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JORDI FOLCH-PI, Program Chairman

The Chemistry of Gangliosides: A Review[1]

ROBERT LEDEEN, The Saul R. Korey Department of Neurology and the Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York

Abstract

Gangliosides are lipids which are found primarily in gray matter of the brain, with lesser amounts in white matter and in some tissues outside the central nervous system. At least ten different gangliosides have been isolated from brain, four of which are major components accounting for over 90% of the mixture. Three of these and possibly the fourth possess a common asialo unit consisting of ceramide, glucose, galactose, and N-acetylgalactosamine in the molar ratios 1:1:2:1.

Structural work emanating from the laboratories of Kuhn, Klenk, Svennerholm, and others has shown the major monosialoganglioside to be

$$\text{GAL}(1\rightarrow3)\text{GALNac}(1\rightarrow4)\text{GAL}(1\rightarrow4)\text{GLU}(1\rightarrow1)\text{CERAMIDE}$$
$$\begin{pmatrix} 3 \\ \uparrow \\ 2 \end{pmatrix}$$
$$\text{NANA}$$

The two major disialo species contain this unit plus an additional NANA attached to terminal galactose in one case, and to the first NANA in the other. The major trisialo may also be related to these, though its structure is not yet settled.

The minor gangliosides generally contain fewer carbohydrate units. One of these has been found to resemble the major ganglioside of Tay-Sachs disease, which is a monosialo species lacking the terminal galactose of the major normal brain types. A number of neurological disorders, including other sphingolipidoses, have characteristic alterations in both pattern and level of brain gangliosides.

Introduction

GANGLIOSIDES ARE A COMPLEX group of glycosphingolipids characterized by the presence of sialic acid in the carbohydrate chain. Much of the credit for their discovery is due Ernst Klenk, who studied them first as the storage lipids of disease (1,2), and later achieved their isolation from normal brain tissue (3). The name he introduced reflected their high concentration in the so-called "ganglion" cells, or neurons. Gangliosides are now known to exist outside the central nervous system as well, though major interest has focused on the large group which occurs primarily in the brain. Their accumulation and pattern alteration in certain neurological diseases has provided additional impetus to the study of their chemistry and metabolism.

The physiological role of the gangliosides is poorly understood at present. Chemical knowledge, however, has progressed at a rapid pace, particularly in the last few years, and will be the main subject of this review. While some of the material to be presented has already been covered in two recent reviews (4,5), the emphasis will be on developments since appearance of the latter.

The presence of both lipophilic and hydrophilic groupings in the structure results in dual solubility properties for most gangliosides. On the one hand, they are extractable from tissue by typical lipid solvents, such as chloroform-methanol, after which they may be separated from the majority of other lipids by virtue of their solubility in water. The latter property is due to the carbohydrate moieties, and in particular the highly polar sialic acid units, which also impart acidity to the structure. Most brain gangliosides also contain neutral sugars and hexosamine.

"Sialic acid" is the group term for the several derivatives of neuraminic acid, the parent substance. All naturally occurring sialic acids contain an acyl group on nitrogen, and in some cases on hydroxyl as well (6,7). N-acetylneuraminic acid (NANA),[1] the predominating species in brain gangliosides, is shown in Figure 1. It is derived biosynthetically from phosphorylated N-acetyl-D-mannosamine and pyruvate (8,9). It occurs predominantly in the cyclic form and is generally linked to other carbohydrates through the hemiketal grouping. Only one of the two possible configurations at that center is shown. Gangliosides have been found outside the nervous system which contain N-glycolylneuraminic acid (NGNA). The majority of procedures for estimating ganglioside levels in tissues are based on colorimetric determination of lipid-bound sialic acid.

[1] Abbreviations used: NANA, N-acetylneuraminic acid; NGNA, N-glycolylneuraminic acid; Glu, glucose; Gal, galactose; GalNac, N-acetylgalactosamine; GluNac, N-acetylglucosamine; Cer, ceramide; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

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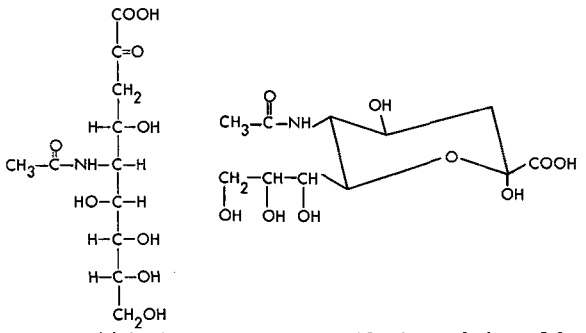


FIG. 1. D(-)-N-Acetylneuraminic acid. Open-chain and hemiketal forms.

Nomenclature and Symbols

The term "strandin" was proposed by Folch et al. (10) for a brain lipid complex subsequently shown to be quite similar in composition to gangliosides (11). A similar parallel was found for the "mucolipid" of Rosenberg and Chargaff (12,13). Some differences in composition among the three were noted, however. These could have been due to varying amounts of sialic acid being split off during isolation, or possibly to varying degrees of contamination by other brain lipids. Originally, there was thought to be a more basic structural difference, strandin and mucolipid comprising polymeric forms of the monomeric ganglioside (14). Peptides were often found in association and were believed to be integral components of the polymer. "Peptide-strandin" fractions have been isolated which are relatively enriched with peptide (11,15).

Klenk and Gielen (16), as well as others (17–19) subsequently showed that the high molecular weights obtained for gangliosides in aqueous solution were due to micelle formation, whereas sedimentation constants determined in organic solvents gave values of 1000–2000, corresponding to the monomer. This appeared to many to have settled the debate of polymer versus monomer, and eliminated the need for separate terminology. Recently, however, a brief note by Kuhn and Müldner (20) reported the isolation of a ganglioside-protein complex from dog brain. The protein portion was split off with cold chloroform-methanol, leaving what was described as a ganglioside polymer. The latter was decomposed with base or hot chloroform-methanol into the characteristic monomers. Ester bonds were believed to be partially involved in the linking of monomers. In this regard, it may be recalled that the original paper of Rosenberg and Chargaff (13) claimed evidence for the presence of ester units in mucolipid. The ganglioside-protein complex of Kuhn and Müldner was described as having both ATPase and acetylcholinesterase activities. The latter finding is of some interest in view of reports suggesting synaptic location of some gangliosides of the central nervous system, either in the vesicles (21) or the nerve-ending membrane (22).

