GANGLIOSIDE ANALYSES IN BRAIN SECTIONS AND ISOLATED NEURONES

GANGLIOSIDE ANALYSES IN SERIAL SECTIONS OF BRAIN AND IN ISOLATED NEURONES

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by

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GANGLIOSIDE ANALYSES IN SERIAL SECTIONS OF BRAIN AND IN ISOLATED NEURONES

There is now considerable evidence to support the specific neuronal localization of gangliosides. The distribution of gangliosides in subcellular fractions from adult and developing rat brain shows that gangliosides are concentrated in nerve-ending particles and dendritic membrane fractions. The ganglioside concentration in mature white matter is one-sixth to one-tenth that of grey matter. However, the gangliosides in white matter do not appear to be a part of myelin but a constituent of the axonal space or axoplasm.

To clarify further ganglioside localization in brain, total ganglioside content in 24 μ serial cryostat sections through Lorente de Nó's area CA2 of the ox hippocampus and folia of the ox cerebellum has been analysed by a fluorometric method for N-acetylneuraminic acid. A purified unfractionated ox ganglioside preparation was used as a standard. Sections were cut parallel to the layers of area CA2 of the hippocampus from the ventricular surface through to the stratum granulare of the fascia dentata. Each section was freeze-dried and weighed on a quartz fibre microbalance before extraction and analysis. Sections perpendicular to the layers and immediately adjacent to the tissue blocks were stained with eosin-methylene blue for comparison of histology with the cryostat sections used for analysis.

The results obtained for the hippocampus showed that the highest concentration of gangliosides were found in the stratum pyramidalis, stratum radiatum and stratum granulare. A striking feature of the ganglioside distribution was the wide variations in content even in adjacent sections. This was found particularly in the stratum radiatum.

Separate analyses of the ox hippocampus showed good agreement of general distribution but exact correspondence of ganglioside content at any particular level in the tissue blocks could not be made.

Sections through a folium of the ox cerebellum also showed wide differences in ganglioside content. The highest ganglioside levels were found in the molecular and granular layers. These results suggest that gangliosides are not uniformly distributed in neurones but occur in local concentrations which may represent variations in concentration of synaptic endings on cell bodies and dendrites.

To show clearly that the wide differences in ganglioside content even in adjacent sections was related to the cytoarchitecture of the tissue, a series of sections were cut through area CA2 of the ox hippocampus perpendicular to the layers. A marked reduction of the differences in ganglioside content of adjacent sections was found.

To obtain direct evidence that gangliosides are located specifically in neurones, neurones and glial clumps were isolated from the Deiters' nucleus of the ox by methods essentially similar to those of Hyden. Gangliosides were found in the isolated neurones and also in the neuropil immediately surrounding the neuronal cell body. When neurones and clumps of the surrounding glia were examined together, much lower ganglioside contents were found which indicated that gangliosides are not only in the neuronal and dendritic membrane, but also in axon terminals.

Finally, in the Appendix the results of ganglioside analysis of the brain and spleen of a case of Late Infantile Systemic Lipidosis are presented.

The following abbreviations are used throughout the text:

NANA	N-acetylneuraminic acid
GABA	gamma-aminobutyric acid
rf	retardation factor
DABA	3,5-diaminobenzoic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
M	molar (mole/liter)

The following abbreviations are used in diagramatic representation of polymers or sequences:

glc	glucose
gal	galactose
galNAc	N-acetylgalactosamine
NANA	N-acetylneuraminic acid
NGNA	N-glycolylneuraminic acid

Chapter I

REVIEW OF THE CHEMISTRY AND FUNCTION OF GANGLIOSIDES

1. Introduction

In 1939 Klenk (104) isolated a complex water soluble lipid from the brain of a child with neuronal lipid storage. This lipid had distinctly acid properties, and was found to give a characteristic purple colour with Bials' orcinol reagent. It was called "gangliosid" because it was thought to occur only in neurones. The chemistry of gangliosides has advanced considerably since Klenk's discovery of "substance X". Molecular structures were assigned to the ganglioside preparations on the erroneous assumption that they represented a relatively pure single substance. However, recent chromatographic techniques have shown that gangliosides consist of a mixture of components.

In the earlier literature the definition of the term "ganglioside" was largely dependent on the techniques of isolation and purification used in its preparation from the original tissue. These substances have been called strandin (47, 48, 50), mucolipids (190, 191), aminoglycolipids (13, 14, 15), and polygangliosides (109). The common feature of these ganglioside preparations was that they were water soluble acidic lipids which contained, besides N-acetylneuraminic acid (NANA), N-acetylgalactosamine, glucose, galactose and sphingosine fatty acid amide (ceramide). It is characteristic of ganglioside preparations that they are not only water soluble but also are non-dialyzable through cellophane membranes which readily permit diffusion of substances with molecular weights of 10,000 or below.

There are at present two concepts regarding the structures of gangliosides in water solutions. Bogoch (13), has postulated that a high molecular weight polymer of approximately 250,000 as determined in the ultracentrifuge, exists in aqueous solutions with a repeating unit composed of fatty acid,sphingosine, glucose, galactose, N-acetylgalactosamine and NANA. Most other workers (47, 239, 191, 112, 85, 54) have interpreted these results on the basis of a micellar rather than a polymer concept of ganglioside solutions. Molecular weights of 180,000 to 250,000 or more have been measured for gangliosides in aqueous solutions, (47, 239, 191), from which it has been concluded that gangliosides aggregate in aqueous media to form micelles as a result of the presence of both hydrophobic and hydrophilic groups in the molecules. This has been confirmed by the decrease in size of ganglioside units observed in non-aqueous or only partly aqueous solutions which is attributed to the dissociation of the micelles into individual molecules or small groups of molecules. For example, Klenk, Gielen and Padberg, (112) found for one preparation a molecular weight of about 1500 in dimethyl-formamide solution. Howard and Burton (85) found that the molecular weight of their beta-ganglioside as measured by vapour pressure depression in a number of different solvents was equivalent to 1665, corresponding to a structure containing the basic ganglioside structure and two NANA residues.

Gammack (54) using surface tension measurements found that the critical micellar concentration, that is the concentration at which the ganglioside molecules do not form micelles, was around 0.015%. Howard and Burton (85) obtained comparable results using vapour pressure

depression measurements. Whatever their original state within the tissue, the evidence is rather compelling that gangliosides as obtained by the usual extraction and fractionation procedures are monomers, with the capacity of forming micellar aggregates.

Chemical knowledge of the ganglioside monomeric forms has developed rapidly in recent years (130, 198, 228, 207, 162, 121, 255, 68, 21, 28). All gangliosides contain a sphingosine base in an amide linkage to a fatty acid, with various oligosaccharide chains glycosidicaly linked to the primary hydroxyl of the sphingosine. Stanacev and Chargaff (210, 211) found that the principal bases in gangliosides were sphingosine (2-amino-4-octadecene-1,3-diol) and icosisphingosine (2-amino-4-eicosene-1,3-diol). They found that bovine brain gangliosides contained 50% sphingosine, 46.5% icosisphingosine and 3.5% of the dihydrosphingosine. Rosenberg and Stern (193) have recently reported icosisphingosine to be absent in fetal brain gangliosides but it appears rapidly during development. Svennerholm (231) also found that sphingosine is predominant in fetal gangliosides but, with increasing age, icosisphingosine increases and constitutes about 60-70% of the total sphingosines in senile brain gangliosides. Sambasivarao and McCluer (200) studied the gangliosides of several mammalian species and found both bases present in all cases. There is some doubt about the general distribution of icosisphingosine in different species as Klenk and Gielen (110) found none in human brain. It is not present in other sphingolipids of the brain or in gangliosides outside the central nervous system.

In 1939 Klenk (104) demonstrated that stearic acid is the major fatty acid of brain gangliosides. More recently Klenk and Gielen (110) reported quantitative data for beef brain gangliosides in which the C18 fatty acid made up 94% of the total fatty acids. Other workers (231, 239,

300, 199, 101) have found that 80-90% of the fatty acids in brain gangliosides are stearic with minor amounts of palmitic and arachidic. Two striking features of the fatty acids of brain gangliosides are that there are no unsaturated fatty acids and no hydroxy fatty acids.

The nomenclature for the individual monomeric forms is generally based on the carbohydrate portion of the molecule. Bonding positions between the units of the oligosaccharide chain are usually studied by two well established techniques of carbohydrate chemistry: periodate oxidation and permethylation. The former has been widely used because of its mildness and specificity. In general, however, the earlier applications of permethylation to gangliosides were not very fruitful; part of the difficulty lying in the reaction itself and part in the fact that mixtures of gangliosides rather than pure compounds were studied. Due to improved methods of separation the latter problem has been greatly reduced. In recent years, much progress has been made with both the permethylation and periodate oxidation techniques (118, 119, 130). Whereas most workers determine the carbohydrate structure from the saccharides split off from the ceramide moiety, Svennerholm (224) has instead determined the carbohydrate sequence by analyzing the different ceramide-saccharides isolated from partial acid hydrolysis of the major normal brain gangliosides.

