review

The gangliosides

LARS SVENNERHOLM

Department of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden

SUMMARY The results of recent studies on the complexities of gangliosides from brain, spleen, and erythrocyte stroma are described, and ganglioside nomenclature is discussed. A description of the chemistry and methods of estimation of component groups (sialic acids, hexosamine and hexoses, fatty acids, and sphingosine bases) is followed by an account of the detailed investigation of isolated mono-, di-, and trisialogangliosides. Methods for the isolation of gangliosides from tissues and for their analysis are critically reviewed, and reference to the physiology of gangliosides is made.

In 1942 Klenk (1) isolated from beef brain a new class of carbohydrate-rich glycolipids, which he named gangliosides. Among the hydrolysis products, Klenk identified fatty acids, sphingosine, galactose, glucose, and most significantly, sialic acid (neuraminic acid). Besides these components most gangliosides also contain hexosamine. As early as 1936, Blix (2) had found 9% hexosamine in a brain lipid preparation, but more than 10 years elapsed before the hexosamine of gangliosides was isolated and identified as D-galactosamine (3).

For several years brain gangliosides were considered to be either low molecular lipids with a homogeneous carbohydrate moiety (4) or macromolecular lipid complexes built up by repeating units bound together with covalent bonds (5). Because of the varying composition of different ganglioside preparations and the possibility of their fractionation on cellulose columns, Svennerholm (6, 7) suggested the occurrence of gangliosides with different carbohydrate moieties. The chromatographic separation of brain gangliosides was confirmed by Kuhn (8), who also stated that the slower moving gangliosides had a considerably more complex structure than the faster moving ones. During the last 3 years reports have appeared from many laboratories on the complexity of brain gangliosides. From human and calf brain Svennerholm and Raal (9) isolated gangliosides containing one or two sialic acid residues per molecule. Accordingly, these were termed mono- and disialogangliosides respectively. Kuhn, Wiegandt, and Egge (10) found one monosialoganglioside, two disialogangliosides, and one trisialoganglioside in human and calf brains. Dain et al. (11) also isolated four main gangliosides from beef brain, but their carbohydrate composition was different from that of the gangliosides described by Kuhn and Wiegandt (12).

A number of partially correct chemical structures for brain gangliosides have been proposed in different laboratories. Recent studies by Kuhn and Wiegandt (12) have given conclusive evidence that the "parent" ganglioside has the structure shown in Fig. 1. One or two molecules of sialic acid can be bound to this monosialoganglioside to give disialo- and trisialogangliosides, respectively.

Although gangliosides are characteristic components of the nerve cell, they have also been isolated from spleen (13, 14) and red blood cell stroma (15, 16). Whether the types of gangliosides found in the nervous system also occur in these sources cannot yet be decided. Klenk (14) has presumed this to be the case in bovine spleen, and has obtained evidence for the occurrence of a ceramide-dihexoside-sialic acid in the stroma of canine and equine erythrocytes (17, 18). In human spleen Svennerholm (19) found predominantly gangliosides of the same composition as in the erythrocytes.

NOMENCLATURE

Klenk (1) assigned the name *gangliosides* to phosphorus-free glycosphingolipids containing sialic acids. Folch, Arsove, and Meath (20) proposed the term *strandin* for a high molecular weight lipid complex of ox brain which contained the same components as gangliosides

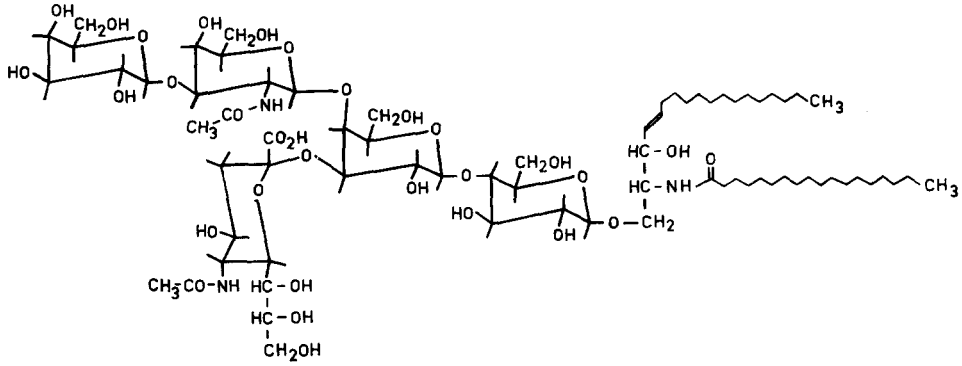

FIG. 1. Structure of ganglioside G_{M1} (G_I). From Kuhn and Wiegandt (12).

but was said to be devoid of sialic acids. This claim was, however, later retracted (21). Folch-Pi and Lees (22) have called attention to the great similarities between strandin and the gangliosides, but also pointed out some differences, for example, a greater concentration of sialic acid in strandin and a larger amount of strandin than of gangliosides in different types of brain material. The last mentioned discrepancy has been shown by Svennerholm (23) to be due to a considerable loss of gangliosides during quantitative estimation by the method of Klenk and Langerbeins (24). The higher concentration of sialic acid in strandin than in gangliosides can now be explained by the different isolation procedures used. In Klenk's original procedure (1) sialic acid is probably split off during isolation, while in the method developed by Folch et al. (20) some of the sialic acid-poor gangliosides are lost.