Whatever their original state within tissue, the evidence is rather compelling that gangliosides as obtained by the usual extraction and fractionation procedures are monomers, and the term as generally used today applies to such units. A large number of discrete structures have been isolated and characterized, including four major gangliosides from normal brain and a somewhat larger group of minor components. As the number continues to grow with new discoveries, the problem of symbol designation becomes increasingly confused. Each investigator has been inclined to introduce his own system, and there are now at least six currently in the literature. These are summarized in Tables I and II.

The structural formulas in Table I for the four major gangliosides of normal brain are those proposed by Kuhn and Wiegandt, but each fraction has also been isolated and to some extent characterized by other workers, who have assigned the symbols shown. Kuhn and Wiegandt's original system (23) assigned numeral subscripts according to thin-layer migratory rates, but in a recent report on minor gangliosides of the brain (24) they employed carbohydrate abbreviations. Svennerholm's nomenclature is based on composition (25), a letter subscript denoting sialic acid content and a numeral subscript the subdivisions within each group. In Figure 2 Svennerholm's system (S) and that of Kuhn and Wiegandt (K & W) are both depicted, while in Figures 3 and 4 Svennerholm's alone is used. The majority of symbols throughout the text are from one or the other of these systems, and in many instances the two have been used conjointly where it was felt clarification would be aided. In a few cases other symbols have had to be used, particularly when the ganglioside in question has not been clearly correlated with any described by these authors. The multiplicity of nomenclature systems is regrettable, and it is to be hoped that the workers in this field will soon agree on a common system by which the well-characterized gangliosides may be given unambiguous symbol designations.

Isolation and Purification

Gangliosides are generally extracted from tissue in combination with other lipid classes. A variety of organic solvents have been employed, the chloroform-

TABLE I

Major Gangliosides of Normal Brain (Human and Beef)

Proposed structure (Kuhn and Wiegandt)	Symbol	Reference	
Gal(1→3)GalNac(1→4)Gal(1→4)Glu(1→1)Cer (3, ↑2) NANA	G_{M1} G_1 1-G G_4 I	Svennerholm Kuhn & Wiegandt Johnson & McCluer Korey & Gonatas Dain, et al.	(25) (23) (36) (37) (18)
Gal(1→3)GalNac(1→4)Gal(1→4)Glu(1→1)Cer (3, ↑2) NANA (3, ↑2) NANA	GD_{1a} G_{II} B_1 2-G G_3 II	Svennerholm Kuhn & Wiegandt Klenk & Gielen Johnson & McCluer Korey & Gonatas Dain, et al.	(25) (23) (56) (36) (37) (18)
Gal(1→3)GalNac(1→4)Gal(1→4)Glu(1→1)Cer (3, ↑2) NANA(8←2)NANA	GD_{1b} G_{III} 3-G G_2 C	Svennerholm Kuhn & Wiegandt Johnson & McCluer Korey & Gonatas Dain, et al.	(25) (23) (36) (37) (18)
Gal(1→3)GalNac(1→4)Gal(1→4)Glu(1→1)Cer (3, ↑2) NANA (3, ↑2) NANA(8←2)NANA	G_{T1} G_{IV} 4-G G_1 D	Svennerholm Kuhn & Wiegandt Johnson & McCluer Korey & Gonatas Dain, et al.	(25) (23) (36) (37) (18)

methanol (2:1)[2] system of Folch et al. (10) being favored by many. Booth (26) has studied a number of variables and concluded that a 1:1 ratio of chloroform-methanol was preferred for extracting gangliosides from brain tissue. Prior extraction with acetone increased the yield and decreased contaminants. Methanol proportions above 50% resulted in more contamination by protein. Svennerholm, on the other hand, claimed it was necessary to extract with both 1:1 and 1:2 chloroform-methanol mixtures in order to quantitatively remove the di- and trisialogangliosides (25).

Phenol (23) and hot methanol (27) have also been used as extraction solvents. Kuhn and Wiegandt (23) used aqueous phosphate buffer, but gave no yields. Water has not generally been found suitable for extracting gangliosides from most tissues, even though the isolated material is soluble in this medium. An interesting exception is frog brain (28), from which gangliosides are apparently completely extractable with water.

Separation of gangliosides from other extracted lipids is usually acheived by solvent partitioning or column techniques. In the method of Folch et al. (29) the total mixture in 2:1 chloroform-methanol is treated with one-fifth volume of water, causing the gangliosides to partition into the upper aqueous phase; they are accompanied by small amounts of other lipids. The latter can be reduced to a minimum by using salt solution in place of water, though this also results in suppression of certain of the lower-molecular weight gangliosides into the lower phase (30). Trams and Lauter (19) utilized tetrahydrofuran—aqueous phosphate buffer for extraction, and added diethyl ether to cause partitioning. The yields appear to be somewhat low by this method, particularly for the trisialo-ganglioside (31).

An isolation method has been described by Svennerholm (32) which is reported to give nearly quantitative recovery of gangliosides. The total lipid extract is chromatographed on a cellulose powder column, which is eluted first with chloroform-ethanol-water mixtures. Gangliosides, being highly polar, remain on the column while the other lipids are washed through. The former are then eluted with polar mixtures of chloroform-methanol-water. Rouser (33) has also developed a method based on cellulose chromatography for quantitative isolation of gangliosides. Subsequently, a DEAE cellulose column was utilized for this purpose by Rouser et al. (34).

[2] All solvent compositions, unless otherwise specified, are in volume ratios.

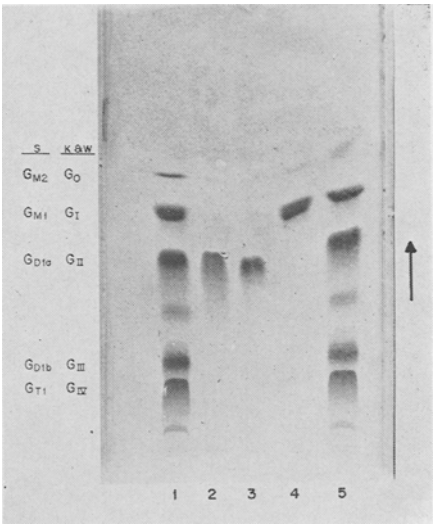


FIG. 2. Ascending TLC. 1 and 5 = ganglioside mixture from normal human brain; 2 = G'Lact; 3 = GD1a; 4 = GM1. Plate: 20 x 40 cm, coated with silica gel G, 250 μ thick; activated 40 min. at 110C. Solvent: chloroform-methanol-2.5 N ammonia (60:40:9), two seven-hour runs at room temperature. Spray: resorcinol.

