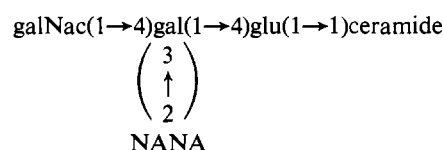


Structure of the Tay-Sachs' Ganglioside. I*

Robert Ledeen and Kenneth Salsman

ABSTRACT: Purified ganglioside from Tay-Sachs' brain was hydrolyzed in aqueous acid to give three glycolipids: glucocerebroside, ceramide-lactoside, and asialo-ganglioside. The latter was shown to have the structure, *N*-acetylgalactosamine(1→4)galactose(1→4)glucose(1→1)ceramide. Brief refluxing of the ganglioside with methanolic HCl gave "hematoside" as one of the products, with the structure *N*-acetylneuraminic acid (2→3)galactose(1→4)glucose(1→1)ceramide. The linkages in both cases were determined by identification of the fragments from the sequence: periodate-borohydride-acid hydrolysis. The results establish for the first time the location of NANA at C-3 of galactose and also serve to confirm the structure of the asialo derivative proposed by Makita and Yamakawa on the basis of permethylation studies. The ganglioside struc-

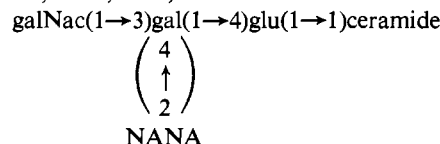
ture was shown to be



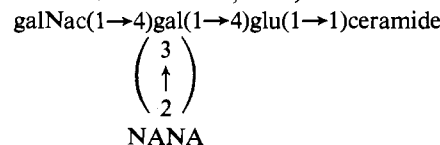
Sphingosine components included four long-chain bases, similar to those of normal brain gangliosides. Stearate comprised 90.5% of the fatty acid composition, and 19 others accounted for the remainder. A carefully purified sample showed no amino acids or peptides. The ganglioside itself was resistant to neuraminidase, but the hematoside derived from it was readily degraded to ceramide-lactoside.

The accumulation of ganglioside in brain tissue of Tay-Sachs' disease was first noted some 25 years ago (Klenk, 1939) and has since been confirmed by several laboratories (Cumings, 1960; Lees and Folch-Pi, 1961; Korey *et al.*, 1963; Wagner *et al.*, 1963; Saifer, 1964). Recently, this dramatic effect was shown to be due to proliferation of one type of ganglioside (Svennerholm, 1962; Wagner *et al.*, 1963), though others are present at much lower levels (Svennerholm, 1963; Wagner *et al.*, 1963; Saifer *et al.*, 1963). An NANA¹-containing glycolipid with similar composition has been isolated in minor amounts from normal human brain (Kuhn and Wiegandt, 1963; Svennerholm, 1963; Johnson and McCluer, 1963), but its suggested identity to the Tay-Sachs' material has yet to be demonstrated. Both compounds are thought to possess ceramide, glucose, galactose, *N*-acetylgalactosamine, and NANA in equimolar ratios. This sets them apart from the four major gangliosides of normal human brain, which have additional galactose and in most cases additional NANA as well (Kuhn and Wiegandt, 1963; Kuhn *et al.*, 1961; Svennerholm, 1963; Klenk and Gielen, 1963; Wolfe and Lowden, 1964; Johnson and McCluer, 1963).

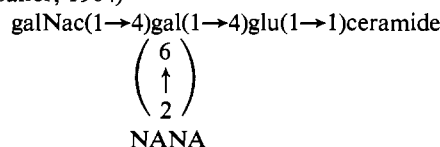
Sequence and substitution positions within the carbohydrate chain of the Tay-Sachs' ganglioside, as well as stereochemistry of the glycosidic bonds, are still unsettled questions. The following three structures have been proposed.

1. (Klenk, *et al.*, 1963)

II. (Makita and Yamakawa, 1963)



III. (Saifer, 1964)



This paper presents new evidence for the carbohydrate sequence and bonding positions, based mainly on periodate-borohydride studies; these were carried out on the Tay-Sachs' ganglioside itself and on two glycolipids derived from it by partial acid hydrolysis. The fatty acids and long-chain bases were analyzed

* From the Saul R. Korey Department of Neurology and the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, N. Y. Received March 26, 1965; revised June 7, 1965. The study was supported by NIH grants (NB-03356, NB-04834, and NB-01006).

¹ Abbreviations used: NANA, *N*-acetylneuraminic acid; galNac, *N*-acetylgalactosamine; glu, glucose; gal, galactose; hematoside, ceramide-glu-gal-NANA.

by gas-liquid partition chromatography, and the preparation of a sample free of associated peptides is also described.

Experimental Procedures

Ganglioside Purification. Crude ganglioside (200 mg) from Tay-Sachs' brain² was chromatographed on 40 g of activated silicic acid³ in a column 3 × 15 cm. The sample was applied in 5 ml of 20% methanol-chloroform⁴ to the column initially in chloroform. Elution with 1500 ml of 20% methanol-chloroform removed most of the lipid impurities, and subsequent elution with 30% methanol-chloroform brought through the major ganglioside. Sixteen fractions of approximately 250 ml each were collected with this solvent and were analyzed qualitatively with thin layer system II (see below). Of these 16 fractions, 4-10 gave a single resorcinol-positive spot on thin layer chromatography and were combined for subsequent use.

