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LIPID COMPONENTS OF SIALOSYLGALACTOSYLCERAMIDE
OF HUMAN BRAIN AND STUDIES OF
GANGLIOSIDES OF BOVINE ADRENAL CORTEX
AND ADRENAL MEDULLA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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The Ohio State University
1969

Approved by

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ABBREVIATIONS

C:M Chloroform:Methanol
C:M:W Chloroform:Methanol:Water
Gal Galactose
GalNHAc N-Acetylgalactosamine
Glc Glucose
GLC Gas-Liquid Chromatography
HMDS Hexamethyldisilazane
NANA N-acetylneuraminic acid
NGNA N-glycolyneuraminic acid
LCB Long-Chain Base
TLC Thin-Layer Chromatography
TMCS Trimethylchlorosilane
TMSi Trimethylsilyl ether
LC Lactosylceramide (Ceramidedihexoside)
GnLC N-acetylgalactosaminyllactosylceramide (Ceramidetrihexoside)
GGnLC GalactosylN-acetylgalactosaminyllactosylceramide (Ceramide-
tetrahexoside)
CHAPTER I.

STATEMENT OF THE PROBLEM

Gangliosides are glycosphingolipids which contain sialic acid and were first isolated by Klenk in 1935 from a Nieman-Pick's disease brain. Gangliosides have now been isolated from almost all mammalian tissues. Nine different brain gangliosides have been isolated and well characterized. In the four most abundant brain gangliosides, the carbohydrate sequence found is Gal-GalNHAc-Gal-Glc. There is one or more sialic acid residues attached to this carbohydrate chain.

The research reported in this dissertation consists of characterization of sialosylgalactosylceramide from human brain and studies of bovine adrenal gangliosides.

A. Sialosylgalactosylceramide (SGC)

Kuhn and Wiegandt (1964 b) and also Wiegandt and Baschang (1964) isolated a minor component of brain gangliosides that yielded 3'-neuraminosylgalactose upon ozone degradation. This indicated that the parent ganglioside, Ggal (sialosylgalactosylceramide), is unusual in that galactose is linked directly to sphingosine whereas the major brain gangliosides are derivatives of lactosylceramide in which the glucose is the residue linked to sphingosine. Because the sialosylgalactosylceramide (SGC) was present in such small amounts in the ganglioside mixture, the possibility was considered that
it might be derived from adult brain cerebroside. The fatty acid and long-chain base composition of brain gangliosides and cerebrosides are clearly distinct; cerebrosides contain a high proportion of hydroxy fatty acids (O'Brien, Fillerup, and Mead, 1964) and no C_{20}-sphingosine (4-eicosasphingenine) (Sambasivarao and McCluer, 1964) whereas gangliosides contain mostly stearic acid and approximately equal quantities of C_{18}-(4-sphingenine) and C_{20}-sphingosines (4-eicosasphingenine). Thus characterization of the ceramide moiety of SGC should contribute to an understanding of the metabolic relationship of this compound to the major brain gangliosides. Isolation of small quantities of SGC from the human brain and characterization of the lipid moieties is reported.

B. Adrenal Gland Gangliosides

The adrenal glands in mammals including man really consist of two glands united together. These two component parts, the cortex and the medulla, are developmentally, histologically, and fundamentally unrelated and are derived from two distinct and different parent tissues which unite only secondarily. As a matter of fact, in the lower vertebrates such as the fishes, the two parts are permanently separated. It is only as we ascend the scale of animal life into amphibia, reptiles, and birds that they become more closely associated. In mammals, they finally unite to form a single glandular structure. The cortex in common with the other glands is mesodermal in origin and is derived from the ventral portion of the celomic epithelium. The medulla cells are ectodermal in origin. They are
intimately connected with the nervous system and developed from the neural crest. The cells of medulla are in fact modified ganglion cells which remain in intimate contact with preganglionic fibers of the sympathetic nervous system (Stoffer et al., 1961).

The gangliosides of the central nervous system are distinct from those of visceral organs. The major gangliosides of the brain are more complex, whereas the major gangliosides of visceral organs are primarily hematosides (sialosyllactosylceramides) (Hakomori, 1965). Characterization of cortex gangliosides and medulla gangliosides might provide some insight into the role of these unusual acidic glycosphingolipids. Brain gangliosides contain C\textsubscript{18}- and C\textsubscript{20}-sphingosines and mostly stearic acid (Sambasivarao and McCluer, 1964) whereas gangliosides obtained from peripheral organs contain only C\textsubscript{18}-sphingosine and the major fatty acids are lignoceric and nervonic (Hakomori, 1965; Handa and Handa, 1965).

It was therefore expected that the medulla may have gangliosides like brain gangliosides which are more complex and cortex may have gangliosides like other organs which are mostly hematosides. Gangliosides from the cortex and medulla were isolated and their compositions and chemical structures studied.
CHAPTER II  
HISTORICAL REVIEW

A. Introduction

Gangliosides are glycosphingolipids which contain sialic acid. Klenk (1935, 1941) first isolated these substances from the brains of patients suffering from amaurotic familial idiocy and Niemann-Pick's disease. Earlier, these lipids had been detected by Landsteiner and Levene (1925) and by Thierfelder and Walz (1927) by their reaction to form a color complex with p-dimethylaminobenzaldehyde and orcinol. Blix (1938) reported the regular occurrence of these lipids in the normal brain. Klenk (1942) named these substances gangliosides, as he suspected that they are localized in ganglion cells. Gangliosides are composed of long-chain base, mostly 4-sphingenine (C18 sphingosine) or 4-eicosasphingenine (C20 sphingosine), hexoses, fatty acid and sialic acid. Sialic acid, i.e. an N-acylated or an O-acylated neuraminic acid (5-amino-3,5 dideoxy D-glycero-β-D-galacto-nonulosonic acid), is an aldol condensation product between pyruvic acid and N-acetylmannosamine.

Folch et al. (1951) proposed the term "strandin" for a high molecular weight lipid complex of ox brain which contained the same components as gangliosides but was said to be devoid of sialic acid. However in 1956, Folch et al. retracted their claim. In 1959, Folch-Pi and Lees called attention to the great similarities between strandin and the gangliosides, but also pointed out some differences,
for example, a great concentration of sialic acid in strandin. Rosenberg and Chargaff (1958) proposed the term "mucolipid" for the complex lipid polymers that contain sialic acid. Bogoch (1958) thought that strandin and mucolipid comprised polymeric forms of the monomeric gangliosides. Saifer et al. (1963) have isolated "Peptide-strandin" fractions which are relatively enriched with peptide. Kuhn and Muldner (1964 a) isolated a ganglioside-protein complex from dog brain. Ganglioside polymer was isolated when this complex was reacted with cold chloroform-methanol. With hot chloroform-methanol, or base, this polymer gave characteristic monomers. They believe that partial ester bonds are involved in the linkage of monomers. This ganglioside-protein complex was reported to have ATP-ase and acetylcholine esterase activity. Thus the original paper of Rosenberg and Chargaff (1958) can be recalled, which claimed evidence for the presence of ester units in mucolipid.

Wagner et al. (1961) showed that the brain ganglioside fraction is heterogeneous on TLC. Klenk and Gielin (1960), Trams and Lauter (1962), and Gammack (1963) have reported that gangliosides form micelles in aqueous solution. The sedimentation constants observed in organic solvents range from 1,000-2,000, which correspond to the monomer. It was thus recognized that gangliosides are low molecular weight compounds. Nine different brain gangliosides have been isolated and well characterized. In the four most abundant brain gangliosides, the carbohydrate sequence found is Gal-Gal/NHAc-Gal-Glc. There is one or more sialic acids residues attached to this carbohydrate chain. Structure of these gangliosides were elucidated
primarily by Kuhn and Wiegandt (1963) by use of acetolysis or ozonolysis to obtain intact carbohydrate moieties and subsequent Smith degradation which involves periodate oxidation followed by reduction with borohydride, hydrolysis and identification of products. Stereochemistry was solved by enzymatic studies and optical rotation.

Gangliosides are found in the brain, spleen, erythrocytes, leukocytes, serum, kidney, adrenal glands, placenta, milk, blood vessel walls, intestines, lungs, and the lens of the human eye.

The chemistry of gangliosides has been reviewed by several investigators (Svennerholm, 1964; Ledeen, 1966; Klenk, 1968; Wiegandt, 1968).

B. Nomenclature and Symbols

In 1961, Wagner et al. performed TLC of gangliosides on silica gel G plates. The plate was developed in chloroform-methanol-water (60:35:8) and spots were visualized with Blais' reagent. These authors found five spots and named them a-d in the ascending order. Thus it was found that the brain gangliosides are heterogeneous; this also started the nomenclature of gangliosides based on their chromatographic behaviour. Every investigator has introduced his own system. Table I shows nine brain gangliosides, some of the most common nomenclature used and abbreviations to be employed in this dissertation. Kuhn and Wiegandt (1963) originally assigned numeral subscripts according to their migration on TLC plates, but to the minor gangliosides, they have assigned carbohydrate abbreviations (Kuhn and Wiegandt, 1964). Svennerholm's (1963) nomenclature is based on composition, a letter subscript denoting sialic acid
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<td></td>
<td>GGGNSLC</td>
<td>Gal(1→3)GalNHex(1→4)Gal(1→4)Glc(1→1)Cer</td>
<td>G^GNT^2b</td>
<td>G^N^II_3</td>
<td>C^4</td>
<td>0^2</td>
</tr>
<tr>
<td>SialosylgalactosylN-acetylgalactosaminyl (dialosyl) lactosylceramide</td>
<td>GGNSILC</td>
<td>NANA(2→3)Gal(1→3)GalNHex(1→4)Gal(1→4)Glc(1→1)Cer</td>
<td>G^GNT^3a</td>
<td>G^N^III_4</td>
<td>C^3</td>
<td>0^1</td>
</tr>
</tbody>
</table>

*aAbbreviations: S=sialosyl; G=galactosyl; L=lactosyl; Gn=N-acetylgalactosaminyl; C=ceramide.
content and a numeral subscript for the subdivision within each group.
Abbreviations used in this dissertation are based on chemical structure.

C. Isolation and Purification

Gangliosides are extracted from tissue with other lipids by use of the Folch solvent, i.e., chloroform-methanol (2:1). Acetone powder gives less contaminants, but the yields are low. The relatively nonpolar gangliosides are lost by the acetone powder method. Fifty per cent methanol yields more protein. Svennerholm (1963 b) used chloroform-methanol (1:1) and 1:2 to get quantitative extraction of gangliosides. Kuhn and Wiegandt (1963) described three different solvent systems, aqueous phosphate buffer, 50% phenol, and chloroform-methanol, to extract gangliosides. When they used aqueous phosphate buffer and 50% aqueous phenol, GGnSLC was not extracted, Trams and Lauter (1962) used tetrahydrofuran-aqueous phosphate buffer for extraction and added diethyl ether to cause partitioning. By this method the yield of trisialoganglioside is decreased. Van Heyningen et al. (1963) used water for the isolation of gangliosides from frog brains.

The method most commonly used for preparation of mixed ganglioside fractions is that of Folch et al. (1957) which essentially involves extraction of total lipids with chloroform-methanol (2:1) and the addition of one-fifth volume of water which causes the gangliosides to partition into the upper aqueous-methanol phase. This preparation gives some contamination of other lipids, which is decreased by using salt solutions instead of water, but this forces
non-polar gangliosides to go into the lower phase. Suzuki (1965) has described quantitative extraction of brain gangliosides using a modified method of the Folch system. Brains are homogenized with nineteen volumes of chloroform-methanol (2:1). After filtration the residue is further homogenized with ten volumes of chloroform-methanol (1:2) with the addition of five percent water. Both filtrates are combined and chloroform-methanol concentration adjusted to a 2:1 ratio and 1/5 volume of 0.88% KCl added; gangliosides partition into the upper phase. The lower phase is washed once with the theoretical upper phase containing potassium chloride and once with the theoretical upper phase containing no salt. Upper phases are concentrated and dialysed against distilled water at 4°C and lyophilized. This method gives over 90 percent recovery of all the gangliosides. Spence and Wolfe (1967) reported that if brain tissue is depleted of monovalent cations by dialysis, chloroform-methanol (2:1) extracts only 28% of the total gangliosides. When the Na⁺ or K⁺ or both ions are added back to ion-deficient tissue, chloroform-methanol extracts all the gangliosides from the brain tissue.

