Krabbe disease: a galactosylsphingosine (psychosine) lipidosis

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Abstract The primary genetic defect underlying Krabbe disease or globoid cell leukodystrophy is considered to be a deficiency of galactosylceramide-β-galactosidase. In the present study of the brains from 18 patients who had died from Krabbe disease at 7–37 months of age, the concentration of galactosylceramide of cerebral and cerebellar white matter was severely reduced to 10–20% of that in age-matched controls. The lowest values were found in the most long-standing cases. Lactosylceramide was reduced to about 50% of normal, while globotriaosylceramide, globotetraosylceramide and III^3-α-fucosylneolactotetraosylceramide were increased 10 to 100-fold. Two glycosphingolipids, which have never before been isolated from normal human brains were now isolated and characterized: galactosylsphingosine (psychosine) and galactosylβ1 → 4galactosylceramide. We were unable to identify galactosylsphingosine in normal human brains with certainty. We estimate its concentration in the cerebral white matter in Krabbe disease to be increased at least 100-fold (higher than normal). Psychosine was isolated also from the cerebral cortex in Krabbe disease after derivatization to the N-acetyl form. Its concentration there was 1 nmol/g tissue compared with 6–10 nmol/g in the white matter. All the neutral glycosphingolipids were isolated and their structure proved by the quantitative determination of their components, degradation by acid and specific glycohydrolases and permethylation and gas–liquid chromatographic–mass spectrometric assay of the methylated sugars. The paradoxical findings of a severely reduced concentration of galactosylceramide and a primary deficiency of cerebroside-β-galactosidase can be explained by the present finding of the accumulation of galactosylsphingosine in the brains from patients who had died from Krabbe disease. The enzyme has a broad specificity and it normally also degrades galactosylsphingosine. Because of competitive inhibition by the accumulated galactosylceramide its lysosomal hydrolysis will be blocked. The concentration of psychosine will steadily increase and reach toxic levels and kill the oligodendroglial cells. This results in an arrest of the galactosylceramide biosynthesis. Therefore, we feel that galactosylsphingosine and not galactosylceramide is the primary storage substance in the brain in Krabbe disease and that the disease is a psychosine lipidosis.—**Svennerholm, L., M-T. Vanier, and J-E. Månsson.** Krabbe disease: a galactosylsphingosine (psychosine) lipidosis. *J. Lipid Res.* 1980. **21:** 53–64.

Supplementary key words lactosylceramide · galactosylβ1 → 4 galactosylceramide · globotriaosylceramide · globotetraosyl-ceramide · III^3-α-fucosylneolactotetraosylceramide · N-acetyl-psychosine

In the first chemical study of the brain in Krabbe disease in 1949 Brante (1) drew attention to the association between the extreme loss of myelin lipids and the histological finding of myelin breakdown. This early finding is not mentioned in recent comprehensive reviews, in which interest has been focused mainly on Austin's observation (2) of an increase of cerebrosides in the globoid cells. It is true that galactosylceramides are stored in the relatively few multinucleated globoid bodies, but there is no convincing evidence that the mononuclear globoid cells store any noteworthy amounts of cerebrosides. Austin's finding (3, 4) of a relatively larger increase of cerebrosides than of cholesterol is artifactual. In a recent extensive study of brains in 18 typical cases of Krabbe disease (5), we found the molar ratio of cerebrosides to cholesterol in cerebral white matter to be 1:8 compared with 1:3–4 in age-matched controls. We ascribed the main lipid changes of white matter partly to a severe myelin loss leading to an extensive reduction of cerebrosides, sulfatides, and cholesterol, and a moderate loss of phosphoglycerides and, partly, to replacement of neural tissue by cells of mesenchymal origin.

Suzuki and Suzuki (6) have attributed Krabbe disease to a deficient activity of galactosylceramide-β-galactosidase and postulated that accumulation of galactosylceramide leads to destruction of oligodendroglia, the cells which produce myelin. It was difficult to ascertain whether the slightly cytotoxic galactosylceramide caused the disappearance of oligodendroglial cells and this led Miyatake and Suzuki (7, 8) to advance the psychosine hypothesis.

A minor portion of the galactosylceramide was not degraded to ceramide and galactose but first de-acylated to psychosine, which is known to be highly cytotoxic. Some years later we succeeded in isolating psychosine from cerebral and cerebellar white matter of deceased Krabbe disease patients (9, 10). This initial finding has now been confirmed in a larger series of brain material, with a procedure that eliminates the risk of hydrolysis of galactosylceramide to psychosine during the isolation step and that also allows isolation of psychosine from cerebral cortex of patients with Krabbe disease.

The replacement of neural tissue by cells of mesenchymal origin could be expected to give rise to glycosphingolipids of visceral type, as first suggested by Eto and Suzuki (11). Several reports about these glycosphingolipids (9, 12, 13) have appeared, but the isolated substances have not been adequately characterized. In the present study we isolated the major oligohexosides in pure form and determined their structure from several preparations.

EXPERIMENTAL PROCEDURES

Brain material

Krabbe brains. Specimens of brain material were obtained at autopsy of 17 children who had died from Krabbe disease at 7–27 months of age, and from one patient, L.B., who had died at 37 months. The clinical diagnosis was confirmed by neuropathological examination of cerebral sections, and the determination of cerebroside-β-galactosidase in cerebral grey and white matter. Data on the patients and their brain lipid composition have been reported previously (5, 14). For isolation of the sphingolipids, cerebral cortex and cerebral white matter were carefully dissected and treated separately. From the 17 cases with a typical course of the disease, three cerebral cortex preparations weighing 250–1150 g, and four white matter preparations (55–260 g) were made of tissue specimens from 6 to 10 different brains in each batch. Also two preparations of whole cerebellar tissue (45 and 180 g) were studied. Cerebral white matter from L.B., who had had a slower course of the disease, was analyzed separately, and simultaneously with that in a case (F.L.) with a typical clinical history.