Once removed from other cellular constituents, the ganglioside mixture must be separated into individual components prior to structural work. This already formidable task is further complicated by the lability of certain of the gangliosides with respect to loss of sialic acid during fractionation. Of the various column substrates described, silica gel appears to cause more difficulty in this regard than does cellulose, though it generally gives better resolution than the latter. Svennerholm has called attention to the importance of chromatographing gangliosides in their salt form rather than as free acids, in order to minimize sialic acid cleavage (25). Column procedures with silica gel have been used by Klenk and Gielen (16,35), Johnson and McCluer (36), Dain et al. (18), and Svennerholm (25). Kuhn and Wiegandt (23) obtained pure fractions by using both silica gel and cellulose powder columns, the latter being eluted with butanol-pyridine-water (6:2:2). Mono-, di-, and trisialo fractions were obtained by Svennerholm using a pressurized paper roll column which was eluted with propanol-water combinations (25).

Thin-Layer Chromatography

The complexity of ganglioside mixtures and the difficulty of fractionation make imperative an effective

TABLE II
Minor Gangliosides of Normal Brain (human primarily studied; beef to lesser extent).

Proposed structure	Symbol	Reference	
NANA(2→3)Gal(1→1)Cer	G_{Gal}	Kuhn & Wiegandt	(24)
NANA(2→3)Gal(1→4)Glu(1→1)Cer	G_{M3}	Svennerholm	(25)
	G_{Lact}	Kuhn & Wiegandt	(24)
	B_2	Klenk & Gielen	(56)
GalNac(1→4)Gal(1→4)Glu(1→1)Cer (3, 2 → NANA)	G_{M2}	Svennerholm	(25)
	Go	Kuhn & Wiegandt	(23)
	G_5	Korey & Gonatas, Suzuki	(37,44)
	FM	Johnson & McCluer	(36)
NANA(2→8)NANA(2→3)Gal(1→4)Glu(1→1)Cer	G'_{Lact}	Kuhn & Wiegandt	(24)
GalNac(1→4)Gal→(1→4)Glu(1→1)Cer (3, 2 → NANA) } 1NANA	G'_{GNTrII}	Kuhn & Wiegandt	(24)
Gal(1→3)Gal(1→3)Gal(1→1)Cer } 2NANA(?)	D	Klenk & Gielen	(50)

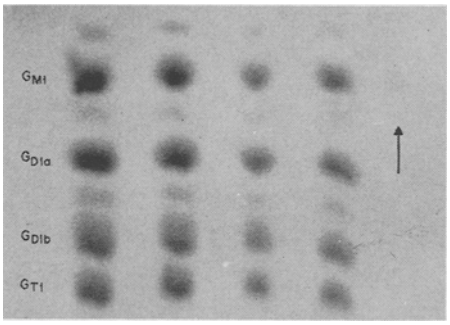


FIG. 3. Descending TLC. Ganglioside mixture from normal human brain; spotted (left to right) 200, 150, 100 and 100 micrograms. Plate: 20 x 20 cm, coated with silica gel G, 250 μ thick; activated one hour at 100C. Solvent: n-propanol-water (7:3), one descending run at 5C, 18 hours. Spray: resorcinol.

method for monitoring homogeneity. As in other areas of lipid research, TLC has proved indispensable for this purpose. Electrophoresis and ultracentrifugation were used previously on the assumption of a high molecular weight polymer, but a single moving boundary for the ganglioside micelle has been shown to be an invalid criterion (4).

TLC has also become an important separation tool in its own right. Though more tedious than column procedures, the superior resolving power often makes the extra effort quite profitable. Dilute aqueous solutions of rhodamine 6G or bromthymol blue are useful nondestructive sprays, revealing the gangliosides (and other lipids) clearly in ultraviolet light. Elution from the silica gel sometimes proves difficult, particularly for the polysialo compounds; we have found the solvent mixture, methanol-chloroform-water pyridine (56:40:12:2), described by Korey and Gonatas (37) to be effective for most fractions. A final clean-up with a small silica gel column is often required to remove the dye and any organic impurities deriving from the thin-layer silica.

A number of TLC systems have been described specifically for gangliosides. Several of these employ chloroform-methanol-water in various proportions, e.g., the (60:35:8) system of Wagner et al. (38). Wherrett and Cumings (39) found this combination more useful when 2.5 N ammonia replaced water. Klenk and Gielen (40) employed n-butanol-pyridine water (3:2:1), while the n-propanol-water (7:3) system of Kuhn et al. (41) has been widely used. The technique of two successive solvents was employed by Jatzkewitz (42) in which chloroform-methanol-water (14:6:1) was followed by n-propanol-aqueous 12.5% ammonia (4:1).

The above procedures all employ silica gel coated plates, run ascending in closed tanks. Eichberg et al. (43), in comparing the ganglioside patterns of subcellular fractions, utilized an ascending system which allowed continuous evaporation of the solvent as it reached the top of the plate. Another technique giving excellent resolution is the descending system of Korey and Gonatas (37). The propanol-water (7:3) solvent is conducted from a trough to the top of the plate by means of a paper wick. Results with this system are shown in Figure 3. Suzuki has utilized this descending technique in his micromethod for quantitative determination of ganglioside patterns (44). The separated fractions are analyzed directly for sialic acid without prior elution from the silica gel. Recovery is close to 100%. This simple and elegant method has been applied to the quantitative determination of each fraction in developmental and pathological studies.

Another TLC modification found to be very advantageous in the reviewer's laboratory is the use of double length (20 × 40 cm) silica gel plates, with chloroform-methanol 2.5 N ammonia (60:40:9) as solvent. Two successive ascending runs of approximately seven hours apiece, with an hour's drying period in between, generally results in good resolution of the four major and some of the minor components (Fig. 2).

The use of two systems is sometimes essential in the TLC characterization of a particular ganglioside. A case in point is one of the minor components of normal brain shown in column 2 of Figure 2. This material appears, on the basis of analysis, to be the G'_{Lact} reported by Kuhn and Wiegandt (24); it contains ceramide-lactoside with two sialic acids. In the system shown it runs parallel to the disialoganglioside G_{D1a}, whereas in the descending propanol-water system it coincides with the monosialo molecule G_{M1}. Either system alone would have failed to detect or identify this particular species.

Spray reagents for nonpreparative work are those used for quantitative determination of sialic acid: orcinol (45), resorcinol (46), and p-dimethylaminobenzaldehyde (47). Charring after spraying with 50% sulfuric acid is equally sensitive, but it does not differentiate sialic acid-containing lipids from other classes.