Svennerholm's (1956 b) quantitative method is based on extraction of total lipids which are put on a cellulose column. Non-polar lipids are eluted first and gangliosides are eluted with chloroform-ethanol-water. Rouser et al. (1963) used a DEAE-cellulose column for quantitative isolation of gangliosides. Siakotos and Rouser (1965) also employed a Sephadex column for the isolation of gangliosides. Svennerholm (1968) described a method for the isolation of gangliosides from total lipid extracts using silica gel G column. The
method involves extraction of total lipids from the brain with chloroform-methanol-2N KOH in water (50:50:1). The lipid extract is dried and further purified by extraction with chloroform-methanol (1:1). This extract is chromatographed on a silica gel G column. All lipids except gangliosides are eluted with fifteen volumes of chloroform-methanol-water (65:25:4) and then the gangliosides are eluted with fifteen volumes of chloroform-methanol-water (60:35:8).

To isolate individual gangliosides silica gel has been used by Klenk and Gielen (1960, 1961), Dain et al. (1962), McCluer and Penick (1966) and Svennerholm (1963 a). Kuhn and Wiegandt (1963) obtained pure fractions by the use of both silica gel and cellulose powder columns. Preparative TLC is also used for the separation of gangliosides (Korey and Gonatas, 1963; Ledeen, 1966). Plates are developed in solvent systems of choice; the gangliosides are visualized with rhodamine or iodine vapors. The sections of silica gel containing one band are scraped off the plates, and the gangliosides are eluted with chloroform-methanol (1:1), chloroform-methanol-water (50:50:10). Since it is difficult to elute trisialogangliosides fractions, Korey and Gonatas (1963) used methanol-chloroform-water-pyridine (56:40:12:2) which is effective for most fractions.

D. Thin-Layer Chromatography (TLC)

TLC has been extensively used for identification of different gangliosides, separation of gangliosides, and to check the homogeneity. Different investigators have used different thin-layer chromatographic solvents for establishing notation systems, and it has resulted in some confusion when work from one laboratory is related to reports from
other laboratories. Relative mobilities of the gangliosides change in different solvent systems and no single solvent completely resolves all of the components of mixed ganglioside preparation.

Several solvent systems are used for TLC of gangliosides. All of these procedures employ silica gel coated plates and ascending development in closed tanks: Chloroform-methanol-water (60:35:8) Wagner et al. (1961), chloroform-methanol-2.5N NH$_4$OH (60:35:8) Wherrett and Cumings (1963), N-butanol-pyridine-water (6:2:1) Klenk and Gielen (1961), n-propanol-water (7:3) Kuhn et al. (1961), n-propanol-conc. NH$_4$OH (7:3) Tettamanti et al. (1964 a). Jatzkewitz (1961) also used two successive solvents, chloroform-methanol-water (14:6:1) followed by n-propanol-aqueous 12.5% ammonia (4:1). Korey and Gonatas (1963) used descending TLC. Ledeen (1966) employed double length (20 x 40 cms) silica gel plates with chloroform-methanol-2.5N ammonia (60:40:9) which give excellent separation of individual gangliosides. Penick, Meisler and McCluer (1966 a) reported that chloroform-methanol-2.5N NH$_4$OH (60:35:8) has the greatest resolving power.

The following sprays are used for identification of sialic acid and gangliosides on analytical TLC plates: orcinol (Klenk, 1942), resorcinol (Svennerholm, 1957 b), and p-dimethylaminobenzaldehyde (Wernér and Odin, 1952).

E. Components of Gangliosides

1. Sialic Acid

There are two sialic acids found in gangliosides, N-acetyl or N-glycolylneuraminic acid (NANA or NGNA). Brain gangliosides contain
one N-acetylneuraminic acid molecule or more. Tettamanti et al. (1964 a) have reported evidence for the presence of small amounts of N-glycolylneuraminic acid (NGNA) in beef brain gangliosides. Sialic acid residues are connected to a sugar moiety singly or as a dimer interconnected via 2\(\rightarrow\)8 bond.

Klenk and Gielen (1936 b) described an unusual ganglioside from normal brain which contains three galactose molecules and two sialic acid molecules; one of the sialic acid molecules moved ahead of NANA on paper chromatography, and this was believed to be an O,N-diacetylneuraminic acid. This sialic acid, when treated with 0.1 N HCl for ten minutes at 100°C, moved with NANA on paper chromatography. Wiegandt (1968) reported that when two molecules of sialic acid are connected by ketoside linkage (2\(\rightarrow\)8) an inner ester is formed. The lactones formed are unstable and can be cleaved by hydrolysis and can be detected by a titration reaction with hydroxamic acid and paper electrophoresis. Sialic acids are relatively strong acids with pKa values of 2-3 and therefore, gangliosides can be titrated.

In 1969, Yu and Ledeen assigned "alpha-ketosidic" bonding to sialic acid in gangliosides. The ketosidic bond is equatorial and the carboxyl group axial to a pyranose ring.

\[ \text{\textalpha-Ketoside of N-acetylneuraminic acid} \]
Of the alpha and beta ketosides of sialic acid, only the alpha anomers are attacked by neuraminidase. (Meindl and Tubby, 1965), (Kuhn and Lutz, 1966). Neuraminidase hydrolyzes all of the sialic acid from gangliosides except the sialic acid residue which is attached to the galactose molecule with N-acetylgalactosamine attached at the adjacent C-4 position, i.e. in ganglioside GnSLC and in the higher gangliosides. The sialic acid is not released due to the steric hindrance of the N-acetylgalactosamine. Kuhn and Wiegandt (1963) first showed that when the hexosamine of neuraminidase-resistant gangliosides is removed, the sialic acid will be released by neuraminidase. Kolodny et al. (1969) have recently reported neuraminidase from rat and human tissue which cleave sialic acid from Tay-Sach's ganglioside (GnSLC). This enzyme was demonstrated in the brain, kidney, and small intestine.

Gangliosides are detected by colorimetric determination of sialic acid with orcinol (Bial's reagent, Klenk, 1942), resorcinol (Svennerholm, 1957 b), p-dimethylaminobenzaldehyde (Ehrlich's reagent, Werner and Odin, 1952), thiobarbituric acid (Warren, 1959), diphenylamine (Kukai, 1962), or 3,5-diaminobenzoic acid (Hess and Rolde, 1964). Sialic acid can also be determined quantitatively by GLC as a silyl ether derivative (Craven and Gehrke, 1968).

2. Hexoses and Hexosamines

The following sugars have been found in gangliosides: glucose, galactose, N-acetylgalactosamine, and N-acetylglucosamine. In the brain gangliosides, only N-acetylgalactosamine is found.
The quantitative estimation of hexosamines can be carried out with the modification of the Elson and Morgan reaction (Svennerholm, 1956 a) or the indole method of Dische and Borenfreund (1950). Total hexoses can be quantitatively determined by phenol-sulfuric acid (Hodge and Hofreiter, 1962). Individual hexoses can be identified and quantitative determination done by GLC (Sweeley and Walker, 1964, Penick and McCluer, 1966 b). Hexosamines can be determined following acetylation according to the procedure C of Sweeley and Walker (1964) or by the GLC method described by Jones, Jones and Perry (1962).

3. Fatty Acids

Fatty acids are present in amide linkage with the sphingosine in the gangliosides. Gangliosides are hydrolyzed by methanolic-HCl to obtain fatty acid methyl esters which are identified by GLC (Penick and McCluer, 1966 b). Brain gangliosides contain mostly stearic acid and small amounts of arachidic acid (Sambasivarao and McCluer, 1964). Saifer et al. (1963) reported stearic, palmitic, and oleic acids and small amounts of arachidic acid in brain gangliosides. Rosenberg and Stern (1966) investigated fatty acids of gangliosides from rat brains of different ages. In the rat brain, stearic acid decreased from 79% to 62% in four months and stayed constant up to one year. Palmitic acid stayed constant up to four months and then increased to 23% in one year, whereas oleic acid increased from 10% in two weeks to 26% in four months and went back to 13% in one year. In the fetal human brain ganglioside, stearic acid content is high and undergoes a decrease with age.
4. Sphingosine

Sphingosines of gangliosides can be estimated after periodate oxidation as aldehydes by the method of Sweeley and Moscatelli (1959), as trimethylsilyl derivatives (Gaver and Sweeley, 1965), or as aldehydes after using the ozonolysis, the method of Evans and McCluer (1969). Sambasivarao and McCluer (1964) reported the sphingosine composition of human brain gangliosides. Brain gangliosides are unique in sphingosine content; they contain about equal amounts of 4-sphingenine and 4-eicosasphingenine and traces of sphinganine and eicosasphinganine. Rosenberg and Stern (1966) investigated long-chain base content of rat brain gangliosides and showed 4-sphingenine at birth and a negligible amount of 4-eicosasphingenine. During development, 4-sphingenine decreases and 4-eicosasphingenine increases. In about 6 weeks, equal amounts of 4-sphingenine and 4-eicosasphingenine are found. Gangliosides of fetal human brain are practically devoid of 4-eicosasphingenine, but it increased with the age.

F. Determination of Ganglioside Structure

To characterize a molecular species, it is necessary to determine the nature of the components, molar ratios, sequence of its components, linkage of substituents, and the stereochemistry. The structural determination of gangliosides involves the following steps: qualitative determination of hexoses, hexosamine, sialic acids, sphingosine, and fatty acids; their molar ratios must also be determined. Gangliosides can be treated with neuraminidase to yield asialoganglioside and sialic acid. If gangliosides are resistant to neuraminidase enzyme,
asialoganglioside can be obtained by mild acid hydrolysis and qualitative identification of sialic acid and asialoganglioside (neutral glycolipid) can be made. Partial hydrolysis of asialoganglioside gives simple glycosphingolipids and simple sugars. Kuhn and Wiegandt (1963, 1964) in their classic papers have described in detail the structural elucidation of gangliosides. Their method involves mild acid hydrolysis, partial acid hydrolysis, acetylosis which is followed by Smith degradation, permethylation and enzymatic hydrolysis to study the configuration of glycosidic bonds. Since sialic acids are labile to aqueous hydrolysis, acetylation of gangliosides is done which yields large numbers of acetylated oligosaccharides, many of which still contain sialic acid. Acetyl groups are removed and Smith degradation is applied to the compounds. This will elucidate the sugar sequence, substitution pattern and some aspects of stereochemistry (Kuhn and Wiegandt, 1963). The ozonolysis procedure of Wiegandt and Baschang (1965) appears to be particularly useful for obtaining the intact carbohydrate moieties of gangliosides. Hakomori (1966) has developed a similar technique for obtaining the carbohydrate moiety from glycosylceramides by employing osmium-catalyzed periodate oxidation followed by treatment with mild acid. The bonding position within the oligosaccharide is established by periodate oxidation or permethylation. In the case of periodate oxidation, after oxidation the aldehyde groups are reduced to alcohol groups by sodium borohydride, followed by acid hydrolysis and identification of the products formed. Permethylation is also very useful tool. Adams and Gray (1968) have described permethylation of micro-quantities of glycolipids. Permethylation is followed by acid hydrolysis and identification of sugars, after
silylation, by GLC. Permethylation of asialoganglioside gives position of sialic acid on carbohydrate molecule by comparing the permethylated products from intact gangliosides.

The problem of stereochemistry can be solved by enzymatic specificity to differentiate alpha and beta isomers. Optical rotatory dispersion and nuclear magnetic resonance tools for carbohydrate stereochemistry have not been applied to gangliosides.