2. Methods of Extraction and Purification of Gangliosides

Gangliosides are extracted from tissue in combination with other lipid classes. A variety of organic solvents have been employed, the chloroform:methanol (2:1) system of Folch, et al (51) is used most commonly. However, when gangliosides are the only subject of investigation, many minor modifications of the original method have been used. Folch,

et al (47, 48) extracted all the tissue lipids with chloroform:methanol 2:1 (v/v) and from this extract the gangliosides were partitioned into an aqueous upper phase. The gangliosides isolated by this technique are slightly contaminated with polar lipids (228, 219). Booth (17) initially treated acetone-dried brain tissue with chloroform:methanol (1:1 v/v) to improve the yield of gangliosides: however, Svennerholm (227) found that chloroform:methanol 1:1 still resulted in incomplete extraction of the more polar gangliosides and recommended the extraction with a chloroform:methanol mixture having at least a 67% methanol. Suzuki (213) concluded from his study of the thin layer pattern of gangliosides that when a study is concerned only with gangliosides, double extraction (chloroform:methanol 2:1 v/v and then 1:2 v/v) or a single extraction with a chloroform-methanol mixture with higher than 60% methanol, followed by partition without salt, would insure maximal extraction of all gangliosides. Suzuki (213) also concluded that when the amount of tissue is limited and the analysis for other lipids, including proteolipids, has to be carried out on the same sample as in a cerebral biopsy, the original procedure of Folch, Lees, Sloane-Stanley (51) is an excellent compromise. This is particularly true in view of the fact that this method does not alter significantly the final ganglioside pattern from that obtained by the more complete extraction procedure.

Thus far the discussion of the composition and structure of gangliosides has avoided the problem of whether these substances as found in brain tissue are devoid of attached protein or peptide. Saifer (198) produced the results of an amino acid composition of normal and Tay-Sach's peptide-strandin, as obtained by automatic analysis with the ion exchange chromatographic system of Moore and Stein. Generally speaking his results resemble the amino acid analysis of the mucolipids by Rosenberg and

Chargaff (192) and also confirm the finding that in Tay-Sach's brain ganglioside there are only traces of amino acids present. Svennerholm (228) has suggested that ganglioside in their micellar form could act as protecting colloids for other lipids or water soluble compounds of lower molecular weight, such as amino acids and peptides. As another approach to this problem, Bernheimer and van Heyningen (9) have found that amino acids are not present in gangliosides extracted from acetone dehydrated brain. These findings were also confirmed by Gammack (54), Svennerholm (222) and Booth (17). The material of Folch and Lees (50) contained 18% amino acids. Klenk's (106) original procedures for isolation of brain gangliosides were based on a selective extraction by hot organic solvents. This kind of treatment would tend to denature any protein or peptide moiety associated with glycolipid, which would account for the fact that his preparations, after strong acid hydrolysis yield little or no amino acids. Many workers have used hot chloroform-methanol extraction of gangliosides from brain tissue whereas other investigators have favoured milder extraction conditions during which the temperature of the solvents was kept at or below room temperature as was originally proposed by Folch (47). The latter procedure almost invariably reveals the presence of a peptide moiety as an integral part of the ganglioside preparations which upon acid hydrolysis yield a variety of alpha amino acids.

A very important finding with respect to the extractibility of gangliosides is that of Spence and Wolfe (208), in which they demonstrated that after 30 minutes of extraction with chloroform:methanol (2:1) at 60°C, only 30% of the gangliosides from the tissue were removed if the monovalent cations had been previously removed by dialysis of the tissue. Restoration

of the ions to the tissue resulted in a recovery of 80% of the glycolipid NANA. The mechanism underlying the failure to extract gangliosides from ion deficient tissue is not known. Perhaps the removal of cations from the carboxyl group of the N-acetylneuraminic acid allows the gangliosides to strongly associate with basic proteins. It is interesting to note that the quantity of gangliosides extractible from tissue slices kept in cold or under anoxic conditions decreases markedly (151, 152). It has been postulated that this is due to a migration of the basic proteins (259). It is possible that the potassium or other cation changes in the slices could be accounted for in part by a loss of ganglioside extractibility.

Trams and Lauter (239) extracted gangliosides from fresh brain tissue by homogenizing with tetrahydrofuran containing phosphate buffer. Lipids were removed by ether extraction, and ionic substances including proteins, were removed by the passage of the aqueous phase through a mixed bed ion exchange column. The "crude" ganglioside preparation was further purified by precipitation as the barium salt and then regenerated with sulfuric acid followed by dialysis and rotary evaporation at room temperature. The yields appear to be somewhat low by this method particularly for the trisialoganglioside (258). Kuhn and Wiegandt (121) have extracted gangliosides from the brain tissue with phosphate buffer and phenol, but it is not possible to calculate the yields from the data (228).

There is still much to be desired in the clean separation of the individual ganglioside types. Column chromatography has been used for the separation of gangliosides from other lipids as well as the separation into individual ganglioside types. An isolation method which gives nearly quantitative recovery of gangliosides under mild conditions was introduced by Svennerholm (219). A total lipid extract is applied to a cellulose

power column and the bulk of the lipids other than gangliosides are eluted with chloroform containing small amounts of alcohol and water. The gangliosides are retained on the column and can be eluted with alcohol and water enriched solvents. This method was later applied to the quantitative isolation of brain gangliosides (196, 197) and recently for the isolation of trisialogangliosides (258). A still better separation of the gangliosides into mono, di and trisialogangliosides was obtained on paper roll columns with propanol water mixtures, Svennerholm (227). Other investigators have used different methods to purify the crude ganglioside preparations obtained either by the method of Klenk or Folch. Silicic acid column chromatography has been employed by Svennerholm to remove other lipid components from gangliosides. Kuhn and Wiegandt obtained pure fractions by using both silica gel and cellulose powder columns, the latter being eluted with butanol-pyridine-water. Penick, Meisler and McCluer (163) have used silicic acid (Anasil S) to obtain nine different gangliosides in quite pure form. When the purified gangliosides are spotted on thin layer chromatography in four solvent systems each runs as a single spot.

Thin layer chromatography has served as the fundamental criteria of heterogeneity of ganglioside preparations and as a guide for the isolation of the various molecular species. The total number of different gangliosides reported has now exceeded thirteen and it has become apparent that any single thin layer chromatographic system is not sufficient to distinguish or characterize all the various molecular species.

The thin layer chromatographic mobilities of nine human brain ganglioside preparations were studied in four commonly used solvent systems by Penick, et al, (163). These solvent systems were: chloroform:methanol: water (60:35:8, v/v/v); chloroform:methanol:2.5N ammonium hydroxide

(60:35:8, v/v/v); N-propanol-water (7:3 v/v) and N-propanol:concentrated ammonium hydroxide:water (6:2:1, v/v/v). They found that no thin layer chromatographic system is sufficient to clearly separate all of the components in a mixed ganglioside preparation but they found the chloroform-methanol-ammonium hydroxide system had the greatest resolving power as did Wherrett, Lowden and Wolfe (254). The data of Johnson and McCluer (96) and Penick, Meisler and McCluer (163) are in complete agreement with those published by Svennerholm (227) except for differences in nomenclature used and for the fact that Svennerholm's ganglioside preparation yielded only six fractions in sufficient quantities for analysis of their composition. The complex situation with respect to the heterogeneity of ganglioside preparations in chromatographic systems has been somewhat clarified as a result of recent publications by Johnson and McCluer (96); Svennerholm (182), Ledeen (130); Kuhn and Wiegandt (121); Penick, Meisler and McCluer (163) and Wiegandt (255).

Almost all workers until 1963 routinely used ascending thin layer chromatography to separate the brain gangliosides. Korey and Gonatas in 1963 (115) described a descending thin layer chromatographic system which was able to separate the gangliosides into seven components. To separate the gangliosides other workers such as Jatzkewitz (94, 95) have applied the technique of two successive solvents in which chloroform-methanol-water was followed by N-propanol-aqueous ammonia. Ledeen (130), in order to separate the gangliosides even more, has used double length silica gel plates (20 x 40 cm.) with chloroform:methanol:2.5 Normal ammonia as the solvent. He used two successive ascending runs of approximately seven hours each, with an hour's drying period in between.

The gangliosides can be visualized on thin layer chromatographic plates by spraying with specific reagents for sialic acid, such as orcinol, resorcinol (227, 253), or p-dimethylaminobenzaldehyde in hydrochloric acid

(121). The ganglioside plates can also be sprayed with a general lipid spray reagent such as bromothymolblue before, and perchloric acidmolybdate (247) after the specific sialic acid reagent. Charring after spraying with 50% sulphuric acid is equally sensitive, but it does not differentiate sialic acid containing lipid from other classes.

The wide application of gas liquid chromatography to the identification of the fatty acid methyl esters and the aldehyde forms of the long chain bases i.e., sphingosine and icosisphingosine has already been mentioned. Qualitative identification of carbohydrate however, following acid hydrolysis, is usually accomplished by paper chromatography. Recently, gas liquid chromatography has come to be recognized as a powerful tool in this area. The work of Sweeley and co-workers (233, 234) has shown the trimethylsilyl ethers to be highly suitable derivatives for both qualitative and quantitative determinations. Feldman and Feldman (44) applied this to identify the gangliosides found in lens. Penick and McCluer (162) claim an accuracy of 5-6% in the determinations of glucose and galactose using the trimethylsilyl ethers in the gas liquid chromatography. Paper chromatography has been used by some workers (25, 54, 242) but has been found to be generally unsatisfactory.

The Craig counter current distribution apparatus has been used by Svennerholm (222); Meltzer (153) for the isolation and separation of gangliosides. Results of the investigation by Meltzer who employed a three-phase counter current distribution technique indicated that strandin may consist of from 8-15 components depending on its degree of purity.

3. Classification of Gangliosides

The evidence is rather compelling that gangliosides as obtained by the usual extraction and fractionation procedures are monomers. A large number of discrete structures have been isolated and characterized, including four major gangliosides (Table I) and in a larger group of minor components from normal brain (Table 2). As the number continues to grow with new discoveries, the problem of single designation becomes increasingly confused. In Table I and 2 the designations by the different authors have been listed with each structural component that has been reported. Each investigator has been inclined to introduce his own system, and there are now at least eight currently in use in the literature and two authors at least have introduced two separate forms of symbol designation. There is a need for an international agreement on nomenclature to clarify the mounting confusion as new ganglioside types are found. The notation system of Korey and Gonatas (115) will be used in this thesis because of its simplicity and because a descending thin layer system has been used to separate gangliosides which is similar to that of Korey and Gonatas (115) and Suzuki (212, 213, 214, 215). Where another name or symbol has been used, the equivalent ganglioside, if it is known, from the Korey and Gonatas (115) system will be included in brackets.

