

Glycolipid abnormalities in a myoclonic variant of late infantile amaurotic idiocy

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ABSTRACT Glycolipids were isolated from the brain of a patient with a myoclonic variant of late infantile amaurotic idiocy. There was an abnormal glycolipid pattern in gray and white matter. The observed high concentration of gangliosides was due to a uniform accumulation of all four major gangliosides and was not limited to one species such as ganglioside A₁, as in Tay-Sachs disease, or ganglioside A₂, as in gangliosidosis-G_{M1}. Two additional stored substances were identified as ceramide lactoside and ceramide tetrahexoside. Partial and total hydrolysis of these ceramide hexosides revealed that their ceramide moiety is identical with the ceramide portion of gangliosides. The sequence of hexoses in the carbohydrate chain of the ceramide dihexoside and ceramide tetrahexoside further suggests a metabolic and chemical relation to gangliosides. Some implications of these findings for the theories of the metabolic defects in gangliosidoses are discussed.

SUPPLEMENTARY KEY WORDS Tay-Sachs disease · gangliosidosis-G_{M1} · gangliosides · ceramide lactoside · ceramide tetrahexoside · gray matter · white matter

IN 1935 KLENK (1) isolated a water-soluble glycolipid from the brain of a patient diagnosed as having had infantile amaurotic idiocy (Tay-Sachs disease) combined with Niemann-Pick disease. He identified this glycolipid as the storage material typically found in Tay-Sachs disease (2). He also isolated this lipid, although in lesser yield, from normal human brain (3), and named it ganglioside because of its glycolipid character and its occurrence in ganglion cells. Further investigations (4–8) revealed that the term “ganglioside” comprised a complex mixture of substances, which could be separated

into four main components whose structures have been elucidated in the following manner: ganglioside A₂, B₁, C₁, and C₃ (9–14).¹ In infantile amaurotic idiocy, however, 70–80% of the accumulated material has been shown to be identical with ganglioside A₁ (16–18), which, because of its low concentration in normal brain, is not readily identified with the major gangliosides. During recent years several special forms of gangliosidoses have been reported to be distinguishable from Tay-Sachs disease both morphologically and with regard to the storage material. Thus, in some cases of infantile amaurotic idiocy ganglioside A₂, but not the Tay-Sachs ganglioside A₁, was found to be stored (19–24). This disease has been termed generalized gangliosidosis (due to the fact that visceral organs in addition to the nervous system are also involved), or gangliosidosis-G_{M1} (a more systematic and unambiguous nomenclature, by which the prefix indicates the stored ganglioside) (23). Probably the only known case of gangliosidosis-G_{M3} has been reported by Pilz, Sandhoff, and Jatzkewitz (25). In addition to elevated levels of ganglioside A₁, A₂, or A₀₂, gangliosidoses are characterized by the concurrent accumulation of glucocerebroside (26), ceramide lactoside (25), ceramide digalactoside (27), *N*-acetylgalactosaminyl (1 → 4) galactosyl (1 → 4) glucosylceramide (27, 28), and ceramide tetrahexoside (23, 29). In one case (29) the storage of ceramide tetrahexoside was only suggestive because the specimen had been preserved in formalin for a prolonged period. The ceramide tetrahexoside could have been an artifact formed by the breakdown of normal gangliosides which are labile in formalin-fixed tissues (29–31). This report is concerned with yet another form of gangliosidosis, a myoclonic variant of late infantile amaurotic idiocy.

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; NAN, *N*-acetylneuraminic acid.

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¹ In this report, the nomenclature of gangliosides is that of Klenk. The Svennerholm nomenclature (15) is given below in the following parentheses: A₁(G_{M2}); A₂(G_{M1}); A₀₂(G_{M3}); B₁(G_{D1a}); C₁(G_{D1b}); C₃(G_{T1}).