Normal brains. Pooled samples of cerebral grey and white matter from 9 infant brains (age range 2–27 months) were studied in parallel. The total sample consisted of 230 g of cerebral cortex and 130 g of white matter, about half of which had been dissected from brains in 9–12-month-old children (15). The control material included also the brains from a 4 month-old and a 12 month-old infant, in whom lipid composition for age (16) had previously been found to be normal.

Chemicals

All the organic solvents used were of analytical quality. Light petroleum, (boiling range 42–43°C) used for GLC of the fatty acid and long-chain bases was redistilled and tested for purity as previously reported (17). Silicic acid, <100 mesh, was obtained from Mallinckrodt A.R., and Biosil-A, 200–400 mesh, from Bio Rad Laboratories, Richmond CA. Silica gel H and G were purchased from Fluka A.G., Buchs, Switzerland; Florisil, 200–300 mesh, from Floridin Co, Talahassee, FL and precoated Silica gel TLC plates from Merck, Darmstadt, Germany; Whatman DE 22 DEAE-cellulose from Balston Ltd, Maidstone, England; Selectacel TEAE-cellulose from Brown Co, Berlin, NH; Sephadex G-25, fine and DEAE-Sephadex A-25 from Pharmacia, Uppsala, Sweden. Reference galactosylsphingosine (psychosine) was prepared by alkaline hydrolysis of galactosylceramide isolated from human brain (18). D-Galactose, D-glucose, D-mannose, L-fucose, and D-galactosamine hydrochloride were bought from Pfanstiehl Laboratories (Waukegan, IL), and D-glucosamine hydrochloride from Mann Laboratories (NY). Sphingenine hydrochloride was a gift from Dr. K. A. Karlsson, Göteborg, Sweden. Sodium taurocholate, A grade, was obtained from Calbiochem, San Diego, CA. α-Fucosidase of bovine kidney was purchased from Boehringer, Mannheim, Germany; α-galactosidase isolated from fig and β-galactosidase and β-N-acetylhexosaminidase from jack bean were gifts from Dr. Y-T. Li, Tulane University, LA.

Characterization of isolated glycolipids

Determination of components. The methods used for quantitative determination of total neutral hexoses (19), hexosamine (20), and sphingosine (21) have been described earlier. The fatty acid and sphingosine compositions of the glycolipids were determined after hydrolysis in 1.0 M HCl in methanol–water 82:18 (22). The quantitative composition of the carbohydrate moiety was determined as alditol acetates by GLC, as described previously (17), with the exception that 2.5 M trifluoroacetic acid was used for the hydrolysis of the glycolipids instead of HCl.

Partial acid hydrolysis. The glycolipids were partially degraded by hydrolysis with 0.2 M HCl in methanol–water 4:6 for 15 min at 100°C. After neutralization and partition against chloroform, the resulting glycolipid mixture was analyzed on TLC plates developed with chloroform–methanol–water 60:

32:7, and stained with the orcinol reagent (19). Galactosylceramide and glucosylceramide were separated on borate-impregnated silica gel TLC plates with chloroform–methanol–2 M ammonia in water 40:10:1 as developing solvent (23). The isolated fucolipids were defucosylated by hydrolysis in 0.1 M trichloroacetic acid at 100°C for 2 hr (24).

Sequential determination of the carbohydrate chain with specific hydrolases. α-L-Fucose was removed from the fucolipids by degradation with α-L-fucosidase for 48 hr at 37°C. The incubation mixture contained 25 nmoles of fucolipid, 0.2 mg of sodium taurocholate, and 0.2 units of α-L-fucosidase in 0.1 ml of sodium acetate buffer, 0.2 M, pH 4.5. After solvent partition, the reaction mixture was analyzed by TLC as described earlier (25).

Permethylation studies. Permethylation of the glycolipids was performed with the method of Hakomori (26). The permethylated glycolipids were isolated by partition against chloroform and purified further by preparative TLC with chloroform–methanol 96:4. The substances were hydrolyzed with 0.35 M sulfuric acid in acetic acid–water 80:20 for 20 hr at 80°C (27) and thereafter neutralized, reduced and peracetylated. The partially methylated alditol acetates were determined with a Perkin–Elmer F22 gas chromatograph and a LKB 2091 gas chromatograph–mass spectrometer. The neutral sugars were separated on 3% ECNSS-M at 150°C or 3% OV-225 at 170°C. The amino sugars were separated by using 3% ECNSS-M at 190°C or 3% OV-17 at 180°C.

Isolation of the sphingolipids

Lipid extraction. The brain tissue was homogenized in a Waring blendor with 8 vol of chloroform–methanol (C–M) 1:2, and reextracted with 8 vol of C–M 2:1. The combined lipid extracts were evaporated and redissolved in 1 vol of C–M 1:2. After standing overnight, the insoluble residue was removed by centrifugation. The removal of nonlipid contaminants by evaporation of the extract, resolubilization in C–M 1:2, and centrifugation was repeated until no sediment appeared within 24 hr. All the steps were performed at room temperature.

Purification of the extract and crude separation of the sphingolipids. For the brain specimens from patients with Krabbe disease, our original method was modified (15) in order to improve the isolation of glycolipids. Since one of the major aims of our studies was to isolate psychosine quantitatively, particular care was taken to avoid the solvent partition step, which incurs the risk of a loss of psychosine in the upper phase, as well as of alkaline hydrolysis of the phosphoglycerides in the presence of galactosylceramide.