Kuhn, Wiegandt and Egge (123) designated the four crystalline gangliosides isolated from beef brain as G_1, G_2, G_3 and G_4 (G_4, G_3, G_2 and G_1 of Korey and Gonatas respectively) in order of decreasing rf values on both paper and thin layer chromatograms. These four major gangliosides of normal brain are shown in Table I. They differ only in respect to the number and attachment sites of the NANA groups. The monosialo compound (G_4) is postulated as the basic structure unit for all four compounds.

TABLE 1

MAJOR GANGLIOSIDES OF BRAIN

NOMENCLATURE	REFERENCES
1. Major monosialoganglioside	
Sialylhexosaminyltrihexosylceramide	Penick, et al, 1966.
Ceramide-sialo-ganglio-N-tetrahexoside	Wiegandt, 1966.
$G_{GNT}1$	Wiegandt, 1966.
G_1	Kuhn & Wiegandt, 1964.
GM1	Svennerholm, 1964.
1-G	Johnson & McCluer, 1964.
1a	Tettamanti, et al, 1964.
A_2	Klenk & Gielen, 1963.
HG-1	Penick, et al, 1966.
G_4	Korey & Gonatas, 1964.

Ceramide (1←1)Glc(4←1,β)Gal(4←1,β)GalNAc(3←1,β)Gal
└(3←2)NANA

2. Disialoganglioside I	
Disialylhexosaminyltrihexosylceramide I	Penick, et al, 1966.
Ceramide-disialo-ganglio-N-tetrahexoside	Wiegandt, 1966.
$G_{GNT}2a$	Wiegandt, 1966.
G_{11} $G_{GNT}11$	Kuhn & Wiegandt, 1964, 1963.
GD1a	Svennerholm, 1964.
2G	Johnson & McCluer, 1964.
1b	Tettamanti, et al, 1964.
B1	Kuhn & Wiegandt, 1964, 1963.
HG-2	Penick, et al, 1966.
G_3	Korey & Gonatas, 1963.

Ceramide(1←1)Glc(4←1,β)Gal(4←1,β)GalNAc(3←1,β)Gal
└(3←2)NANA (3←2)NANA

TABLE 1 (Cont'd)

	NOMENCLATURE	REFERENCES
3.	Disialoganglioside II	
	Disialylhexosaminyltrihexosylceramide II	Penick, et al, 1966.
	G_{GNT}^{2b}	Wiegandt, 1966.
	G_{III}	Kuhn & Wiegandt, 1964, 1963.
	GD1b	Svennerholm, 1964.
	3G	Johnson & McCluer, 1964.
	IIIb	Tettamanti, et al, 1964.
	HG-4	Penick, et al, 1966.
	G_2	Korey & Gonatas, 1963.

Ceramide(1←1)Glc(4←1,β)Gal(4←1,β)GalNAc(3←1,β)Gal
└(3←2)NANA(8←2)NANA

4.	Trisialoganglioside I	
	Trisialylhexosaminyltrihexosylceramide I	Penick, et al, 1966.
	Ceramide-disialo-sialo-ganglio-N-tetrahexoside	Wiegandt, 1966.
	G_{GNT}^{3}	Wiegandt, 1966.
	G_{IV}	Kuhn & Wiegandt, 1964, 1963.
	GT1	Svennerholm, 1964.
	4-G	Johnson & McCluer, 1964.
	HG-5	Penick, et al, 1966.
	IIIe	Tettamanti, et al, 1964.
	G_1	Korey & Gonatas, 1963.

Ceramide(1←1)Glc(4←1,β)Gal(4←1,β)GalNAc(3←1,β)Gal(3←2)NANA
└(3←2)NANA(8←2)NANA

TABLE 2

MINOR GANGLIOSIDES OF BRAIN

	NOMENCLATURE	REFERENCES
1.	Tay-Sach's ganglioside	
	Sialylhexosaminyldihexosylceramide	Penick, et al, 1966.
	Ceramide-sialo-ganglio-N-trihexoside	Wiegandt, 1966.
	G_{GNTr11}[1]	Wiegandt, 1966.
	G_o, G_{GNTr11}	Kuhn & Wiegandt, 1964, 1963.
	GM2	Svennerholm, 1964.
	FM	Johnson & McCluer, 1964.
	HG-D	Penick, et al, 1966.
	A_1	Klenk & Gielen, 1963.
	G_5	Korey & Gonatas, 1963.

Ceramide(1←1)Glc(4←1,β)Gal(4←1,β)GalNAc
└(3←2)NANA

2.	Hematoside	
	Sialyldihexosylceramide	Penick, et al, 1966.
	Ceramide-sialo-lactoside	Wiegandt, 1966.
	G_{Lact}[1]	Wiegandt, 1966.
	G_{Lact}	Kuhn & Wiegandt, 1964.
	GM3	Svennerholm, 1964.
	G_6	Suzuki, 1964, 1965.
	HG-C	Penick, et al, 1966.
	G_6	Korey & Gonatas, 1963.

Ceramide(1←1)Glc(4←1,β)Gal(3←2)NANA

TABLE 2 (Cont'd)

	NOMENCLATURE	REFERENCES
3.	Disialyldihexosylceramide	Penick, et al, 1966.
	Ceramide-disialo-lactoside	Wiegandt, 1966.
	$G_{Lact}2$	Wiegandt, 1966.
	HG-1a	Penick, et al, 1966.
	G^1Lact	Kuhn & Wiegandt, 1964.
	G_{3a}	Ledeen, 1966.

Ceramide(1⟵1)Glc(4⟵1)Gal(3⟵2)NANA(8⟵2)NANA

4.	Second Trisialoganglioside	
	Trisialylhexosaminyltrihoxosylceramide II	Penick, et al, 1966.
	HG-6	Penick, et al, 1966.
	G_0	Korey & Gonatas, 1963.

Structure undetermined.

5.	Tetrasialoganglioside	
	GV	Kuhn & Wiegandt, 1964, 1963.
	IVb	Tettamanti, et al, 1964.

Structure undetermined.

6.	Tetrahexosedisialoganglioside	Tettamanti, et al, 1964.
	IVa	Tettamanti, et al, 1964.

Structure undetermined.

7.	Disialylhexosaminyldihexosylceramide	Penick, et al, 1966.
	Ceramide-N-triose-di-NANA	Spence, 1966.
	G^1_{GNTrII}	Kuhn & Wiegandt, 1964, 1963.

Ceramide(1⟵1)Glc(4⟵1)Gal(4⟵1)GalNAc

(3⟵2)NANA(8⟵2)NANA ?

Other NANA location not known .

TABLE 2 (Cont'd)

	NOMENCLATURE	REFERENCES
8.	Disialyltrihexosylceramide	Penick, et al, 1966.
	D	Klenk & Gielen, 1963.
	Ceramide(1←1)Gal(3←1)Gal(3←1)Gal	
	Location of the two NANA's unknown.	
9.	Sialylhexosylceramide	Penick, et al, 1966.
	G_{Gal}	Kuhn & Wiegandt, 1964.
	Ceramide(1←1)Gal(3←2)NANA	
10.	Three fast running gangliosides of unknown structure.	
	HG-A	Penick, et al, 1966.
	HG-B	Penick, et al, 1966.
	HG-E	Penick, et al, 1966.

The two major disialogangliosides (G_2, G_3) contain this unit plus an additional NANA attached to the terminal galactose in one case and to the first NANA (refer to Table I) in the other. So far only D-galactose has been found to be directly attached to the sialic acid in gangliosides (228). Brain gangliosides were at first generally considered to be complex glycolipids or aminoglycolipids of uniform chemical composition. Svennerholm (219) was the first to separate human gangliosides into two fractions with different carbohydrate composition by chromatography on cellulose. Further confirmation of the correctness of these structures for various human brain ganglioside fractions is furnished by the studies of the hydrolytic products formed during acid or enzymatic, i.e. neuraminidase (sialidase) action. The relationship between the products formed from the various normal ganglioside components when acted upon by sialidase was first postulated by Kuhn, et al (123, 120, 121) as follows:

$$G_3 \xrightarrow{\text{sialidase}} G_4 \xleftarrow{\text{sialidase}} G_2 \xleftarrow{\text{sialidase}} G_1$$

Sialic acid in brain gangliosides is attached to the two main positions: C-3 of galactose and C-8 of another sialic acid. Other attachment sites have been suggested (228, 198, 97, 85) but the evidence for these is not yet as convincing.

A phenomena which originally stimulated the search for different linkages was the repeated observation that treatment of total brain gangliosides with neuraminidase splits off approximately half the sialic acid. The resistance of the remainder was thought possibly due to a different bond type. Kuhn and Wiegandt (120) were the first to present evidence showing that the reason that neuraminidase acts differently on Tay-Sach's ganglioside and hematoside was due to steric factors. Tay-Sach's

ganglioside (see Table 2) has one NANA residue which is resistant to neuraminidase but the "hematoside" (see Table 2) derived from it by selective removal of the terminal N-acetylgalactosamine was quite susceptible to the enzyme. Part of the reason for the resistance to neuraminidase appears to be the presence of a large grouping at the four axial hydroxyl of galactose which hinders the approach of the enzyme to the adjacent NANA bond (132).

A list of the minor brain gangliosides so far reported in the literature together with the nomenclature and symbols used is presented in Table 2.