Rosenberg and Chargaff (25) proposed the group designation of *mucolipid* for the complex lipid polymers that contain sialic acid or a related substance as the most significant group. They have included only two subgroups, gangliosides and strandin, but mentioned the related substances *globoside* (26), which has been shown (27, 28) to be a ceramide-trihexoside-N-acetylalactosamine, and *hematoside* (15), which is the hexosamine-free ganglioside isolated from red blood cell stroma (15, 17, 18).

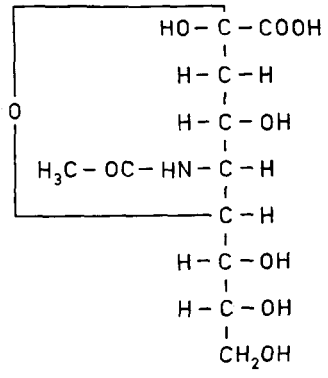

FIG. 2. β-D(−)-N-acetylneuraminic acid.

Klenk's original definition for gangliosides is still valid and the term ganglioside is a convenient trivial group name for sialic acid-containing glycosphingolipids. Strandin cannot be considered a new or well characterized glycolipid. In carbohydrate chemistry there is an endeavor to omit the prefix "muco" for amino sugar-containing saccharides (mucosamine was an old name for hexosamine), and there is little reason to introduce mucolipid as a group name for a vaguely defined lipid group. Much confusion would be prevented by dropping the names strandin and mucolipid.

COMPONENTS OF GANGLIOSIDES

Chemistry of Sialic Acids

Sialic acid (Fig. 2)—the unique constituent of gangliosides—can be considered to be a product of an aldol condensation between N-acylmannosamine and pyruvic acid. Since the chemistry of sialic acids has recently been reviewed by Gottschalk (29) only one point will be mentioned here. At that time the steric configuration at C_4 of sialic acid was not conclusively established, but Kuhn and associates (30, 31) have now shown that the hydroxyl group at C_4 is situated on the right in the Fischer projectional formula, instead of on the left as earlier suggested.

As to the nomenclature, Blix, Gottschalk, and Klenk (32) suggested that the name *neuraminic* acid be reserved for the unsubstituted structure $C_9H_{17}O_8N$. All naturally occurring sialic acids are N-acylated and some of them are in addition O-substituted. They are designated according to the nature and linkage of substituent(s), e.g. N-acetylneuraminic acid. *Sialic acid* is the group name for all acylated neuraminic acids. This nomenclature has been accepted by all workers in the field except Kuhn and collaborators, who use the name lactaminic acid for N-acetylneuraminic acid.

No O-substituted sialic acid has been found in gangliosides. In brain gangliosides, the presence of only N-acetylneuraminic acid has been indicated (33–36), but in gangliosides of equine erythrocyte stroma exclusively the glycolyl type, in which the sialic acid is linked through its potential keto group glycosidically to another sugar residue, seems to be present (35). N-Glycolylneuraminic acid has also been isolated from bovine spleen (37).

So far only D-galactose has been found to be directly attached to sialic acid in gangliosides.

In mammalian tissue the immediate precursors of sialic acids are phosphoenolpyruvate and N-acyl-D-mannosamine (38, 39). The biosynthetic reaction is catalyzed by three enzymes; a kinase, a condensing enzyme, and a dephosphorylating enzyme. The sialic acids can be activated by condensation with cytidine triphosphate to cytidine-5′-monophospho-N-acylneuraminic acid (40, 41). The compound itself has been isolated from *Escherichia coli*, K235 (42), but it has not yet been found in the mammalian system (41). An enzyme that catalyzes the condensation has, however, been found in several mammalian tissues (sheep submaxillary gland, hog intestinal mucosa, hog liver, and rat mammary gland [41]).

Estimation of Lipid-Bound Sialic Acid

As the sialic acids are very unstable toward both alkaline and acidic reagents, a quantitative isolation of sialic acids has not been achieved. The quantitative color reactions for sialic acids have therefore been, and still are, of great importance in investigations of the chemical structure of gangliosides.

Bial's orcinol reaction was the earliest method used for the estimation of gangliosides. The property of certain glycolipids (prepared from horse kidney, ox brain, and spleen) to produce a violet color when heated with Bial's orcinol reagent was first described by Landsteiner and Levene (43) and Walz (44). Klenk (45–47) used the same method when he demonstrated that subjects with Niemann-Pick and Tay-Sachs diseases had increased amounts of glycolipids of this type in brain. Blix (2) showed that a crystalline acid from bovine submaxillary mucin (48), later termed sialic acid (3), produced the same color in Bial's orcinol assay as that given by these glycolipids from brain (2).

Using this reagent Klenk and Langerbeins (24) developed a method for the quantitative determination of sialic acid, which has later been modified by several workers (49–52). The orcinol reagent was originally introduced for the estimation of pentoses and hexoses (53), and one could, therefore, suspect that these carbohydrates would interfere with the color formation of the sialic acids. Svennerholm (51) found, however, that at the absorption maximum of the sialic acids the absorbancy indices of the other sugars in gangliosides were low and could be further reduced if resorcinol replaced orcinol (54). The reproducibility and specificity of the reaction was further increased by the extraction of the colored material in butyl acetate–butanol (55) instead of amyl alcohol used in the original method (49).