G. Major and Minor Gangliosides of Brain

The main gangliosides of the brain are GGnSLC, SGGnSLC, GGnSSLC, and SGGnSSLC. There are several minor gangliosides normally present in the brain in smaller quantities. Tay-Sach's ganglioside GnSLC, contains one less galactose from the parent major ganglioside GGnSLC. This ganglioside accumulates in an infantile form of amaurotic idiocy of Tay-Sach's type disease and therefore is also called Tay-Sach's ganglioside. Tay-Sach's ganglioside with a second molecule of sialic acid (GnSSLC) is also found in small quantities in human and bovine brain gangliosides (Kuhn and Wiegandt, 1964 b; and Wiegandt and Baschang, 1965). Klenk and Naoi (1968) established the structure of GnSSLC; the second NANA is attached to the eight position of the first NANA residue. Sialosyllactosylceramide (SLC) has also been found in very small quantities in the human brain by Svennerholm (1963 a) and by several other investigators (Klenk and Gielen, 1963 a; Kuhn and Wiegandt, 1964; Penick, Meisler and McCluer, 1966 a). Kuhn and Wiegandt (1964) reported that disialosyllactosylceramide (SSLC) is a minor component of brain gangliosides and presented evidence that the second NANA residue is linked in the eight position of the first
Kuhn and Wiegandt (1964 b) and Wiegandt and Baschang (1965) isolated a minor component of brain gangliosides that yielded 3'-neuraminosylgalactose upon ozone degradation and treatment with alkali. This ganglioside is sialosylgalactosylceramide (SGC). SGC has also been isolated by Klenk and Georgias (1967 a) and Siddiqui and McCluer (1968). When it is treated with neuraminidase, NANA is released. On permethylation, followed by hydrolysis 2,4,6-tri-O-methylgalactose is obtained; this suggests that sialic acid is attached to carbon number three of galactose (Klenk and Georgias, 1967 a). This ganglioside has been designated Ggal by Kuhn and Wiegandt (1964 a), A01 by Klenk and Georgias (1967 a) and previously named HG-B in our laboratory. Klenk and Gielen (1963 a) found a ganglioside in the human brain which contains stearic acid, sphingosine, galactose, and sialic acid in approximate molar ratios of 1:1:3:2. This ganglioside was completely susceptible to neuraminidase. A sialic acid free glycolipid obtained by neuraminidase treatment was subject to partial hydrolysis and the disaccharide identified as galactosyl (1→3) galactose and galactosylceramide were identified as products. Permethyllation studies supported the structure given below, but NANA's positions have not been assigned

\[(\text{NANA})_2\text{Gal(1→3)Gal(1→3)Gal(1→1)Cer}\ .\]

Several other brain gangliosides not listed in Table I, and not thoroughly characterized have been reported. A ganglioside which migrat below the major trisialoganglioside (SGGnSSLC) was reported by Kuhn and Wiegandt (1964 b). Because the material upon treatment with neuraminidase gave rise to a component migrating as a trisialosylganglioside, it was
(G\textsubscript{y}). Penick and McCluer (1965) isolated a material with similar chromatographic properties but analytical data indicated it to be a trisialosylganglioside. Klenk et al. (1966, 1967b) have also reported a second trisialosylganglioside (C\textsubscript{2}) which lost one galactose residue when subjected to periodate oxidation like trisialoganglioside examined by Johnson and McCluer (1963). A tetrasialoganglioside (C\textsubscript{4}) was also reported which may be the same as G\textsubscript{y}.

H. Gangliosides and Diseases

Sach (1896) coined the term "amaurotic idiocy" (now known as Tay-Sach's disease) for a disease which is characterized by blindness (amaurosis) and extreme mental deficiency (idiocy). Vogt (1909) suggested that amaurotic idiocy occurs in several forms all characterized by neuronal lipid accumulation, distinguishable simply by the age of onset. Accordingly, contemporary medical writers recognize a congenital, infantile, late infantile, juvenile, and adult form of amaurotic idiocy. Klenk first showed that Tay-Sach's disease is characterized by alterations in brain gangliosides. However, only the infantile and late infantile involve accumulation of gangliosides.

1. Gangliosidosis*

*These above diseases have been reported by several other workers who have given them different nomenclatures. Tay-Sach's (Saifer, 1964; Cumings, 1960), GM\textsubscript{2} gangliosidosis (Sandhoff et al., 1968) GM\textsubscript{1} Gangliosidosis Type I (Derry et al., 1968; Okada and O'Brien, 1968), Late infantile systemic lipidosis (Cumings, 1960; Ledeen, Salsman, and Gonatas, 1965b) Generalized gangliosidosis (O'Brien et al., 1965a; Suzuki et al., 1969), Tay-Sach's disease with visceral involvement (Norman et al., 1959), Familial neurovisceral lipidosis (Landing et al., 1964), A variant of Hurler's syndrome (Craig et al., 1959), Pseudo-Hurler's disease (Landing et al., 1964) Biochemically special form of infantile amaurotic idiocy (Jatzkewitz, Pilz, and Sandhoff, 196 GM\textsubscript{1} gangliosidosis Type II (Derry et al., 1968).
a. GM$_2$(GnSLC) gangliosidosis (Tay-Sach's disease).

b. GM$_2$(GnSLC) gangliosidosis arises from hexoaminidase deficiency (Sandhoff et al., 1968).

c. GM$_1$(G GnSLC) gangliosidosis Type I (Derry et al., 1968) were reported to be $\beta$-galactosidase deficiency (Okada and O'Brien, 1968).

d. GM$_1$(GGnSLC) gangliosidosis Type II (Derry et al., 1968).

2. Gaucher's Disease

Glucosylceramide accumulates primary in reticuloendothelial cells (Fredrickson, 1966). Brady, Kanfer, and Shapiro (1965) have demonstrated a pronounced deficiency of the activity of the glucocerebrosides cleaving enzyme in spleens and livers obtained from patients with Gaucher's disease. Phillippart and Menkes (1964) reported a ten-fold increase in sialosyllactosylceramide (SLC) in the spleen of Gaucher's disease patients.

3. Niemann-Pick's Disease

Sphingomyelin is accumulated in this disease. Diminished activity of sphingomyelin-cleaving enzyme is exhibited by patients. Booth, Goodwin, and Cumings (1966), examined by TLC, extracts of cortex and white matter from cases of gargoylism and Niemann-Pick's disease and noted two abnormal fast moving bands of gangliosides. The fastest of these was reported to contain no glucose and to have NANA, sphingosine, and galactose in a molar ratio of approximately 1:1:2. Presumably, this ganglioside is sialosyl digalactosylceramide. However other workers (Phillippart et al., 1969; Kishmoshita et al., 1969) reported the two abnormal fast moving gangliosides seen in Niemann-Pick's disease to be SLC and GnSLC.
4. Subacute Sclerosing Leukoencephalitis

While studying a case of subacute sclerosing leukoencephalitis, Ledeen, Salsman, and Cabrera (1966) have noted an increased level of gangliosides having TLC characteristics of disialosyllactosylceramide (SSLC). The same authors (1968 b) have described isolation and fatty acid composition of nine fractions of gangliosides from subacute sclerosing leukoencephalitis. They have found increased amounts of sialosyllactosylceramide (SLC), disialosyllactosylceramide (SSLC), N-acetylgalactosaminylsialosyllactosylceramide (GnSLC), and perhaps N-acetylgalactosaminyldisialosyllactosylceramide (GnSSLC).

Kishimoto et al. (1967) reported that in the plaques of patients with multiple sclerosis, ganglioside concentration is below normal.

I. Biosynthesis and Metabolism of Gangliosides

Sphingosine is synthesized by the microsomal particles (Brady and Koval, 1958) in mammalian tissue.

\[
\text{Palmitoyl coenzyme A} + \text{serine} + \text{Mn}^{++}, \text{pyridoxal phosphate} \rightarrow 3-\text{Ketodihydro-sphingosine} + \text{CO}_2
\]

\[
3-\text{Ketodihydrosphingosine} + \text{TPHN} + \text{H}^+ \rightarrow \text{Dihydrosphingosine} + \text{TPN}
\]

\[
\text{Dihydrosphingosine} \rightarrow \text{Sphingosine} + 2\text{H}
\]

Brady in 1962 and Cleland and Kennedy in 1960 reported the biosynthesis of galactocerebroside catalyzed by enzyme localized in microsomal preparation of brain tissue from young animals.

\[
\text{Sphingosine} + \text{uridine diphosphate galactose (UDP-galactose)} \rightarrow \text{galactosylsphingosine (psychosine)} + \text{uridine diphosphate (UDP)}
\]
Galactosylsphingosine + fatty acylcoenzyme A $\rightarrow$ galactocerebroside + coenzyme A (Co A).

Basu et al. in 1969 reported the synthesis galactocerebroside with hydroxy fatty acid-ceramide and UDP-galactose in homegenates of 19-20 day old chick brains.

$$\text{N-acylsphingosine (ceramide) + UDP-galactose} \quad \xrightarrow{^{14}\text{C}} \quad \text{galactocerebroside + UDP.}$$

This reaction works only with ceramide containing hydroxy fatty acids; when ceramide contains non-hydroxy fatty acid, galactocerebroside was not synthesized. Morell and Radin in 1969 synthesized galactocerebroside using crude microsomal fraction from brains of young mice. Only ceramide which contains hydroxy fatty acids accept galactose from uridine diphosphate galactose to form galactocerebroside. Ceramide with non-hydroxy fatty acid does not form cerebroside.

Basu in 1968 in a preliminary report noted that non-hydroxy fatty acid ceramide is precursor to glucosylerceramide, an intermediate in ganglioside biosynthesis.

$$\text{nFA ceramide + UDP glu} \quad \xrightarrow{\text{glucosylerceramide + UDP.}}$$

Maker and Hauser (1967) injected labelled glucose in the brain slices of rats from 2-26 days of age; they observed the incorporation of label in ganglioside, cerebroside, and total lipids. Hauser and Hildebrand (1969) reported that the kidney of mice contain enzyme which converts glucosylerceramide and UDP-gal to lactosylerceramide and triglycosylerceramide.

$$\text{glucosylerceramide + UDP-gal} \quad \xrightarrow{\text{lactosylerceramide + UDP}}$$
lactosylceramide + UDP-gal $\rightarrow$ triglycosylceramide + UDP

When galactosylsphingosine or galactocerebroside were used, galactose was not transferred. Stepwise biosynthesis of gangliosides. See Figure 1.

J. Subcellular Localization and Physiological Function

Gangliosides are localized in neuronal membranes in the cell body, dendrites, axons, and synaptic junctions, i.e., in exactly those elements which are involved in the transmission of nerve impulses. When gray matter is homogenized with great care, it is possible to retain some of the nerve endings (synasptosomes) which become detached from the nerves. These nerve endings can be purified by differential and density-gradient centrifugation (De Robertis et al., 1962). They consist of an outer membrane and inner small mitochondria and vesicles characteristic of synapses. The synaptic vesicles contain the chemical substances responsible for the transmission of impulses along nerves. They occur in great numbers on presynaptic membranes. In hypotonic medium, (De Robertis et al., 1963) the synaptosomes burst and their components are released. The fragments can be separated by centrifugation. Burton et al. (1964) have reported that gangliosides and bound acetylcholine are higher in gray matter than in white. They have found parallel distribution of both these in crude microsomal fractions and pinched off nerve ending fractions. Nerve ending on osmotic shock gave synaptic vesicle fractions which also contain gangliosides and acetylcholine. They have suggested that gangliosides play a role in the transportation of acetylcholine from synaptic vesicles through the synaptic membranes. However, Wiegandt (1967) reported that the ganglio-
Figure 1  Biosynthesis of the Brain Gangliosides SGGnSLC and SGGnSSLc.
sides are concentrated in subcellular fractions containing pinched off nerve endings. Synaptic vesicles which can be obtained by opening the nerve ending under the influence of a hypoosmotic condition do not contain gangliosides. Acetylcholine estimated in this fraction is also very low. The gangliosides in microsomes showed a bimodal distribution, the highest content being in the light fraction. Wiegandt (1967) also did not find gangliosides in mitochondria.

A neutral detergent, Triton X100 (isooctylphenoxyethoxyethanol) dissolves gangliosides out of the membranes only to a small extent. Total microsomes fractions from gray matter or the osmolyzed nerve ending fraction freed from mitochondria and synaptic vesicles is suspended in weakly alkaline Triton X100; the various types of membranes are attacked with different intensities. Analysis of the membrane components that remain undissolved shows that they are the ones with a high ganglioside content (Wiegandt, 1968). Rambourg et al. (1967) have shown in their electron micrograph of nerve endings, acidic carbohydrates on the outside of axonal membranes and in particular directly at the synaptic gap. This localization of gangliosides suggests that they carry electric charges and are involved in the impulse conduction of neurons, and many observations on the physiological action of these lipids support this hypothesis.