Carbohydrate Composition

Ganglioside structures may be considered from the standpoint of their two major groupings: the lipophilic ceramide unit and the hydrophilic oligosaccharide chain. The latter is joined by a glycosidic bond to the terminal primary hydroxyl of sphingosine or a similar base. Molecular differentiation is generally based on the carbohydrate portion. This applies to nomenclature systems as well as experimental parameters such as thin-layer separation. Virtually all gangliosides studied to date show considerable heterogeneity in lipid components; these will be considered in a later section.

Tables I, II and III summarize the current state of knowledge of the ganglioside carbohydrate structures. Some of those listed are supported by considerable experimental evidence, while others are less certain. All gangliosides (by definition) contain sialic acid, and the large majority are seen to contain hexosamine as well. Glucose and galactose are the only neutral sugars, and both are present in most molecular types. Galactosamine, in the N-acetylated form, has been the only hexosamine yet found in brain gangliosides. Outside the nervous system, however, a different situation may prevail; the one hexosamine-containing ganglioside thus far characterized was found to have N-acetylglucosamine (48). This was obtained by Kuhn and Wiegandt from bovine milk and erythrocytes. In contrast to the pattern of brain gangliosides, it had the carbohydrate structure of lacto-N-neotetraose from human milk, studied earlier by Kuhn and Gauhe (49). An additional departure was the presence of NGNA in place of NANA.

Although NANA is known to be the major sialic acid in brain gangliosides, recent papers indicate others might be present to a very limited extent. Klenk and Gielen (50) have described an unusual ganglioside from normal brain containing 3 galactose and 2 sialic acid units as the only carbohydrates.

TABLE III
Gangliosides from Sources Other than Brain

Proposed structure	Source	Reference
NGNA(2→3)Gal(2→4)Glu(1→1)Cer	Erythrocytes (equine)	(66,68)
NANA(2→3)Gal(1→4)Glu(1→1)Cer	Erythrocytes (canine)	(67)
Gal(1→4)GluNac(1→3)Gal(1→4)Glu(1→1)Cer NGNA	Spleen, liver (human) Erythrocytes, milk (bovine)	(4,69) (48)

One of the acids migrated ahead of NANA on paper chromatography and was believed to be an O,N-diacetyl neuraminic acid. Tettamanti and co-workers (51) reported evidence for the presence of small amounts of NGNA in beef brain gangliosides. Stanacev and Chargaff (52) also found trace amounts of this acid in ox brain mucolipid. Since an NGNA-containing ganglioside has now been isolated from bovine erythrocytes (see above), the possibility must be considered that this sialic acid originated in blood rather than brain tissue.

Sialic acid in brain gangliosides is attached to two main positions: C-3 of galactose and C-8 of another sialic acid. Other attachment sites have been suggested (4,5,53), but the evidence for these is not yet convincing. The fact that NANA linkages are labile to acid but stable toward base supports the prevailing view that these units are attached to other carbohydrates through ketosidic bonds. On the other hand, Kanfer and Brady (54) obtained results with the major monosialoganglioside which they interpreted as suggesting an ether-type linkage. Their observation was that bound NANA reacted with lithium borohydride, presumably through reduction of a (free) hemiketal. An argument against that interpretation is the fact that normal ether linkages are much less susceptible to acid cleavage than the NANA bond.

There are, however, other reports which suggest that the situation may be more complex than is now assumed, at least for certain sialic acid bonds. Gammack found (17) that only 70% of the NANA carboxyl groups in brain gangliosides could be titrated, both before and after mild hydrolysis with dilute acid. Wolfe and Lowden's study (31) of the trisialo fraction (see below) showed the sialic acid of this material to be considerably more labile to acid hydrolysis than NANA of the other fractions. Evidence for ester linkages has already been discussed. A fact which may be relevant to some of these "anomalies" is the facility with which the sialic acid carboxyl undergoes ester formation (6). Such bonds, either inter- or intramolecular, could conceivably form spontaneously with available free hydroxyls, and as such would bear no relation to original structure. These uncertainties relating to special situations should not obscure the main point that the weight of present evidence strongly supports ketosidic linkages for most NANA units.

A phenomenon which originally stimulated the search for different NANA linkages was the repeated observation that treatment of total brain gangliosides with neuraminidase splits off approximately half the sialic acid. The resistance of the remainder was thought as possibly due to a different bond type. However, Kuhn and Wiegandt have presented good evidence that the explanation lies rather in steric factors (23). In comparing the reactivities of several NANA-containing oligosaccharides derived from their monosialo G_1 fraction, they found that a NANA linked to the 3-hydroxyl of galactose is resistant when the galactose is also substituted at the 4 position. It is perhaps significant that the 4-hydroxyl of galactose is the only functional group on the pyranose ring with an axial configuration. Support for Kuhn and Wiegandt's hypothesis was obtained by the reviewer in connection with the Tay-Sachs ganglioside (see below). The NANA of this molecule is known to be resistant to neuraminidase, but the "hematoside" derived from it by selective removal of the terminal N-acetylgalactosamine (linked to the 4-hydroxyl of galactose) was quite susceptible to the enzyme.

Major Gangliosides of Normal Brain

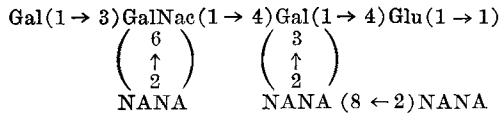
The structures depicted in Table I were proposed by Kuhn and Wiegandt (23,55). They differ only in respect to the number and attachment sites of NANA groups. The monosialo compound (G_1, G_{M1}) is postulated as the basic structural unit for all four compounds. Treatment of the di- and trisialogangliosides with neuraminidase transforms these to products which show the same migratory rates on TLC as G_1. Additional support for a common basic structure, at least for certain of the major components, comes from a large body of analytical data.

Kuhn and co-workers were the first to isolate and analyze the four major gangliosides of normal brain (41). Their molar ratios for galactose-glucose-N-acetylgalactosamine were 2:1:1, and this finding characterized all four fractions. Sialic acid values indicated two isomeric disialo compounds, and one each of the mono- and trisialo types. This was the first indication of two galactose units in the oligosaccharide chain, in contrast to the prevailing opinion at that time which favored one galactose. Substantially the same carbohydrate ratios were later reported by Svennerholm (25). Additional support for certain of these fractions has come from the work of Johnson and McCluer (36), Klenk and Gielen (56) and Korey and Gonatas (37). Dain et al. (18) also isolated four major fractions which agreed in carbohydrate ratios with Kuhn's data, except for sialic acid which was similar for all four fractions.