Owing to the assumed large errors in the orcinol assay, several other methods, such as the direct Ehrlich, the diphenylamine, and the thiobarbituric acid reactions have been applied to the determination of sialic acid of gangliosides. Gottschalk (29) has concluded that the direct Ehrlich reaction is the most characteristic color reaction for sialic acids, but in the reviewer's opinion this statement is dubious. The direct Ehrlich reaction is essentially a pyrrole reaction and cannot be used on tissues or fluids containing pyrroles or acetaminofurans either present as such or arising from 2-amino sugars by alkali treatment. In our hands lipid-bound sialic acid did not react stoichiometrically in the direct Ehrlich reaction as described by Werner and Odin (49) or Booth (56). The diphenylamine reaction (57) has not been widely used and we have never been able to elaborate a method suitable for gangliosides. The thiobarbituric acid method (58) is the most sensitive of all the sialic acid methods and it is claimed to be most specific. As recently shown (59), the thiobarbituric acid method is unsuitable in its present form for the quantitative determination of sialic acid in gangliosides, as only free sialic acid reacts in the method and the percentage release of sialic acid from different gangliosides shows large variations. The reliability of the sialic acid figures found with the thiobarbituric acid method must, therefore, be seriously questioned.

Another difficulty in the determination of sialic acid in gangliosides has been the lack of pure standard substances. In general N-acetylneuraminic acid prepared from human serum (60) and methoxyneuraminic acid from submaxillary mucin (61) have been used. Klenk and Gielen (62) found the latter to give a higher molar absorption coefficient in Bial's orcinol reaction.

Hexosamine and Hexose

Most gangliosides also contain a hexosamine. In brain gangliosides only D-galactosamine (3, 63) has been found. The hexosamine occurs in acetylated form. Hexosamine-containing gangliosides have been reported in organs other than brain, e.g. spleen (14), but the hexosamine has not been conclusively identified. The quantitative estimation of hexosamine has been carried out with modifications (63, 64) of the method of Elson and Morgan (65) and the indole method of Dische and Borenfreund (66). The two methods have given practically identical figures (11).

TABLE 1 FATTY ACID COMPOSITION OF BRAIN GANGLIOSIDE (68)

	14:0	16:0	18:0	20:0	22:1*	22:0	24:2*	24:1*	24:0
Beef		3	82	4		2	2	3	4
Monkey		2	89	7		2	1		
Man			86	10		3			
Pig		4	82	7		2	1	2	3
Turkey			96	2		2			
Porpoise		1	80	14	1			2	1
Shark		5	72	1	4	5		13	
Alligator	1	7	79	4		2		7	

Values are percentages areas on the gas–liquid chromatogram.
* Identification of each methyl ester is on the basis of retention time on one column and is tentative.

Galactose and glucose are the only two hexoses detected in gangliosides. Galactose was identified in 1942 by Klenk (1) as the methylphenylhydrazone, while glucose has been identified by means of paper chromatography, optical rotation, and glucose oxidase. The quantitative figures found for the sugars have in general been too low, and have led to erroneous suggestions for the structure of gangliosides (10). By adopting more suitable hydrolysis conditions and adequate reference samples of sugars, values corresponding to theoretical figures for hexoses have been obtained (12, 59).

Fatty Acids

Klenk (1) showed that the major fatty acid of brain gangliosides is *n*-octadecanoic (stearic) acid. By the use of gas–liquid chromatographic techniques many laboratories (9, 10, 62, 67, 68) have confirmed that stearic acid constitutes 80–90% of total fatty acids of brain gangliosides, not only from mammals but also from other vertebrates (68). In Table 1 is given the fatty acid pattern found by Trams et al. (68). Other than brain, only the gangliosides of canine and equine erythrocytes have been analyzed for their fatty acid composition by gas–liquid chromatography (17, 18). *n*-Tetracosanoic (lignoceric) acid constituted about 75% of the fatty acids in equine erythrocytes, while in canine erythrocytes (18) *n*-tetracosenoic (nervonic) acid predominated over *n*-tetracosanoic acid and they together constituted about 80% of the total. In both sources there was also a considerable amount of *n*-docosanoic (behenic) acid.

Sphingosine

Klenk (1) isolated sphingosine sulfate from brain gangliosides. In the sphingolipid fraction of equine and bovine brain, a sphingosine homologue with 20 carbon atoms was detected (69). Klenk and Gielen (62) found that in bovine brain gangliosides the C_{20}-homologue of sphingosine constituted about 50% but they could not detect it in human brain gangliosides. Stanacev and Chargaff (70) found in bovine brain 50.0% sphingosine, 3.4% dihydrosphingosine, and 46.5% C_{20}-sphingosine. They proposed the name "icosisphingosine" for the new substance and the following structure:

$$CH_3(CH_2)_{14}CH=CHCH(OH)CH(NH_2)CH_2OH$$

Minute amounts of a C_{20}-dihydrosphingosine were also indicated.

CHEMICAL STRUCTURE OF MAJOR BRAIN GANGLIOSIDES

A uniform nomenclature for the gangliosides has not been adopted. In this review the symbols chosen by us (59) have been used, and in Table 2 the corresponding symbols suggested byKuhn and Wiegandt (12) are shown. In Fig. 3 the thin-layer chromatographic pattern of human brain gangliosides is shown. By enzymic hydrolysis with sialidase (neuraminidase), the disialogangliosides G_{D1a} and G_{D1b} and the trisialoganglioside G_{T1} were all transformed to the monosialoganglioside G_{M1} (12, 59, 71).