MATERIAL AND CASE HISTORY

Part of the cerebrum used for this examination was obtained postmortem and stored at -40°C until analysis. The male patient died at the age of 7 years, 10 months. He exhibited fits and psychic degeneration at the age of 5. On admission to the hospital he was 7 years, 6 months old, bedridden, and physically retarded. In addition to a profound dementia, amaurosis with optic atrophy, increased tonus with pyramidal signs, and contractions, there were generalized myoclonias with motoric effect which were considered characteristic of this disease. Brain biopsy confirmed the diagnosis of a myoclonic variant of late infantile amaurotic idiocy. Two sisters of this patient had already died, and they had had a similar clinical picture. Further details of the clinical, histochemical, and electron microscopic observations are reported elsewhere (32). As a control, a normal brain from an 8-year-old patient was analyzed simultaneously with the pathological specimen.

METHODS

Extraction and Isolation of Gangliosides and Ceramide Hexosides

Gray and white matter were separated, weighed, and extracted with 19 volumes of chloroform-methanol 2:1 at room temperature for 5 min in a mechanical homogenizer (33). The extract was filtered through a sintered-glass funnel. The gangliosides were partitioned into the aqueous phase with the addition of 0.2 volume of 0.58% NaCl. The upper phase was removed, and the lower phase was washed twice with the theoretical upper phase containing no salt. The combined upper phases were dialyzed against several changes of distilled water for 24 hr at 4°C . The contents of the dialysis bag were lyophilized and assayed for neuraminic acid by the orcinol reaction (34, 35). Prior to the isolation of ceramide hexosides, the crude total lipid extract of the lower phase was dialyzed at room temperature against petroleum ether through a rubber membrane (36). TLC of the concentrated dialysate was carried out on Silica Gel G (E. Merck AG, Darmstadt, Germany) with *n*-hexane-ethyl acetate 4:1 as the solvent system. The lipids were located by charring the plate with 50% H_2SO_4 and were identified as cholesterol, cholesteryl esters, and triglyceride. The nondialysable lipids were fractionated by column chromatography on silicic acid (100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.), which had been activated for 20 hr at 115°C . The lipids, dissolved in chloroform, were eluted with a stepwise gradient of methanol in chloroform. Aliquots were taken and analyzed by TLC on Silica Gel G in chloroform-methanol-water 65:25:4. Ceramide hexosides and their corre-

sponding chromatographic standards were located by spraying the TLC plates with 50% H_2SO_4 followed by charring at 150°C . The ceramide tetrahexoside was obtained, after column chromatography, as a pure homogeneous compound. The ceramide dihexoside fraction contained large amounts of phosphatidyl ethanolamine which was separated from the ceramide dihexoside by preparative TLC. Both ceramide dihexoside and ceramide tetrahexoside were white, water-insoluble substances. Dissolved in hot chloroform, the ceramide hexosides crystallized when the solutions were made slightly turbid by dropwise addition of methanol and kept at 4°C for 24 hr. The total amount of reducing sugars present in the isolated glycolipids was determined according to Somogyi (37) and calculated as galactose. Conditions of hydrolysis: 2 *N* HCl, sealed tube, 3 hr at 100°C . The Blix modification (38) of the Elson and Morgan test (39) was used for the hexosamine determination. Conditions of hydrolysis: 2 *N* HCl, sealed tube, 16 hr at 100°C .

Test Substances

Test substances, derived from ganglioside A_2 , were kindly provided by Professor E. Klenk (ceramide lactoside) and Dr. W. Gielen (ceramide trihexoside and ceramide tetrahexoside).

Products of Acid Methanolysis

Fatty Acids. The methyl esters of fatty acids were obtained by refluxing the lipids with 30 volumes of 5% methanolic HCl for 3 hr at 75°C . The hydrolysate was extracted three times with petroleum ether (boiling range $35-60^{\circ}\text{C}$) to remove methyl esters. The combined petroleum ether extracts were washed with water and dried over Na_2SO_4 for 12 hr (under N_2). The solutions were then filtered and carefully evaporated to a small volume. Portions of each sample were analyzed by GLC on a Barber-Colman Model 10 gas chromatograph with argon as carrier gas. Column temperature was 160°C ; stationary phase, 15% ethylene glycol succinate polyester on Gas Chrom P, 80-100 mesh (Applied Science Laboratories Inc., State College, Pa.).