Generally speaking, the major gangliosides make up approximately 90% of the total brain gangliosides so that by definition a minor ganglioside would include any ganglioside in the remaining 10%. By far the most work has been done on the Tay-Sach's ganglioside (G_5) which was the ganglioside originally isolated by Klenk from the brain of a child with Tay-Sach's disease. Essentially the Tay-Sach's ganglioside (G_5) has the same structure as the major monosialoganglioside except that the terminal galactose which is attached to the N-acetylgalactosamine is not present (224, 131). The second most studied minor ganglioside is more commonly known as hematoside (G_6) and has been called by Kuhn, et al (119), G-lactose, and by Svennerholm, et al, GM_3 (227). Penick and McCluer (163) call it HG-C. This ganglioside makes up less than 1% of the total brain ganglioside sialic acid and is found generally speaking to be much more plentiful outside the central nervous system.

The other trisialoganglioside (G_0) described by Penick and McCluer (163), may have two sialic acids attached to the terminal galactose, and one to the central galactose but this has not been established. Kuhn and

Wiegandt (121) described a still more complex ganglioside containing four molecules of NANA which on mild acid hydrolysis converts to the trisialoganglioside but there is little information available and it may be a second trisialoganglioside. On the other hand, Tettamanti (238) described a ganglioside isolated from pig brain which on molar ratios appears to be a tetrasialoganglioside and they believed it to be identical with that described by Kuhn and Wiegandt (121). Kuhn and Wiegandt (122) have isolated another ganglioside related to the Tay-Sach's type, but possessing an additional sialic acid. The location of the additional NANA was not determined.

In the Korey and Gonatas (115) system it is thought that the G_{3a} is the same as hematoside with an additional NANA (130). Klenk and Gieland (109) have also isolated a trigalacto-disialoganglioside which showed unusual lability of its sialic acid bonds.

Table 3 gives the structures of the gangliosides that have been found outside the central nervous system and the source from which they have been found. Gangliosides have also been isolated from spleen (107) and from red blood cell stroma (260, 107). Whether the types of gangliosides found in the nervous system also occur in these sources cannot yet be determined. Klenk (107) obtained evidence for the occurrence of a ceramide-dihexsoside-sialic acid in the stroma of canine and equine erythrocytes (112). In the human spleen Svennerholm (225,226) saw that the predominant ganglioside had the same composition as the ganglioside in erythrocytes. He also has found that when the concentration of gangliosides is expressed in percentage of total lipids or glycolipids, placenta contains more gangliosides than all other organs. This ganglioside (G_6) found in placenta was the same as that found in the spleen. Gangliosides

TABLE 3

GANGLIOSIDES OUTSIDE THE CENTRAL NERVOUS SYSTEM

1.

Ceramide(1←1)Glc(4←1)Gal(3←2)NANA

Source:	Erythrocytes, canine	Klenk & Huer, 1960. Handa & Yamakawa, 1964.
	Spleen, human	Svennerholm, 1964.
	Placenta, human	Svennerholm, 1963.

2.

Ceramide(1←1)Glc(4←1)Gal(3←2)NGNA

N-glycolyl containing ganglioside.

Source:	Erythrocytes, equine	Klenk & Padberg, 1962. Yamakawa & Suzuki, 1951. Handa & Yamakawa, 1964.

3.

Ceramide(1←1)Glc(4←1)Gal(3←2)NGNA(8←2)NGNA

Di-glycolyl containing hematoside. Handa & Handa, 1965.

have also been isolated from lung (53) and from the human lens by Feldman and Feldman (44). The ganglioside of human lens was found to be very similar to the ganglioside found in the spleen (G_6). Gangliosides outside the central nervous system appear to be characterized by longer chain fatty acids. Lignoceric acid comprises about 75% in hematoside from horse erythrocyte (112), while that from canine erythrocytes contain C24 fatty acids. The species of gangliosides from horse erythrocytes contains N-glycolylneuraminic acid while those from canine erythrocytes and human spleen (70) contain N-acetylneuraminic acid (226, 230). Handa and Handa found that the hematoside isolated from cat erythrocytes contained two residues of N-glycolylneuraminic acid (69).

4. Methods Used to Determine Gangliosides

Elemental analyses are of limited value, as large admixtures of other glycolipids, will give only small deviations of the analytical results. Nitrogen determination can be valuable in the analyses of sphingosine in sphingolipids, but in gangliosides the nitrogen content is of small significance because the nitrogen can be derived from sphingosine, sialic acid, galactosamine and nitrogenous impurities (228). Gangliosides have been estimated by the quantitative analyses of one or more of their carbohydrate constituents. Of these the most logical choices are hexosamine, i.e., N-acetylgalactosamine and the sialic acid N-acetylneuraminic acid.

Bial's orcinal reaction was the earliest method used for the estimation of gangliosides (128, 250). Klenk (103, 104, 105) used the same method when he determined that subjects with Nieman-Pick's and Tay-Sach's disease had increased amounts of glycolipid of this type in the brain. Using Bial's orcinol reagent, Klenk and Langerbeins (106) developed

a method for the quantitative determination of sialic acids which has been modified by several workers (137, 221, 154). The molar extinction coefficient of the orcinol reaction has been reported to be 4,340 (251) and 5,895 (221). The discrepancy between the two figures is probably due to the difference in purity of the NANA preparation.

In 1957 Svennerholm (221) replaced the orcinol with resorcinol. He found that the molar extinction coefficient when calculated on the amyl alcohol phase was 9,500. He, therefore, stated that the sensitivity of the new method was 50% greater than that of the orcinol method. The reproducibility and specificity of the reaction was further increased by the extraction of the coloured material into butylacetate-butanol instead of amyl alcohol used in the original method (154), but there was no increase in the molar extinction coefficient. Warren (251) reported that the molar extinction coefficient of the resorcinol reaction of Svennerholm with N-acetylneuraminic acid was only 4,700.

Recently, Suzuki (212) has devised a method using Svennerholm's resorcinol technique with the modifications of Miettinen and Takki-Luukkainen (154) to determine sialic acid directly from silica gel removed from thin layer plates. In each instance, duplicate determinations of sialic acid content of the ganglioside mixtures were carried out to check the sialic acid recovery and in almost all instances it was between 96 and 100%. This is a fast and simple procedure to perform and applicable to very small samples of brain tissue.

In 1959 Warren (251) described the thiobarbituric acid assay as reproducible, sensitive, [molar extinction coefficient of 57,000 for N-acetylneuraminic acid (251) and 70,700 (3)] and considerably more specific

than the other methods. The thiobarbituratic acid method and alkali-Ehrlich methods (3) only measure unbound sialic acid. The molar extinction coefficient of the alkali-Ehrlich method is however, only 7,700 (3). Hence by carrying out the assay on hydrolyzed and unhydrolyzed samples, the levels of free, bound and total sialic acid in biological materials can be determined. The sialic acid content of gangliosides is usually determined by hydrolyzing the gangliosides with 0.1 normal sulfuric acid for two hours at 80° along with a standard ganglioside, and then by extrapolation determining the amount of ganglioside present in the unknown sample. The method has the disadvantage that when the individual ganglioside monomers are being determined for the sialic acid content the amount of sialic acid released from the different monomers is quite variable as has been mentioned previously. Whereas the orcinol and resorcinol reactions determine the total amount of sialic acid whether in the bound or free form.

Dische in 1930 (39) described the diphenylamine reaction for sialic acid. The molar extinction coefficient was reported to be 4,040 by Werner and Odin (252) and the same by Warren (251). Svennerholm (228) was unable to make the direct Ehrlich reaction to react stoichiometrically with the lipid bound sialic acid. Whereas Warren (251) found that the direct Ehrlich reagent gave a molar extinction coefficient of 2.030 for N-acetyl-neuraminic acid.

In 1964 Hess and Rolde (79) reported the development of a new fluorometric technique for the measurement of sialic acid in ganglioside. Since fluorometric methods usually possess a sensitivity 100-1000 fold that of spectrophotometric methods, it was possible now to measure the ganglioside content of samples containing less than 0.3 micrograms of sialic acid which is the limit of the colorometric reactions. The application of this technique to the analysis of gangliosides in small tissue sections of brain is the main topic of this thesis. The N-acetylneuraminic acid of

brain gangliosides is a 9-carbon alpha-keto acid, and such compounds readily undergo decarboxylation on treatment with hot mineral acids (252, 79). The desoxy-sugars and in general aldehydes of the type $R\text{-}CH_2\text{-}CHO$ will react with 3,5-diaminobenzoic acid when heated in mineral acids to yield highly fluorescent quinaldines (see Fig. 1). The method is based on the reaction of the sialic acid with the 3,5-diaminobenzoic acid in hot dilute HCl to yield a product with an intense green fluorescence. Bound and free sialic acids react equally as in the resorcinol reaction. Ox brain gangliosides of grey and white matter and whole rat brains were analyzed by the fluorometric technique and by microadaptions of the resorcinol HCl and thiobarbituric acid procedures (79). The three methods agreed closely as was also found by Booth (18).

Most methods of determining gangliosides, as has been shown above, have been colorometric. The NANA residue, most characteristic of this lipid class, has been the subject of most of these analytical procedures. In 1965 Kishimoto and Radin (101) described a new method for the determination of brain gangliosides measuring stearic acid, the chief fatty acid of gangliosides, in brain extracted by the Folch procedure. The method depends on the simple composition of ganglioside fatty acids. The values obtained with this technique agree well with the values obtained with the colorometric techniques by Lowden and Wolfe (141) and James and Fotherby (93).