Monosialoganglioside G_{M1}

By stepwise acid degradation and isolation of the acyl sphingosine saccharides Svennerholm (71) showed that

TABLE 2 CARBOHYDRATE COMPOSITION OF HUMAN BRAIN GANGLIOSIDES (59)

Symbol* 1	Symbol* 2	Compound	Galactosamine as Free Base	Hexose	N-Acetyl Neuraminic Acid	Ceramide: Hexosamine: Hexose: N-Acetylneuraminic Acid
			%	%	%	*Molar ratio*
		Normal brain				
	G_{M3}	Ceramide–lactose–N-acetylneuraminic acid	0.6	28.1	24.0	1:0:2:1
G_O	G_{M2}	Ceramide–N-triose–N-acetylneuraminic acid	12.8	27.4	21.4	1:1:2:1
G_I	G_{M1}	Ceramide–N-tetrose–N-acetylneuraminic acid	11.9	35.5	20.6	1:1:3:1
G_{II}	G_{D1a}	Ceramide–N-tetrose–di-acetylneuraminic acid	9.5	28.6	31.8	1:1:3:2
G_{III}	G_{D1b}	Ceramide–N-tetrose–di-acetylneuraminic acid	9.3	29.0	31.5	1:1:3:2
G_{IV}	G_{T1}	Ceramide–N-tetrose–tri-N-acetylneuraminic acid	7.5	23	38	1:1:3:3
		Tay-Sachs brain				
	G_{M2}	Ceramide–N-triose–N-acetylneuraminic acid	13.1	26.2	21.8	1:1:2:1

* Symbols in column 1 used by Kuhn and Wiegandt (12); in column 2 by Svennerholm (59)

the sequence of components in G_{M1}, from which sialic acid had been split off, was acyl–sphingosine–glucose–galactose–N-acetylgalactosamine–galactose. This finding did not agree with the prevailing view, according to which the hexosamine was assumed to be terminal, as in the neutral aminoglycolipid (globoside) isolated from erythrocyte stroma (15, 27). The then prevailing view had been arrived at from experiments involving mild acid degradation of gangliosides. Bogoch (5) reported that more galactosamine than galactose was released by weak acid hydrolysis, and Klenk and Gielen (72) isolated a hexosamine-containing disaccharide which was suggested to be identical with O-α-N-acetyl-D-galactosaminoyl(1→3)-galactose first isolated by Coté and Morgan (73). Svennerholm (71) found two galactosamine-containing disaccharides after mild acid hydrolysis of G_{M1}. They were both composed of galactose and galactosamine: in one of the sugars the galactosamine moiety had a free-reducing group, while in the other this was contained in the galactose. The former disaccharide was at the same time independently isolated by Klenk, Hendricks, and Gielen (74), and later by Kuhn and Wiegandt (12). It was shown to be O-β-D-galactopyranosyl (1 → 3)-2-acetamido-2-deoxy-D-galactose and identical with a disaccharide of human blood group substance, the structure of which had shortly before been established by Morgan and co-workers (75). The proposed chemical structure was based on the following data: The disaccharide was hydrolyzed by crystalline β-galactosidase (12) (but not by α-galactosidase [75]) to galactose and N-acetylgalactosamine. After hydrogenation with sodium borohydride and acid hydrolysis, only galactose was identified by means of aniline phthalate. The sugar was very labile in alkaline environment and was degraded at room temperature to free galactose and a "direct" Ehrlich chromogen (76). Oxidation of the disaccharide by sodium metaperiodate before and after borohydride reduction gave results consistent with those obtained by simple oxidation according to Malaprade (75). Some N-acetylgalactosamine remained intact if the periodate treatment was not allowed to proceed for more than a few hours, thus making linkage through the 5 or 6 position of galactose to galactosamine less probable (77, 78).

Kuhn and Wiegandt (12) also isolated a disaccharide of ganglioside G_{M1} which was shown to be identical with lactose. The yield was not reported. In our hydrolysis experiments only very small amounts of a disaccharide with the chromatographic behavior of lactose were found. As there are many different gangliosides the isolation of small amounts of lactose cannot prove that this lactose is a component of ganglioside G_{M1} because it can be derived from other gangliosides contaminating the preparation of G_{M1}. Kuhn and Wiegandt (12) have, however, also degraded G_{M1} by acetolysis. The ganglioside was hydrolyzed with acetic acid–acetic anhydride containing a little sulfuric acid (12). This treatment released sialic acid-containing oligosaccharides from which the sialic acid could be split off by mild acid hydrolysis. One tetrasaccharide, two trisaccharides, two disaccharides and a monosaccharide to which sialic acid was bound, were demonstrated by paper chromatography. The oligosaccharides were termed as shown in Table 3.