Van Heyningen (1959, 1961) showed that gangliosides combine with tetanus toxin eg. SGnSLC, fixes about 0.6 moles of toxin (M.W. = 70,000 Such combinations presumably contribute to the transportation or action of toxin in producing tetanus. Inhibitory pathways are blocked at the synaptic junction in the spinal cord (Eccles, 1964). Mellanby and
Van Heyningen et al. (1965) examined subcellular fractions for their ability to combine with tetanus toxin; fractions containing nerve terminals showed marked activity. Lowden and Wolfe (1964) showed the distribution of \( \gamma \)-aminobutyric acid; the presumed transmission substance of the inhibitory synapses resembles that of gangliosides. Gangliosides produce specific antibodies when injected into the blood. The serologic specificity of gangliosides is localized in the carbohydrate residue; sialic acid also plays an important role. Dodd, Bigley and Geyer (1960) showed that crude gangliosides containing NANA and NANA alone from several sources inhibit specific agglutination by Rho (D) antibody. Dodd, Bigley, Johnson and McCluer (1964) showed that gangliosides in the Rho (D) antigen-antibody system are found to be carriers of blood group properties. In serologic tests, lacto-N-neotetraose from gangliosides GLNt\(^1\) NGNA [Gal (\( \beta 1,4 \)) GlcNHAc (\( \beta 1,3 \)) Gal (\( \beta 1,4 \)) Glc] show specificity as regards to the nature of the pneumococcus type XIV polysaccharide. Varon and McIlwain (1966) showed that gangliosides lower the concentration of sodium and potassium in the non-inulin space by reducing the sodium space, increasing the potassium space by depressing the concentration of potassium in the potassium space. Meisler and McCluer (1966) showed that gangliosides can reverse the heat stabilization of DNA by histones.

Five-hydroxytryptamine (serotonin) is a neurotransmitter in the central nervous system. Wooley and Gommi (1965) showed that the serotonin-receptor must contain sialic acid. When stomach muscle is treated with neuraminidase, the serotonin response is lost but can be restored by adding gangliosides particularly SSIC. Gielen (1966)
suggested that an equimolecular complex is formed between SLC and serotonin. When Ca\(^{++}\) ions were added to this mixture, no complex was formed between serotonin and gangliosides, since gangliosides fix Ca\(^{++}\) stronger than serotonin. In summary 1) gangliosides may be involved in the storage and release of acetylcholine at nerve endings, 2) gangliosides have been implicated as a component of the receptor site for neurohumoral agents such as serotonin, 3) the rapid metabolism of gangliosides just preceding myelination and the diminuation of this activity as myelin formation ceases suggesting that the turn over of gangliosides is somehow involved in myelination 4) immunochemical reactions involving gangliosides have been implicated in certain demyelination conditions since it has been observed that sera from some patients with multiple sclerosis and amyotrophic lateral sclerosis contain low but definitely detectable antiganglioside antibody activity (Yokoyama et al., 1962), 5) finally excessive quantities of gangliosides accumulate in neural tissues of patients with Tay-Sach's disease and GM\(_1\)-gangliosidosis (GGnSLC).

K. Gangliosides of Peripheral Organs

Gangliosides were first investigated by Klenk (1935, 1942) in the central nervous system. There has been a small amount of research done in the peripheral nervous system (PNS). Folch et al. (1958) reported a very low level of gangliosides in sciatic nerve (PNS). In 1964 Lowden and Wolfe reported that sciatic nerves do not contain any ganglioside; they also reported that sympathetic chain do not contain any gangliosides. In 1967, O'Brien et al. reported that intradural spinal roots does not contain gangliosides. Since Folch's extraction
method most of the relatively non-polar gangliosides go in the lower phase, the above should be reinvestigated.

In 1951, Yamakawa and Suzuki first isolated a ganglioside-like lipid from equine red blood cells stroma, which they termed hematosides. Hematosides consist of lignoceric acid, sphingosine, two moles of hexoses (galactose and glucose) and N-acetylenuraminic acid (Yakakawa, 1956). Klenk and Heuer (1960), Klenk and Padberg (1962), and Handa and Yamakawa (1964) established that the sialic acid was linked to the 3-position of a lactose residue as follows:

\[
\text{NGNA (2} \rightarrow 3\text{) Gal (1} \rightarrow 4\text{) Glc (1} \rightarrow 1\text{) Cer.}
\]

Handa and Yamakawa (1964) have reported that equine hematoside contain NGNA attached to the third position of galactose molecule of ceramide lactoside, where as both N-acetyl (73%) and N-glycolyl (27%) neuraminic acids were attached to ceramide lactoside in dog hematoside. Sialic acid moieties could easily be split from both hematosides by mild acid hydrolysis or neuraminidase. Dog hematoside contains large amounts of stearic and nervonic acid than equine hematoside.

Svennerholm, (1963 b, 1965) isolated sialosyllactosylceramide (SLC) from human spleen, liver and placenta. In human placenta, it was found to be the most abundant glycolipid and contained only NANA (1965).

Handa and Handa (1965) isolated the main glycolipid from cat erythrocytes and was identified as disialosyllactosylceramide (SSLC), which contain only NGNA. The structure of this glycolipid is proposed to be \(\text{N-glycolylneuraminosyl (2} \rightarrow 8\text{) N-glycolylneuraminosyl (2} \rightarrow 3\text{) galactosyl (1} \rightarrow 4\text{) glucosylceramide. Both sialic acids were released by}\)
Neuraminidase or mild acid hydrolysis. The constituent fatty acids were mainly lignoceric and nervonic acid. Svennerholm (1965) has reported that a similar compound is also a component of placentai gangliosides. Wiegandt and Baschang (1965) reported the presence of sialosyllectosylceramide (SLC) in bovine milk and erythrocytes stroma which contains NGNA. A ganglioside which contains glucosamine was isolated from bovine erythrocytes and spleen by Kuhn and Wiegandt (1964 b). The oligosaccharide obtained by ozonolysis was treated with mild acid, and sialic acid free residue was shown to be identical with lacto-N-neotetraose Gal (β 1→4) GlcNHAc (β 1→3) Gal (β 1→4) Glc. The position of the attachment of sialic acid (identified as NGNA) was not determined, but the intact ganglioside was susceptible to neuraminidase hydrolysis. This compound has also been found in human milk by the same authors.

Vance et al. (1966) recently have found a ganglioside in intestinal tissue and lens of the human eye that contains sphingosine, fatty acids, sialic acid, glucose, and galactose in the ratio of 1:1:1:1:2.

Feldman, Feldman and Rouser (1966) have also isolated ganglioside from the lens of the human eye. They have reported two types of monosialogangliosides; the major ganglioside contains long-chain base, fatty acids, glucose, galactose, neuraminic acid in the ratio of 1:1:1:2:1. The minor ganglioside has ratio of 1:1:1:1:1. Dihydrosphingosine was the major base, and major fatty acids were palmitate and nervonate. Hakomori and Murakami (1968) have reported on the glycolipids of hamster kidney fibroblasts and derived malignant
transformed cell lines. These tissue culture cells contained SLC as the major ganglioside and smaller amounts of more complex gangliosides which exhibited interesting immunological properties.

Ledeen, Salsman, and Cabrera (1968 a) have investigated the gangliosides of bovine adrenal medulla, about 90% of which consist of two sialosylgalactosylglucosylceramides which contain NANA or NGNA. The remainder consist of at least four more complex gangliosides.
CHAPTER III

EXPERIMENTAL PROCEDURES

A. Instrumentation

The instrumentation used for the GLC assays were a) 5000 series equipment manufactured by Barber-Coleman, Rockford, Illinois. The detection system was the hydrogen flame detector sold by the same company. The recorder was equipped with disc integrator for measuring the areas under the chromatographic peaks, b) Packard Gas chromatographs series 7600-7800, with hydrogen flame detector. The recorder was an Electronik 17, Howell, Honeywell, Philadelphia, Pa.

B. Material

All solvents used were A.C.S. reagent grade; those used in analytical procedures were redistilled. Ratios of solvent mixtures are on a volume basis.

C. General Methodology

1. Thin-Layer Chromatography (TLC)

Thin-layer plates were prepared with silica Gel G or HR (E. Merck A.G., Darmstadt, West Germany). For analytical TLC plates, 250 microns of silica gel was spread, dried in air for 10-20 minutes. The plates were activated at 110 degrees, for 30 minutes when silica gel G plates were used and 2-3 hours when silica gel HR were used, cooled to room temperature and used immediately.
For preparative TLC plates, 2mm thick plates were spread with silica gel G HR, dried in air over night and finally activated at 110 degrees for 30 minutes.

For the preparation of borate plates, one percent borate solution was used instead of water and treated same as the above plates.

The solvent systems referred to in this dissertation have been numbered as follows: I, chloroform-methanol-2.5 N NH₄OH (60:35:8); II, chloroform-methanol-water (60:35:8); III, n-propanol-water (7:3); IV, n-propanol-conc. NH₄OH (7:3); V, chloroform-methanol-water (65:25:4); VI, chloroform-methanol-water (65:30:5); VII n-propanol-1N NH₄OH-water (6:2:1).

Galactosyl and glucosylceramides, and lactosylceramide were distinguished on borate-impregnated plates developed with chloroform-methanol-water-15.0N NH₄OH (280:70:6:1) as described by Kean (1966).

N-acetylneuraminic acid and N-glycolylneuraminic acid were distinguished on silica gel G plates developed with n-propanol 1N NH₄OH-water (6:2:1) (Granzer, 1962).

The developing reagent used routinely for gangliosides or sialic acid was the resorcinol Cu⁺⁺ reagent prepared according to Svennerholm (1957 a). After the plates were sprayed, a clean glass plate was laid over the absorbant layer, held in place by clamps, and the color develop by heating at 120-130°C for twenty minutes.

For visualizing non-ganglioside compounds, the plates were sprayed with forty percent sulfuric acid and heated at 130°C for several hours.

2. Gas-Liquid Chromatography (GLC) of Fatty Acids and Carbohydrates

Samples of SGC were treated in 0.5N anhydrous methanol-HCl as
described for the analysis of glucose and galactose (Penick and McCluer, 1966 b). The heptane extracts were concentrated and used for GLC or TLC analysis of the fatty acid methyl esters. The normal and hydroxy esters were separated by preparative TLC with diethyl ether-heptane (15:85) (Vioque and Holman, 1962). After development of the plates, esters were made visible with iodine vapor. When all color had disappeared, appropriate areas of the silica gel were scraped off and fatty acid esters were eluted with diethyl ether.

Normal fatty acid esters were identified by GLC at 180°C on a 6 ft. x 1/8 inch column packed with 3% SE-52 (methylpolysiloxo gum, General Electric) on Anakrom ABS, 110-120 mesh, (Analabs, Inc., Hamden, Conn.) with a helium flow rate of 30ml per minute. The identity of the esters was confirmed by analysis before and after hydrogenation, on 15% ethylene glycol succinate polyester (EGS). Standard fatty acids were obtained from Applied Science Laboratories, Inc., State College, Pa.

Hydroxy fatty acids were identified as their trimethylsilyl ether (TMSi) derivatives, (Wood, Raju, and Reiser, 1965). The derivatives were prepared with Sweeley's reagents (Sweeley, Bentley, Makita, and Wells, 1963) and analyzed by GLC with SE-52 column referred to above, operated at 202°C with a helium flow rate of 25 ml per minute. Methyl-α-hydroxytetrasanoate and methyl-α-hydroxydocosanoate (Applied Science Laboratories) as well as the hydroxy fatty acid esters prepared from the "beef brain cerebrosides" served as standards. Additional confirmation of the identity of TMSi derivatives of the hydroxy fatty acid methyl esters was provided by their mass spectra.
The total fatty acid methyl ester fraction was treated with the TMSi reagents and analyzed on the 3% SE-52 column with a temperature program of 2°C per minute from 135 to 202°C and at a helium flow rate of 25 ml per minute. Mass spectra of the components of this total methyl ester fraction were obtained by Dr. Charles C. Sweeley (Michigan State University) with an LKB 9000 instrument fitted with a 6 ft. x 1/8 inch column packed with 3% OV-1 (polar silicone phase) (Supelco, Inc., Bellefonte, Pa.). These data provided confirmatory evidence of the identity of the fatty acids and led to the identification of di(2-ethylhexyl) phthalate as a contaminant in this fraction. A sample of di(2-ethylhexyl) phthalate was obtained from Dr. P. P. Nair.

The methanolic phase from the above hydrolysate was used for GLC of carbohydrates. This was dried with N₂ at 37°C and trimethylsilyl ether was prepared as described by Penick and McCluer (1966 b) and analyzed by GLC at 180°C on a 8 ft. x 1/8 inch column packed with 3% SE-52 on Anakrom ABS 110-120 mesh, with a helium flow rate of 30 ml per minute. Mannitol was used as an internal standard for quantitative carbohydrate determination. Other standards used were GGnSLC and cerebroside.

Molar ratios and percent content of glucose, galactose, and total fatty acids were determined by the methanolysis-silylation GLC procedure (Sweeley and Walker, 1964) as previously described (Penick and McCluer, 1966 b).