The concept of a common structural unit for the four major gangliosides is thus not only an attractive hypothesis but appears to have some basis in experiment. There are, however, certain findings which conflict with this picture, especially in regard to the trisialoganglioside. Wolfe and Lowden (31) isolated this fraction by cellulose column chromatography and found a galactose-glucose ratio of 3:1; their other carbohydrate analyses were consistent with Kuhn and Wiegandt's formulation. They suggested the high galactose content might be explained by the presence of a second unresolved ganglioside, such as the trigalacto species of Klenk and Gielen (50). It is noteworthy that Korey and Gonatas (37) reported for their trisialo fraction (G_1 according to their system) a hexose-hexosamine ratio of 4.6. An important observation of Wolfe and Lowden was that all the sialic acid of their trisialo fraction was liberated by three hours hydrolysis at 80C with 0.1 N HCl, whereas the total ganglioside mixture required 24 hours for complete removal. This is obviously difficult to reconcile with the concept of a common monosialo unit.

The trisialo fraction of Johnson and McCluer (4-G

in their system) showed similar composition to that of Kuhn and Wiegandt (53). However, it consumed six moles of periodate. (This did not include glucose, which reacted very slowly under their conditions.) In addition, periodate destroyed half the galactose. The periodate results are clearly incompatible with the Kuhn and Wiegandt structure, which would reduce four moles of periodate and retain all its galactose. Johnson and McCluer proposed a new structure consistent with their data:



Their attachment of NANA to C-6 of galactosamine, however, was tentative. Recent reports suggest the possibility of more than one trisialo species, and possibly a tetrasialo (see below) (23,4,57). The current ambiguities in this area are reminiscent of those originally plaguing the entire field, and may derive similarly from the difficulty of isolating pure fractions.

Kuhn and Wiegandt's proposals for the other gangliosides appear to be on firm ground (23,55). The monosialo was first shown to have the indicated carbohydrate sequence by Svennerholm (58), who analyzed the family of glycolipids obtained by partial hydrolysis. Kuhn's group later reported a thorough study of this molecule using the periodate, permethylation and partial acetolysis techniques (23,59). Only certain aspects of stereochemistry appear to remain undetermined.

Regarding the two disialogangliosides, the evidence indicates these to be isomers differing only in the attachment site of the neuraminidase-labile NANA. The one which migrates ahead on TLC (G_{II}, G_{D1a}) has this group at the 3-hydroxyl of the terminal galactose. Klenk and Gielen (56) established this point by permethylation, which gave rise to 2, 4, 6-trimethylgalactose, and also by the fact that periodate had no effect on galactose. Kuhn and co-workers used substantially the same methods (55,59) with similar results. Klenk and Gielen's initial uncertainty as to the substitution sites of galactosamine and the neuraminidase-resistant NANA was cleared up by a later report (60) which identified 2, 3, 6-trimethylgalactose as a product from permethylation of the asialo derivative. This established the GalNac(1→4)Gal linkage, and brought their structure into full accord with that of Kuhn and Wiegandt.

The slower migrating disialoganglioside (G_{III}, G_{D1b}) has the neuraminidase-labile NANA joined to the 8-hydroxyl of the other NANA, according to the proposal of Kuhn and Wiegandt (55). Their evidence was the non-destruction of one sialic acid with periodate, along with the formation of only one mole of formaldehyde. The periodate studies of Johnson and McCluer fully support this structure (53).

Minor Gangliosides

The presence of a very small amount of a polysialoganglioside (G_V) in normal brain which migrates on TLC behind the trisialo was first reported by Kuhn and Wiegandt (23). By implication, it was a tetrasialo species since brief neuraminidase treatment gave rise to G_{1V}, the trisialo, as well as G_I and G_{III} (or rather, substances which corresponded to these on TLC). Analytical data were not given so the precise compositoin of this G_V remains uncertain. Svennerholm reported obtaining small amounts of a similar material from fetal and infantile brains (4). A ganglioside recently described by Penick and McCluer (57) migrated behind the trisialo and might have corresponded to Kuhn and Wiegandt's G_V. Analysis showed a hexose/hexosamine ratio of 3, but the sialic acid percentage was considerably less than expected for a tetrasialo compound. Whether it is a new trisialo or a tetrasialo which lost some labile NANA during purification is not known.

The trigalacto-disialo ganglioside of Klenk and Gielen discussed above (Table II) was unusual in the high lability of its sialic acid bonds. The attachment sites of these units were not determined. The absence of both glucose and hexosamine is a marked departure from the other structures, as is the reported presence of a sialic acid different from NANA. The yield of purified material from total ganglioside mixture was approximately 7%, but the total percentage was believed to be much higher. Hence, its classification as a minor ganglioside may have to be revised, pending further data. The existence of such a ganglioside is consistent with the earlier report by Klenk and Gielen (16) of a disaccharide containing only galactose resulting from mild hydrolysis of ganglioside mixture. Neither this disaccharide nor the ganglioside itself, however, has been reported as yet by other laboratories. The proposed composition is of some interest in view of the ceramide-digalactoside found by Gatt and Berman (61) in Tay-Sachs brain.

Ganglioside G_{M2} (G_O) has been described by Svennerholm (25) as constituting 3–6% of normal human brain mixture. A similar value has been reported by Suzuki (44). The special interest which attaches to this compound is its apparent similarity to the ganglioside which accumulates in Tay-Sachs disease. This purported relation is based on similar compositions (23,25,36) and on some structural work (53), though the latter is incomplete. A considerable amount of data has been reported for the Tay-Sachs material (58,62–65). The structure shown in Table II was first proposed by Makita and Yamakawa (63) on the basis of permethylation studies. The substitution point of NANA, however, was not established. A recent study in the author's laboratory (65) has determined this to be the 3-hydroxyl of galactose, based on periodate treatment of the derived hematoside. The latter was formed by selective cleavage of the terminal N-acetylgalactosamine with methanolic HCl. The Tay-Sachs ganglioside differs from the major gangliosides of normal brain in the absence of terminal galactose. The remaining sugars are identical in composition, sequence, and substitution points. Bond configurations, however, have not yet been determined.

A ganglioside related to the Tay-Sachs type, but possessing an additional sialic acid (Table II; G'_{GNTr11}) was isolated from normal human brain in minor amounts (24). The location of the additional acid was not determined.