For preparation of highly purified ganglioside, material from fractions 4-10 above was chromatographed preparatively with thin layer system II (see below). Each plate containing a milligram or less was subjected to two consecutive runs of 7 hours each, with an hour or more drying in between. Ganglioside was detected by spraying with a dilute aqueous solution of Rhodamine 6G and viewing with ultraviolet light. Elution from the silica gel was accomplished with methanol-chloroform-pyridine-water (56:40:2:12), and the residue after filtration and evaporation was rechromatographed on a Unisil column. Methanol-chloroform (20%) removed rhodamine and any remaining residue of phosphatidylserine (Rouser *et al.*, 1961), while the ganglioside itself was eluted with 30% methanol-chloroform.

Paper, Thin Layer, and Gas Chromatography. Whatman 3MM paper was used for all paper chromatograms. Paper system A consisted of 1-butanol-pyridine-water (6:4:3), and system B was ethyl acetate-pyridine-acetic acid-water (5:5:1:3); the first was descending and the second ascending. Spots were developed by a modification of the alkaline silver nitrate dipping technique (Treveleyn *et al.*, 1950) which proved satisfactory for glycerol, threitol, erythritol, and sialic acid as well as the reducing sugars.

Three thin layer chromatography systems were used: I, chloroform-methanol-water (70:30:4); II, chloroform-methanol-2.5 N ammonia (60:40:9); III, heptane-diethyl ether (2:1). For systems I and III, regular 20 × 20 cm plates were used, while for system II double length (40 × 20 cm) plates were employed. Plates were coated with silica gel G⁵ at a thickness of 250 μ, and activated 40 minutes at 110°.

² A gift from the late Dr. Saul R. Korey.

³ Unisil, 200-325 mesh; Clarkson Chemical Co., Inc., Williamsport, Pa.

⁴ All solvent compositions are expressed as ratios or percentages by volume.

⁵ E. Merck, A.G. Darmstadt, Germany.

Gas-liquid partition chromatography was carried out with an F & M Model 1609 instrument, equipped with flame detector. A coiled 1.8-m column, 0.64-cm diameter, was packed with JXR silicone polymer⁶ coated 3% on Gas-Chrom P⁶; a similar column was prepared with 15% EGSS-X.⁶ Helium gas was used as the carrier with an inlet pressure of 30 psi.

Analytical Procedures. A. QUALITATIVE IDENTIFICATION OF CARBOHYDRATES. One milligram or less of glycolipid was hydrolyzed in 1 ml of 1 N HCl at 100° for 14 hours. The cooled mixture was shaken with 1 ml of chloroform, the aqueous phase was passed through Dowex 1-X4 (acetate) anion-exchange resin, and the effluent was evaporated to dryness with nitrogen. Chromatography with paper system A served to identify neutral sugars and hexosamine. Milder hydrolytic conditions were used for the partial liberation of hexosamine and sialic acid in their acylated forms, *i.e.*, 0.1 N HCl at 100° for 1 hour. The cooled mixture was brought to 2 ml with water, treated with 10 ml of chloroform-methanol (2:1), shaken, and centrifuged. The upper phase was evaporated to dryness and the residue was chromatographed with paper systems A and B.

B. QUANTITATIVE ANALYSIS OF CARBOHYDRATES. An aqueous stock solution (0.5 ml) containing 1.5 mg of ganglioside was mixed with an equal volume of 2 N HCl and heated in a sealed tube at 100° for 14 hours. After extraction with 1 ml of chloroform to remove lipids (see below), the aqueous phase was made up to 2.5 ml with water. Four aliquots of 0.2 ml were used for hexosamine determination according to the Elson-Morgan method as applied by Boas (1953). No effort was made to remove the acid or neutral sugars as these were found not to interfere.

The remaining 1.7 ml was passed through 3 ml of mixed bed resin Amberlite MB-3 and a total of 10 ml of effluent collected. This solution of neutral sugars was analyzed for glucose with glucostat, a glucose oxidase preparation,⁷ and for total hexose by the orcinol procedure (Svennerholm, 1956). Neuraminic acid was determined by the resorcinol method (Svennerholm, 1957) using small aliquots of the original ganglioside stock solution; blank runs were carried out with an equivalent amount of asialoganglioside.

C. LIPID ANALYSES. The chloroform extract from above (B) was evaporated to dryness with nitrogen and the residue was shaken in the cold with hexane. After prolonged centrifugation the hexane was removed to another tube and the procedure was repeated. The combined hexane solutions contained the fatty acids and, provided the operation was done in the cold, very little sphingosine bases. After evaporation of the hexane, the residue was refluxed briefly in 1 ml of boron trifluoride-methanol⁶ to form the methyl esters (Metcalf and Schmitz, 1961). These were extracted with hexane, purified by thin layer system III, and analyzed by gas-liquid partition chromatography. A Disc integrator

⁶ Applied Science Laboratories, Inc., State College, Pa.

⁷ Worthington Biochemical Corp., Freehold, N. J.

attached to the recorder was used to quantify individual fatty acids; this was also done by cutting out and weighing the peaks. The detector response was assumed to be linear with respect to carbon number, and values reported are weight rather than molar percentages. Peaks were identified by standards and log retention time plots.