The following micromodification of the procedure has been used more recently. Approximately 0.05 μM of adrenal ganglioside, 5.0 μg of mannitol, and 5.0 μg C₂₁-methyl heneicosanoate are placed in a small
conical glass vial, dried and dissolved in 0.3 ml of 0.5N anhydrous methanol-HCl. The tube is flushed with N₂, sealed and heated at 78°C for 24 hours. The contents are diluted with an equal volume of methanol and extracted three times with n-heptane to remove fatty acid esters. The methanol phase is passed over Dowex-50 (H⁺) if hexosamine is present. The solution is dried under a stream of N₂ in a small conical vial and silylated with 20 μl of HMDS:TMCS:pyridine (5:1:10) at 60°C for 1 hour. (These silylating conditions are necessary for complete solubilization and reaction of mannitol.) The reaction mixture is evaporated to dryness, and the products are dissolved in 20 μl of CH₂Cl₂ or freshly distilled CS₂. It is usually necessary to evaporate the sample to dryness 3 times to remove residual pyridine. Trimethylsilyl ethers of sugars are examined directly by GLC on 3% OV-101 column (8 ft, x 1/8 in) coated on Anakrom-ABS, 110-120 mesh (Analab, North Haven, Conn.), at 175°C column temperature, N₂ flow rate 20 ml/min. Heptane extract was used for fatty acid analysis. The conditions for GLC of fatty acids were the same as described for carbohydrates except a program temperature run of 2° per minute from 175°C to 210°C with a nitrogen flow rate of 20 ml per minute was employed. A standard fatty acid mixture was used for the identification of fatty acids. Fatty acids were quantitated with C₂₁ fatty acids as the internal standard. Peak areas were calculated by multiplication of height by width at half-height. Quantitative results with fatty acid standard RM-3 (Supelco) agreed with the stated composition data with a relative error less than one percent for major components (>10% of the total mixture) and less than 0.5% for minor components (<10% of the total mixture).
3. Spectrophotometric Assays

(a) Sialic Acid

Ganglioside sialic acid was determined by the resorcinol method of Svennerholm (1957 b) as modified by Miettinen and Takki-Luukkainen (1959). The NANA content was calculated according to the method of McCluer et al. (1962). A micromodification of the resorcinol method, employing 1/5 volume of all reagents, was used for quantitative determination of sialic acid in adrenal gangliosides. Ganglioside containing 2-4 μg of sialic acid was used for each determination. NANA and NGNA were used as standards.

(b) Hexosamine

SGC was subjected to hydrolysis, and the hexosamine was determined in the hydrolysate according to method B of Svennerholm (1956 a). N-acetylgalactosamine was used as a standard.

(c) Hexoses

Total hexoses were determined by the phenol-sulfuric acid assay (Hodge and Hofreiter, 1962) of the ganglioside hydrolysate. SGC was hydrolysed according to the condition described by Johnson (1963). Galactose was used as a standard.

(d) Long-Chain Base (LCB)

Long-chain bases were determined according to Lauter and Trams (1962).

D. Procedures for Sialosylgalactosylceramide

1. Preparation of Mixed Gangliosides of Human Brain
Normal human brains were obtained at autopsies and stored in frozen state. Thawed or fresh brains were homogenized in two volumes of acetone and filtered through cheese cloth and the residue was re-extracted with two volumes of acetone. The acetone powders were then extracted twice with two volumes of anhydrous diethyl ether, and the residue was dried under a stream of nitrogen. This residue was exhaustively extracted with chloroform-methanol (1:2) in a Soxhlet apparatus and finally extracted with absolute methanol. The combined extracts were dried and the lipids subjected to a three-tube double-withdrawal countercurrent-distribution using chloroform-methanol-0.9% aqueous NaCl (8:4:3). The insoluble interphase material that forms during the distribution was collected and extracted with chloroform-methanol (2:1). This extract was examined by TLC and found to contain a significant quantity of the minor gangliosides as well as some non-ganglioside lipids. The extract of the insoluble interphase material was added to all mixed human brain ganglioside preparations used for the isolation of SGC.

2. Isolation of SGC

4.75g of mixed human brain gangliosides was applied to an Anasil S column (900 g, 5.2 x 102 cm) which had been previously equilibrated with chloroform-methanol-water (65:30:5). Elution was begun with the same solvent system and 57-ml fractions were collected. Fractions were examined for gangliosides by TLC with solvent system I. Fractions 35-63, which contained SGC (HG-B) and other minor gangliosides, were pooled and evaporated to dryness to yield 325 mg of material. This mixture was applied to an Anasil S column (80 g, 2 x 61 cm) and eluted
with chloroform-methanol-water (65:30:5); 8.5-ml fractions were collected. Although there was overlapping of the different gangliosides, and no attempt was made to recover all of the SGC, fractions 30-44 showed only a single resorcinol-positive spot in solvent system I. These fractions were pooled, concentrated, and examined by TLC in several solvent systems. Solvent systems III and V revealed the presence of a nonganglioside organic contaminant. The material was further purified on an Anasil S column (80 g, 2 x 60 cm) developed with chloroform-methanol-water (65:25:4). The fractions containing SGC were pooled and evaporated to dryness, and the material was rechromatographed with chloroform-methanol-water (65:25:4) as before. SGC fractions that were free of the nonganglioside contaminant were pooled, evaporated to dryness, and dissolved in 70% methanol. No attempt was made to obtain a dry weight of the sample, but it was calculated from the total sialic acid present that 6 mg of SGC (calculated average mol. wt. 1089) was obtained.

3. GLC of Long-Chain Bases (LCB)

To characterize the sphingosine, SGC was hydrolyzed in 1N aqueous methanol-HCl at 75°C for 22 hours (Gaver and Sweeley, 1965). The sphingosine was isolated and subjected to periodate oxidation according to the procedure of Sweeley and Moscatelli (1959). The resulting aldehydes after purification on silicic acid were analyzed by GLC on a 6 ft x 1/8 inch column packed with 15% EGS on Anakrom ABS 110-120 mesh (Analabs). Sphingosine prepared from mixed gangliosides and "Beef brain cerebroside" served as standards.
E. Procedures for Adrenal Gland Gangliosides

1. Isolation of Crude Ganglioside Preparations

Fresh beef adrenal glands obtained from the slaughter house were carefully dissected into cortex and medulla. The tissue was lyophilized and then reconstituted with water to the original wet weights and extracted essentially according to the procedure of Suzuki (1965) as shown in Figure 2. The suspensions were homogenized with 19 volumes of C:M (2:1), filtered and re-extracted with 10 volumes of C:M (1:2) containing 5% water. The extracts were combined and chloroform added to make a final C:M ratio of 2:1 and 0.2 volume of 0.88% KCl added. After separation of the phases, the lower phase was extracted once with "Folch pure upper phase" with salt and once with "Folch pure upper phase" without salt. The combined upper phases were concentrated at 40°C in a rotary evaporator, dialyzed, passed over Dowex 50 x 2H⁺ and lyophilized to yield dark brown preparation.

2. Methods of Structural Analysis

(a) Permethylation Studies

Permethylation procedure of Adams and Gray (1968) for the analysis of gangliosides.

The procedure is applicable to 0.2 to 1.0 mg of sample and involves methylation with methyl iodide in dimethylformamide in the presence of finely ground barium oxide and barium hydroxide. In some instances, the methylated products were purified by preparative TLC (Silica gel G developed with Benzene:MeOH (85:15). The methylated product is subjected to methanolysis in 0.2 ml of 0.5N anhydrous MeOH-HCl for 4 hours at 90°C. The solution is evaporated to dryness, residu
Figure 2. Extraction of adrenal cortex or medulla gangliosides. F.P.U.P. refers to "Folch pure upper phase" (Folch, Lees and Sloane-Stanley; 1957).
dissolved in 0.5 ml of hexane and placed on a small "Unicil" column. Liberated lipids are eluted with 10 ml of petroleum ether-diethyl ether (9:1) and permethylated sugars are eluted with 10 ml of absolute methanol. (In some cases, it was necessary to rechromatograph the samples on Unicil in order to remove all traces of interfering lipids). The methyl ethers are examined directly by GLC on 3% ECNSS-M column (6 ft. x 1/4 in. glass coil) at 125°C and are also silylated with HMDS:TMCS:pyridine (5:1:10) and analyzed on the 3% ECNSS-M column at 110°C and on a 15% EGS column (6 ft. x 1/4 in.) at 150°C. Conditions for permethylation were checked and identification of methyl ethers was accomplished by methylation of methylglucoside, methylgalactoside, glucosylceramide, lactosylceramide, lactose, melibiose, and 3'neuraminosyllactose. Analysis of standard gangliosides, sialosyl (2→3) lactosylceramide (SLC), N-acetylglactosaminyl (1→4) (sialosyl 2→3)) lactosylceramide (GnSLC), galactosyl (1→3) N-acetylglactosaminosyl (1→4) (sialosyl 2→3)) lactosylceramide (GGnSLC), and the asialo compound galactosyl (1→3) N-acetylglactosaminyl (1→4) lactosylceramide (GGnLC) have allowed us to identify the 2,6 dimethylgalactoside and 2,3,6 trimethylgalactosides. The hexosamine derivatives formed under these methylation conditions do not interfere and are eluted from the ECNSS-M column as multiple peaks at 170°C.

(b) Determination of Long-Chain Bases by Ozonolysis

The microozonolysis procedure was used to examine the sphingosine moiety of the ganglioside. An apparatus similar to that described by Beroza and Bierl (1967) is used. Ozone is passed through 0.4 ml of ethanol containing 0.05 to 0.2 mg of gangliosides for five minutes at
-70°C. The reaction mixture is allowed to warm to room temperature, mixed, flushed with N₂ and the residue dissolved in 0.4 ml of 97% MeOH. The aldehydes are extracted with 0.6 ml of hexane and the hexane is washed once with water. The aldehydes are identified on a 15% EGS column (6 ft. x 1/4 in.) at 150°C with a carrier gas flow of 80 ml per minute.
CHAPTER IV

RESULTS

A. Sialosylgalactosylceramide (SGC)

1. Chromatographic Purity

SGC (HG-B) was not observed on TLC plates when mixed human brain gangliosides were analyzed but was easily seen in mixtures of minor gangliosides eluted from Anasil S columns before GGnSLC. The appearance of mixed human brain gangliosides and mixed minor gangliosides when analyzed in solvent system I is shown in Figure 3. A thin-layer chromatogram of purified SGC and other minor gangliosides in solvent I is shown in plate B of Figure 3. The chromatographic mobilities of SGC relative to GGnSLC (R_{GGnSLC}) in solvent systems II, III, IV, V were 1.42, 1.13, 2.32, and 4.34, respectively. Only one spot was detected in all solvent systems with the purified SGC, whether the plates were sprayed with resorcinol reagent or 50% H_2SO_4.

2. Composition

Analytical data, presented in Table II, provided further evidence of purity and showed that the material contained equimolar ratios of sphingosine, galactose, and sialic acid.

3. Neuraminidase Treatment of SGC

0.1 mg of SGC was treated for 24 hours at 37°C with neuraminidase (EC 3.2.1.18) (Vibrio cholerae, General Biochemicals, Chargin Falls, Ohio) in 0.05M acetate buffer, pH 5.5, containing one percent NaCl and
Figure 3  TLC of mixtures of human brain gangliosides developed with C:M:2.5N NH₄OH (60:35:8). Plate A: MHE, Mixed Human Gangliosides; MMG, Mixed Minor Gangliosides. Eleven resorcinal positive spots (HG-A through HG-E and HG-1 through HG-6) are discernable. Plate B: Purified preparations of SGC (HG-B), GGnSLC (HG-1), SLC (HG-C), GnSLC (HG-D), HG-E (unknown) and N-acetylneuraminic acid (NANA).
<table>
<thead>
<tr>
<th>Component</th>
<th>Content (\mu\text{moles/\mu\text{mole NANA}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hexose(^a)</td>
<td>1.12</td>
</tr>
<tr>
<td>Galactose(^b)</td>
<td>1.05</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>0.98</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.08</td>
</tr>
</tbody>
</table>

NANA, N-acetylneuraminic acid.
\(^a\)Phenol-sulfuric acid assay.
\(^b\)GLC assay; no detectable glucose.
0.1% CaCl₂. N-acetylneuraminosyllactose (General Biochemicals) was used as a standard substrate. The reaction products were examined in solvent systems I-V and in the Kean system. The following standards were used for TLC: galactosylceramide, Glucosylceramide, and N-acetylneuraminic acid. In all cases, spots corresponding the NANA and galactosylceramide standard were observed.