This hydrolysis could cause a minute hydrolysis of the cerebrosides and then give rise to artifactual psychosine. The procedure used in the preparations V, L.B. and F.L. are described below in detail. A schematic flow chart of the method is given in **Fig. 1**.

The total lipid extract was passed through a Sephadex G-25 column (1 g Sephadex for 5–10 g of tissue) for removal of low molecular weight nonlipid contaminants (28). Neutral lipids were separated from the acidic ones by ion-exchange chromatography on DEAE-Sephadex A-25 in acetate form (1 g for 10–20 g tissue) according to the procedure of Yu and Ledeen (29). A crude separation of the neutral sphingolipids was achieved by column chromatography on 100–200 g of silicic acid (Biosil-A, 200–400 mesh, 1 g silicic acid for 0.5–2.0 g tissue), using the following elution scheme: chloroform, 10 vol (per g. adsorbent); C–M, 9:1, 10 vol; C–M, 4:1, 10 vol; C–M, 2:1, 10 vol; C–M, 1:1, 10 vol; methanol, 20 vol. Fractions of approximately 20 ml were collected and monitored by TLC.

The sphingolipids were eluted in the following order: ceramide, glucosylceramide, galactosylceramide, lactosylceramide, digalactosylceramide, globotriaosylceramide, globotetraosylceramide, neolactotetraosylceramide and fucosylneolactotetraosylceramide. Galactosylsphingosine showed variations in its pattern of elution, with its main fraction appearing either between globotriaosylceramide and globotetraosylceramide, or together with neolactotetraosylceramide. With optimal loading of the column, ceramide, monohexosylceramides, and the lactosylceramide were well separated and eluted before ethanolamine phosphoglycerides. In the subsequent fractions, the glycosphingolipids were eluted together with large amounts of phosphoglycerides and with considerable overlapping. These pooled fractions were saponified overnight at room temperature by addition of 0.5 M KOH in methanol–water 1:1. After adjustment of the pH to 5 with 2 M HCl in water, the extract was evaporated to dryness. The lipids were redissolved in chloroform–methanol–water 60:30:4.5, the extract was freed from salts on a Sephadex G-25 column, and the lipids were rechromatographed on a silicic acid column, as described above.

An alternative procedure was used for the analysis of the 12-month-old control brain and the brain from F.L. which was affected by Krabbe disease. The pooled fraction, from the appearance of ethanolamine phosphoglycerides until that of sphingomyelin, was subjected to peracetylation (30). The peracetylated lipids were chromatographed on a Florisil column (30), which gave a complete separation between the
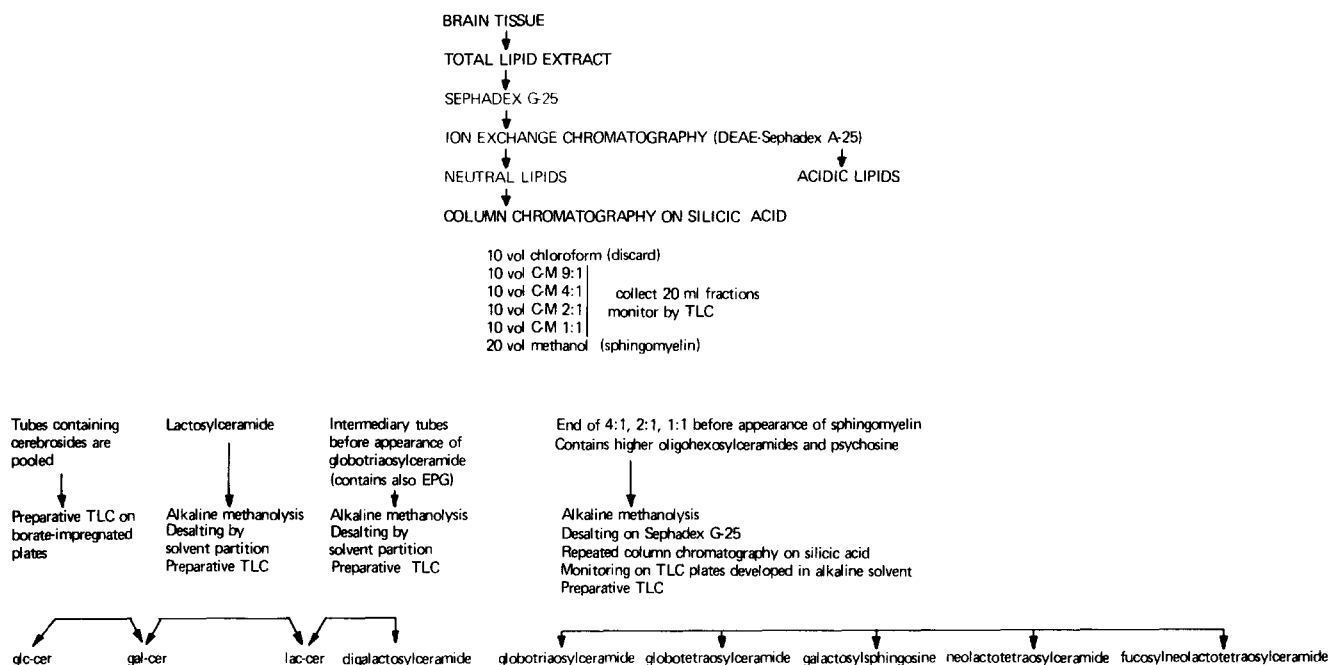
Fig. 1. Flow chart of the scheme used for preparation of the neutral sphingolipids and of psychosine in cerebral tissue from patients with Krabbe disease. EPG, ethanolamine phosphoglycerides; glc-cer, glucosyl-ceramide; gal-cer, galactosylceramide; lac-cer, lactosylceramide.

glycolipids and phospholipids. The glycolipid fraction was dissolved in C–M 1:2; an equal volume of 0.2 M of NaOH in methanol–water 8:2 was added and the hydrolysis was continued for 30 min at room temperature. This treatment completely removed the O-acetyl groups, but left intact the N-acetyl group in N-acetyl-psychosine.