Sphingosine. After extraction of the fatty acid methyl esters, the hydrolysate was made alkaline (pH 11) with $\text{Ba}(\text{OH})_2$, and sphingosine was extracted with ethyl ether. The sample was chromatographed by TLC on Silica Gel G using chloroform-methanol-2 *N* NH_4OH 40:10:1 as solvent. Sphingosine was identified after the plates were sprayed with 0.2% ninhydrin in butanol.

Hexoses. After the sphingosine was removed, barium was precipitated by addition of 2 *N* H_2SO_4 . The excess acid was removed by shaking the aqueous alcoholic solution with Lewatit M 600 (Bayer, Leverkusen, Germany), and the solution was evaporated to dryness. The methylglycosides in the residue were hydrolyzed with

1 N HCl in sealed tubes at 100°C for 2 hr. After hydrolysis methanol was added to the aqueous solution which was then carefully evaporated. This procedure which removed the acid was repeated several times. The concentrated solution was lyophilized. The mixture of hexoses was applied to Schleicher and Schuell 2043 b paper for descending paper chromatography in ethyl acetate–pyridine–water 10:7:4. The reducing sugars were located by spraying the chromatogram with an alkaline solution of silver nitrate (40, 41), and their molar ratios were determined quantitatively by photometry in a Beckman Analytrol.

Sequence Analysis of Carbohydrates. The sequence of hexoses in the isolated ceramide oligosaccharides was determined as follows. Ceramide tetrahexoside and ceramide dihexoside (as well as corresponding test substances) were partially hydrolyzed in sealed tubes with 0.1 N HCl at 100°C for 20 min. The hydrolysate was dialyzed for 24 hr against distilled water, and the dialysate was neutralized and lyophilized. Monosaccharide in the dialysate was identified by chromatography as described above. The other product of hydrolysis, a ceramide hexoside with its carbohydrate chain shortened by one hexose, was separated from the remaining unhydrolyzed starting material by preparative TLC on plates containing Silica Gel G with chloroform–methanol–water 65:25:4 as solvent. Ceramide hexosides were located by brief exposure of the plates to iodine vapor. Zones of silica gel were scraped off the plates and were extracted with chloroform–methanol 2:1 at room temperature. The extracts were evaporated to dryness, and the residue was chromatographed on thin-layer plates together with chromatographic standards as described for the isolation of ceramide hexosides. The carbohydrate composition of the recovered ceramide hexosides was qualitatively and quantitatively determined after hydrolysis of aliquots with 5% methanolic HCl and descending paper chromatography of the reducing sugars as outlined above. Under these conditions the ceramide dihexoside gave rise almost quantitatively to a glucocerebroside, galactose being the only monosaccharide cleaved off. A glucocerebroside was also the only ceramide monohexoside found after sequential removal of monosaccharide units from ceramide tetrahexoside.

RESULTS

A comparison of the concentration of individual lipids in white and gray matter of the myoclonic variant with the normal control brain is summarized in Table 1. The myoclonic variant of amaurotic idiocy is characterized by an elevated level of gangliosides and the accumulation of ceramide dihexoside and ceramide tetrahexoside,

TABLE 1 LIPIDS IN GANGLIOSIDOSIS AND A NORMAL CONTROL BRAIN

| Lipid | Gangliosidosis | | Normal Control Brain | |
|------------------------|----------------|--------------|----------------------|--------------|
| | Gray Matter | White Matter | Gray Matter | White Matter |
| Galactocerebroside | 0.6 | 11.2 | 0.9 | 12.8 |
| Glucocerebroside | <0.1 | <0.1 | — | — |
| Ceramide lactoside | 1.4 | 0.8 | <0.2 | — |
| Ceramide tetrahexoside | 1.3 | 0.9 | <0.1 | — |
| Gangliosides | 2.8 | 1.9 | 1.2 | 0.3 |
| Gangliosides* | 2.9 | 1.7 | 1.2 | 0.35 |
| Lecithins | 9.2 | 8.2 | 9.5 | 8.3 |
| Sphingomyelins | 2.3 | 4.8 | 2.1 | 4.8 |
| Total Lipids | 29.2 | 65.5 | 27.3 | 63.2 |

The results are expressed as weight per cent based on the dry weight of tissue.