The residue from the above hexane extraction, composed of long-chain bases, was dissolved in chloroform-methanol-concentrated ammonia (60:30:2), filtered, and evaporated to dryness. The sphingosines were purified with the thin layer system described for this purpose (Sambasivarao and McCluer, 1963a). Periodate oxidation gave a mixture of aldehydes, as previously reported (Sweeley and Moscatelli, 1959), which were identified by gas-liquid partition chromatography.

An alternative procedure used for fatty acid analysis was direct methanolysis using boron fluoride-methanol (Morrison and Smith, 1964). Ganglioside (1–4 mg) was dissolved in 1.5 ml of the latter and heated 90 minutes at 100°; a tube with Teflon-lined screw cap was used. Water (1 ml) was added and the mixture was extracted four times with 3 ml of hexane in the cold. The combined hexane layers were washed with water and evaporated, and the residue was chromatographed preparatively with thin layer system III. Best results were obtained with silica gel HR^s without binder. Standards of methyl stearate and methyl 2-hydroxystearate were used to delineate two zones. Normal fatty acid esters were clearly visible on spraying the developed plate with water, but the Tay-Sachs' ganglioside gave rise to no distinct spots corresponding to the 2-hydroxy fatty ester. Both zones were nevertheless eluted and examined by gas-liquid partition chromatography at high sensitivity for detection of trace components.

Quantitative analyses of the two lipid classes were carried out on aliquots of the original ganglioside aqueous stock solution. A sample containing 1.5 mg was evaporated to dryness with nitrogen and treated with 10 ml of methanolic HCl (8 ml of concentrated HCl + 80 ml of methanol). After 14 hours of reflux, the cooled mixture was extracted three times with hexane to remove fatty acid esters, which were analyzed by the hydroxamate method (Snyder and Stephens, 1958). The methanolic lower phase was analyzed for total long-chain bases by the methyl orange procedure (Lauter and Trams, 1962).

D. AMINO ACIDS AND PEPTIDES. Approximately 4 mg of ganglioside was hydrolyzed in 6 N HCl at 100° for 16 hours. After extraction with chloroform, the aqueous solution was filtered and evaporated to dryness. The residue was dissolved in citrate buffer, pH 2.2, and the amino acids were determined with a Phoenix automatic analyzer. Under the operating conditions 0.01 μ mole were easily detectable.

Partial Acid Hydrolysis. To prepare the ceramide mono-, di-, and trihexosides, ganglioside was heated in aqueous 0.1 N HCl at 100° for 15 minutes. The cooled mixture was shaken with a fivefold volume of chloroform-methanol (2:1), and the upper phase was dis-

carded. The residue, after evaporation of the lower phase, was chromatographed preparatively with thin layer system I, which gave good separation of the three ceramide hexosides and left the small amount of unhydrolyzed ganglioside at the origin. Spots were detected by spraying with water and eluted from the silica gel with chloroform-methanol (2:1). After filtration and evaporation, the remaining solid was purified by chromatography on Unisil.

To prepare ceramide-dihexoside-NANA, ganglioside was refluxed 12–15 minutes in methanolic HCl (1 ml of concentrated HCl + 23 ml of methanol). The cooled solution was brought to pH 13 with aqueous NaOH, allowed to stand 2 hours at room temperature, and then brought to neutrality with HCl. Chloroform and methanol were added to give a mixture of 4 parts of chloroform, 2 parts of methanol, and 1.2 parts of aqueous sodium chloride. After shaking and phase separation, the lower phase was evaporated to give a residue which was chromatographed on Unisil. A small amount of asialoganglioside was eluted with 14% methanol-chloroform, while the desired product came through with 18% methanol-chloroform. The latter could also be purified by preparative thin layer chromatography using system II.

Periodate-Borohydride. This reaction sequence was applied to the ganglioside, ceramide-trihexoside (asialoganglioside), and ceramide-dihexoside-NANA (hematoside). A solution of approximately 1 μ mole of glycolipid in 1 ml of methanol was treated with 0.2 ml of freshly prepared 0.6 M sodium metaperiodate, and the mixture was placed in the dark at room temperature for at least 100 hours. Excess periodate was reduced with 1 drop of ethylene glycol, the resulting precipitate was removed by centrifugation, and the supernatant was added to 1 ml of cold solution containing 50 mg of sodium borohydride in methanol–0.001 aqueous sodium hydroxide (1:1). After standing 2 hours in the cold, the solution was brought to pH 4–5 with 2 N HCl to destroy excess borohydride. A few milliliters of water were added and the mixture was dialyzed in prewashed cellulose tubing against distilled water in the cold for 2–3 days. The bag contents were lyophilized to dryness and the residue was hydrolyzed in a sealed tube with aqueous 1 N HCl at 100° for 8 hours. The cooled mixture was extracted with chloroform and passed through Dowex 1X4 (acetate) anion-exchange resin, and the effluent was evaporated to dryness. A portion of the residue was chromatographed with paper system A, and the remainder was acetylated several hours with acetic anhydride-pyridine (1:1). The resulting acetates were analyzed by gas-liquid partition chromatography using the JXR column described above at 130°. A known mixture of threitol and erythritol acetates were separated by this column into two distinct, though somewhat overlapping peaks.⁸

⁸ Separation of threitol and erythritol by gas-liquid partition chromatography of the acetates was described by Dr. C. C. Sweeley, personal communication.