Final purification of the sphingolipids. The sphingo-lipids were finally purified by preparative TLC. The TLC plates were developed in the following solvent systems: for ceramide, chloroform–methanol–acetic acid 90:5:5, for the glycolipids, chloroform–meth-anol–water 65:25:4, and for psychosine also chloro-form–methanol–4 M ammonia in water 60:35:8. Glucosyl- and galactosylceramides were separated on borate-impregnated plates made with a 2.5% solution of sodium tetraborate in water, and de-veloped in chloroform–methanol–water 65:25:4 for 2 hr. Under these conditions 5–6 μmoles of the monohexosylceramide mixture could be applied to one plate with excellent separation of the glucosyl-ceramide. The plates were sprayed with bromthymol blue reagent (31) or water. The gel corresponding to the lipid fractions was scraped out and eluted on small columns with C–M 2:1 containing 5% water.

Quantitative determination of the glycolipid content. The amount of individual glycolipids was measured by quantitative determination of their hexose content (19) and in some instances by quantitative densi-

tometry of the glycolipids separated on thin-layer plates. Portions of the isolated glycolipids were chromatographed on precoated thin-layer plates with standard mixtures of 2.5 and 5.0 nmoles of each individual glycolipid. The plates were sprayed with 0.15 M cupric acetate in 1.4 M phosphoric acid in water (32) heated for 25 min at 140°C, and absorbances were then recorded with a Zeiss KM3 or Vernon PH16 chromatogram scanner at 450 nm.

RESULTS

Characterization of the sphingolipids

Monoglycosylceramides. The monohexoside fraction was separated into glucosyl- and galactosylceramide on borate-impregnated plates. The substances mi-grated on TLC plates like authentic standards of spleen glucosylceramides and brain galactosylcera-mides. GLC of alditol acetates showed the occurrence of only glucose and galactose, respectively.

Diglycosylceramides. The diglycosylceramide fraction migrated in three bands at TLC in chloroform–methanol–water 65:25:4, the slowest migrating one had a migration rate of 0.84 in relation to lactosyl-ceramide with C_{18}-fatty acid. This band contained galactose as the only sugar, and had a sphingosine: galactose ratio of 1:2 (**Table 1**). After acid transmeth-ylation only 2-hydroxy fatty acids were recovered by

TABLE 1. The molar ratio of carbohydrates and sphingosine of neutral glycosylceramides and galactosylsphingosine from brain tissue in Krabbe disease.

Glycosphingolipid	Brain Tissue	Sphingosine	Gal	Glc	Fuc	GalNAc	GlcNAc
Lactosylceramide	C C[a]	1.00	0.99	0.96			
	Wh M[b]	1.00	1.16	0.80			
	Cbl[c]	1.00	1.10	0.81			
Digalactosylceramide	C C	1.00	2.06				
	Wh M	1.00	2.08				
	Cbl	1.00	2.35				
Globotriaosylceramide	C C	1.00	1.76	1.00			
	Wh M	1.00	1.92	1.00			
	Cbl	1.00	1.70	0.95			
Globotetraosylceramide	C C	1.00	2.15	1.04		1.21	
	Wh M	1.00	2.15	1.09		1.16	
	Cbl	1.00	1.89	0.90		1.01	
III^3-α-fucosylneolacto-tetraosylceramide	C C	1.00	1.87	0.99	1.08		0.80
	Wh M	1.00	1.75	0.98	1.00		0.91
	Cbl	1.00	2.08	1.23	1.06		1.01
Galactosylsphingosine	Wh M	1.00	1.08				
	Cbl	1.00	1.13				

[a] Cerebral cortex.
[b] White matter.
[c] Cerebellum.

GLC. Enzymic hydrolysis with β-galactosidase gave galactosylceramide and galactose. Permethylation analysis of the partially methylated alditol acetates showed two peaks with the same migration rates as 2,3,4,6-tetra-O-methylgalactitol and 2,3,6-tri-O-methylgalactitol.

The two fast-moving bands were isolated together. They contained galactose, glucose, sphingosine, and fatty acids. The ratio sphingosine:glucose:galactose was 1.00:0.80:1.15 in white matter and cerebellum. This suggested that the substance consisted of a small portion of digalactosylceramide besides galactosylglucosylceramide. Hydrolysis with β-galactosidase gave, besides free galactose, ca. 5% galactosylceramide, 80% glucosylceramide, and 15% unreacted dihexoside. This dihexoside was resistant to α-galactosidase. Analysis of the permethylated sugars from the two fast-migrating dihexosylceramide fractions by GLC and GLC–MS gave two major peaks, 2,3,4,6-tetra-O-methylgalactitol and 2,3,6-tri-O-methylglucitol, and a small amount of 2,3,6-tri-O-methylgalactitol.

Taken together, these data indicate that the dihexoside fraction consisted of lactosylceramide and a small amount of digalactosylceramide. Since only the methyl esters of unsubstituted aliphatic fatty acids were detected at the fatty acid assay by GLC this digalactosylceramide fraction migrated faster than the other because it contained normal (unsubstituted) fatty acids.

The results suggest that the diglycosylceramide fraction of brains in Krabbe disease consists of galβ1 → 4glc-ceramide and a minor portion of galβ1 → 4gal-ceramide.