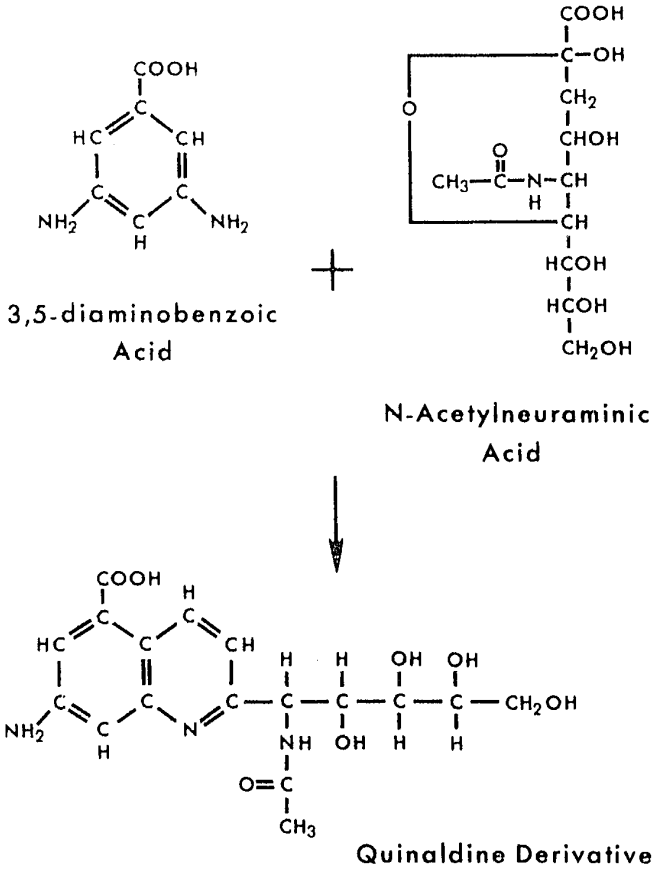
5. Location of Gangliosides

There have been three surveys of the ganglioside content of the different regions of the central nervous system (141, 214, 127). Lowden and Wolfe (141) measured the amount of glycolipid N-acetylneuraminic acid extracted with chloroform:methanol (2:1) in the different regions of the

FIGURE 1

Scheme proposed for chemical reaction of 3,5-diaminobenzoic acid reaction with N-acetylneuraminic acid to form quinaldine derivative.

Proposed Chemical Reaction of 3,5-diaminobenzoic Acid Reaction with N-Acetylneuraminic Acid



nervous system. Generally the results indicated that gangliosides are restricted to the regions of brain containing neurones. The values are high in the cerebral cortex, cerebellum, caudate nucleus and thalamus, while they are low in the centrum semiovale, corpus callosum and optic tracts. Gangliosides were not found in the optic or sciatic nerves or in the sympathetic chain. The presence of gangliosides in all layers of the cerebral cortex and the demonstration of greater amounts in the superficial layers suggested that the gangliosides may be concentrated in dendritic processes (141).

Suzuki (214) and Landolt, Hess and Thalheimer (127) found that the regional distribution of glycolipid NANA is in good agreement with the data found by Lowden and Wolfe (141). Suzuki found the various anatomical regions of the human brain revealed significantly different ganglioside patterns which are laterally symmetrical and consistent from one brain to another. As compared to the frontal region, the uncal area contains a higher amount of less polar gangliosides, whereas the visual and cerebellar cortices are much richer in trisialoganglioside. The thalamus and caudate nucleus show contrasting patterns in that the pattern of the thalamus is more polar than that of the other basal ganglia. Suzuki suggested in view of all the findings discussed and the preliminary observation that significant pattern differences existed among various animal species, that it was extremely important that animal species, age and the area of the brain, are specified whenever an analysis of ganglioside pattern is carried out.

The distribution of gangliosides in the different regions of the central nervous system strongly suggests that gangliosides are located specifically in the neurones of the central nervous system; either in the

cell bodies or dendrites, or both. It has been found (142) that the histological demonstration of the loss of neurones without a corresponding loss of glial cells was associated with a decrease in the amount of gangliosides that could be extracted from the cerebral cortex in pathological conditions, such as hypoxia and hypoglycemia. The absence of gangliosides from glial tumors as reported by Lowden and Wolfe (141) and Svennerholm (222), also support the neuronal localization. No antibodies to ganglioside were found in the sera from rabbits immunized with optic or sciatic nerve but were demonstrated in rabbits immunized with ganglioside extracts from cerebral cortex (205).

Svennerholm (229) found that the ganglioside patterns on thin layer chromatography of the sixteen fetal brains analyzed had a similar pattern to that in the cerebral cortex of a normal adult. All of the material was obtained by caesarean section. However, in all the brains of premature and full term babies born by normal delivery, the ganglioside pattern showed a big difference from that of the fetuses. The trisialo and disialoganglioside were markedly diminished. It was clear that G_3 is the predominant ganglioside of new born human brains. Most workers have found a linear increase in the total gangliosides extracted from rat brains between the day zero to 20 days (176, 93, 193, 194). Suzuki (214), however, found striking changes in the pattern on thin layer chromatography of G_1 and G_3; that is, the trisialoganglioside and the faster moving disialoganglioside respectively. At birth, the molar ratio of G_1 and G_3 is one. However, G_1 undergoes a rapid decline up until the 18th and 20th day and then gradually increases to the adult level in rat brain. Conversely, G_3 increases rapidly until 18 days and then comes down to adult level. Thus, a clearly inverse relationship between G_1 and G_3 exists. It is well known that the

total dendritic surface area increases between 10 and 20 days, the basal dendrites especially markedly increasing in the 10 to 20 day old period. Cell bodies stay about the same. These parameters were measured in rabbit brain cortex (152). From the foregoing facts, it is possible that gross analyses of the total gangliosides are hiding individual differences in the monomers occurring in regional and distributional studies. There may be anatomical structures which contain only gangliosides of certain types and the rapid increase in G_3 found in the developing rat brain by Suzuki represents the proliferation of certain very specific anatomical structures in the central nervous system.

Spence and Wolfe (209) found that a subcellular fraction enriched in ganglioside could be isolated from new born rat brains. On an electron microscopic examination this fraction consists almost entirely of membrane elements, possibly derived from the plasma membrane or the smooth surface of the endoplasmic reticulum. The results supported the hypothesis that gangliosides are localized in the neuronal membrane elements. Burton, Howard, Baer and Balfour (27) suggested a functional role for gangliosides in the transport of acetyl choline from the synaptic vesicles through the pre-synaptic membrane because gangliosides and bound acetyl choline are higher in the grey matter than in the white, and also the subcellular distribution of these two compounds appears to be parallel in rat brain. Norton and Autilio (158) suggested that the very small amounts of gangliosides found in their purified myelin fractions was due to contamination.

6. Possible Functions of Gangliosides in Brain

Gangliosides do not affect impulse transmission in the profused superior cervical ganglion of the cat, and injected intravenously, do not alter blood pressure in cats (16). Also, North and Dorrey (157) found all

preparations of gangliosides injected into experimental animals intravenously had no detectable ill affects.

McIlwain and co-workers (151) have found that the response to the electrical stimulation of cerebral slices was abolished when the tissues were kept in a cold media or when a basic protein, such as protamine, was added to the medium without cold incubation. Gangliosides were found to be extremely potent in blocking the inhibition of response produced by cold incubation or by added basic protein. The work of Wolfe and McIlwain (259) suggested that when cerebral slices were kept in the cold, nuclear histones migrated to the subcellular membranes which contained the gangliosides. McIlwain (151, 152) has suggested that these properties support a picture of ion transport in which the gangliosides are membrane constituents but are not mobile carriers, constituting rather a hydrophilic path for cationic movement through the lipid membrane. Protamine could inhibit cation transport in such a pore by combining with the acidic group at its mouth.

Many amino acids and other agents applied iontophoretically to cerebral and spinal motor neurones depolarize them (117). Hillman and Hyden (82) found that a ganglioside preparation had the effect of increasing cellular polarization of isolated neurones as it did in cerebral slices (80). The values obtained of the membrane potentials at room temperature then fell within the range found at 37° in cells identified by antidromic stimulation as pyramidal motor neurones.

In the central nervous system, membranes of adjacent cells in mammalian cerebral cortex remain at least 200 angstroms apart. It has been suggested that there is an outer coating or gap substance (45) which occurs as an envelope some 100 to 200 angstroms in thickness beyond the lipid membrane. In neurones, mucopolysaccharides or mucoproteins are among the

substances proposed as present beyond their outer surfaces. It is possible gangliosides are part of or connected to the gap substance. Hence it has been suggested by McIlwain on the basis of the work mentioned above that the gangliosides are involved in selective cation transport across cell membranes (151).

The regional distribution of gangliosides in the central nervous system closely parallels that of gama-aminobutyric acid (GABA). Both gangliosides and GABA occur only in very small amounts or not at all in peripheral nerve, sympathetic chain, optic nerve, pineal gland, corpus callosum and white matter in general. That gangliosides specifically combine with tetanus toxin has been amply substantiated (241, 242). Physiological studies show that the action of this toxin is at inhibitory synapses in the cerebral cortex and in the spinal cord (40, 22). So that there is a certain amount of evidence to suggest as Lowden and Wolfe have done in 1964 (141), that gangliosides might be specific membrane components involved in synaptic inhibition in the central nervous system.

Chapter II

QUANTITATIVE HISTOCHEMISTRY OF THE CENTRAL NERVOUS SYSTEM

The founder of quantitative histochemistry, Linderstrøm-Lang (136), said in 1939, "The elements of this procedure (quantitative histochemistry) are the removel of small pieces of tissue or cells with known size and well defined histological structure, and the investigation of these pieces by means of well known chemical methods modified for the specific purpose of estimating small amounts of chemical compounds in small amounts of material". In 1951 Holter and Linderstrøm-Lang (84) reviewed all the microchemical methods and their application to tissues and cells. Recent reviews of this new and exciting approach to the study of the central nervous system chemistry include Hyden (86), Krivánek (116), Robins (180), Sidman (206), Pope (170) and Lowry (145). The methods used in correlating chemical composition with the histological structure of the central nervous system fall into four main groups (167). Firstly, there is the classical microscopic histochemistry which has been reviewed by Sidman (206), in which the histological sections are prepared in such a way that the substance for analysis reacts with the chemical agent used, producing a stain whose activity can be studied by means of the microscope. Histochemistry produced preparations accurately defined histologically but their quantitative analysis is hindered by diffusion and other artifacts. Secondly, there are histophysical techniques which apply physical methods for quantitative analysis in preparations similar to those used in histochemistry. The most important of these techniques are ultra-violet microscopy and absorption histospectroscopy developed by Caspersson for

quantitative analysis of proteins and nucleic acids. The method is based on measuring absorption at those wave lengths characteristic to a given substance in situ in histological preparations. Thirdly, there is cytochemistry of subcellular particles which are separated and collected by high speed centrifugation methods. Finally, there are the methods of quantitative histochemistry with their application to the central nervous system.