* In these analyses *N*-acetylneuraminic acid was determined by the method of Warren (59).

normally absent or present only in trace amounts in gray matter (23, 25, 42).

Gangliosides

The higher concentration of gangliosides in gray and white matter of a myoclonic variant of amaurotic idiocy is due to a generalized accumulation of all major gangliosides. The gangliosides present and their relative amounts do not differ from a ganglioside mixture obtained from normal brain (Fig. 1). The storage of only one species of

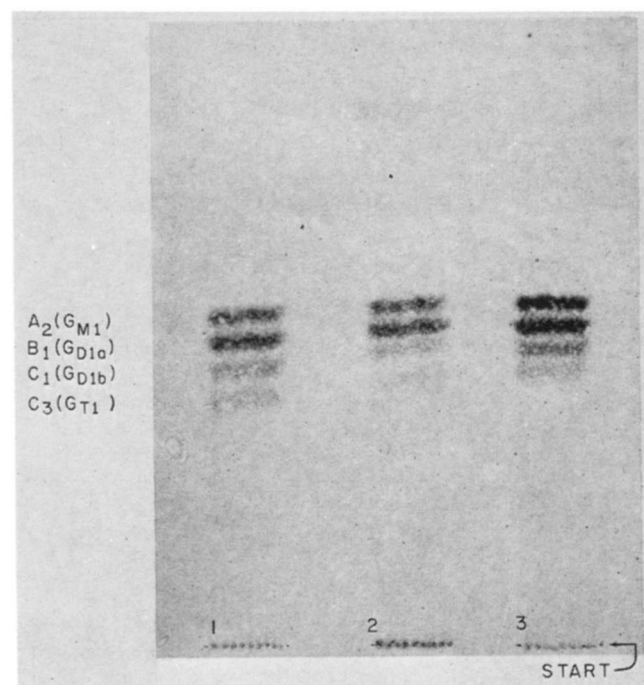


FIG. 1. Thin-layer chromatogram of gangliosides on Silica Gel G. Solvent, *n*-butanol–pyridine–water 6:5:4; spray, Bials reagent (developed at 120°C, 15 min). 1, gangliosidosis, gray matter; 2, gangliosidosis, white matter; 3, normal gray matter.

ganglioside such as ganglioside A₁ in Tay-Sachs disease, or ganglioside A₂ in gangliosidosis-G_{M1} can thus be excluded. The gangliosides isolated from both normal and pathological brain contained 32.3% *N*-acetylneuraminic acid.

Ceramide Dihexoside and Ceramide Tetrahexoside

The lower phase of the chloroform-methanol extract of gray and white matter from the pathological brain contained, in addition to galactocerebrosides, phosphorus-free substances which were isolated and tentatively characterized by chromatographic comparison with known standards as ceramide dihexoside and ceramide tetrahexoside. Fig. 2 depicts the hexoses obtained after acid methanolysis of the isolated ceramide hexosides. Partial hydrolysis of the ceramide dihexoside produced glucocerebroside and galactose. In conformity with the results of the total analysis, the ceramide dihexoside was identified as ceramide lactoside (Table 2). The examination of the sequential arrangement of hexoses in the carbohydrate chain of the ceramide tetrahexoside characterized this compound as well as the ceramide tetrahexoside prepared from ganglioside A₂ as galactosylgalactosaminylgalactosylglucosylceramide. The possibility that the amino sugar of the tetrahexoside is present in the form of its *N*-acetyl derivative could not be excluded. The sphingosine bases of ceramide lactoside and ceramide tetrahexoside showed the same thin-layer chromatographic pattern as authentic sphingosines from normal gangliosides.

GLC analyses of fatty acids from the glycolipids are summarized in Table 3. The fatty acids of the galactocerebroside isolated both from pathological and normal brain were identical. In addition to stearic acid and other unsubstituted fatty acids, α -hydroxyacids, which are characteristic of this glycolipid, were also found.