The remaining gangliosides listed in Table II are hexosamine-free. The compound containing one unit each of glucose, galactose, and NANA (G_{M3}, G_{Lact}) is similar to the ganglioside isolated from equine and canine erythrocytes and human spleen (69) contain by Yamakawa and Suzuki (68). The species from horse erythrocytes contains NGNA while those from canine erythrocytes and human spleen (69) contains NANA. Longer chain fatty acids predominate, principally 24:0 and 24:1 (see below). The corresponding material from brain (24,25,56) has not been carefully analyzed with respect to these units. It is, how-

ever, a truly minor component, the level being well below that of the Tay-Sachs type.

A closely related minor ganglioside of brain (G'_{Lact}), discussed earlier, contains the above structure with an additional sialic acid. The latter was reported to be joined to the 8-hydroxyl of the first sialic acid (24). At least one disease state is now known in which this ganglioside builds up to much higher levels in brain tissue (see below).

Methods of Structure Determination

Chemical characterization of a given molecular species generally includes the following determinations: (a) qualitative composition; (b) quantitative analysis; (c) carbohydrate sequence; (d) substitution positions, (e) stereochemistry. The stages leading to total structure elucidation often follow approximately in the sequence listed. Several of the colorimetric methods used for quantitative work have recently been evaluated (4,25). Saifer has compiled a comprehensive summary of most ganglioside analyses to date (5).

Qualitative identification of carbohydrates, following acid hydrolysis, is usually accomplished by paper chromatography. Recently, GLC has come to be recognized as a powerful tool in this area. The work of Sweeley and co-workers (70,71) has shown the trimethylsilyl ethers to be highly suitable derivatives for both qualitative and quantitative determinations. GLC remains the method of choice for analyzing the fatty acid and long-chain base components (see below).

Carbohydrate sequence is frequently solved through the technique of partial acid hydrolysis. Comparison of the rates of liberation of the various sugars is one source of information, but a more usual procedure is to isolate and analyze the oligosaccharide products. A variation of this approach was utilized by Svennerholm (58) who isolated the water-insoluble products, e.g., the family of ceramide hexosides.

One disadvantage in using aqueous hydrolysis is that groupings containing bound sialic acid are seldom obtained, due to its preferential cleavage in this medium. Also, units larger than disaccharides are not usually obtained in workable yields. Kuhn and co-workers have solved both problems by the technique of partial acetolysis in which the ganglioside is treated under mild conditions with acetic acid-acetic anhydride containing a little sulfuric acid. A large group of acetylated oligosaccharides are produced, many of which still contain bound NANA. The acetyl groups are then removed with methanolic ammonia. In this manner, Kuhn and Wiegandt (23) obtained from the major monosialoganglioside a number of di-, tri-, and tetrasaccharides, some of which had NANA still attached. These served to elucidate the sugar sequence, substitution pattern, and some aspects of stereochemistry.

When the sample size is limited, an obvious advantage is gained in working with the glycolipid products rather than the lower molecular weight saccharides. In the author's experience it has been possible to obtain reasonable yields of glycolipids with sialic acid still attached by using methanolic-HCl in place of aqueous hydrolysis. This medium keeps the carboxyl group of NANA in esterified form, thereby removing a relatively strong acid group from close proximity to the ketosidic bond. This is believed to result in relative stabilization of the latter, permitting the cleavage of other glycosidic bonds to proceed on a more competitive basis.

Bonding positions between units in the oligosaccharide chain are usually studied by two well-established techniques of carbohydrate chemistry: periodate oxidation, and permethylation. The former has been widely used because of its mildness and specificity. Any unit within the oligosaccharide chain destroyed by this reagent may be reasonably certain to possess a vicinal glycol group (or a closely related grouping, such as an α-ketol or an α-amino alcohol). The utility of the method is enhanced when the oxidation step is followed by borohydride reduction. Acid treatment then liberates either the original carbohydrate, or a smaller fragment derived from it. Thus, glucose substituted at the 4, but not the 2 or 3 positions, gives rise to erythritol, while galactose in a similar setting generates threitol. Either sugar substituted only at the 2-hydroxyl yields glycerol, as do unsubstituted pyranose rings in a terminal position. Separation of threitol and erythritol has been a major problem in applying this method to glycolipids. A paper chromatographic system was used by Kuhn and Wiegandt (23) for this purpose, but details were not given. In the author's experience the most reliable method has been GLC of the tetraacetates, a technique originally described by C. Sweeley (personal communication).

Formic acid and formaldehyde are frequently products of periodate oxidation, arising respectively from a vicinal triol grouping, and a diol which includes a primary hydroxyl. Valuable information is often obtained from their quantitative measurement. The amount of periodate consumed is determined most conveniently by U.V. spectrophotometry (72). Both over- and under-oxidation are problems which sometimes complicate this method. Johnson and McCluer (53) have shown that ganglioside carbohydrates with the free vicinal diol are readily oxidized, with the exception of glucose joined to ceramide. Cleavage of the 2, 3-diol within this unit requires high periodate concentration and long reaction time for complete oxidation. This was attributed to conformational effects and micellar structure.

Permethylation as a tool in carbohydrate chemistry dates back to the classical studies of Purdie and Haworth. The method involves conversion of the free hydroxyls of the polysaccharide chain to methyl ethers, and identification of the partially methylated monosaccharides following acid hydrolysis. Methyl iodide or dimethyl sulfate are the usual methylating agents, while for the catalyst a wide assortment of bases has been used. Some of the latter were introduced specifically for gangliosides by Kuhn and co-workers, who also made the valuable discovery that dimethylformamide and dimethylsulfoxide are superior solvents for the reaction (59,73).

In general, however, the earlier applications of permethylation to gangliosides were not very fruitful. Part of the difficulty lay in the reaction itself, and part in the fact that mixtures of gangliosides rather than pure compounds were studied. Later, when pure fractions became available, difficulty was encountered in bringing about complete methylation. Degradative side reactions also complicated the picture, while separation and identification of the methylated sugars often proved an additional problem. In recent years, however, much progress has been made in all these areas. Several combinations of solvent, catalyst, and methylating agent were recently described and evaluated (74). A new technique described by Hakomori (75) utilizes methylsulfinyl carbanion as catalyst, and shows much promise for application to glycolipids in general.

Kuhn and Egge (59) methylated their gangliosides G_I and G_{II} with methyliodide and silver oxide in dimethylformamide solvent. Decomposition products were removed by silica gel chromatography and the methylated monosaccharides identified by GLC. This latter technique appears to be the ideal solution to the problem of identification. TLC has also been used for the galactose unit of sulfatides (76).