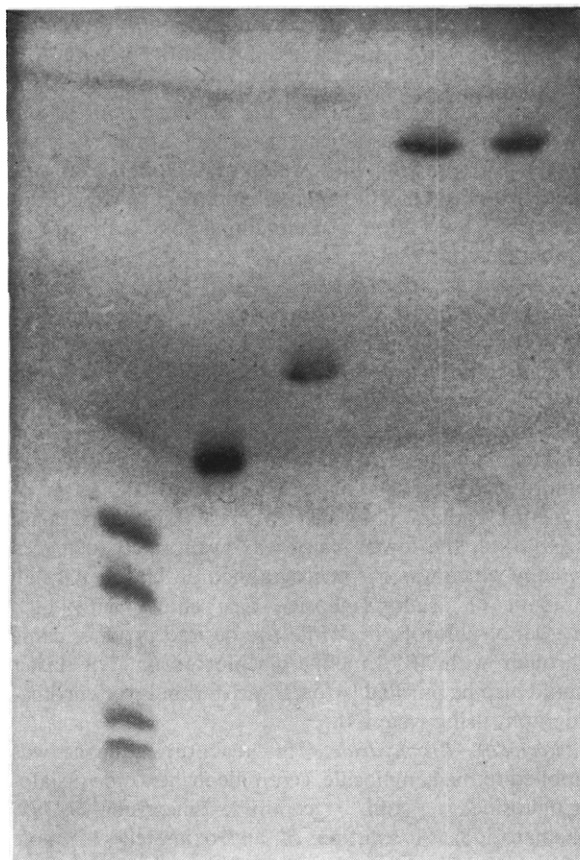


FIGURE 1: Thin layer chromatography of gangliosides and derived products. (1) Normal human brain gangliosides, (2) Tay-Sachs' ganglioside, (3) ceramide-lactoside-NANA (hematoside) derived from Tay-Sachs' ganglioside, (4) product from neuraminidase treatment of derived hematoside, (5) ceramide-lactoside (from acid hydrolysis of Tay-Sachs' ganglioside).

Neuraminidase. This enzyme⁹ was incubated with the purified Tay-Sachs' ganglioside and the ceramide-dihexoside-NANA derived from it. To 0.2 ml of an aqueous solution containing 0.5 mg of glycolipid were added 0.2 ml of enzyme and 0.4 ml of calcium-Versene solution (0.005 M calcium chloride and 0.0005 M disodium ethylenediaminetetraacetate). Toluene was overlaid and the mixture was incubated at 37° for 3 days, during which time two additional 0.2-ml portions of enzyme were added. The mixture was evaporated to dryness with nitrogen, and the residue was extracted with chloroform-methanol (2:1). The filtered solution was reduced in volume and applied to thin layer plates, which were run in systems I and II.

Results

Ganglioside Purification. The Tay-Sachs' ganglioside

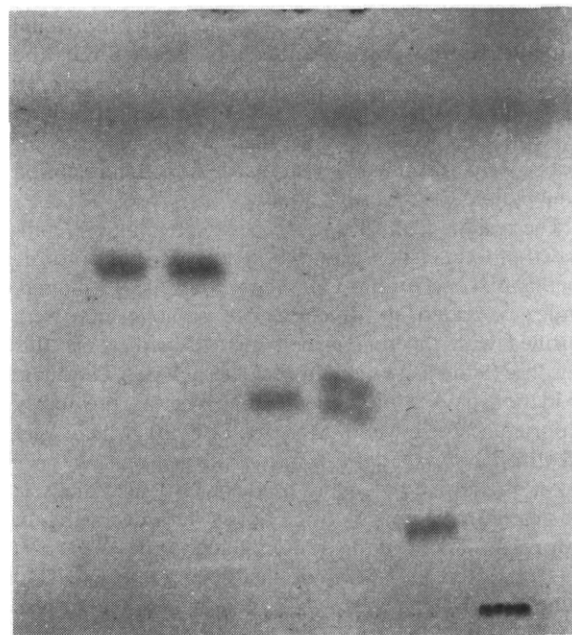


FIGURE 2: Partial hydrolysis products (aqueous HCl) from Tay-Sachs' ganglioside. (1) Ceramide-glucoside, (2) galactocerebroside standard,¹⁰ (3) ceramide-lactoside, (4) cytolipin H standard,¹¹ (5) ceramide-tri-hexoside, (6) Tay-Sachs' ganglioside.

which was purified by Unisil chromatography contained 0.08% phosphorus, while the sample further purified by preparative thin layer chromatography had a phosphorus content below 0.005%. Figure 1 shows the thin layer chromatography behavior of the Tay-Sachs' ganglioside. As previously described, it migrates ahead of the four major gangliosides of normal brain and parallel to a minor component of the latter.

Carbohydrate Composition. Paper chromatography of the products from the more vigorous acid hydrolysis revealed glucose, galactose, and galactosamine as the only carbohydrates from the Tay-Sachs' ganglioside. Sialic acid did not survive the vigorous conditions, but could be observed by paper chromatography after mild hydrolysis; comparison in paper systems A and B with standards of NANA and *N*-glycolylneuraminic acid showed correspondence with the former. Mild hydrolysis also showed galactosamine to be present in the *N*-acetyl form.