DISCUSSION

The study of the products of partial hydrolysis of ceramide lactoside and ceramide tetrahexoside, i.e. carbo-

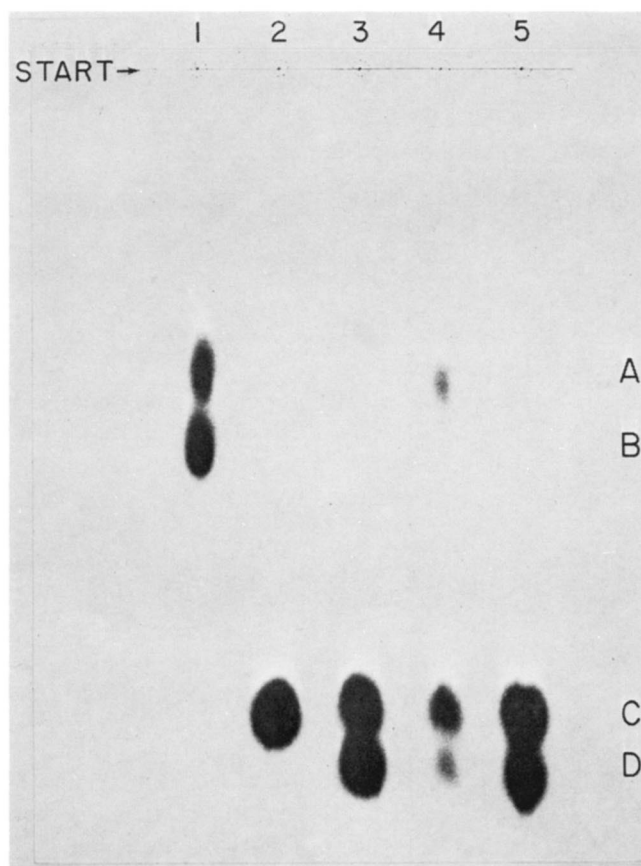


FIG. 2. Descending paper chromatogram of the reducing sugars from ceramide hexosides. Solvent: ethyl acetate-pyridine-water 10:7:4; spray, alkaline solution of silver nitrate. 1, galactosamine and glucosamine (test substances); 2, reducing sugars from cerebrosides (gangliosidosis); 3, reducing sugars from ceramide lactoside (gangliosidosis); 4, reducing sugars from ceramide tetrahexoside (gangliosidosis); 5, galactose and glucose (test substances). A, galactosamine; B, glucosamine; C, galactose; D, glucose.

hydrates, sphingosine, and fatty acids, suggests that these hexosides are chemically related to gangliosides, although the limited yield of each glycolipid did not permit the elucidation of the carbohydrate linkages. The galactocerebroside found in this disease, however, is identical with galactocerebroside from normal brain. It is chem-

TABLE 2 ANALYSES OF CERAMIDE HEXOSIDES

| Ceramide Hexoside | C | | H | | Reducing Sugars | | Galactosamine | | Molar Ratios | | |
|---|-------|-------|-------|-------|-----------------|--------|---------------|-------|--------------|------|--------------------|
| | Calc. | Found | Calc. | Found | Calc.* | Found* | Calc. | Found | Glc | Gal | GalNH ₂ |
| | % | | % | | % | | % | | | | |
| Lactocerebroside (gangliosidosis) | | | | | | | | | | | |
| C ₄₃ H ₉₁ N ₁ O ₁₃ | 64.75 | 63.35 | 10.31 | 10.29 | 40.5 | 38.9 | | | 1 | 1.05 | |
| Lactocerebroside (test substance) | 64.75 | 64.19 | 10.31 | 10.42 | 40.5 | 38.2 | | | 1 | 1.03 | |
| Ceramide tetrahexoside (gangliosidosis) C ₆₀ H ₁₁₂ N ₂ O ₂₂ | 59.30 | 59.26 | 9.30 | 9.60 | 59.4 | 54.6 | 14.7 | 13.6 | 1 | 1.9 | 0.92 |
| Ceramide tetrahexoside (test substance) | 59.30 | 59.57 | 9.30 | 9.49 | 59.4 | 55.1 | 14.7 | 13.4 | 1 | 2.05 | 0.89 |