1. The Laboratory of Lowry

Lowry in 1953 (144) followed the general approach of Linderstrøm-Lang (136) and Holter (84) in describing quantitative histochemical approaches to analysing different structures of the central nervous system. The tissue is quick frozen to keep ice crystals as small as possible, and then sectioned on a microtome inside a box held at a temperature of -20° centigrade or so. The sections are next dried at -40° centigrade under vacuum. One of the advantages to using the frozen dried material is that substrates or soluble materials do not diffuse after drying and even biologically unstable substrates, such as ATP, are not affected by reasonable periods of exposure to room temperature. The tissue slices during the freeze-drying are placed in holders which are made of drilled aluminum blocks sandwiched between glass slides. Although the freeze-dried tissue has not been stained or inbedded, it is possible, working under a microscope, to dissect out even parts of the cells such as nuclei.

The dissecting tools are of simple construction and some of these will be described more fully in the next chapter. The knives used to trim away the cytoplasm of single nerve cells in order to isolate nuclei from freeze-dried tissue are usually made of splinters of razor blades.

In order to determine the sample size, three methods have been suggested by Lowry (146, 144). These are protein content, the dry weight, or the sample volume. In 1953 Lowry (144) described a "fishpole" balance which was a modification of the original quartz torsion balance described by Lowry in 1944 (143). The balances used are made of fine quartz fibres. The smallest made to date is capable of weighing one red blood cell with an accuracy of 10% (145). The sample is loaded onto the tip of the fibre with a fine hair point, and the displacement is read through a micrometer ocular of a microscope or cathetometer. A wide field microscope is used to supervise the loading. At that time (1953) (144), it was suggested that to decrease static electrical effects a thin film of platinum should be placed on the inside of the glass tubing, and also ten to twenty gama of radium salt, suitably painted on a small piece of metal, should be placed inside. Since that time it has been found that the platinizing of the inside of the tube is unnecessary. The question arises whether the exceedingly porous tissue may not be so hygroscopic as to give a falsely high dry weight when weighed in room air. To check this point, Lowry (144) used freeze-dried brain sections weighed on a fishpole balance mounted in a vacuum desicator. After determining the weight <u>in vacuo</u>, air of varying degrees of humidity was admitted and the new weight measured. The samples reached each new weight within a few minutes and there was no subsequent drift. Hence, although frozen freeze-dried sections weighed in room air tend to absorb moisture and gases of the air, the increment in weight is consistent, and by making suitable allowance the true dry weight may be estimated to within two or three percent. Under the usual laboratory conditions, a 6% correction is appropriate. For a particular fishpole balance the useful range is not very great, so therefore, a series

of balances are needed. For samples weighing 0.1 micrograms or more, a pan of very thin glass is needed to hold the sample. Smaller samples will stick to the fibre tip without a pan. For a given fibre the sensitivity increases as the cube of the length, but there is a critical length beyond which sagging due to the weight of the fibre itself becomes excessive. Paradoxically, for fibres used at their critical lengths, the sensitivity varies inversely as the cube of the length. One way of estimating the critical length is to make the fibre long enough so that without any load the weight of the fibre itself will bend the tip a distance equal to about one-third of the fibre length (145).

Lowry (145) has described a new technique developed recently in order to measure substrates of reactions. It became necessary at the early stages of the analysis to work with volumes which were too small for regular micro test tubes. It was found possible to greatly reduce the scale of operations by working with minute droplets under oil in small holes drilled in a block of teflon. As many procedures as are necessary, including a cycling step, can be carried out in the original oil well by adding successive reagents at the proper time. Finally, the sample is easily transferred to a regular fluorometer tube and the fluorescence measured.

Lowry has chosen fluorometry as his method of chemical analysis because of its general applicability and high sensitivity. Almost every substance in living cells can be caused to either oxidize NADH or NADPH, or reduce NAD^+ or $NADP^+$. The reason that the pyridine nucleotides have been so useful as analytical tools is that NADPH and NADH are naturally fluorescent and $NADP^+$ and NAD^+ can be made highly fluorescent with strong alkali. The way these chemical properties may be put to use is illustrated by the following example of a two enzyme system:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

$$\text{glucose-6-P} + \text{NADP}^+ + \text{glucose-6-P} \xrightarrow[\text{dehydrogenase}]{} \text{6-P-gluconate} + \text{NADPH}$$

These two reactions can usually be combined into a single analytical step, and the NADPH formed becomes a measure of either the glucose, ATP, or hexokinase, depending upon which one of those components is omitted.

In the cycling process $NADP^+$ serves as a catalyst for a two-enzyme system. The reagent contains ammonia, alpha-ketoglutarate, and the enzyme glutamic dehydrogenase, which quickly oxidizes the NADPH to $NADP^+$ and forms glutamate. The reagent also contains glucose-6-P and its dehydrogenase, which quickly reduces the $NADP^+$ back to NADPH and forms 6-P-gluconate.

$$\text{NADPH} + \text{alpha-ketoglutarate} + NH_4 \xrightarrow[\text{dehydrogenase}]{\text{glutamic}} \text{glutamate} + NADP^+$$

$$\text{NADPH} + \text{6-P-gluconate} \xleftarrow[\text{dehydrogenase}]{\text{glucose-6-P}} \text{glucose-6-P} + NADP^+$$

Enough of each of the two enzymes is added so that each molecule of NADPH goes around the cycle as many as 10,000 times in 30 minutes. Therefore, for every $NADP^+$ molecule, 10,000 molecules of each product are formed. At this time the enzymes are inactivated with heat, and one of the products, 6-P-gluconate, is measured by adding 6-P-gluconate dehydrogenase and an excess of new $NADP^+$.

The overall result is that for each original molecule of NADPH there are now 10,000 molecules. If this is still not enough, any remaining excess of the added $NADP^+$ is destroyed as before with alkali and the NADPH is cycled with new reagent. This can result in another 10,000-fold multiplication. With double cycling, the sensitivity, at least in principle, is sufficient to measure one molecule of any enzyme of moderate turnover.

When dealing with small quantities of tissue and reagents it is necessary to introduce a number of modifications. So-called constriction pipettes are used. The lumen is narrowed to 0.1-0.2 mm. and this holds back the meniscus of the titrated liquid by surface tension, thus defining the volume. Calibration is either gravimetric (volumes over 20 µl) or colorimetric.

2. The Laboratory of Pope

For some years in Pope's laboratory (166, 167, 168, 169, 170, 172, 173, 175) the principles and methods of quantitative histochemistry have been used for studies on the chemical pathology of the nervous system. A particular effort has been made to describe the biochemical architecture of rat and human cerebral cortex, primarily because of its intrinsic neurological importance and the implications of its function for behaviour. The overall purpose of the work is to build up a body of information which would be helpful in assessing the possible metabolic or structural alterations of human cortex in certain nervous and mental diseases. A biochemical analysis at successive intra-cortical levels has provided quantitative information on the cortical chemoarchitectonics, and has provided a basis for the correlation between the chemical constituents analysed, and the characteristic stratification of its anatomical components.

In Pope's (166, 167) technique the brain is extracted after decapitation, frozen with dry ice and then warmed to -12° centigrade. Cylindrical pieces of cortex, usually somotosensory are taken, two millimeters in diameter and two millimeters high, from which sections 20 microns thick are prepared in a cryostat at -12° centigrade, cut in direction from pia to white matter. The first section is reserved for histological study by the Nissl method (structural control of chemical analyses). The second

section is used to analyse enzymatic activity. The third section is used as either an analytical control, or for parallel analyses of other enzyme activities. The fourth section is used for analysis of dry matter or proteins.

3. The Laboratories of Hyden and Edström

Hyden (86, 87, 88) in Gothenburg uses a stainless steel thread, 15 or 18 microns in diameter and sharpened to approximately two microns, to lift out nerve cells from the lateral vestibular nucleus (Deiter's) of the rabbit. Sections are prepared from an animal, usually rabbits, sacrificed by air embolism. Single cells are isolated by hand under a binocular microscope at a magnification of around 60 to 100 times. Usually a very small amount of methylene blue in the isotonic sucrose solution is applied to the cut surface for some seconds. The stain is taken up by the synapses, which are seen as a finely dotted border around the area occupied by the unstained nerve cell. The cell is removed before it takes up the dye, and transferred to a substrate or to the sucrose solution, where it is freed from the adhering glia by gentle manipulation. The oligodendrocytes closely surrounding the nerve cell will easily come off in the sucrose and adhere to each other, and the collection of glial cells may assume a spherical form. It can be easily trimmed to approximately the same volume as that of the nerve cell to which it belongs. The collection of glial cells is freed from parts of axons or dendrites. More will be said about contamination of glial clumps in later chapters. Nerve cells and glia isolated in this way are not vitally damaged, as it has been shown by Hyden that it is possible to culture them in his laboratory for periods up to three months.