The following molar ratios were obtained, related to hexosamine as unity: glucose:galactose:NANA:hexosamine 0.95:0.95:1.08:1.00. Galactose was calculated as the difference between total hexose and glucose.

Partial Hydrolysis. For determination of carbohydrate sequence, mild acid hydrolysis in aqueous medium was used to produce the three ceramide-hexosides shown in Figure 2. Cerasin¹⁰ and cytolipin H¹¹ are shown for

⁹ Prepared from *Vibrio cholerae*; General Biochemicals, Chagrin Falls, Ohio.

¹⁰ Gift of Dr. W. T. Norton.

¹¹ Gift of Dr. Maurice Rapport.

comparison. Following vigorous acid hydrolysis the carbohydrates of each were characterized by paper chromatography (system A): monohexoside—glucose; dihexoside—glucose, galactose; trihexoside—glucose, galactose, galactosamine. No NANA was detected by the resorcinol reaction in any of these. These results are in agreement with an earlier study (Svennerholm, 1962) and point to a linear sequence of ceramide-glucose-galNac. However, a branched structure for these units (exclusive of NANA) could not be excluded on this evidence alone. Such an arrangement, *e.g.*, with galactose and *N*-acetylgalactosamine both joined to glucose, would have the possibility of producing a second ceramide-dihexoside containing glucose and galactosamine. Such a product was carefully sought on the thin layer plates used for preparative isolation of the ceramide hexosides by eluting the regions above and below the major ceramide-dihexoside, even though no spots were actually visible in these zones. Vigorous acid hydrolysis followed by paper chromatography failed to show any trace of galactosamine. Even more compelling evidence for the linear arrangement came from the periodate-borohydride experiments described below.

When partial acid hydrolysis was carried out, as above, in aqueous medium, no NANA-containing glycolipids were obtained, indicative of the relatively greater lability of the NANA glycosidic bond under these conditions. When, however, the medium was changed to methanolic HCl, a reasonable yield of a product containing ceramide, glucose, galactose, and NANA could be isolated. This is similar in composition to a ganglioside isolated in minor amounts from normal human brain (Svennerholm, 1963; Klenk and Gielen, 1963; Kuhn and Wiegandt, 1964), and in somewhat elevated amounts from a gargoylism brain (Taghavy *et al.*, 1964). The same material appears to accumulate in the spleen in Gaucher's disease (Philippart and Menkes, 1964). The term "hematoside" was assigned by Yamakawa and Suzuki to a very similar substance isolated from erythrocyte stroma (1951; Klenk *et al.*, 1962). The migratory rate on thin layer chromatography of the synthetic material obtained above was virtually identical with that of the natural ganglioside from brain. As seen in Figure 1, it moves ahead of the Tay-Sachs' ganglioside, consonant with its loss of one sugar unit. Isolation of this product eliminated the possibility of an NANA-galNac bond, and also led to an unequivocal placement of the NANA attachment site (see below).

Periodate-Borohydride. Periodate oxidation followed by borohydride reduction and acid hydrolysis was applied to the Tay-Sachs' ganglioside, the ceramide-trihexoside (asialoganglioside), and the ceramide-dihexoside-NANA (hematoside). Products were identified by a combination of paper and gas-liquid partition chromatography; these are shown in Table I opposite the carbohydrate in the original compound from which each was derived.

In both compounds containing NANA, the latter was destroyed by periodate, consistent with its terminal linkage. Galactose was the only carbohydrate unaffected

TABLE I: Products from Periodate-Borohydride Treatment.

	PRECURSOR	PRODUCT
Tay-Sachs ganglioside	glucose galactose galactosamine sialic acid	erythritol galactose glycerol (destroyed)
asialoganglioside	glucose galactose galactosamine	erythritol threitol glycerol
hematoside	glucose galactose sialic acid	erythritol galactose (destroyed)

by periodate in the original ganglioside and also in the hematoside. The galactose unit of the ceramide-trihexoside, however, was converted to threitol. In all three compounds glucose was transformed by the reaction sequence to erythritol. The conversion of *N*-acetylgalactosamine to glycerol confirmed its terminal location. Identification of the four-carbon polyols was feasible when it became known that the tetraacetates could be separated by gas-liquid partition chromatography, according to the method of Sweeley.⁸ Earlier attempts with the trimethylsilyl ethers had failed.

In some runs small amounts of unchanged glucose were still evident on paper chromatography, even after a week of periodate oxidation. Glucose within the ganglioside molecule is known to undergo much slower oxidation than the other carbohydrates with this reagent (Johnson and McCluer, 1964), but a more likely explanation is felt to lie in the sloughing of small cellulose fragments from the dialysis tubing. Thus, blank runs with water alone frequently showed similar small quantities of glucose. The magnitudes were such, however, as to leave no doubt of the destruction of the large majority of ganglioside glucose.