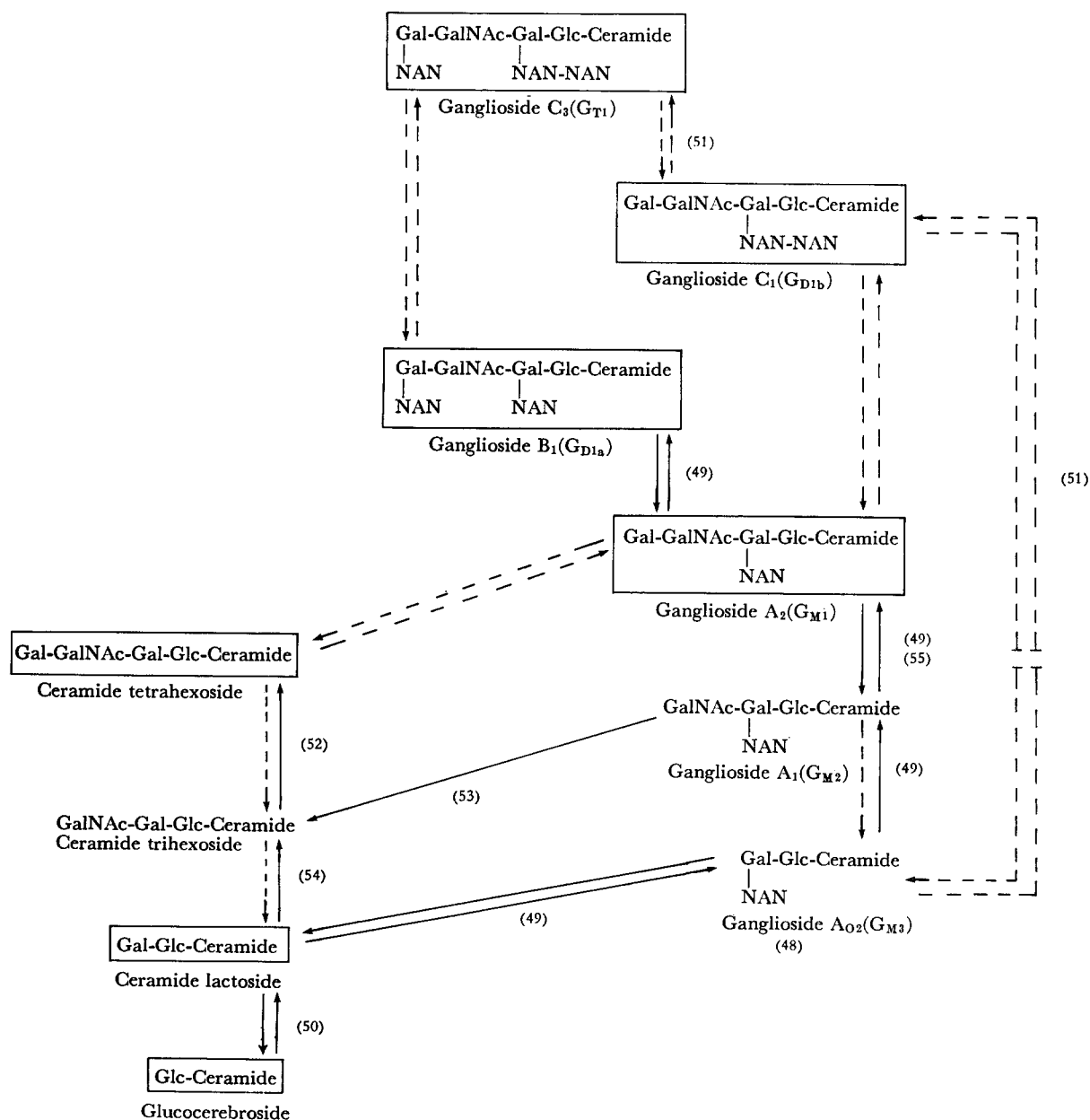
* Calculated as galactose.