Stereochemistry, the final consideration in determining total structure, pertains, in the case of the oligosaccharide portion, to the configurations of the glycosidic bonds. Kuhn and Wiegandt (23) applied Hudson's isorotation rules to the oligosaccharides split off from their G_I ganglioside by partial acetolysis. They also used enzymatic specificity to differentiate alpha and beta anomers. Optical rotatory dispersion and nuclear magnetic resonance are tools for determining carbohydrate stereochemistry but have not yet been applied to gangliosides specifically. Regarding sialic acid, there is still very little evidence for the configuration of the bond joining this unit to other carbohydrates. This fact is worth emphasizing in view of the frequent references in the literature to an "alpha-ketosidic" bond, a term without precise meaning.

Lipid Components

Gangliosides contain fatty acid joined in amide linkage to a sphingosine-like base, forming the characteristic ceramide unit. Vigorous acid hydrolysis is required to separate these components from each other and from the oligosaccharide chain. GLC is generally employed to identify the acids (as methyl esters), as well as the long-chain bases; the latter are first converted to volatile aldehydes by periodate oxidation (77). Such studies have revealed heterogeneity in both the acid and base groupings.

In their study of the sphingosine fraction of beef brain gangliosides, Klenk and Gielen (78) noted that oxidation with permanganate produced myristic and palmitic acids. From this they concluded that a C_{20} homologue of sphingosine was present in amounts approximately equal to sphingosine itself. This was later confirmed by Stanacev and Chargaff (79) who proposed the name "icosisphingosine" for the homologue. The presence of this unit in the sphingosine fraction of brain had been reported earlier (80), though its localization in gangliosides had not been established. To date, no other type of sphingolipid has been found to have this long-chain base.

Sambasivarao and McCluer (81) studied the gangliosides of several mamalian species and found both bases present in all cases. This conflicted with the report of Klenk and Gielen (78) that the C_{20} unit was present in beef brain but not in human brain gangliosides. Sambasivarao and McCluer obtained close to equal ratios for the two unsaturated bases in the latter case, and these together accounted for approximately 84% of the total. In calf brain the C_{18} to C_{20} ratio was 48:34. Rosenberg and Stern (82) have recently reported icosisphingosine to be absent in fetal brain, but to rise rapidly with development. A stereochemical study of this base was carried out by Majhofer-Oresčanin and Prostenik (83), who tentatively assigned it a trans-erythro configuration similar to that of naturally occurring sphingosine. Small amounts of the dihydro derivatives of each base have also been found in gangliosides (52,81).

It was demonstrated very early by Klenk (3) that stearate is the major fatty acid of brain gangliosides. More recently, Klenk and Gielen (35) reported quantitative data for beef brain gangliosides, 18:0 comprising 94%. Also present were 16:0, 20:0, and 22:0, of stearate, though slightly lower percentages are usually obtained. Trams et al. (84) found stearate to comprise 86% in human brain gangliosides, and between 72% and 96% for other species. Sambasivarao and McCluer (81) found approximately 89% stearate for normal human brain and slightly higher values for rabbit, dog and calf. A large number of minor fatty acids are also present, among which 20:0 predominates. The earlier mucolipid preparation of Rosenberg and Chargaff (13), from which crystalline lignoceric acid was isolated, appears to have been qualitatively different from the more recent preparation of Stanacev and Chargaff (52) which contained mainly stearate.

Most fatty acid studies have been carried out on total ganglioside mixtures. Some variation among individual fractions is suggested by the finding of Dain et al. (18) that fraction D, their slowest migrating, had equal amounts of 22:0 and 18:0, whereas the other three fractions contained predominantly 18:0.

An unusual fatty acid pattern was reported by Saifer et al. (15) for their strandin preparations. Their normal brain sample contained 16:0, 15.2%; 18:1, 23.8%; 18:0, 51.1%; while a similarly prepared fraction from Tay-Sachs brain contained 16:0, 16.3%; 18:1, 31.5%; 18:0, 37.2%. In both cases, other minor components were also present. The reason for these marked departures from the usual ganglioside pattern is not known; they may reflect different molecular species, though other possibilities cannot yet be ruled out. These strandin preparations contained large proportions of peptide and unusually high ratios of galactose:glucose.

Gangliosides outside the central nervous system appear to be characterized by longer chain fatty acids. Lignoceric acid comprised about 75% in hematoside from horse erythrocytes (66), while that from canine erythrocytes had 24:1 and 24:0 with the unsaturated predominating (67). Little data, however, are available for other non-brain gangliosides.

2-Hydroxy fatty acids, which characterize certain other brain sphingolipids, do not appear to be present in gangliosides (85). Sambasivarao and McCluer (81) reported very minor amounts in human brain gangliosides, though contamination by cerebroside was mentioned as a possible explanation. A study of the Tay-Sachs ganglioside in the author's laboratory revealed no trace of hydroxy fatty acids (65).

Relatively few comparative studies have been reported for ganglioside fatty acids in different disease states. An exception is Tay-Sachs, for which a number of investigators have found stearate to be the major component (62,63, 65). In general, its percentage is somewhat higher than for normal brain gangliosides. This may be a reflection of retarded development, in accord with the finding of Rosenberg and Stern (82) that the percentage of 18:0 decreases during development.

to the extent of 1%, 4%, and 1%, respectively. Several GLC studies have confirmed the predominance

Gangliosides and Diseases

Klenk's pioneering work first established the fact that Neimann-Pick and Tay-Sachs diseases are characterized by alterations in brain gangliosides (2,86). It is now recognized that virtually all the sphingolipidoses involve ganglioside changes of one type or

another, though in many cases such changes appear to be secondary to those of other lipids. Furthermore, interesting abnormalities in these compounds are now being found in other categories of neurological disorder.

It is useful to differentiate between alterations in tissue level of total gangliosides, on the one hand, and pattern changes relating to individual molecular species, on the other hand. Earlier studies dealt primarily with the former. While these were useful in establishing gangliosides in general as storage substances, the methods now available for separating and characterizing individual fractions permit much greater precision in defining the chemical abnormalities of each disease. One result of this has been more effective use of chemical analysis as a diagnostic tool (87).

Ganglioside accumulation is seen most dramatically in Tay-Sachs disease (5,88). Both gray and white matter show increases several-fold above normal levels, but that of white appears to be greater proportionately (15). The entire elevation is now known (58) to be due to a single molecular species (G_{M2} G_O), which constitutes 6% or less of normal brain gangliosides (25,44).