4. Fatty Acids of Sialosylgalactosylceramide (SGC)

Examination of the fatty acid methyl esters obtained from SGC in the TLC system described by Vioque and Holman (1962) revealed spots corresponding to hydroxy and normal fatty acids esters. Hydroxy and normal fatty acid ester fractions were obtained by preparative TLC and examined by GLC. Normal and hydroxy fatty acid esters were confirmed by their mass spectra. The normal ester fraction contained palmitic, stearic and oleic acid (Table IV). The composition of hydroxy fatty acid esters (Table V) was similar to that reported for cerebrosides (O'Brien, Fillerup, and Mead, 1964).

In a determination of the relative amounts of hydroxy and non-hydroxy fatty acids, the total fatty acid methyl ester fraction was trimethylsilylated and subjected to GLC. The ratio of hydroxy to non-hydroxy fatty acids was calculated as 0.9 (Table III) by comparison of the total peak areas corresponding to the fatty acids, no correction for the contribution of the trimethylsilyl groups to the GLC response was made.

In addition to the fatty acids listed in Table IV and V the total methyl ester fraction gave on the GLC analysis an additional peak not observed in the hydroxy or normal fatty acid ester fractions.
### TABLE III PERCENT OF NORMAL FATTY ACIDS AND HYDROXY FATTY ACIDS OF SGC AND BEEF BRAIN CEREBROSIDES

<table>
<thead>
<tr>
<th></th>
<th>SGC</th>
<th>Beef Brain Cerebrosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Fatty Acids</td>
<td>53.7</td>
<td>39.5</td>
</tr>
<tr>
<td>Hydroxy Fatty Acids</td>
<td>46.3</td>
<td>60.5</td>
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</table>
TABLE XV  COMPOSITION OF NORMAL FATTY ACIDS OF SGC AND BEEF BRAIN CEREBROSIDES

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SGC</th>
<th>Beef Brain Cerebrosides % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>27.7</td>
<td>1.5</td>
</tr>
<tr>
<td>18:1</td>
<td>25.1</td>
<td>-</td>
</tr>
<tr>
<td>18:0</td>
<td>47.3</td>
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<tr>
<td>20:0</td>
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<td>-</td>
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<td>24:1</td>
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<td>26:0</td>
<td>-</td>
<td>5.9</td>
</tr>
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</table>

Fatty acids are designated by chain length: no. of double bonds.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SGC</th>
<th>Beef Brain Cerebrosides</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:0</td>
<td>tr.</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td>2.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>22:0</td>
<td>6.3</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>23:0</td>
<td>21.7</td>
<td>10.6</td>
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</tr>
<tr>
<td>24:1</td>
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<tr>
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<td>41.9</td>
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<tr>
<td>25:1</td>
<td>-</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>25:0</td>
<td>10.7</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>26:1</td>
<td>tr.</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>26:0</td>
<td>-</td>
<td>7.7</td>
<td></td>
</tr>
</tbody>
</table>

Fatty acids are designated by chain length.
: no of double bonds.
obtained by preparative TLC. This peak had an equivalent chain length of 22.1 on 3% SE-52 and 23.9 on 15% EGS. The mass spectrum (Fig. 4) and chromatographic behavior of this material were similar to that found by Dr. Nair for di(ethylhexyl)phthalate. A sample of the phthalate was obtained from Dr. Nair and was found to have the same retention time as the unknown on 1% SE-52 and 6% OV-1. Since this substance is a commonly used plasticizer, it was considered to be a contaminant, not a natural fatty ester.

5. Long-Chain Base Analysis of SGC

Analysis of the long-chain base fraction by periodate oxidation and GLC analysis of the resulting aldehydes showed that more C\textsubscript{20} than C\textsubscript{18} sphingosine was present (Table VI).

B. Adrenal Gland Gangliosides

1. Isolation

(a) Crude Ganglioside Fractions.

Crude gangliosides were prepared as described by Suzuki (1965). The yield of crude mixed gangliosides from the cortex was 0.68% whereas 0.91% was obtained from medulla (Table VII). After extraction of lipids with C:M (2:1) and C:M (1:2) with 5% water, the residual material which was extracted with 50% methanol did not show any resorcinol positive spots on the TLC plates.

(b) Column Chromatographic and Preparative TLC.

(i) Cortex Gangliosides

\textsuperscript{aNair, P. P. Personal communication. Sinai Hospital of Baltimore Inc., Belverdere Avenue at Greenspring, Baltimore, Maryland 21215.}
Figure 4. Mass Spectrum of Di(2-ethylhexyl)phthalate
### Table VI: GLC Analysis of the Long-Chain Bases as Aldehydes

<table>
<thead>
<tr>
<th></th>
<th>4-Sphingenine&lt;sup&gt;b&lt;/sup&gt; (C&lt;sub&gt;18&lt;/sub&gt;)</th>
<th>Sphinganine&lt;sup&gt;a&lt;/sup&gt; (C&lt;sub&gt;18&lt;/sub&gt; dihydro-)</th>
<th>4-Ericosa-sphingenine&lt;sup&gt;a&lt;/sup&gt; (C&lt;sub&gt;20&lt;/sub&gt;)</th>
<th>Eicosasphinganine&lt;sup&gt;a&lt;/sup&gt; (C&lt;sub&gt;20&lt;/sub&gt; dihydro-)&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed gangliosides</td>
<td>47</td>
<td>2</td>
<td>49</td>
<td>2</td>
</tr>
<tr>
<td>SGC</td>
<td>31</td>
<td>4</td>
<td>55</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as percentage of known aldehydes; these include the derivatives of sphinganine and eicosasphinganine and 3-0-methyl-4-sphingenine.

<sup>b</sup>Figures represent the sum of peaks from 4-sphingenine and 3-0-methyl-4-sphingenine.
| TABLE VII YIELDS OF CRUDE GANGLIOSIDES OBTAINED FROM ADRENAL CORTEX AND MEDULLA |
|---------------------------------|-----------------|-----------------|
|                                 | Cortex          | Medulla         |
| Wet Weight                      | 584.0g          | 166.0g          |
| Dry Weight                      | 130.2g          | 36.4g           |
| % Water                         | 76.0            | 78.1            |
| Crude Mixed Ganglioside         | 883.0mg         | 332.0mg         |
| % Yield of Crude Gangliosides   | 0.68            | 0.91            |
| $\mu$ Moles of Gangliosides per Gram Dry Weight* | 5.7             | 7.6             |

*$\mu$ Moles were calculated assuming an average molecular weight of ganglioside as 1200, which is sialosyllactosylceramide (SLC).
800 mg of crude cortex gangliosides were placed on 1200 x 15 mm Anasil S column and eluted with 1200 ml of C:M:W: (60:35:8), 900 ml of C:M:W: (60:40:10) and with 800 ml of methanol. Ten ml fractions were collected (Fig. 5). The fractions were analyzed by TLC in propanol:NH₄OH (solvent IV) and pooled to make four preparations C-A₁, C-B₁, C-C₁ and C-D₁ as shown in Figure 5. The TLC behaviour of these fractions are shown diagramatically in Figure 6. Components of fraction C-A₁ were purified by preparative TLC as shown in Figure 7. Gangliosides C-5 (60.4 mg), C-6 (4.0 mg) were obtained pure. No attempt was made to isolate C-1, C-2, C-3, and C-4 in pure form. C-1 through C-4 are minor gangliosides in the cortex. C-7 ganglioside was isolated, and it showed non-ganglioside impurity. No further purification was carried out. (Figure 7 shows purification procedures for C-5 and C-6 gangliosides). The nomenclature of cortex and medulla gangliosides adopted here is similar to that adopted by Ledeen et al. (1968 a). This is based on their mobilities in solvent system I, the fastest being C-7 and the slowest being C-1.

(ii) Medulla Gangliosides

300 mg of medulla gangliosides were similarly treated as cortex gangliosides. Four fractions obtained were M-A₁, M-B₁, M-C₁ and M-D₁. (Figure 8). Figure 6 shows the TLC behaviour of these preparations. Note that the cortex preparation C-A₁ has a component not seen in the corresponding medulla M-A₁ preparation. The resorcin positive components were numbered according to their behaviour in solvent system I in a manner analogous to that used by Ledeen et al.
Figure 5. An elution pattern of adrenal cortex gangliosides from silicic acid column. Ten-ml fractions were collected and a small aliquot of each fraction spotted on TLC plate and sialic acid detected with resorcinol reagent. Fractions were pooled into four groups: C-A, C-B, C-C, C-D.
Figure 6  Diagramatic representation of adrenal gland gangliosides TLC mobilities. Fractions were obtained from Unasil S column as shown in Figure 5 and 8. Spots were developed with resorcinol reagent.
Figure 7 Isolation of adrenal cortex gangliosides.
Figure 8  An elution pattern of adrenal medulla gangliosides from silicic acid column. Ten-ml fractions were collected and a small aliquot of each fraction spotted on TLC plates and sialic acid detected with resorcinol reagent. Fractions were pooled into four preparations: M-A₁, M-B₁, M-C₁, M-D₁.
Components M-A₁ (M-5 and M-6) were further purified by rechromatography on Anasil S column as shown in Figure 9. Ganglioside M-5 (50.0 mg) and M-6 (22.0 mg) were obtained chromatographically pure. No attempt was made to purify other gangliosides of medulla. Note the absence of ganglioside in the medulla which corresponds to C-6 in the cortex.

2. Chromatographic Purity

(a) Cortex Gangliosides

Crude cortex gangliosides showed seven distinct resorcinol positive components. The final preparation of C-5 was chromatographically pure in all five solvent systems (I through V). C-5 runs slower than GnSLC (Tay-Sach's) in all the five solvent systems. C-7 which was not obtained pure runs ahead of GnSLC in all the five solvents and runs similar to M-6 and presumably is the same. C-6 was homogenous in solvent III and IV, showed three resorcinol spots in solvent II and two spots in solvent I. C-6 shows unusual chromatographic behaviour; that is, in solvent II, it migrates ahead of M-6 and GnSLC, while in solvent IV, it migrates below GnSLC and M-6. Figure 10 shows TLC mobilities of C-5, C-6 in solvent I. Figure 11 illustrates TLC mobilities of C-5 and C-6 in all five solvent systems. Rf values of C-5 and C-6 are summarized in Table VIII.

(b) Medulla Gangliosides

Gangliosides M-5 and M-6 were chromatographically pure in all the five solvent systems. M-5 runs slower than GnSLC (Tay-Sach's) in all the five solvent systems and chromatographically is similar to C-5.
Figure 9 Isolation of adrenal medulla gangliosides.
Figure 10  TLC plate of gangliosides SnSLC (TS), C-5, C-6, M-5, M-6 and STD. Mixture (Standard mixture containing GnSLC, GGnSLC, SGGnSLC, GGnSSLc and SGGnSSLc). Adsorbent was silica gel G, developed in C:M:2.5N NH₄OH (60:35:8). Spots were visualized with resorcinol reagent.
Figure 11  TLC mobilities of GnSLC, C-5, C-6, M-5, M-6.
<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>C-M-NH₄OH (60:35:8)</th>
<th>C-M-W (60:35:8)</th>
<th>C-M-W (65:25:4)</th>
<th>n-propanol-NH₄OH (7:3)</th>
<th>n-propanol (7:3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-5 or M-5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>C-6</td>
<td>1.16</td>
<td>3.59</td>
<td>30.00</td>
<td>0.65</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>1.24</td>
<td>3.69</td>
<td>27.00</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-6</td>
<td>1.49</td>
<td>2.62</td>
<td>9.00</td>
<td>1.09</td>
<td>1.39</td>
</tr>
</tbody>
</table>
M-6 runs slower than GnSLC in all the solvents and is chromatographically similar to C-7. Figure 10 shows TLC mobilities of M-5 and M-6 in solvent I. Figure 11 illustrates TLC mobilities of M-5 and M-6 in all solvents. Rf values are summarized in Table VIII.