Triglycosylceramides. The trihexoside fraction contained sphingosine, glucose, and galactose in the ratio of 1.0:1.0:1.8. The galactose value was slightly low, but at the alditol acetate assay of sugars no aminosugar or other sugar, except galactitol and glucitol, could be detected. Enzymic hydrolysis with α-galactosidase yielded galactose + lactosylceramide; and with α-galactosidase plus β-galactosidase, galactose + glucosylceramide as products. Permethylation and GLC assay of partially methylated alditol acetates showed 2,3,4,6-tetra-O-methylgalactitol and 2,3,6-tri-O-methylglucitol. These data suggest that the only triglycosylceramide of brains in Krabbe disease has the structure galα1 → 4galβ1 → 4glc-ceramide.

Tetraglycosylceramides. In white matter two fractions occurred in the area where the tetrahexosides migrate at TLC. In cerebral cortex only the fast migrating band was detected. It migrated at TLC as globotetraosylceramide of human red cells. It contained sphingosine:glucose:galactose:galactosamine in the proportions 1:1:2:1. Stepwise degradation of the glycolipid with β-N-acetylhexosaminidase, α-galactosidase, and β-galactosidase gave the following glycolipids as products: gal-gal-glc-cer, gal-glc-cer and glc-cer. Analyses of the partially methylated alditol acetates gave 2,4,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylglucitol, and 3,4,6-tri-

TABLE 2. The relative migration rate by TLC of galactosyl-sphingosine in different solvent systems compared with that of lactosylceramide with C_{18} fatty acids.

Solvent	
Chloroform–methanol–water 65:25:4	0.33
Chloroform–methanol–water–acetic acid 75:25:2:1	0.33
Chloroform–methanol–14 M ammonia in water 70:30:5	0.78
Chloroform–methanol–2.5 M ammonia in water 60:40:9	0.95

O-methyl-2-deoxy-2-N-methylacetamidogalactitol. Permethylation analysis of the triglycosylceramide, isolated by preparative TLC after partial acid hydrolysis of the tetraglycosylceramide, showed 2,3,4,6-tetra-O-methylgalactitol, 2,3,6-tri-O-methylgalactitol, and 2,3,6-tri-O-methylglucitol. No methylated aminosugar could be detected. The results of the structural investigation shows that the faster migrating tetraglycosylceramide has the same structure as globotetraosylceramide.

The other tetraglycosylceramide migrated on TLC as neolactotetraglycosylceramide. Its components were sphingosine, glucose, galactose, and glucosamine in the ratio of 1:1:2:1. Degradation of the glycolipid with β-galactosidase gave a product that migrated like glc$N$Ac-gal-glc-cer on TLC. Incubation first with β-galactosidase, then heat-inactivation followed by an incubation with β-N-acetylhexosaminidase resulted in the formation of a substance with a TLC mobility corresponding to that of lactosylceramide. The combined action of β-galactosidase and β-N-acetylhexosaminidase gave glucosylceramide as a product. This suggests that the sequence and anomeric configuration of this tetraglycosylceramide is galβglc-NAcβgalβglccer.

Higher oligoglycosylceramides. Only one neutral glycolipid with more than four sugars was isolated in the materials investigated. It contained sphingosine, glucose, galactose, N-acetylglucosamine, and fucose in the ratio 1:1:2:1:1. The fucolipid showed only one band by TLC in chloroform–methanol–2.5 M ammonia in water 40:80:25. Partial acid hydrolysis gave a mixture of glycolipids with thin-layer chromatographic behavior as gal-glc$N$Ac-gal-glc-cer, glc$N$Ac-gal-glc-cer, gal-glc-cer, and glc-cer. Treatment of the fucolipid with α-fucosidase converted it to a tetraglycosylceramide with the same TLC mobility as neolactotetraglycosylceramide. Incubation of the tetraglycosylceramide with β-galactosidase gave a triglycosylceramide with the same mobility as glc$N$Ac-gal-glc-cer. Further degradation of the triglycosylceramide with β-N-acetylhexosaminidase converted it into lactosylceramide. The combined action of β-galactosidase and β-N-acetylhexosaminidase gave glucosylceramide as end product. Analyses of the permethylated sugars from the fucolipid revealed that it contained 2,3,4-tri-O-methylfucitol, 2,3,4,6-tetra-O-methylgalactitol, 2,4,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylglucitol, and 6-O-methyl-2-N-methylacetamidoglucitol. Permethylation analyses of the defucosylated substance, isolated by preparative TLC after weak acid hydrolyses, gave as a result 2,3,4,6-tetra-O-methylgalactitol, 2,4,6-tri-O-methylgalactitol, 2,3,6,-tri-O-methylglucitol and 3,6-di-O-methyl-2-deoxy-2-N-methylgalactitol, 2,3,6-tri-O-methylglucitol and 3,6-di-O-methyl-2-deoxy-2-N-methylacetamidoglucitol. No 2,3,4,-tri-O-methylfucitol could be detected. The results of the degradation studies and the permethylation analyses suggest that the fucose-containing glycolipid of brain in Krabbe disease has the following structure: galβ1 → 4(fucα1 → 3)glc$N$Acβ1 → 3galβ1 → 4glc → cer.

Galactosylsphingosine. The mobilities on TLC of galactosylsphingosine in different solvent systems are given in **Table 2.** Peracetylation of the isolated psychosine and subsequent mild saponification yielded a substance that migrated by TLC in several different solvents like authentic N-acetylgalactosylsphingosine. Galactosylsphingosine contained sphingosine and galactose in the molar ratio of 1:1. Galactose was the only sugar found in the alditol acetate analysis. The substance was ninhydrin-positive and no unsubstituted or 2-hydroxy fatty acid methyl esters could be detected after acid transmethylation. Permethylation of the galactosylsphingosine gave 2,3,4,6-tetra-O-methylgalactitol as the only partially methylated sugar.