Lipid Analysis. Colorimetric determination of total fatty acids and long-chain bases gave a molar ratio of 1.10 for these two classes, while the NANA to base ratio was 0.84. Both the sphingosine and fatty acid fractions proved to be heterogeneous on gas-liquid partition chromatography analysis. Stearate constituted over 90% of the latter. However, the ganglioside used initially for this analysis contained 0.08% phosphorus, making it necessary to consider the possibility that the minor fatty acids were derived in part from the small amount of lipid contaminants. A portion of ganglioside was therefore purified additionally by preparative thin layer chromatography with system II, which separated it from all lipids except phosphatidylserine. This contaminant could be removed by a silicic acid column from which it was eluted with 20%

methanol-chloroform (Rouser *et al.*, 1961). The resulting ganglioside had a phosphorus content of <0.005%. The fatty acids obtained from methanolysis of this repurified material are shown in Table II.

TABLE II: Fatty Acid Composition of Tay-Sachs' Ganglioside.

Fatty acid	Percent comp.	Fatty acid	Percent comp.
14:0	tr	21:0	0.01
15:0	0.03	21:1	tr
16:0	0.48	22:0	0.60
16:1	0.04	22:1	0.09
17:0	0.08	23:0	0.10
18:0	90.50	23:1	0.01
18:1	0.18	24:0	0.02
19:0	0.13	24:1	1.02
20:0	6.80	25:1	tr
20:1	tr	26:0	tr

An effort was made to detect trace amounts of 2-hydroxy fatty acids, as described in the experimental section, but none were found.

The sphingosine fraction contained primarily four long-chain bases: sphingosine, C₂₀ sphingosine, and the dihydro derivative of each. The possibility of minor amounts of other bases cannot yet be excluded. It was estimated that 80% or more of the mixture was divided about evenly between the two unsaturated bases. The four aldehydes generated from the mixture by periodate were easily separated by gas-liquid partition chromatography, according to the method of Sweeley and Moscatelli (1959). However, estimation of the original bases by quantification of the aldehyde peaks involves some error due to alterations at the allylic hydroxyl during acid hydrolysis (Weiss, 1964). The results are therefore to be taken only in a qualitative sense.

Peptides and Amino Acids. The sample of ganglioside showing small but measurable contamination (0.08% P) gave relatively minor amounts of amino acids after acid treatment. Analysis on an automatic analyzer of the hydrolysate from 2.80 μ moles of Tay-Sachs' ganglioside gave the following amino acids (in μ moles): aspartic (0.016), serine (0.048), glutamic (0.025), glycine (0.034), alanine (0.016), leucine (0.022). The relatively larger amount of serine may have reflected contamination by phosphatidylserine. It was of interest to determine whether or not the ganglioside could be ultimately divested of even these small quantities; a similar analysis was therefore carried out on the highly purified sample containing <0.005% P, and no amino acids were detected.

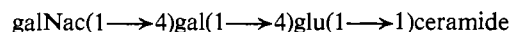
Neuraminidase. The ganglioside itself proved totally resistant to this enzyme. On the other hand, the hematoside derived from it was readily cleaved, as seen in Figure 1. The thin layer system shows both the disap-

pearance of hematoside and the formation of a product running parallel to ceramide-lactoside.

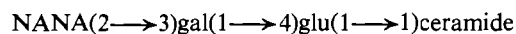
Discussion

The analytical values obtained here are in close agreement with the equimolar ratios for each of the four carbohydrates which have been reported previously (Svennerholm, 1962; Klenk *et al.*, 1963; Makita and Yamakawa, 1963; Berman and Gatt, 1962). Agreement on this point, however, has not been universal (Rosenberg and Chargaff, 1959; Saifer *et al.*, 1963; Samuels *et al.*, 1963). Fatty acids and long-chain bases, when analyzed as total groupings, were also approximately equimolar with sugars. The compositions of the glycolipids from partial hydrolysis similarly indicated one unit each for the four carbohydrates. The absence of an asialoganglioside with two galactose units agrees with the concept of the Tay-Sachs' ganglioside differing from the major species of normal brain in lacking the terminal galactose; a similar observation has been made by Svennerholm (1962).

The composition of the three glycolipids produced by aqueous hydrolysis indicated a linear arrangement of ceramide-glucose-galactose-galactosamine, and the failure to detect a dihexoside containing galactosamine argues against a branched arrangement. More conclusive evidence on this point came from periodate-borohydride treatment of the ceramide-trihexoside (asialoganglioside), which gave as products erythritol, threitol, and glycerol. A branched structure would require two of the three carbohydrates to form glycerol. This result also established two of the substitution points and required the asialoganglioside to have the structure



Preservation of galactose on periodate-borohydride treatment of the ganglioside showed that NANA must be joined at the 2 or 3 position of galactose. Application of the same reaction sequence to the hematoside produced erythritol and left galactose unchanged, thus establishing the structure



Combining the results for these two compounds permits assignment of the ganglioside structure, shown in Figure 3.

Determination of the bonding site of NANA was made possible by isolation of the hematoside after selective removal of terminal galactosamine. As discussed above, this product could not be obtained when the hydrolysis medium was aqueous, since NANA was preferentially split off. The reason methanolic HCl proved successful is possibly related to the fact that this medium would keep the carboxyl group of NANA in esterified form. Considering the mechanism which has been proposed for acid-catalyzed cleavage of glycosidic bonds (Armour *et al.*, 1961), it is easy to visualize how

an acidic carboxyl group adjacent to a ketosidic bond could accelerate hydrolysis of the latter. Esterification might serve, therefore, to reduce the relative lability of that particular bond, allowing cleavage of other units to proceed on a more competitive basis.