3. Composition and Structural Analysis

(a) Medulla Gangliosides

(1) M-5 Ganglioside

Essentially equimolar ratios of glucose, galactose, sialic acid and fatty acids were found (Table IX). M-5 (20-25 µg of sialic acid) was dissolved in 0.4 ml of 0.1M sodium acetate buffer, pH 5.0, 0.2 ml neuraminidase (Clostridium perfringens, 1 mg per 1 ml) added and the mixture incubated at 37°C for 48-72 hours. After incubation 0.4 ml of water was added followed by 4 ml of C:M (2:1) to make Folch's solvent. Lower phase was used for TLC with solvent V. The plate was sprayed with resorcinol reagent first and then with H₂SO₄ to visualize non-resorcinol positive spots. Standard used were cerebroside, lactosylceramide (LC) (ceramide dihexoside), N-acetyl galactosaminyllactosylceramide (GnLC) (ceramide trihexoside), galactosy N-acetylgalactosaminyllactosylceramide (GGnLC) (ceramide tetrahexoside) The upper phase which presumably contains sialic acids, proteins, salt and ganglioside resistant to neuraminidase were lyophilized and run through Dowex 2-X8 (1 x 1.25 cm) acetate column (50-100 mesh). Protein, salts, and gangliosides were eluted with 10 ml water. This solution was lyophilized and extracted with C/M (2:1) and subjected to TLC with solvent IV. The sodium salt of sialic acid was eluted with 2 ml 0.5M
**TABLE IX  COMPOSITION OF C-5, C-6, M-5 AND M-6 GANGLIOSIDES**

<table>
<thead>
<tr>
<th>GANGLIOSIDES</th>
<th>COMPONENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>C-5</td>
<td>1.00</td>
</tr>
<tr>
<td>C-6</td>
<td>1.00</td>
</tr>
<tr>
<td>M-5</td>
<td>1.00</td>
</tr>
<tr>
<td>M-6</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Expressed as molar ratios relative to glucose. All values except "Sialic Acid (resorcinol)" were determined by GIC.*
sodium acetate. To obtain free sialic acid, the material was passed through a column of Dowex 50 W x 2(H⁺) (3 x 1 cm), and immediately frozen and lyophilized. This residue was subjected to TLC with the n-propanol-1N NH₄OH-water (6:2:1) system (solvent VII) according to the method of Granzer (1962). NGNA, NANA were used as standards.

M-5 ganglioside was hydrolyzed by the neuraminidase to yield NGNA and lactosylceramide (LC) which were identified by TLC.

A quantity of M-5 ganglioside (about 20 µg of ganglioside sialic acid) was dissolved in 0.5 ml of 0.03 N HCl (aqueous) and heated at 85°C for 2 hours. After hydrolysis, 2 ml C:M (2:1) was added. The lower phase was used for TLC with solvent system V. Standards used were lactosylceramide (LC), N-acetylgalactosaminyllactosylceramide (GnLC) and galactosylN-acetylgalactosaminyllactosylceramide (GGnLC). The upper phase was neutralized with Na₂CO₃, dried and analyzed by TLC with solvent system VII. The standards used were NANA and NGNA. GnSLC was hydrolyzed under the same conditions. It was thus shown that M-5 ganglioside yielded NGNA and LC as a result of mild acid hydrolysis.

M-5 ganglioside contains only normal fatty acids, the major fatty acids found are stearic (18:0), behenic (22:0) and palmitic (16:0) acids (Table X).

Ozonolysis of M-5 ganglioside showed tetradecenal as a major product no hexadecenal was detected. Therefore long-chain base of M-5 is primarily 4-sphingenine (sphingosine). Figure 12 shows GLC of the aldehyde obtained from M-6 which is similar to M-5 ganglioside.

Permethylation of M-5 gave methyl-2,4,6-tri-0-methylgalactosides and methyl-2,3,6-tri-0-methylglucosides without silylation (Figure 13).
<table>
<thead>
<tr>
<th></th>
<th>C-5</th>
<th>C-6</th>
<th>M-5</th>
<th>M-6</th>
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<tbody>
<tr>
<td>16:1</td>
<td>2.7</td>
<td>4.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16:0</td>
<td>29.1</td>
<td>32.2</td>
<td>21.0</td>
<td>9.0</td>
</tr>
<tr>
<td>17:0</td>
<td>2.6</td>
<td>tr.</td>
<td>2.9</td>
<td>1.9</td>
</tr>
<tr>
<td>18:1</td>
<td>4.3</td>
<td>10.3</td>
<td>-</td>
<td>6.0</td>
</tr>
<tr>
<td>18:0</td>
<td>26.9</td>
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<tr>
<td>19:0</td>
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<td>tr.</td>
<td>7.4</td>
<td>1.7</td>
</tr>
<tr>
<td>20:1</td>
<td>-</td>
<td>tr.</td>
<td>tr.</td>
<td>-</td>
</tr>
<tr>
<td>20:0</td>
<td>3.8</td>
<td>3.5</td>
<td>4.3</td>
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<td>22:1</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>22:0</td>
<td>16.8</td>
<td>10.3</td>
<td>16.5</td>
<td>33.4</td>
</tr>
<tr>
<td>23:0</td>
<td>5.9</td>
<td>4.9</td>
<td>1.9</td>
<td>3.0</td>
</tr>
<tr>
<td>24:1</td>
<td>3.8</td>
<td>9.3</td>
<td>-</td>
<td>6.2</td>
</tr>
<tr>
<td>24:0</td>
<td>6.2</td>
<td>6.5</td>
<td>2.4</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Fatty acids are designated by chain length.
: no. of double bonds.
Figure 12  GLC (on 15% EGS) of aldehydes obtained by ozonolysis of M-6 ganglioside. Peaks identified as: (1) tetradecanal (2) unidentified. Similar results were obtained from M-5 and C-5 gangliosides.
Figure 13 (top) GLC of permethylated sugars of C-5 ganglioside on 3% ECNSS-M column (6 ft x 3/4") column temp. 125°C. Peaks identified as: (1) and (3) Methyl-2,3,6-tri-0-methylglucosides; (2) and (3) Methyl 2,4,6-tri-0-methylgalactosides. Similar results were obtained from M-5 and M-6.

Figure 14 (bottom) GLC of permethylated and silylated sugars of C-5 ganglioside on 3% ECNSS-M column (6 ft x 1/4") column temp. 110°C. Peak identified as: (1) Methyl-2,3,6-tri-0-methyl-4-trimethylsilylglucoside, (2) Methyl-2,4,6-tri-0-methyl-4-trimethylsilylgalactoside, and (3) Methyl-2,3,6-tri-0-methyl-4-trimethylsilylglucoside. Similar results were obtained from M-5 and M-6.
Permethylated and silylation gave methyl-2,3,6-tri-O-methyl-4-trimethylsilylgalactosides and methyl-2,4,6-tri-O-methyl-4-trimethylsilylgalactosides (Figure 14). Thus the structure N-glycolylneuraminosyl (2→3) lactosylceramide can be assigned to this ganglioside. Table XI shows relative retention values of standard methylated hexoses.

(ii) M-6 Gangliosides

Essentially equimolar ratios of glucose, galactose, sialic acid and fatty acids were found (Table IX). M-6 ganglioside was hydrolyzed by neuraminidase and mild acid hydrolysis to yield NANA and LC. Major fatty acids of M-6 are behenic (22:0) and stearic (18:0). (Table X). Long-chain bases are primarily 4-sphingenine. Permethylation of M-6 gave methyl-2,4,6-tri-O-methylgalactosides and methyl-a,3,6-tri-O-methylglucosides (Figure 13). Therefore the structure of M-6 is N-acetylneuraminosyl (2→3) lactosylceramide.

(b) Cortex Gangliosides

(i) C-5 Gangliosides

C-5 ganglioside gave equimolar ratios of glucose, galactose, sialic acid and fatty acids. It is hydrolyzed by neuraminidase and mild acid hydrolysis to yield NGNA and LC. The long-chain bases (LCB) are primarily 4-sphingenine.

Major fatty acids of C-5 are palmitic (16.0), stearic (18.0) and behenic (22.0). Permethylation studies indicated the structure to be N-glycolylneuraminosyl (2→3) lactosylceramide.

(ii) C-6 Ganglioside

Essentially equimolar ratios of glucose, sialic acid and fatty acids were found (Table IX). The galactose molar ratio is less
<table>
<thead>
<tr>
<th>Compounds</th>
<th>3% ECNSS-M 125°C</th>
<th>3% ECNSS-M 110°C (TMSi)</th>
<th>15% EGS 150°C (TMSi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-2,3,4,6-tetra-0-Methylgalactosides</td>
<td>1.00 0.92</td>
<td>1.00 ---</td>
<td>1.00 0.93</td>
</tr>
<tr>
<td>Methyl-2,3,4,6-tetra-0-Methylglucosides</td>
<td>0.74 0.47</td>
<td>0.76 0.48</td>
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</tr>
<tr>
<td>Methyl-2,3,6-tri-0-Methylgalactosides</td>
<td>2.74 1.95</td>
<td>0.50 0.44</td>
<td>--- ---</td>
</tr>
<tr>
<td>Methyl-2,3,6-tri-0-Methylglucosides</td>
<td>2.95 2.00</td>
<td>0.60 0.35</td>
<td>0.49 0.31</td>
</tr>
<tr>
<td>Methyl-2,4,6-tri-0-Methylgalactosides</td>
<td>3.00 2.50</td>
<td>0.52 ---</td>
<td>0.40 ---</td>
</tr>
<tr>
<td>Methyl-2,3,4-tri-0-Methylglucosides</td>
<td>2.20 1.35</td>
<td>0.43 0.26</td>
<td>0.34 0.23</td>
</tr>
<tr>
<td>Methyl-2,6-di-0-Methylgalactosides</td>
<td>--- ---</td>
<td>0.41 0.25</td>
<td>0.21 0.14</td>
</tr>
</tbody>
</table>
than one. The sialic acid value is slightly higher than one, this could be due to the presence of NGNA which gives higher response than NANA with the resorcinol assay. No correction was made for the presence of NGNA in this ganglioside. This ganglioside was resistant to neuraminidase under the conditions which hydrolyzed M-5, M-6, and C-5 gangliosides. Ganglioside C-6 was also resistant to mild acid hydrolysis (0.03N HCl, aqueous, 85°C, 2 hours) under the conditions which hydrolyzed M-5, M-6 and C-5 gave sialic acid and asialoganglioside. A small percent of C-6 was hydrolyzed with 0.05N H2SO4 at 90°C for 90 minutes. There were three resorcinol positive spots observed in solvent VII, two spots corresponded to NANA and NGNA. One unknown resorcinol positive spot which travelled ahead of NANA was not identified. The major fatty acids of C-6 ganglioside are palmitic and stearic.

A small quantity of C-6 ganglioside (5 µg of ganglioside sialic acid) was treated with mild base (0.02 ml 1N KOH, 37°C, 16 hours). After hydrolysis it was neutralized with HCl. A resorcinol positive spot which migrated with C-5 in solvent system II was observed. Thus treatment with alkali produced a product which migrated much slower than the original ganglioside.

100 µg of C-6 ganglioside was dissolved in 0.2 ml water. The hydroxylamine reaction was carried out according to the method of Hestrin (1949). Ethyl-n-butyrate was used as standard. C-6 gave a positive hydroxamic acid test and is presumably a lactone or ester of SLC.
CHAPTER V

DISCUSSION

A. Sialosylgalactosylceramide (SGC)

The SGC reported here was isolated from a large sample of mixed gangliosides of normal adult brain prepared by a procedure involving a Folch solvent distribution, and no attempt was made to isolate all the SGC from brain. In fact, we have evidence that some of this ganglioside remains in the lower phase of the solvent partitions. It is possible, therefore, that there may have been selective losses based on solubility properties and that the lipid composition reported here does not truly represent that of total brain SGC. Nevertheless, the fatty acid and long-chain base compositions found are sufficiently distinctive to be significant.

The presence of C_{18} and C_{20}-sphingosine in SGC suggests a metabolic relationship with the major brain gangliosides rather than with the brain cerebrosides. However, the composition of the fatty acid fraction complicates this interpretation. The composition of the hydroxy fatty acid fraction resembles that reported for brain cerebroside (O'Brien et al., 1964), while the normal fatty acid fraction is clearly distinct from the normal fatty acids of adult brain cerebrosides. O'Brien and co-workers (1964) report that 24:0 and 24:1 constitute the major fatty acids of cerebrosides in both the normal and hydroxy series. The high percentage of oleic acid
also distinguishes the normal fatty acid pattern from that of the major brain gangliosides.