TABLE 3 OLIGOSACCHARIDES ISOLATED FROM GANGLIOSIDE G_{M1} (12)

Name	Structure
Ganglio-N-tetrose	$Gal(1\xrightarrow{\beta}3)GalNac(1\xrightarrow{\beta}4)Gal(1\xrightarrow{\beta}4)Gl$
Ganglio-N-triose I	$Gal(1\xrightarrow{\beta}3)GalNac(1\xrightarrow{\beta}4)Gal$
Ganglio-N-triose II	$GalNac(1\xrightarrow{\beta}4)Gal(1\xrightarrow{\beta}4)Gl$
Ganglio-N-biose I	$Gal(1\xrightarrow{\beta}3)GalNac$
Ganglio-N-biose II	$GalNac(1\xrightarrow{\beta}4)Gal$
Lactose	$Gal(1\xrightarrow{\beta}4)Gl$

The sialyl monosaccharide, which was hydrolyzed by weak acid to galactose and sialic acid, was also obtained by acetolysis of sialyl lactose isolated from cow colostrum and human milk (79–81). The sialic acid is bound to C_3 of galactose, as lyxose was formed after periodate oxidation and subsequent acid hydrolysis of sialyl galactose (12). Of the two sialyl disaccharides, sialyl lactose and sialyl ganglio-N-biose II, the latter compound predominated. Sialic acid was easily liberated from sialyl lactose by sialidase (from *Vibrio cholerae*) but sialyl ganglio-N-biose II, sialyl ganglio-N-triose I + II and

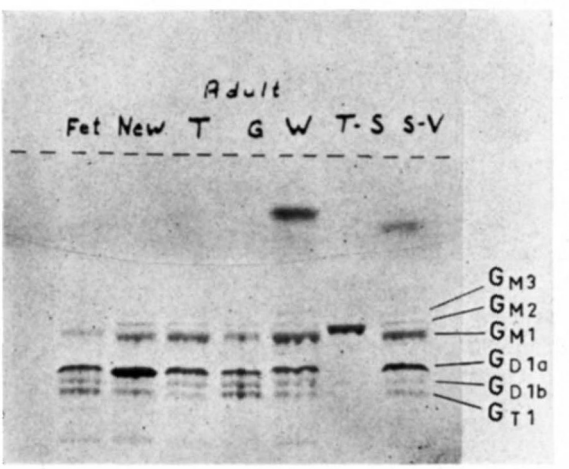

FIG. 3. Thin-layer chromatogram (partition on silica gel) of total ganglioside extracts of different tissues. Fet = fetal brain, New = newborn brain, T = total, adult human brain, G = grey matter of adult human brain, W = white matter of adult human brain, T-S = infantile amaurotic idiocy brain, and S-V = juvenile amaurotic idiocy brain. Solvent: 1-propanol–water, 7:3 (v/v). Spray: resorcinol reagent.

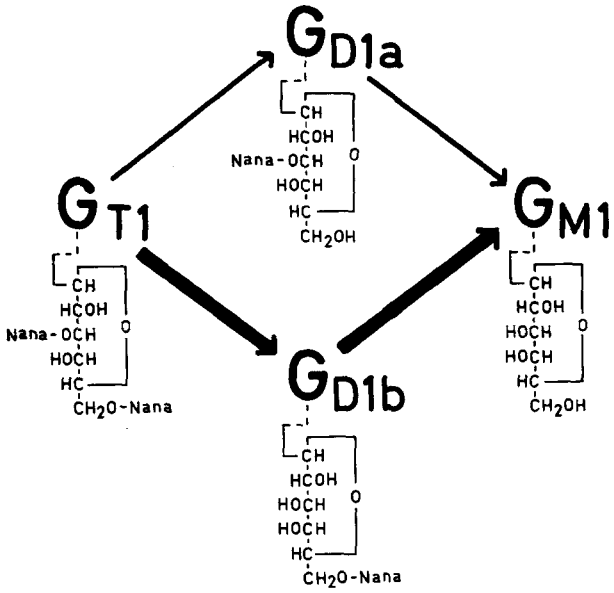

FIG. 4. Pathways for the hydrolysis of brain trisialoganglioside G_{T1} to monosialoganglioside G_{M1} with sialidase from *V. cholerae* (59).

sialyl ganglio-N-tetrose were all quite resistant to the action of this enzyme.

On periodate treatment of ganglioside G_{M1} and ganglio-N-tetrose the terminal galactose was rapidly, and glucose more slowly, destroyed (12). The second galactose unit remained intact. These data indicate that the hydroxyl groups of C_2 and C_3 are not substituted in the glucose moiety and that galactose is bound to C_4. After periodate oxidation of the acyl sphingosine-N-tetrose (59) isolated after mild acid hydrolysis of ganglioside G_{M1}, all the galactose was destroyed and only galactosamine remained. This is a further proof for the binding of sialic acid to the galactose moiety of the lactose in gangliosides.

Kuhn and Wiegandt (12) established the linkage between lactose and ganglio-N-biose I by periodate oxidation and subsequent potassium borohydride reduction of the ganglio-N-tetrose. Erythritol, glycerol, and threitol were chromatographically identified. Erythritol was shown to be derived from glucose, glycerol from galactose in the end position and, consequently, threitol from the other galactose. Threitol and glycerol were also identified from ganglio-N-triose I and ganglio-N-biose II treated in the same manner. This indicated that ganglio-N-biose I is bound to C_4 of the galactose moiety of lactose. Application of Hudson's isorotation rules to the specific rotation of ganglio-N-tetrose and ganglio-N-triose II suggests a β-glycosidic linkage. The final proof for the linkage must await the isolation of ganglio-N-biose II and determination of its structure. This disaccharide is preferentially isolated from ganglioside G_{M2} as it is the only hexosamine-containing disaccharide in this compound. Large amounts of ganglioside G_{M2} can easily be prepared from the brain of patients with Tay-Sachs disease (59, 71).

