N-acetylhexosaminidase, but sufficient evidence has not yet been obtained to warrant such a conclusion. At about the same time, Okada and O'Brien (61) in a study of β-N-acetylhexosaminidase activity in Tay-Sachs patients and normal controls found this same dual-component activity in the controls and labeled these components A and B. These investigators also found an absence of component A in all Tay-Sachs patients studied, thus paralleling Sandhoff's work (60).

These findings suggest that β-N-acetylhexosaminidase assays of Thomey cerebral and visceral tissues would have been of immense value in clarifying the distinction between classical Tay-Sachs disease and Tay-Sachs disease with visceral storage. The data in this study, however, unequivocally identify Thomey as a case of Tay-Sachs disease with visceral involvement and strongly suggest an identity with the exceptional case of Sandhoff, et al. (46,47). Full clarification, however, must await careful enzymatic analysis and characterization of the remaining tissues.

SUMMARY

Glycosphingolipids were isolated from cerebral grey and white matter, kidney, spleen, and liver of Todd Thomey, an 18-month old child who died from a lipid storage disorder diagnosed as classical Tay-Sachs disease. Three different glycosphingolipids accumulated in excessive amounts: (1) normal kidney globoside (galNAc-gal-gal-glc-cer) in the visceral organs, (2) asialo ganglioside G_{M2} (galNAc-gal-glc-cer) in the brain and liver, and (3) ganglioside G_{M2} (galNAc-gal(NANA)-glc-cer) in the brain. These findings classified Thomey as an exceptional case of Tay-Sachs disease with visceral involvement. A striking similarity was noted between this case and a case reported by Sandhoff, et al. (46,47) in which a deficiency of β-N-acetylhexosaminidase was cited as the cause for glycosphingolipid storage in the brain and visceral organs.

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