Ceramide composition. Stearic acid (18:0) was the dominating fatty acid of the higher neutral glycosphingolipids as of the gangliosides (**Table 3**). Other more abundant fatty acids were 24:1, 24:0 and 22:0. Globotriaosyl- and globotetraosylceramides did not show any significant difference between grey and white matter but the fucosylneolactotetraosylceramide contained more 18:0 and less C_{24}-fatty acids in the grey matter. The fatty acid patterns of glucosyl- and lactosylceramide contained significantly less of 18:0 than the other glycolipids in cerebral white matter, and they showed large similarities with the patterns of normal (unsubstituted) fatty acids in galactosylceramide. The fatty acid composition of the hydroxy fatty acids of digalactosylceramide of white matter was the following: $C_{22:0}$ 10%, $C_{23:0}$ 13%, $C_{24:0}$ 52%, $C_{24:1}$ 13%, $C_{25:0}$ 6%, $C_{25:1}$ 2%, $C_{26:0}$ 1% and $C_{26:1}$ 2%.

4-Sphingenine (d 18:1) constituted ca. 90% of the sphingosine (**Table 4**), and 3–5% each of sphinganine (d 18:0), 4,11-sphingadiene (d 18:2) and 4-eicosasphingenine (d 20:1). Lactosylceramide and the fucosylneolactotetraosylceramide both contained sig-

TABLE 3.   Fatty acid composition of neutral glycosylceramide in brain tissue in Krabbe disease.

Glycosphingolipid	Brain Source	Preparation	16:0	18:0	20:0	22:0	23:0	24:0	24:1	>24
Glucosylceramide	C C[a]	I + III	7	40	4	8	10	17	11	3
	Wh M[b]	I + III + IV	6	26	3	10	13	23	17	3
Lactosylceramide	C C	I + III	10	52	3	5	4	9	11	5
	C C	V	7	35	3	7	6	14	19	8
	Wh M	I + III + IV	8	27	3	7	8	18	22	5
	Wh M	V	9	25	3	7	10	16	24	6
	Cbl[c]	V	11	34	4	7	8	13	18	
Globotriaosylceramide	C C	I + III	7	51	3	8	4	8	17	1
	C C	V	6	51	3	10	3	9	15	
	Wh M	I + III + IV	6	55	3	6	4	6	19	1
	Wh M	V	6	52	3	8	5	7	19	
	Cbl	V	8	58	3	8	3	6	12	
Globotetraosylceramide	C C	I + III	6	60	3	9	4	10	24	1
	C C	V	5	48	3	10	3	10	20	
	Wh M	I + III + IV	4	50	3	6	4	6	25	1
	Wh M	V	5	54	3	7	5	8	17	
	Cbl	V	6	59	3	8	4	9	10	
III³-α-fucosylneolacto-tetraosylceramide	C C	V	5	72	3	6	2	5	6	
	Wh M	III + IV	3	51	3	6	4	6	26	1
	Wh M	V	4	52	3	6	4	7	23	
	Cbl	V	5	61	3	5	2	7	16	

[a] Cerebral cortex.
[b] White matter.
[c] Cerebellum.
Values expressed in molar percentage.

nificantly more of 4-eicosasphingenine in grey matter than in white matter.

Quantitative distribution

The concentration of neutral glycosphingolipids in control infant brains and in brains from children who had died from Krabbe disease are given in **Tables 5 and 6.** The control brains contained only galactosylceramide and lactosylceramide in substantial amounts. Only minute amounts of the other glycosphingolipids were found, and because of shortage of material, it was not possible to characterize them completely. From their migration by TLC in several different solvents with authentic samples of the appropriate glycosphingolipid standard the following glycolipids from the control brains were identified: globotriaosylceramide, globotetraosylceramide, neolactotetraosylceramide, and III³-α-fucosylneolactotetraosylceramide. A weak band migrating between globotriaosylceramide and globotetraosylceramide had a R_f similar to that of gangliotriaosylceramide, and there were probably also minute amounts of gangliotetraosylceramide.

The accuracy with which substances occurring in low concentrations can be quantified depends on two major factors, careful separation of grey and white matter, and limitation of the isolation procedure to only a few steps. At the assay of the two individual control infant brains careful dissection was performed and the peracetylation procedure was used, which involves only a few steps. The values found for these brain specimens should be regarded as particularly relevant. The shorter isolation procedure led to slightly higher values for the neutral glycosphingolipids than those previously found in the pooled samples of brain from 2 to 27-month-old children, but no major difference in the glycolipid pattern was observed. In a previous study (9) the fraction designated tetraglycosylceramide was found to contain equal amounts of galactosamine and glucosamine. In retrospect we can assume that this fraction was a mixture of globotetraosylceramide and neolactotetraosylceramide, and that tetraglycosylceramide II was a mixture of gangliotetraosylceramide and III³-α-fucosylneolactotetraosylceramide.

The importance of a strict separation of the brain material into grey and white matter was also observed in preparation V of brains from children with Krabbe disease. Since preparation V also contained most brain material, the values found for it should also be regarded as the most representative ones. Thus, the values noted for galactosylceramide and lactosylceramide in the cerebral cortex (grey matter) in Krabbe disease were essentially normal, while globotriaosylceramide and globotetraosylceramide were increased.

TABLE 4. Sphingosine composition of neutral glycosylceramides and galactosylsphingosine of brain tissue in Krabbe disease.