The permethylation technique was previously applied by other laboratories to this ganglioside. In one case (Klenk *et al.*, 1963) a somewhat different carbohydrate arrangement was proposed, while in the other (Makita and Yamakawa, 1963) the results were similar to those obtained here with periodate-borohydride. The latter workers carried out permethylation of the asialo-ganglioside, and after methanolysis were able to identify the methyl glycosides of 2,3,6-trimethylglucose and 2,3,6-trimethylgalactose. Their structure for the trihexoside was thus identical with that proposed here. However, permethylation of the ganglioside itself led only to identification of 2,3,6-trimethylglucoside. Since no methylated galactoside was characterized, the NANA attachment site could not have been determined. The supposition by these workers that it was the 3-hydroxyl of galactose is experimentally confirmed in the present study.

It should be noted that Klenk *et al.* (1963) stated their uncertainty regarding the attachment sites of NANA and galNAc, due to difficulty in identifying the trimethylgalactose arising from permethylation of the asialo derivative. A more recent publication from Klenk's group (Klenk and Kunau, 1964) identified 2,3,6-trimethylgalactose as a product from the disialo-ganglioside B₁ (after prior removal of both sialic acids). However, no correlation was made between that product and the one from Tay-Sachs' ganglioside. The results of the present study and of Makita and Yamakawa suggest that Klenk's unidentified trimethylgalactose from the Tay-Sachs' ganglioside was in all probability identical with the product from their B₁ fraction.

An argument which has been advanced against an NANA(2→3)gal bond (Saifer, 1964) is the well-known resistance of this ganglioside to neuraminidase. A linkage of this type has been shown to exist at the terminal galactose of a disialoganglioside of normal brain (Kuhn and Wiegandt, 1963; Klenk and Gielen, 1963). Since it is readily hydrolyzed by the enzyme, it was reasoned that a nonsusceptible NANA would necessarily be attached at a different position of galactose. That such need was not the case was shown here by the observation that NANA of the derived hematoside is removed by neuraminidase. The deciding factor appears to be the presence of a large grouping at the 4-axial hydroxyl of galactose, which hinders approach of the enzyme to the adjacent NANA bond. This explanation was advanced earlier (Kuhn and Wiegandt, 1963) when it was observed that sialyllactose and sialylgalactose, obtained by partial degradation of normal brain ganglioside, were hydrolyzed by the enzyme. The possibility that this could have been attributed to removal of the ceramide unit rather than galactosamine is eliminated by the present study. The naturally occurring hematoside of human brain is also known to lose NANA readily on treatment with

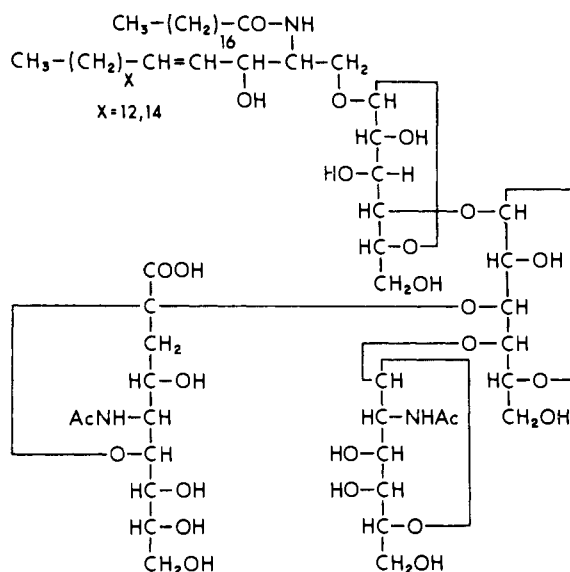


FIGURE 3: Proposed structure of Tay-Sachs' ganglioside.

neuraminidase (Svennerholm, 1963; Kuhn and Wiegandt, 1964).

With regard to the lipid portion of the molecule, gangliosides appear to be unique among the sphingolipids in containing a C₂₀ homolog of sphingosine, besides sphingosine itself (Stanacev and Chargaff, 1962; Sambasivarao and McCluer, 1963b, 1964). In normal brain gangliosides, as well as Tay-Sachs', these two bases were reported to occur in approximately equal proportions, along with much smaller amounts of the two dihydro derivatives (Sambasivarao and McCluer, 1964). On the other hand, Klenk *et al.* (1963) obtained oxidation products from the sphingosine fraction of Tay-Sachs' ganglioside which indicated 88% sphingosine and only 7% of the C₂₀ homolog. Results obtained in this study are qualitatively in accord with those of Sambasivarao and McCluer, though precise quantification was not achieved for the reasons cited earlier.

Heterogeneity of fatty acids has been the rule for most lipids of the nervous system, though gangliosides show more tendency toward uniformity in this component than do the others. The Tay-Sachs' ganglioside has been reported to contain 90% or more of stearic acid (Sambasivarao and McCluer, 1964; Makita and Yamakawa, 1963; Klenk *et al.*, 1963). It was thought desirable to determine the degree of uniformity that would prevail in a sample of high purity. The preparative thin layer technique described above combined with two chromatographies on silicic acid undoubtedly reduced contaminants to a minimum; this was verified by the complete absence of peptides and a phosphorus content below 0.005%. Gas-liquid partition chromatographic analysis showed this material to still contain 9-10% of minor fatty acids (Table II), with stearate contributing 90.5%.