The isolation of the sialic acid-containing sugars and the periodate oxidation have shown that in the basic ganglioside of human and beef brain the N-acetylneuraminic acid is bound to C_3 of the galactose in the lactose part of the tetrose. Thus, the sialic acid moiety will be in the *cis*-position to the ganglio-N-biose I moiety bound to C_4 of the same galactose. This steric configuration will prevent the sialidase from liberating the sialic acid. This suggestion is supported by the fact that not only gangliosides but also all saccharides which have substituents at C_4 of this galactose are not attacked by sialidase. Sialyl (2 → 3) galactose, sialyl (2 → 3) lactose, and ganglioside G_{M3} (19), which have no substituents in position 4 are easily hydrolyzed by the enzyme.

The carbohydrate moiety is bound to the primary hydroxyl group of sphingosine (12, 62). Klenk and Gielen (72) showed this by periodate oxidation of the methyl sphingosine obtained by permethylation of gangliosides, and Wiegandt (cf. reference 12) used ozonide treatment and subsequent fragmentation.

Di- and Trisialogangliosides

In the "disialoganglioside fraction," the two disialogangliosides G_{D1a} and G_{D1b} and the trisialoganglioside G_{T1} are quantitatively transformed to the basic ganglioside G_{M1} with sialidase from *V. cholerae* (12, 59, 71). It could be shown that G_{D1a} was hydrolyzed much faster than G_{D1b}, and that G_{T1} was hydrolyzed first to G_{D1b} and finally to G_{M1} (12, 59). Small amounts of G_{D1a} were, however, also formed from G_{T1} (59). Hydrolysis of G_{T1} with weak acid (0.01 N HCl for 10 min in a boiling water bath) gave about equal amounts of G_{D1a} and G_{D1b}. The results of the hydrolysis experiments with the trisialoganglioside are consistent with the hypothesis of a compound in which the two extra sialic acids are bound in such a way that they are both easily attacked by hydronium ions. They must be attached to different carbon atoms of the hexose, as there is a large difference in the velocity with which the two sialic acids are liberated. Gauhe (cf Kuhn et al. [82]) has isolated sialyl (2 → 6) lactose and shown that in this sugar the sialic acid is more slowly liberated by sialidase than in sialyl (2 → 3) lactose. It was therefore suggested (59) that in G_{D1a} sialic acid was bound to C_3 of the terminal galactose, in G_{D1b} to C_6 of the galactose and in G_{T1} both to C_3 and C_6 of the galactose (Fig. 4). Besides these gangliosides derived from the same "parent ganglioside" Kuhn and Wiegandt (12) have described a still more complex ganglioside, termed G_V, which on mild acid hydrolysis is

converted to trisialoganglioside G_{T1}. In our own studies (59) only small amounts of this ganglioside were observed in human fetal and infantile brains.

Four gangliosides, derived from a tetrose built up from 2 moles of galactose, 1 mole of glucose, and 1 mole of N-acetylgalactosamine, have been isolated from human and cow brains by Kuhn and Wiegandt (12) and by Svennerholm (59). The quantitative figures for the carbohydrate composition of the gangliosides given in these papers are very similar. Dain et al. (11) have also isolated four human brain gangliosides, which are probably identical with those just mentioned, although their preparations of gangliosides yielded lower values for the carbohydrates. The thin-layer chromatograms and molar ratio between the carbohydrate components were, however, similar to those of Kuhn and Wiegandt (12) and Svennerholm (59). The low values are probably due to contamination of the compounds with adsorbents. Klenk and Gielen (83) have isolated one ganglioside with this tetrose chain. It was a disialoganglioside, probably identical with ganglioside G_{D1a}. Permethylation indicated that the second sialic acid was bound to C_3 of the terminal galactose.

There is still rather little known about the distribution of the different gangliosides in tissues. Svennerholm (59) has attempted a quantitative estimation in infantile human brains. It is evident from Fig. 3 that the disialoganglioside G_{D1a} predominates in all the different normal brain materials, but there are also rather large amounts of the monosialoganglioside G_{M1}. Figure 3 also shows that the disialoganglioside G_{D1b} increases with age. The trisialoganglioside occurs only in small amounts. An idea of the ganglioside profile can also be obtained from the carbohydrate composition of the total gangliosides.

OTHER MONOSIALOGANGLIOSIDES

In normal human brains there is between 3 and 5% of a second hexosamine-containing monosialoganglioside G_{M2} (59). It differs from the normal monosialoganglioside in that there is no terminal galactose (12, 59, 71). Its structure has been established from quantitative analysis (59), and by isolation and characterization of acyl sphingosine saccharides and free sugars after acid hydrolysis (59, 71). After mild hydrolysis acyl sphingosine-N-triose, acyl sphingosine lactose and acyl sphingosine glucose with exactly the same R_F-value as comparable compounds from monosialoganglioside G_{M1} (59, 71) are formed. No acyl sphingosine-N-tetrose was found. Klenk and Gielen (62, 72, 84) have reported on a ganglioside with the same components as G_{M2}. Their analytical data (72) and the fact that they report it to be the main component of human brain (83) suggest that they have been dealing with ganglioside G_{M1}. We (9, 23) and Kuhn and collaborators (82) also overlooked the galactose in the end position in previous work.