The dry weight or the protein content of a cell is determined by means of x-ray microspectography at 8-12 A^{o}. In this wave length region the x-ray absorption is proportional to the amount of carbon, nitrogen and oxygen. The specimen, which can be fresh or dry nerve cells or samples of glia, is exposed on aluminum foil together with a reference system having the same wave length dependence as that of the biological material. A scanning and recording microphotometer is used to evaluate the x-radiograms. The instrument divides each cell into a maximum of 12,000 areas each of which is $3\mu^2$, and gives each area a figure from 0 to 99 depending on its absorption. The instrument also gives the integrated figure of all values recorded, that is the dry weight of the whole cell, dendrites included.

This method of isolation has now been extended to isolate fresh membranes from the nerve cell (33). In this technique about 10 nerve cells are rapidly isolated and placed on a glass slide in sucrose solution. A longitudinal cut is made with a stainless steel knife 20 to 30 microns long, along one of the dendrites of the nerve. The flaps of the cells are folded back, and the cell contents are removed partly mechanically and partly by directing a jet of solution onto it from a micro pipette.

Hillman and Hyden (82) have studied the physiological status of isolated neurones of Deiter's nucleus of the rabbit. They have been able to demonstrate resting potentials of 39 millivolts at 23^{o} centigrade or room temperature. These cells are studied at room temperature because first, at this temperature, they had membrane potentials which could be reversibly and significantly changed; second, they did not stick to glass at 37^{o} centigrade and so penetration was very difficult; third, a satisfactory system to prevent the medium around them from evaporating as they were warmed from 23^{o} centigrade to 37^{o} centigrade was not achieved;

fourth, they appeared swollen at 37° centigrade. A ganglioside preparation had the effect of increasing the cellular polarization as it did in cerebral slices (81). The values of the membrane potentials at 23° centigrade then fell within the range found at 37° centigrade in cells identified by antidromic stimulation as pyramidal motor neurones.

Edström (42, 43) described a method for the determination of RNA in μμg amounts. After reacting ribonuclease with the isolated cells or glial samples, the cells are extracted to remove the RNA. The extracted RNA is dried on a glass slide and then dissolved in a microdrop of glycerol-containing buffer. This microdrop, 100-200 microns in diameter, is optically homogeneous and contains all the RNA present in the original sample. It is photographed at 2600 A°, together with a reference system. The extinction at this wave length is evaluated photometically. By this method, amounts of RNA down to 20 μμg can be determined with an accuracy of ±5%.

Using the microelectrophoretic method of Edström (42, 43), it is possible to determine the composition of the RNA. After extracting the RNA from the sample it is hydrolysed with HCL. The purines and pyrimidines are then determined electrophoretically, using a microscopic cellulose fibre. The fibre is photographed at 2600 A°, and the variation of optical density along it is recorded with a microphotometer. From the recorded curves the purine and pyrimidine bases can be estimated as percentage of the total.

4. Brain Regions Studied

For the study of the biochemical properties of various types of, or parts of cells (axons, dendrites, somas) it is necessary to select a portion of the central nervous system which has a characteristic

laminated architecture. The cerebral cortex, Ammon's horn, cerebellar cortex, and the retina all meet these requirements. The biochemical characteristics of these various layers may be attributed to those elements which form the greatest part of each particular layer. The structures are reasonably easy to isolate and histological controls can be easily made. It must be remembered, however, that these preparations are not histologically homogeneous, and that the various layers do not contain one type of element only.

A. Cerebral Cortex

Pope and Hess (173, 172, 71, 74, 75, 76, 77, 78) have chosen this region because the details of its neuronal fine structure and the composition of its axonal and dendritic plexuses have been exhaustively determined by Lorente de Nó (139). Therefore, it is easier to establish correlations between the distribution of biochemical substances and histological composition.

Five main layers in the somotosensory cortex may be distinguished: (1) the plexiform layer, containing a network of tangential myelinated fibres; (2) a layer of the so-called small pyramidal cells; (3) the layer of medium pyramidal cells; (4) stellate or granulare cells; (5) the layer of large pyramidal cells in which the three sublayers can be distinguished according to the density of the cells. A systematic study of enzymatic activity and enzyme distribution in various layers of the cerebral cortex (somotosensory cortex of rats and man), both normal and pathological aspects, is being carried out by Pope, et al (167, 170).

Hess and Pope (76, 172, 173) have determined the distribution of cytochrome oxidase activity within the architectonic layers of human cerebral cortex in biopsy specimens removed during frontal lobotomy.

They concluded that the enzyme was localized chiefly in the cell bodies and dendrites of neurones and in axon terminals.

In studying the intralaminar distribution of dipeptidase activity in human frontal cortex (168, 173) the same workers found that there was a high rate of protein turnover in the brain of man. It was also concluded that the dipeptidase activity is present in all cortical species including neurones and the several types of glia.

A study of intracortical distribution of Ca-ATPase and Mg-ATPase showed a similar pattern for both human (77) and rat cortex (75). The Ca-ATPase activity appeared in the axons and dendrites of the cortex. The Mg-ATPase activity appeared to an important extent in neuronal somata, dendrites and axon terminals.

More recently Hess, et al (80, 73, 135, 126) described a quantitative microchemical scheme for the coordinated assay of gangliosides, RNA, DNA, cerebrosides, proteolipid proteins and residue proteins in microtome-prepared frozen and dried sections of brain. The results of anslyses of the intralaminar distribution of cerebrosides supported a myelin localization.

Robins, et al (183, 185, 184) have compared the distribution of certain substances and enzymatic activity in the visual and motor cortex of monkeys. They have shown that functionally different cortical layers differ in the distribution of proteins and lipid components. All the higher enzymatic activity found in the visual cortex indicated that the overall energy metabolism of the visual cortex is higher than that of the motor region.

B. Ammon's Horn

The horn of Ammon is in the hippocampus. The following layers are observed: 1. alveus which consists mostly of myelinated fibres or white matter; 2. the stratum oriens, which contains nonmyelinated axons and dendrites; 3. the stratum pyramidalis, in which all of the cell bodies are found in this layer; 4. the stratum radiatum, which consists of a dense layer of dendrites; 5. the stratum lacunosum, a mixture of myelinated fibres and dendrites; 6. the stratum moleculariis, containing the terminal arborizations of dendrites and axons and the blood vessels of the pia mater. The above is the histological identification of the stratification of Ammon's horn in the rabbit, as described by Lowry (147). There are certain histological differences between the hippocampus of the ox which was studied in the present work and that found in the rabbit. These differences will be described in greater detail in Chapter III.

Lowry, et al (147) analysed cryostat sections through the layers of Ammon's horn for dry weight protein, total lipids, four lipid fractions, five phosphorus fractions, six enzymes and riboflavin. They found that the cell bodies of the pyramidal cell layer contained low total lipid values. From the enzyme studies it was concluded that dendrites account quantitatively for the bulk of brain metabolism. Substantial quantities of lipids were associated with the dendrites but no conclusion was reached as to whether they were part of the dendrites or part of the neuropil.

C. Cerebellar Cortex

There are two main layers in the cerebellar cortex: 1. the molecular layer, consisting predominantly of dendrites of the Purkinje cells and axons of the granular cells from the granular layer; 2. the

granular layer, containing cell bodies. An outer granular layer can be observed in the animal before the 21st day of birth, when it disappears. Robins, et al (181) found that the granular cortex has the lowest lipid content of the three layers and this was attributed to the presence of densely packed nerve cell bodies. Non-phosphorus containing sphingolipids were low in concentration in the molecular layer. They were present at a somewhat higher concentration in the granular layer. It was suggested that gangliosides could account for the somewhat higher value in the granular layer.

The level of GABA, calculated in terms of dry matter, is highest in the molecular layer, lower in the granular layer and very low in white matter (83). Like the cerebral cortex, the cerebellar cortex shows a high activity of glycolytic enzymes and enzymes of the tricarboxylic acid cycle in the molecular layer which consists primarily of dendrites and axons (24).

D. Retina

The early work on the quantitative histochemistry of the retina was done by Anfinsen (4,5,6) in which many of the histochemical techniques were worked out. Layer one of the retina is the layer of pigment epithelium closely joining the choriod. The first set of neurons (rods and cones) consist of an outer layer (layer 2a) of photoreceptors and an inner layer (2b) of dendritic processes, an outer nuclear layer of closely packed cell bodies (layer 4) and finally layer 5a, composed of nonmyelinated axons. The first set of neurons terminate in layer 5b, synapsing with the dendrites of the bipolar neurons located in the inner nuclear layer (layer 6), together with cell bodies of Muller's glial cells. The bipolar cells synapse with a third set of neurones in the inner plexiform layer (layer 7). These are the ganglion cells, whose large bodies form layer 8; nonmyelinated fibres

(layer 9) connect these cells with the optic nerve. The retina is one of the few tissues of the mammallian organism in which symaptic structures are found in reasonably compact form. Anfinsen (4, 5, 6) found it possible in his studies to obtain sections of retina rich in synaptic material. This author was able to show that the enzyme cholinesterase, and the coenzyme, nicotinamide adenine dinucleotide phosphate (NADP) are far more abundant in the molecular layer than in the nuclear layers thus suggesting the localization of both enzymes in the synaptic junctions of the bipolar cells.

E. Analysis of Cellular Structure

For a review of the analysis of cellular structures see Robins (180). McDougal (150) in Lowry's laboratory has initiated a series of studies on the chemical characterization of well defined central tracts. The ten tracts studied were divided into four groups on the basis of their lipid content. These groups also coincided with the grouping on the basis of histological characteristics: a nonmyelinated tract very low in lipid, a group of lightly myelinated tracts low in lipid, a group of tracts with intermediate degree of myelination and intermediate in lipid content, and a group of heavily myelinated tracts containing much lipid. The degree of myelination and fibre size tend to vary together, so that more heavily myelinated fibres have the larger fibre diameter and vice versa.