From the data presented we have concluded that SGC is not a direct derivative of normal adult brain cerebroside. Because adult brain cerebroside contains no C_{20}-sphingosine, the possibility of a common psychosine precursor appears to be eliminated; the fatty acid differences eliminate consideration of a common ceramide precursor. The fatty acid composition of SGC seems to eliminate the possibility of a ceramide precursor in common with the major brain gangliosides. Thus, the unique ceramide composition of the isolated SGC suggests that this compound has no direct metabolic relationship with either the major brain gangliosides or adult brain cerebrosides. However, O'Brien and Sampson (1965 b) report that the normal fatty acids of cerebroside obtained from infant and child gray matter consist mostly of 16:0, 18:0, and 18:1. Menkes, Phillipart and Concone (1966) report similar data for normal fatty acids from cerebrosides isolated from fetal and 1 day old infant gray matter. Thus, the fatty acid composition of SGC more closely resembles that reported for infant gray matter cerebroside than adult brain cerebroside. It will be of interest to determine the sphingosine composition of infant gray matter cerebroside, which contains a large percentage of these medium-chain normal fatty acids.

B. Adrenal Gland Gangliosides

There were six gangliosides detected in bovine adrenal medulla. Two major gangliosides, M-5 and M-6, were obtained pure and were
shown to contain N-glycolylneuramic and N-acetylneuramic acids respectively, as well as equimolar ratios of glucose, galactose, and fatty acids and thus correspond to AG\textsubscript{5} and AG\textsubscript{6} previously reported by Ledeen et al (1968 a). Permethylation of these materials led to the production of 2,3,6-tri-0-methylglucosides and 2,4,6-tri-0-methylgalactosides identified with and without silylation. Thus the structure sialosyl (2→3) galactosyl (1→4) glucosylceramide can be assigned to these gangliosides. The sphingosine moiety of M-5 and M-6 was examined by microzonolysis procedure. In both cases tetradecanal was the major product obtained, and no hexadecanal was detected. Therefore, the LCB in both these gangliosides is 4-sphingenine. More complex gangliosides of adrenal medulla (M-1 through M-4) were not examined.

Seven gangliosides were detected in bovine adrenal cortex. Gangliosides C-5 and C-6 were obtained pure and examined. C-7 in small amounts still contaminated with non-gangliosides material showed chromatographic behavior similar to M-6 and is presumably N-acetylneuraminosyllactosylceramide, but no further studies have been conducted on this trace component. Ganglioside C-5 which is the major ganglioside component of bovine adrenal cortex (80-90%) was obtained chromatographically pure. Its chromatographic behavior in five solvent systems is identical to M-5. It has been assigned the structure N-glycolylneuraminosyl (2→3) lactosylceramide. Evidence that 4-sphingenine is the major long-chain base present was obtained by ozonolysis.

M-6 migrates ahead of GnSLC and M-5 in all five solvent systems. Both M-5 and C-5 contain N-glycolylneuraminic acid and travel slower
than GnSLC (TS) and M-6, in all the TLC solvent systems. These two
gangliosides have N-glycolyl group instead of an N-acetyl group as
in M-6, and GnSLC has N-acetylgalactosamine in addition to N-acetyl-
neuraminosylactosylceramide. Chromatographic properties of C-5 and
M-5 cannot be explained easily; possibly the configuration of the
molecule accounts for the large effect of the glycolyl hydroxyl
group.

Ganglioside C-6 was obtained chromatographically homogeneous
in n-propanol-NH$_4$OH (7:3) and n-propanol-water (7:3) but showed two
resorcinol spots in C:M:2.5N NH$_4$OH (60:35:8) and three spots in
C:M:W: (60:35:8). It showed unusual chromatographic behavior; in
C:M:W: (60:35:8), it migrates ahead of M-6 and GnSLC while in propanol-
NH$_4$OH (7:3); it migrates below GnSLC (Tay-Sach's) and M-6. This
ganglioside contains roughly equimolar amounts of sialic acid, glucose,
galactose, and fatty acid. No hexosamine was detected. Ganglioside
C-6 gave a positive hydroxamic reaction and is probably a lactone or
an ester derivative of SLC. This might explain its slow migration in
n-propanol-NH$_4$OH (7:3). After treatment of C-6 with weak base (1N KOH,
37°C for 16 hours) a product formed which migrated much slower than
the original ganglioside in C:M:W (60:35:8); it almost migrated the
same as N-glycolylneuraminosylactosylceramide. Therefore, the base
hydrolyzed some group or component which might be responsible for its
atypical chromatographic behavior in different solvents. The hydrol-
yzed component was not investigated. Ganglioside C-6 was resistant
to hydrolysis by Cl.perfringens neuraminidase under conditions which
completely hydrolyzed M-5, M-6, and C-5. This shows that there is
some hindered group which is responsible for its resistance to neuraminidase enzyme. It will be interesting to see the effect of neuraminidase on the product obtained after hydrolysis with a weak base. C-6 is resistant to mild acid hydrolysis, the conditions which hydrolyzed C-5, M-5 and M-6. However, a small percentage of the ganglioside was hydrolyzed by stronger acid hydrolysis conditions (0.05N H₂S₄, 90°C for 90 minutes). After hydrolysis and neutralization, three resorcinol spots were observed, two corresponding to NANA and NGNA on TLC plates and one unidentifiable spot travelling ahead of NANA. This could explain why intact ganglioside shows heterogeneity on some TLC solvent systems.

Fatty acids of C-5, C-6, M-5, and M-6 are very different from brain gangliosides which contain mostly stearic acid (Sambasivarao and McCluer, 1964) or hematosides (SLC) obtained from red cell stroma which contain mostly nervonic (24:1) and lignoceric acids (24:0) (Handa and Handa, 1965). M-6 contains 22:0 and 18:0 as the major fatty acid while in M-5 major fatty acids were 18:00, 16:00, and 22:00. C-5 and C-6 have 16:00, 18:00, and 22:00 as the major fatty acids. Since in our extraction procedures we lost gangliosides in the Folch's lower phase, there could be selective losses of gangliosides with long-chain fatty acids. C-5, C-6, M-5 and M-6 contain 4-sphingenine as the primary LCB.

The glycolipids contribute to the cell surface specificity. The gangliosides of the brain (CNS) are different in quality and quantity from other tissues. Brain gangliosides contain more complex carbohydrate residue, whereas most other tissues contain hematoside as
the major ganglioside in which the carbohydrate is simple lactose. The cortex is mesodermal in origin and the medulla is the only organ which remains intimately connected with the nervous system and develops from the neural crest. But the gangliosides of the medulla are not as complex as brain gangliosides, but they are similar to cortex gangliosides. However, the medulla contains M-5 and M-6 as the major gangliosides, whereas C-5 (which corresponds to M-5) is the major ganglioside of the cortex. Small quantities of C-7 (which corresponds to M-6) were found in the cortex. Since it is rather impossible to completely separate the cortex from the medulla, it could be that C-7 was a contamination of M-6 from the medulla during the separation of the cortex and the medulla tissues. Fatty acids of C-7 could have given some information about the resemblances between the M-6 and C-7. The cortex contains an extra ganglioside C-6 which is missing in the medulla.

Ledeen et al. (1968 a) showed that adrenal medulla does contain small portions of gangliosides that resembles brain gangliosides. Our preliminary results also showed that both cortex and medulla gangliosides have minor gangliosides which migrated with major brain gangliosides on TLC plates. O'Brien et al. (1967) have investigated lipids of intradural spinal roots and reported the absence of gangliosides. Lowden and Wolfe (1964) also reported the absence of gangliosides in sciatic nerve. Micromoles of gangliosides in adrenal medulla, brain gray matter and white matter are 3.09, 7.17 and 1.41 respectively (Ledeen et al., 1968 a). Thus the medulla contains one half concentration of gangliosides as the gray matter and twice that of white matter.
Our results show that cortex and medulla both contain high amounts of gangliosides. It is very difficult to explain why sciatic nerve and intradural spinal roots do not contain any ganglioside. It is possible that these tissues contain hematosides which were lost in the Folch's lower phase.

What physiological functions do these gangliosides have in the cortex and medulla? Derry and Wolfe (1967) have shown that ganglioside occur in neuronal membrane in CNS and small portions are probably associated with glia (Ledeen et al., 1968 b). Subcellular studies with brain have indicated high concentration of gangliosides at the synapses and adjoining membranes (Lapetina et al., 1967) and there is evidence to suggest that they are involved in cation transportation (McIlwain, 1963). In the adrenal medulla, depolarization of the chromaffin cell by acetylcholine is accompanied by an influx of ions, which leads to release of catecholamines from chromaffin granules (Douglas and Poisner, 1962, 1966). By analogy with CNS, it is conceivable that the gangliosides of this tissue might also serve in some capacity related to ion transport, but there is no evidence to support this.
CHAPTER VI

SUMMARY

A. Sialosylgalactosylceramide (SGC)

A ganglioside, previously designated HG-B in our laboratory, was isolated from mixed human brain ganglioside preparations and shown to contain equimolar quantities of sialic acid, galactose, and sphingosine. Treatment of this material with neuraminidase yeilded a galactosylceramide. The ganglioside, now referred to as sialosylgalactosylceramide (SGC), thus appears to be identical with Ggal reported by Kuhn and Wiegandt (1963, 1964). Approximately equal amounts of normal and hydroxy acids were found by gas-liquid chromatography (GLC). Oleic (18:1), palmitic (16:0), and stearic acid (18:1) were the only normal fatty acids present. In the hydroxy series, the C24 and C23 saturated fatty acids were the major components. The ratio of C20 to C18 long-chain base was approximately 5:3.

These data suggest that sialosylgalactosylceramide has no direct metabolic relationship with either the major gangliosides or adult brain cerebrosides.

B. Adrenal Gland Gangliosides

Six gangliosides were detected in the bovine adrenal medulla. Two major gangliosides M-5 and M-6 were obtained pure and they contain sialic acid, glucose, galactose, and fatty acids in equimolar quantities. The structure of these gangliosides was elucidated by
permethylation studies. M-6 was found to be N-acetylneuraminosyl (2→3) galactosyl (1→4) glucosylceramide and M-5 was N-glycolyneuraminosyl (2→3) galactosyl (1→4) glucosylceramide. The gangliosides contain 4-sphingenine (C₁₈-sphingosine) as the long-chain base and no 4-eicosasphingenine (C₂₀-sphingosine) was observed.

Seven gangliosides were detected in the bovine adrenal cortex. Ganglioside C-5 and C-6 were obtained pure. C-5 is the major ganglioside and it contains sialic acid, glucose, galactose, and fatty acid in equimolar quantities. The structure of this ganglioside was elucidated by permethylation and was N-glycolyneuraminosyl (2→3) galactosyl (1→4) glucosylceramide. C-7 still contaminated with non-ganglioside showed chromatographic behavior similar to M-6 and is presumably N-acetylneuraminosylactosylceramide. C-6 ganglioside contains sialic acid, glucose, galactose, and fatty acid in equimolar ratios. This ganglioside was resistant to neuraminidase and was also resistant to mild acid hydrolysis which hydrolyzed C-5, M-5, and M-6 to yield sialic acids. However under stronger acid conditions, C-6 yielded NANA, NGNA and an unidentifiable resorcinol spot on a TLC plate. C-6 gave a hydroxamic positive reaction and is probably lactone or an ester. C-6 shows atypical chromatographic behavior in n-propanol-NH₄OH (7:3), it migrates slower than GnSLC (Tay-Sach's) M-5, M-6, C-5, and C-7, and it migrates ahead of all the above gangliosides in C:M:W: (60:35:8). M-5 and C-5 migrate slower than GnSLC and M-6 in all five TLC solvents, whereas M-6 and C-7 migrate faster than GnSLC. C-6 showed one spot in n-propanol-NH₄OH (7:3), n-propanol-water (7:3), showed two spots in C:M:2.5N NH₄OH (60:35:8) and three spots in C:M:water (60:35:8). C-6, when hydrolyzed with
weak base, gave resorcinol positive spots which migrated with N-glycolylneuraminosyllactosylceramide in C:M:W: (60:35:8). The long-chain bases of C-5 and C-6 were found to be 4-sphingenine. Fatty acids of C-5, C-6, M-5, and M-6 are different from brain gangliosides which mostly contain stearic acid (Sambasivarao and McCluer, 1964) and red blood stroma's hematosides (sialosyllactosylceramide) which mostly contain nervonic (24:1) and lignoceric acid (24:0) (Handa and Handa, 1965). The major fatty acids of C-5, C-6, M-5, and M-6 are 16:0, 18:0, and 22:0, though in different proportions. The differences between bovine adrenal cortex ganglioside and bovine adrenal medulla gangliosides were: the cortex contains C-6, an unusual ganglioside which is missing in the medulla, the major ganglioside of the cortex is N-glycolylneuraminosyllactosylceramide, whereas in the medulla the major gangliosides were N-acetyl- and N-glycolylneuraminosyllactosylceramide.
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