Ganglioside G_{M2} had accumulated in large amounts in the brain of a patient who died from the infantile form of familial amaurotic idiocy (Tay-Sachs) and was also termed Tay-Sachs ganglioside (71). An identification of Tay-Sachs ganglioside with ganglioside G_{M2} has been made only in one case (71), but the analytical figures reported for the ganglioside of Tay-Sachs disease (85–88) strongly support the view that ganglioside G_{M2} is the ganglioside stored in the brain in this disease.

Yamakawa and Suzuki (15) and Klenk and associates (17, 18) have isolated a ganglioside (hematoside) from canine and equine erythrocyte stroma which contains fatty acid–sphingosine–hexose–sialic acid in the molar ratio 1:1:2:1. A ganglioside with the same composition has been found in human spleen (19), liver,[1] and brain (59). The following structure has been reported for this ganglioside (17, 19): N-acetylneuraminic acid (2 → 3) galactose (1 → 4) glucose (1 → 1) N-acylsphingosine.

The sialic acid is easily liberated by sialidase (19). Svennerholm (19) found only glucose attached to sphingosine but Klenk and Padberg (17) reported that in some of the ganglioside molecules the disaccharide was composed of two moles of galactose.

Klenk and associates (84, 89, 90) have described one additional hexosamine-free ganglioside. By an ion exchange method acyl sphingosine–trihexoside–N-acetylneuraminic acid was isolated from human brain (89) in a yield of about 30% of total gangliosides, but this finding has not been confirmed by more recent work (12, 59). Klenk and Gielen (72) supported their structure proposal by the finding of a disaccharide with 2 moles of galactose, but this disaccharide could not be detected by Kuhn and Wiegandt (12) in their preparations. Svennerholm and Raal (9) also suggested the occurrence of the same type of ganglioside as Klenk and Gielen (89) but this suggestion was based mainly on the fact that their hexosamine values were too low and for hexose too high to be consistent with the then prevailing conception of the structure of gangliosides (82).

Although this matter needs further study, it is probable, however that there does occur a brain ganglioside with the structure suggested by Klenk and Gielen (89).

ISOLATION OF GANGLIOSIDES FROM TISSUES

One of the great obstacles in the study of gangliosides has been the lack of easy and reliable methods for the isolation of pure compounds from tissue extracts. Apart

[1] Svennerholm, unpublished results.

from contamination of one ganglioside with another, the gangliosides are also easily contaminated with other polar lipids and with nonlipid impurities. Klenk's original procedure (1) for isolation of brain gangliosides were based on a selective extraction by organic solvents. A similar technique was used by Blix (2). A fairly pure ganglioside mixture is obtained with this method, but a definite disadvantage is the great loss of gangliosides during the isolation. Because the gangliosides are very inhomogeneous with respect to the carbohydrate moiety, it is essential to achieve a high recovery to be able to draw conclusions about the composition of the native substances and their metabolism.

Folch et al. (20) extracted all the tissue lipids with chloroform–methanol 2:1, v/v, and from this extract the gangliosides were isolated, by dialysis against water, into an upper (aqueous) phase from the other lipids which stayed in the lower (chloroform) phase. With this partition dialysis method there is a good recovery of the gangliosides, but the method allows preparation of only small amounts. The ganglioside mixture in the upper phase is also contaminated with polar lipids, such as sulfatides and phosphatidyl serine, and large amounts of nonlipid impurities that are very difficult to remove (91, 92). However, the partition technique introduced by Folch has proved to be an excellent method for the isolation and purification of gangliosides. The combination of this technique with solvent precipitation as in the original procedure of Klenk (1) gave a simple, practical method for the large scale preparation of gangliosides (5, 14, 22). The method has the disadvantage that rather large quantities of monosialogangliosides are lost (59).

An isolation method which gives a nearly quantitative recovery of gangliosides under mild conditions was introduced by Svennerholm (6). A total lipid extract is applied to a cellulose powder column and the bulk of lipids other than gangliosides are eluted with chloroform containing small amounts of alcohol and water. The gangliosides are retained on the column and can be eluted with alcohol- and water-enriched solvents. By this procedure a partial separation of the gangliosides into sialic acid-poor and sialic acid-rich fractions occurs (93). The method has later been applied to the quantitative isolation of brain gangliosides by Rouser and associates (94, 95) and recently for the isolation of trisialogangliosides (96). Klenk and Heuer (18) also achieved a good extraction of the simple ganglioside of canine erythrocyte lipid extracts with this method. A still better separation of the gangliosides into mono-, di-, and trisialogangliosides was obtained on paper roll columns with propanol–water mixtures (59).

Chromatography on silicic acid with chloroform–methanol mixtures has in several instances been found to be superior for the separation of gangliosides and their final purification. The procedure developed by Svennerholm (97) has been successfully applied by Svennerholm and Raal (9) and by Van Heyningen and Miller (98). Similar procedures have been used by Klenk and Gielen (72) and by Dain et al. (11).