Considerably different results were reported for a

peptide strandin preparation from a Tay-Sachs' brain (Saifer *et al.*, 1963), which contained nearly equal proportions of oleic and stearic, and somewhat less palmitic. This same peptide strandin also showed the presence of some 14 amino acids following acid hydrolysis; the amounts, however, were considerably below those of normal brain peptide strandin. Rosenberg and Chargaff (1959) had originally reported this kind of difference between normal brain and Tay-Sachs' preparations. In the present study, amino acids were detected in hydrolysates of the original sample (0.08% P), though at levels considerably below those described in the above reports. When the ganglioside was further purified ($P < 0.005\%$), even these small quantities of amino acids disappeared. This demonstrates the feasibility of isolating ganglioside from Tay-Sachs' brain completely free of peptide. It does not, of course, preclude the existence of other molecular species with ganglioside covalently linked to peptide. Also, there remains the possibility that such moieties are joined *in situ* by weak covalent bonds which are ruptured during extraction and purification. A preliminary report of such a complex from dog brain (Kuhn and Müldner, 1964) may help lead to some clarification of this difficult problem.

The results found here for the "abnormal" ganglioside are in many respects parallel to those reported for the major monosialoganglioside of normal brain (Kuhn and Wiegandt, 1963). Except for the absence of terminal galactose in the Tay-Sachs' material, the remaining carbohydrates are identical in composition, sequence, and attachment points. The term "abnormal" may prove to be inappropriate, in a structural sense, if the same pattern is found to characterize the "Tay-Sachs'-like" ganglioside of normal brain. This latter material has been found to comprise 6% or less of normal brain gangliosides, and has been variously designated as G_{M2} (Svennerholm, 1963), G_0 (Kuhn and Wiegandt, 1963), G_5 (Suzuki, 1964), and FM (Johnson and McCluer, 1963). The important question of stereochemical relationships has received little attention to date and constitutes the necessary third dimension for total structure comparison.

It is interesting to note that the ceramide-trihexoside which also accumulates in Tay-Sachs' brain tissue (Gatt and Berman, 1963) has been found to have the same carbohydrate structure and fatty acid composition as the derived asialo compound (Makita and Yamakawa, 1963). Thus, a single metabolic defect might be invoked to account for both storage lipids. However, the additional accumulation of a ceramide-digalactoside (Gatt and Berman, 1963), dissimilar to these in both carbohydrate and lipid composition, suggests that the disease etiology may prove somewhat more complex.

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Isolation, Characterization, and Synthesis of Pyrimine, an Iron(II)-Binding Agent from *Pseudomonas* GH*

Ross Shiman and J. B. Neilands

ABSTRACT: An iron(II)-binding agent from a soil isolate, designated *Pseudomonas* GH, was crystallized, characterized, and synthesized. The substance may be named L-5(2-pyridyl)-2-amino-5-ketopentanoic acid or L-2(2-pyridyl)- Δ^1 -pyrroline-5-carboxylic acid, respectively, depending on whether the side chain is open or cyclized.

Over the past few years, it has become apparent that the path of iron in microbial metabolism is controlled and directed by the coordination of this element to specific organic structures. Obviously, in order to understand the biochemical mechanisms involved in iron metabolism in living cells, it will be necessary to elucidate the chemical nature of the organic compounds which are found associated with iron within the living cell.

It is a reasonable assumption that naturally occurring binding agents which form *very stable* complexes with iron will play a role in the metabolism of the metal. Most such compounds hitherto studied in this laboratory have, by virtue of the presence of phenolic (Ito and Neilands, 1958) or hydroxamate (Emery and Neilands, 1959) functional groups, displayed a pronounced preference for ferric iron. Thus, in the case of the ferrichrome compounds, six oxygen atoms engage a single metal ion in a spin-five, octahedral complex in

Pyrimine is suggested as the trivial name.

A stable 3:1 complex (ferropyrimine) is formed with ferrous iron at pH values greater than about 2.0. Pyrimine was synthesized by Claisen condensation of methyl picolinate with *N*-trityl-L-glutamic acid dimethyl ester.

which ferric iron is held with a stability constant of about 10^{30} (Neilands, 1964).

Recently our colleagues in the Department of Bacteriology of this University, Dr. George Hegeman and Dr. R. Y. Stanier, brought to the laboratory a microbial culture which they obtained in the course of plating a soil extract on agar medium. The organism and the agar on which it was growing had acquired a brilliant magenta color. Since Hegeman and Stanier had obtained some indication that the pigment contained iron, we concluded that the substance was worthy of investigation. The compound was quickly established to be a ferrous iron coordination derivative and because of the rather unique property of binding divalent iron we set about its isolation and characterization.

The purpose of this paper is to describe the crystallization, characterization, and synthesis of a substance which may alternatively be named L-5(2-pyridyl)-2-amino-5-ketopentanoic acid or L-2(2-pyridyl)- Δ^1 -pyrroline-5-carboxylic acid. We suggest the trivial name pyrimine. At all pH values greater than about 2, the compound affords a stable ferrous complex (ferropyrimine).¹

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¹ The metal-binding characteristics will be the subject of future investigations; it is anticipated that other divalent ions, such as Cu^{II} , are also strongly bound.