Glycosphingolipid	Brain Source	t 18:0	d 18:0	d 18:1	d 18:2	t 20:0	d 20:1
Glucosylceramide	C C[a]		6	94			
	Wh M[b]		4	93			3
Lactosylceramide	C C	1	3	81	6	<1	9
	Wh M	<1	4	88	2	1	4
	Cbl[c]	<1	3	91	2	1	2
HFA-digalactosylceramide	C C	<1	7	89	3	1	1
	Wh M	1	5	86	7		
	Cbl	<1	8	86	2	2	2
Globotriaosylceramide	C C	2	3	86	3	1	4
	Wh M	1	5	86	5	1	2
	Cbl		6	84	7	1	2
Globotetraosylceramide	C C	3	4	86	4	1	2
	Wh M	1	3	90	3	1	2
	Cbl	1	3	92		1	2
III³-α-fucosylneolacto-tetraosylceramide	C C	<1	3	88	2	1	6
	Wh M		2	91	3	1	3
	Cbl	<1	2	92	3	1	2
Psychosine	Wh M		5	91	1		3

[a] Cerebral cortex.
[b] White matter.
[c] Cerebellum.
Values expressed in molar percentage.

In the white matter, the concentration of galactosylceramide was strikingly reduced to 10–15% of that in age-matched control material. But there was also a constant and significant diminution of the lactosylceramide concentration to one-third to one-half of the concentration in age-matched control brains. A particularly low concentration of lactosylceramide was found in L.B., who had survived longer than the other patients. In that patient also, the galactosylceramide concentration was lower than in the other preparations. The concentration of glycosphingolipids in the cerebellum was largely the same as that in the cerebral white matter.

The concentration of the oligoglycosphingolipids in the brain was abnormally high in Krabbe disease. In the white matter the amounts of globotriaosyl-, globotetraosyl- and fucosylneolactotetraosylceramide found were many times the normal values and, though

TABLE 5. Quantitative distribution of the neutral glycosphingolipids in cerebral white matter from control infants and patients who had died from Krabbe disease.

	Controls			Krabbe Disease					
	4 months	12 months	2–27 months	I	III	IV	V	F.L.	L.B.
Weight of sample studied (g)	5	45	130	140	87	55	260	65	100
	nmol/g fresh weight			*nmol/g fresh weight*					
Galactosylceramide	10,700	23,600	21,400	3,700	3,600	2,750	3,050	3,850	2,000
Glucosylceramide	62	130	110	32	91	n.d.	52	97	n.d.
Lactosylceramide	215	360	273	140	150	130	120	140	50
Digalactosylceramide			n.d.[a]	n.d.	n.d.	13	2	8	4
Globotriaosylceramide	0.5	1.4	<1	40	62	33	26	24	18
Globotetraosylceramide	4.0	4.7	2	64	100	64	64	51	59
Neolactotetraosylceramide	1.0	0.8	<1	n.d.	n.d.	n.d.	3	5	n.d.
III³-α-fucosylneolacto-tetraosylceramide	6.5	2.4	n.d.	15	19	23	17	19	15
Galactosylsphingosine	<0.05	<0.05	n.d.	n.d.	10	10	6	9	6

[a] Not determined.

TABLE 6. Quantitative distribution of the neutral glycosphingolipids in cerebral cortex of control infants and patients who had died from Krabbe disease.

	Controls			Krabbe Disease			
	4 months	12 months	2–27 months	I	III	V	F.L.
Weight of sample studied (g)	42	55	230	250	350	1155	100
	nmol/g fresh weight			*nmol/g fresh weight*			
Galactosylceramide	415	455	975	650	780	400	950
Glucosylceramide	8	4	5	16	17	n.d.	23
Lactosylceramide	15	24	30	36	52	20	40
Globotriaosylceramide	0.3	0.8	<1	8	26	6	12
Globotetraosylceramide	1.0	n.d.[a]	1	13	14	14	20
III³-α-fucosylneolacto-tetraosylceramide	1.0	0.6	1	n.d.	8	n.d.	7

[a] Not determined.

not so markedly, that of neolactotetraosylceramide also. An increase in these glycolipids has also been found in other brain storage diseases and in subacute chronic inflammations (33).

The occurrence of two other glycolipids in the Krabbe disease group was unique. The first one was galactosylβ1 → 4galactosylceramide. We were able to isolate only that portion of it which contained hydroxy fatty acids, but indirectly we showed that a second portion contained normal fatty acids. The concentration varied considerably between different preparations. The most important finding was, however, the definite identification of *galactosyl-sphingosine (psychosine)* in the grey and white matter and cerebellum from children that had died from Krabbe disease. The concentration of psychosine from cerebral and cerebellar white matter was constant in all the preparations analyzed, 6–10 nmol/g tissue, and independent of the concentration of galactosyl-ceramide and/or the duration of the disease. With the new isolation method in which psychosine is isolated as N-acetylpsychosine it was possible to demonstrate its occurrence also in cerebral cortex. This raises the question whether galactosylsphingosine exists in measurable amounts in normal brain tissue. We have applied different procedures to find an answer to this question. Both with the old method and the N-acetyl-sphingosine method we have demonstrated material migrating approximately at the same rate as authentic galactosylsphingosine and N-acetylsphingosine, re-spectively, but its concentration is then less than 0.05 nmol/g tissue. It is important to stress that we had to apply to the plates a lipid extract from ca. 5 g of tissue, and there was always a very strong background of other substances which made the identification very uncertain. Therefore, our conclusion must be that we have not been able to identify with certainty

galactosylsphingosine in normal brain tissue, and that its concentration is at least 100 times higher in Krabbe disease.