TABLE 3 FATTY ACID COMPOSITION OF GANGLIOSIDES, CERAMIDE LACTOSIDE, AND CERAMIDE TETRAHEXOSIDE

| Lipid | C _{16:0} | C _{18:0} | C _{18:1} | C _{26:0} |
|---|-------------------|-------------------------------|-------------------|-------------------|
| | | <i>% of total fatty acids</i> | | |
| Gangliosides (gangliosidosis) | 2 | 88 | 1 | 9 |
| Gangliosides (normal control brain) | 2 | 87 | 1 | 10 |
| Ceramide tetrahexoside (gangliosidosis) | 2 | 86 | 2 | 10 |
| Ceramide tetrahexoside (test substance) | 2 | 88 | 1 | 9 |
| Ceramide lactoside (gangliosidosis) | 3 | 86 | 1 | 10 |
| Ceramide lactoside (test substance) | 2 | 86 | 1 | 11 |

ically unrelated to major gangliosides or to the ceramide hexosides mentioned above because of its terminal galactose and its fatty acids, whereas the ceramide portion of the glycolipid stored in the pathological brain is linked to glucose.

The terminal galactose distinguishes the accumulated ceramide tetrahexoside from the globoside found in erythrocyte stroma, which possesses a terminal galactosamine moiety (43). The ceramide tetrahexoside appears to be identical with a glycolipid occurring in the brains of individuals with gangliosidosis-G_{M1} (23, 29). In gangliosidosis-G_{M1}, however, its storage is confined to the gray matter and is not, as in this case of a myoclonic



variant of amaurotic idiocy, extended to the white matter. As in Tay-Sachs disease (23) and in gangliosidosis-G_{M3} (25), the ceramide dihexoside could be identified as ceramide lactoside.

A review (44) of previously reported cases of late infantile amaurotic idiocy reveals that the majority constitutes a subacute and precocious variant of the classical form of juvenile amaurotic idiocy and cannot be regarded as a ganglioside storage disease (44-46). The results of our study suggest that at least our case of a myoclonic variant of late infantile amaurotic idiocy presents the features of a gangliosidosis, characterized by a general increase of all four major gangliosides and an abnormal concentration of a ceramide tetrahexoside and ceramide lactoside. It seems unwarranted however, to propose that the observed glycolipid abnormalities are characteristic for the myoclonic variant of late infantile amaurotic idiocy in general. This caution is justified, because another case, probably belonging to the same category, of which we have biochemical and pathological accounts was not associated with a gangliosidosis (47).

From a theoretical point of view, the abnormal composition of glycolipids might be attributed to one or several defective steps in the biosynthesis or biodegradation of the gangliosides: cleavage of *N*-acetylneuraminic acid, followed by stepwise removal of terminal hexoses during biodegradation or accumulation of intermediary ceramide hexosides during the biosynthesis of gangliosides. The scheme on page 245 depicts these various possibilities. Frames denote substances, which are found to be stored in our case of a myoclonic variant of late infantile amaurotic idiocy. The solid arrow designates the reaction established, while the broken arrow indicates the reaction proposed.

During recent years intensive studies have been directed to the discovery of the enzymatic lesions in lipidoses. Whereas there is still no evidence for primary defects in specific glycolipid transferases, a marked deficiency of galactosidase activity has been found to be responsible for the delayed degradation of ganglioside A₂ in gangliosidosis-G_{M1} (56). In infantile cases of Gaucher's disease an almost total lack of glucosidase leads to the cerebral accumulation of an abnormal glucocerebroside whose fatty acid pattern and sphingosine composition is identical with the ceramide moiety of normal brain gangliosides (57, 58). Thus, the glucocerebroside of the infantile form of Gaucher's disease can possibly be associated with the biosynthesis and biodegradation of gangliosides.

A clear-cut delineation of both cause and relationship of the various types of gangliosidoses is inevitably limited at the present time. Further confirmation of enzyme defects is necessary before a meaningful interpretation of metabolic disturbances in gangliosidoses can be assumed.

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