Although chloroform–methanol mixtures have been generally adopted for the tissue extraction of gangliosides, other solvents have recently been used. Trams and Lauter (36) extracted total lipids from fresh brain tissue with tetrahydrofuran. On addition of ethyl ether to the lipid extract two phases were formed. The gangliosides were contained in the water phase. No figures have hitherto been reported for the recovery of gangliosides with this procedure. Kuhn and Wiegandt (12) have extracted the gangliosides from the brain tissue with phosphate buffer or phenol, but it is impossible from the data given to calculate the yield. The latter workers have also applied several other methods not previously used for the purification and separation of the gangliosides; e.g., chromatography on hydroxylapatite with phosphate solutions, on silicic acid with propanol–water, and on cellulose powder with butanol–pyridine–water. As no experimental details or results are given it is difficult to evaluate the usefulness of their procedures at present. Craig countercurrent distribution (23, 99, 100) has also been applied to the isolation and separation of gangliosides.

ANALYSIS OF GANGLIOSIDES

Although pure gangliosides may be obtained by a combination of modern chromatographic methods there is as yet no ideal method of separating all the individual gangliosides from one another. The isolation of the gangliosides is difficult because they consist of a lipophilic and a large, charged, hydrophilic portion and therefore form micelles in water and organic solvents (36, 83, 92). They may act as protecting colloids for other lipids or water-soluble compounds of low molecular weight, such as amino acids and peptides. Gangliosides have, therefore, sometimes been erroneously assumed to contain peptides (20, 25). Several of the methods that have been used as proof for purity and homogeneity of gangliosides do not fulfill their purpose.

Elemental analyses are of limited value, as large admixtures of, for example, other glycolipids will give only small deviations of the analytical results. Nitrogen determinations can be valuable in the analyses of sphingolipids, but in gangliosides the nitrogen content is of small significance because the nitrogen can be derived from sphingosine, sialic acid, galactosamine, and nitrogenous impurities. Because it is difficult to remove all phospholipids and sulfatides, analyses for phosphorus and sulfur ought to be generally performed. Analyses of glucose,

galactose, galactosamine, and sialic acid with adequate methods and reliable standard substances are, of course, essential for investigation of purity, homogeneity, and chemical structure of gangliosides.

Of the physicochemical methods, electrophoresis and ultracentrifugal analysis are commonly accepted as tests of chemical homogeneity. A single moving boundary in these methods is, however, no proof for homogeneity because the gangliosides form micelles in water solutions. In our experience electrophoretically homogeneous preparations of gangliosides could be separated into five or six different components by thin-layer chromatography.

At present partition chromatography on thin-layer plates of silica gel is, in our opinion, the best aid in the determination of purity and homogeneity of gangliosides. Before the introduction of thin-layer chromatography, partition chromatography on untreated or silica-impregnated papers was used with satisfactory results. Some of the solvents commonly used and the R_F values of the different gangliosides have recently been summarized (59). On thin-layer plates of silica gel these solvents have been found suitable for the separation of gangliosides: chloroform–methanol–water, 60:35:8, v/v (101) (or for separation of monosialogangliosides, 50:50:8, v/v [59]), or propanol–water, 7:3 or 3:1, v/v (12, 59). The gangliosides can be visualized by spraying with specific reagents for sialic acid, such as orcinol (72), resorcinol (59, 101) or *p*-dimethylaminobenzaldehyde in hydrochloric acid (12). The ganglioside plates can also be sprayed with a general lipid spray reagent such as bromothymol blue (102) before, or perchloric acid–molybdate (103) after, the specific sialic acid reagent.

Structural information on brain gangliosides has been obtained by qualitative and quantitative estimations of their constituents (see above), by acid and enzymic degradation, permethylation, and periodate oxidation. Partial acid degradation of total gangliosides was first done by Bogoch (5), who studied the order in which the carbohydrates were released from gangliosides. Klenk and Gielen (72, 84) and Kuhn and associates (82) also used this technique for unseparated or partly separated gangliosides. Nevertheless, it was not until Kuhn and Wiegandt (12) applied the technique to chromatographically uniform gangliosides that the method gave definite and reproducible results. All these workers determined the carbohydrate structure from the saccharides split off from the ceramide moiety. Svennerholm (71) has instead determined the carbohydrate sequence of the different ceramide–saccharides isolated from partial acid hydrolysis of the major normal brain ganglioside and the Tay-Sachs ganglioside.

Karkas and Chargaff (104) and Klenk and Gielen (84) applied permethylation to total ganglioside extracts. The results were difficult to interpret. Kuhn and associates (82) showed that permethylation caused serious degradation of gangliosides. They tried to prevent hydrolysis by using a mixture of the oxide and hydroxide of barium or strontium instead of silver oxide, but the results were rather disappointing.

Periodate oxidation on total gangliosides was applied by Rosenberg and Chargaff (105). The results were difficult to interpret, but they indicated that galactose might be the principal sugars to which sialic acid was attached. On the other hand periodate oxidation has given valuable data when applied to pure gangliosides or saccharides (12, 19, 62, 74).

PHYSIOLOGY OF GANGLIOSIDES

The distribution of gangliosides in nervous tissue under normal and pathological conditions has recently been reviewed (106) and will not be discussed here. The very few reports existing on the biosynthesis of gangliosides (described in reference 106) were made before the present knowledge of the complexity of the gangliosides and can no longer be regarded as adequate.

So far there are only vague speculations on the physiological role of the gangliosides. As they are characteristic components of the neuron, many workers have assumed that gangliosides are engaged in the transmission phenomena in the central nervous system. A recent monograph by McIlwain (107) surveys the chemical basis of excitability, and this topic will not be discussed here.

The remarkable advances in the chemistry of brain gangliosides over the past few years provide a firm basis for investigations of metabolism and physiology.

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