DISCUSSION

A severe genetic deficiency of a lysosomal enzyme will, by definition, lead to a massive storage in the lysosome of its primary substrate (34). In 1970, Suzuki and Suzuki (6, 35) reported a profound lack of cerebroside-β-galactosidase in various tissues, serum, leukocytes and cultured fibroblasts from patients with Krabbe disease. However, a considerable residual cerebrosidase activity has since been shown in leuko-cytes and cultured fibroblasts from such patients (36), and in the same cell types of the obligatory heterozy-gotes (the parents) the cerebrosidase activities have shown a wide range of variation (36). In the central nervous system, a local storage of galactosylceramide has been demonstrated in globoid bodies (2), although the overall cerebroside concentration in the brain is strongly reduced (3–5, 11, 38). No universal or local increase of cerebrosides was found in any visceral organ in our study. Dawson (37) reported highly elevated levels of galactosylceramide in liver in Krabbe disease. Also other results in his publication are controversial. Therefore, we think it difficult to consider galactosylceramide the primary storage substance in Krabbe disease.

In the beginning when we tried to isolate psychosine from tissue samples of 5–10 g of white matter we failed to detect any psychosine, and it was not before we used tissue samples of 50 g or more that we were able to find definitive proof for the occurrence of galactosylsphingosine in cerebral white matter and cerebellum (9). We then repeated the isolation of psychosine in many samples and when it was isolated

as acetylpsychosine 5 g of white matter or cerebellum was sufficient for a quantitative isolation. The psychosine concentration has been remarkably constant in the different brain tissue samples assayed, whether the children survived for 7–12 months (sample IV) or 37 months (L.B.). The psychosine concentration was between 6 and 10 nmol/g fresh cerebral white matter, and this concentration is at least 100-fold that estimated in the control cerebral white matter. We have not been able to identify galactosylsphingosine in normal cerebral white matter with certainty, but in the last preparations the psychosine concentration was less than 0.05 nmol/g fresh weight. In a previous publication (9) we estimated the psychosine concentration in cerebral white matter of control children to be approx. 0.1 nmol/g, but that preparation was isolated from a lipid extract which had undergone alkaline hydrolysis with 1.0 M KOH at 37°C overnight. It has always been assumed that this mild alkaline hydrolysis would not degrade the sphingolipids to corresponding lyso substances, but when this procedure was first devised no methods were available to demonstrate degradation to less than 0.01%. In order to avoid any possible hydrolysis of galactosylceramide to sphingosine during the isolation, the cerebroside was separated from the lipid extract before the alkaline hydrolysis. Therefore, there is barely any risk of psychosine being formed during the isolation.

Since this modification of the procedure was introduced, all attempts to isolate psychosine from control brains have failed. This finding supports our assumption that psychosine does not form from galactosylceramide during the isolation. Since no psychosine was formed in the control brains the risk is still less in Krabbe disease, in which the galactosylceramide concentration is much lower. We can therefore conclude that the accumulation of galactosylsphingosine in the white matter of the brain in Krabbe disease is characteristic of the disease.

When Miyatake and Suzuki (7, 8) advanced their psychosine hypothesis they assumed that the psychosine was formed by the degradation of galactosylceramide. In our first reports (9, 10) of the finding of psychosine we suggested instead that the psychosine was an aberrant formation from the biosynthesis of sphingosine and UDP-galactose, which was normally rapidly degraded. Our hypothesis that the psychosine is a product of an aberrant biosynthesis has been accepted by Suzuki and Suzuki (33) in their recent large review of Krabbe disease. In the present study, the finding of a new dihexosylceramide, galactosylβ1 → 4galactosylceramide in the cerebral white matter in Krabbe disease, lends further support for our hypothesis that under normal conditions aberrant glycosphingolipids are formed but then rapidly hydrolyzed.

However, the mechanism of the psychosine accumulation in Krabbe disease is not yet properly understood. In normal mammalian brain tissue the cerebroside-β-galactosidase has been shown to hydrolyze galactosylceramide, lactosylceramide, monogalactosyldiglyceride, and galactosylsphingosine (psychosine) (33). A low but definite level of residual cerebroside-β-galactosidase activity is a constant observation in Krabbe disease (33, 36), and the finding of the lowest levels of cerebroside and lactosylceramide in the brain of our patient (L.B.) with the longest survival, suggests that considerable hydrolysis of galactosylceramide still occurs in the brain tissue of these patients. The question thus arises why psychosine and digalactosylceramide are hydrolyzed in the brains of normals, but not in patients with Krabbe disease despite this residual activity. A possible explanation might be a competitive inhibition of psychosine hydrolysis by the limited but existing lysosomal storage of galactosylceramide.

In Krabbe disease, one can assume that the concentration of psychosine in the oligodendroglial cells steadily increases, reaches toxic levels, and kills the cells. The selective destruction of the oligodendroglial cells results in an arrest of the myelin formation. From the fatty acid composition of cerebrosides, we have estimated that it will occur approximately at the age of 3–4 months (9). As a consequence of the arrest of the myelin formation and the destruction of the oligodendroglial cells, the concentration of cerebrosides and sulfatides of white matter is significantly lower than in age-matched controls. Also two other glycolipids which normally occur in white matter are reduced, namely glucosylceramide and lactosylceramide. These lipids closely resemble galactosylceramides in composition of unsubstituted fatty acids, which suggests that they are normally formed from the same fatty acid pool (in the oligodendroglial cells). Eto and Suzuki (11) found only traces of lactosylceramide in normal white matter, and thus reported an increase of this lipid in the cerebral white matter in Krabbe disease, but in fact its concentration is reduced to approximately one-half to one-third of normal.

The third glycolipid change, occurring mainly in the white matter of patients with Krabbe disease, is the significant increase of higher ceramide hexosides, particularly globotriaosylceramide and globotetraosylceramide. There is also an increase of a fucose-containing glycolipid, III[3]-α-fucosylneolactotetraosylceramide, which was first isolated by Yang and Hakomori from human adenocarcinomas (39). We are not aware of any previous report about its occurrence in normal mammalian tissue and our finding

that this glycolipid is one of the most abundant higher neutral ceramide hexosides in the white matter of infant brains opens several new interesting aspects.[2]

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