A disease somewhat similar to Tay-Sachs, which has been termed "late infantile systemic lipidosis" (89,90) is also characterized by marked and specific elevation in brain gangliosides. It is very probably identical to the "generalized gangliosidosis" of O'Brien et al. (91), and to other reported cases variously termed "Tay-Sachs disease with visceral involvement" (92), "familial neurovisceral lipidosis" (93), "a variant of Hurler's syndrome" (94), and "pseudo-Hurler disease" (93). The latter two names arose from certain clinical similarities to gargoylism as well as Tay-Sachs. All three diseases can be readily differentiated on the basis of brain ganglioside patterns (Fig. 4). Late infantile systemic lipidosis is characterized by proliferation of a single ganglioside which differs from that in Tay-Sachs. It corresponds rather to the major monosialoganglioside of normal brain (G_{M1}, G_1). A similar ganglioside pattern has been recently reported by Jatzkewitz et al. for a "biochemically special form of infantile amaurotic idiocy" (100).

The question is frequently raised regarding any storage disease of whether the accumulated substance has an altered structure compared to its normal counterpart. Such possibilities are obviously quite relevant to considerations of disease etiology. A structural study was made of the major ganglioside in late infantile systemic lipidosis for the purpose of making such a comparison (89). No differences were found between this structure and the major monosialoganglioside of normal brain, though stereochemical comparisons have yet to be made.

Gargoylism shows a less pronounced elevation in total gangliosides than does Tay-Sachs or late infantile systemic lipidosis (88), but the pattern alteration is quite distinctive (89,90,95). As seen in Figure 4, the slow-moving gangliosides are decreased, while the three faster moving ones are increased relative to normal brain. These three are all monosialo compounds and correspond in composition to G_{M1}, G_{M2}, and G_{M3}, respectively. The elevation of these gangliosides specifically is seen more clearly in Figure 4B which includes the combined upper phases from three successive Folch partitionings (29); the gargoyle sample in Figure 4A came from only the first partitioning.

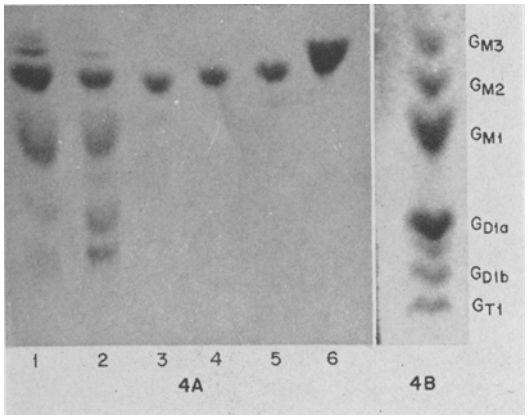


FIG. 4. Gangliosides from normal human brain and three lipidoses. *4A:* 1 = gargoylism, first Folch upper phase; 2 = normal human brain, first Folch upper phase; 3, 4, 5 = major ganglioside from late infantile systemic lipidosis; 6 = major ganglioside from Tay-Sachs' brain. Plate: 20 x 20 cm, coated with silica gel G, 250 μ thick; activated one hour at 100C. Solvent: n-propanol-water (7:3), descending, 11 hours at room temperature. Spray: 50 per cent H_2SO_4. *4B:* Gargoylism brain gangliosides, combined upper phases from three Folch partitionings. Plate and spray same as 4A. Solvent: n-propanol-water (7:3), descending, 13 hours, room temperature.

Mucopolysaccharides also accumulate in gargoylism, brain as well as other organs being affected. Chondroitin sulfate B and heparitin sulfate are the two specific compounds (96,97). It has been frequently pointed out that hexosamine is the unit common to these molecules and gangliosides, suggesting a possible area for the metabolic lesion. However, the fact that G_{M3} (hematoside) is one of the proliferating gangliosides is worth noting since this molecule contains no hexosamine (excepting, of course, that incorporated into sialic acid).

Austin has recently confirmed the above pattern change in gargoylism gangliosides, and also reported the same finding for one case of metachromatic leucodystrophy (98). This latter disease has been considered primarily a sulfatidosis, although mucopolysaccharide storage has also been reported in some instances (88,98). Cumings found raised total hexosamine levels in all of 31 cases (87). A metachromatic leucodystrophy case recently studied by Suzuki (105) showed normal gangliosides in gray matter but a two-fold elevation in white, similar to Svennerholm's results (99). It did not, however, show the pattern change found by Austin; the relative proportions of each fraction were the same as for normal white matter of comparable age.

Niemann-Pick is considered primarily a sphingomyelin storage disease. A less pronounced accumulation of gangliosides (in brain) also occurs (88). Jatzkewitz et al. (100) have recently reported a specific elevation of the Tay-Sachs type ganglioside (G_{M1}, G_O) in this condition. They also claimed a similar finding for the adult and juvenile forms of amaurotic idiocy. Unfortunately, their samples were formalin fixed, and their thin-layer patterns were somewhat difficult to interpret. Svennerholm, on the other hand, found a normal ganglioside pattern for juvenile amaurotic idiocy (25). An unusual case of late infantile amaurotic idiocy was described by Volk et al. (101) in which the fast-moving disialoganglioside (G_{D1a}, G_{II}) is stored. This is one of the rare instances of a gangliosidosis involving accumulation of other than a monosialo species.

An unusual pattern alteration has recently been observed by Norton, et al. (103) in the white matter

specifically of a case of subacute sclerosing leucoencephalitis. Two elevated gangliosides were observed on TLC which corresponded to G_{2A} and G_{3A} (system of Korey and Gonatas—see reference 37); these are normally present in very small amounts. A study currently in progress (104) indicates G_{3A} to consist of ceramide-lactoside with two sialic acids; it would thus appear to correspond to G'_{Lact} obtained by Kuhn and Wiegandt from normal brain (24). The structure of G_{2A} has not yet been determined. This case constitutes an additional example of disialoganglioside accumulation, and brings to approximately six the number of different gangliosides known to be involved in storage diseases. It would seem a justified speculation that further research will add to this list.

The major storage lipid in Gaucher's disease is cerebroside. Cases in which the central nervous systems were involved were reported to have normal ganglioside levels (88), though pattern changes may not have been specifically studied. In the spleen, however, such changes can occur. Philippart and Menkes investigated the splenic lipids of three children and one adult and found ganglioside levels approximately 10-fold above normal (102). The bulk of this was due to an hematoside type (G_{M3}), but a second unidentified species was also detected.

The chemistry of gangliosides has clearly advanced to a considerable degree since Klenk's discovery of "substance X." The detailed structural knowledge now becoming available is adding fresh insight to the specific chemical aberrations of many diseases. Pathological specimens, in turn, have aided structural work in many instances by providing large amounts of minor gangliosides difficult to detect in normal brain. This symbiosis may be expected to yield further benefits to both areas in the future.

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