F. The Analysis of Single Cells

The work of Hyden and Lowry on the analysis of single cells has already been mentioned. Others have used slightly different approaches to the isolation of uncontaminated neurones. Roots and Johnston (98, 99, 187, 188) homogenized the lateral vestibular nuclei of the ox and passed the homogenate in sucrose through a series of progressively smaller sieves.

They were able to concentrate the neurones to the point where it was much easier to pick them out of the solution by hand using small instruments. They say that by this method 300 to 600 neurones can be collected in three hours. No glia are obtained by this technique. The most notable feature of the isolated neurones was the absence of a plasma membrane over the greater part of the soma and dendrites. The typical electron micrographic image of a cell surface membrane was usually seen only where boutons were present. Organelles in the synapses were less well preserved but were still recognizable. The synaptic thickening could be seen, and structures corresponding to the mitochondria and synaptic vesicles were present in the presynaptic bag. No difference was observed between the hand dissected cells by the method of Hyden and those obtained by the method described by Roots and Johnston (99, 188). The interiors of the cells prepared in sucrose were slightly less well preserved than those cells prepared in Ringer-Locke's solution. There were no differences between cells prepared in 0.25 M. and 0.3 M. sucrose. The details of the appearance of these cells in comparison to cells isolated during the work on this thesis will be compared in Chapter VI. Roots and Johnston have been able to demonstrate the presence of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and cholesterol in isolated neurones. The major fatty acids were oleic, stearic and palmitic (99, 188).

Rose (189) used Ficoll to isolate 1.2 times 10^6 neuronal cells containing less than 12% glial contamination (on the basis of carbonic anhydrase). He found that they were able to prepare the neurones within three hours from the rat cortex and the technique described allows the use of up to 15 animals simultaneously. They have shown also that these cells will grow in tissue culture.

Chapter III

METHODS FOR THE ANALYSIS OF GANGLIOSIDES IN SERIAL CRYOSTAT SECTIONS OF BRAIN TISSUE

A. Preparation of Tissue

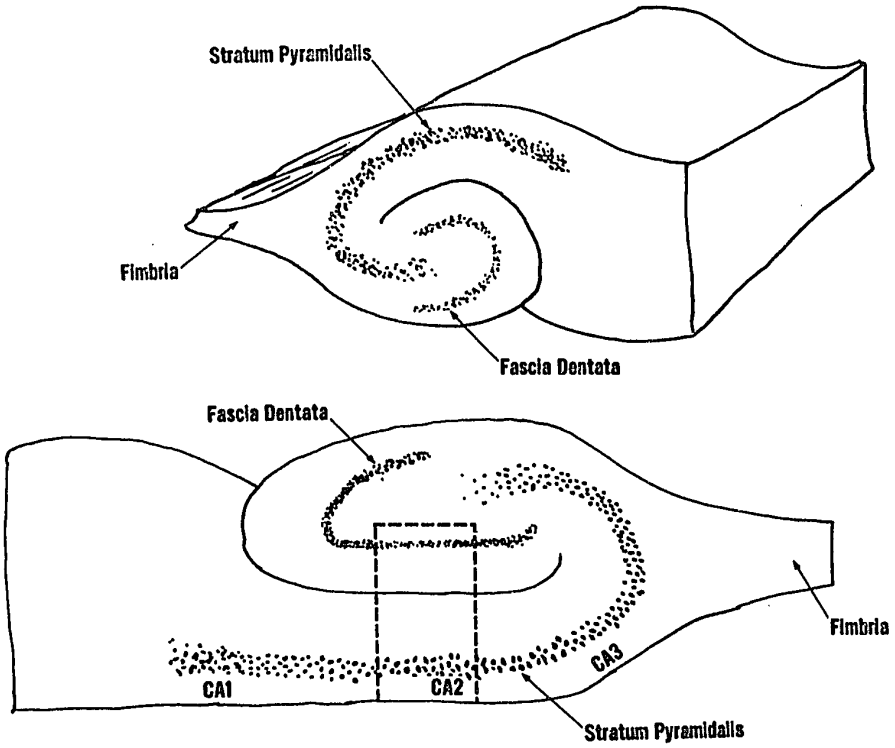
The reason the hippocampus and cerebellar cortex of the ox were chosen for analysis of total gangliosides content in serial cryostat sections was that in these brain regions the neurones and dendrites are arranged in a clearly stratified manner.

Fresh ox brains (kosher killed) were obtained at the slaughter-house and transported to the laboratory in plastic bags on crushed ice. The specific anatomical regions were dissected out immediately from the chilled unfrozen brain. The hippocampus including part of the fimbria and hippocampal gyrus was removed from each hemisphere. Two cuts, 2 cms apart, perpendicular to the long axis of the hippocampus were made (see Fig. 2). This piece of hippocampus including Ammon's horn and the fascia dentata was placed ependymal surface down on a moistened petri dish and adjusted so that all the layers of area CA2 had become flattened parallel to the ependymal surface (Fig. 2). The petri dish was then placed on a block of dry ice and the total tissue block frozen completely. The time for the total procedure was about one minute. With a Stadie blade, a small block of tissue two mm. wide, four mm. long, and 3.5 mm. in depth was cut out of area CA2. The longest dimension was parallel to the long axis of the hippocampus. Without thawing, the tissue block was then transferred with the ependymal surface facing upwards onto the platform of a Lipshaw microtome inside a Harris refrigerated chamber at -20° centigrade. Serial sections 24 microns thick were cut and placed in small aluminum trays as

FIGURE 2

Drawings of the gross appearance of the ox hippocampus. At A is shown the hippocampus after removal from the brain. The central portion is isolated by making two cuts about 2 cms apart. At B is shown the method by which the layers are flattened prior to freezing.

Preparation of Block for Analysis from the Hippocampus



described by Lowry (144, 145) and shown in Figure 3. The aluminum trays were transferred to a Virtis flask without allowing the sections to thaw and the sections rapidly freeze-dried. The freeze-dried sections were stored before weighing in an evacuated dessicator over sodium hydroxide.

In the hippocampus blocks of tissue immediately adjacent to the ones used for analysis on both sides were stained for general structure using Reid's eosin methylene blue method. This method is quick and because of the very short period of formalin fixation (2 minutes) no detectable shrinkage was found when compared with the block analysed. Direct comparison of the histological appearance of the tissue around the block used for chemical analyses was made on all occasions. A drawing of the microscopic appearance of a cross-section of the ox hippocampus is shown in Figure 4.

In Figure 5 is shown a drawing of the histological appearance of area CA2 of the ox hippocampus when stained with eosin and methylene blue and when stained with Cajal silver stains. The principal neuronal components of each layer are listed at the appropriate points.

In order to have a histological control of sections from the cerebellum, small blocks of tissue from each end of the block are analysed. When the microtome slices 10 μ thick were stained with eosin and methylene blue it was found that the distance from pial to pial surface exactly equaled the sum of all sections cut in the block analysed. Therefore little shrinkage of the block had taken place.

During the cutting of the sections for analysis in the cryostat some sections were found to fragment easily, especially in the layers containing no myelin. The most likely reason for this was due to the

FIGURE 3

Photograph of Lowry-type aluminum tray for holding tissue sections. Each slice is placed in one of the wells. Glass slides are clamped onto the top and bottom of the tray. The small space between the slide and the aluminum tray allows the sublimation of the water to take place.

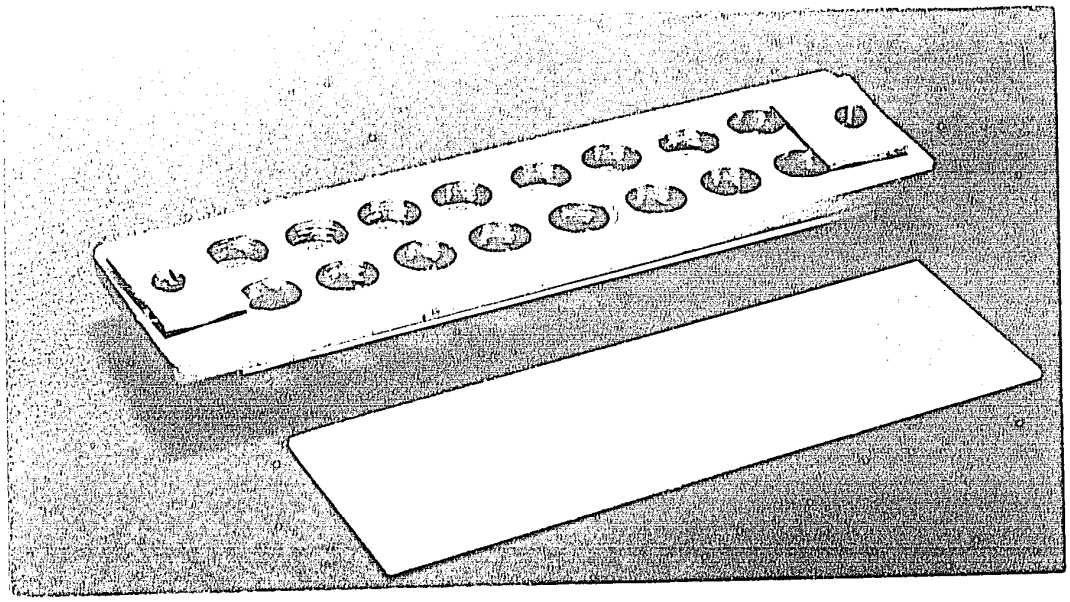


FIGURE 4

Drawing of cross-section of ox hippocampus showing the relationship of area CA2 to surrounding tissue. The principal neuronal components of the different areas are drawn to illustrate the orientation of the pyramidal cells of stratum pyramidalis and the granule cells of the fascia dentata. The axons of the granule cells are shown extending into area CA3 as the mossy fibres.

CROSS SECTION OF OX HIPPOCAMPUS

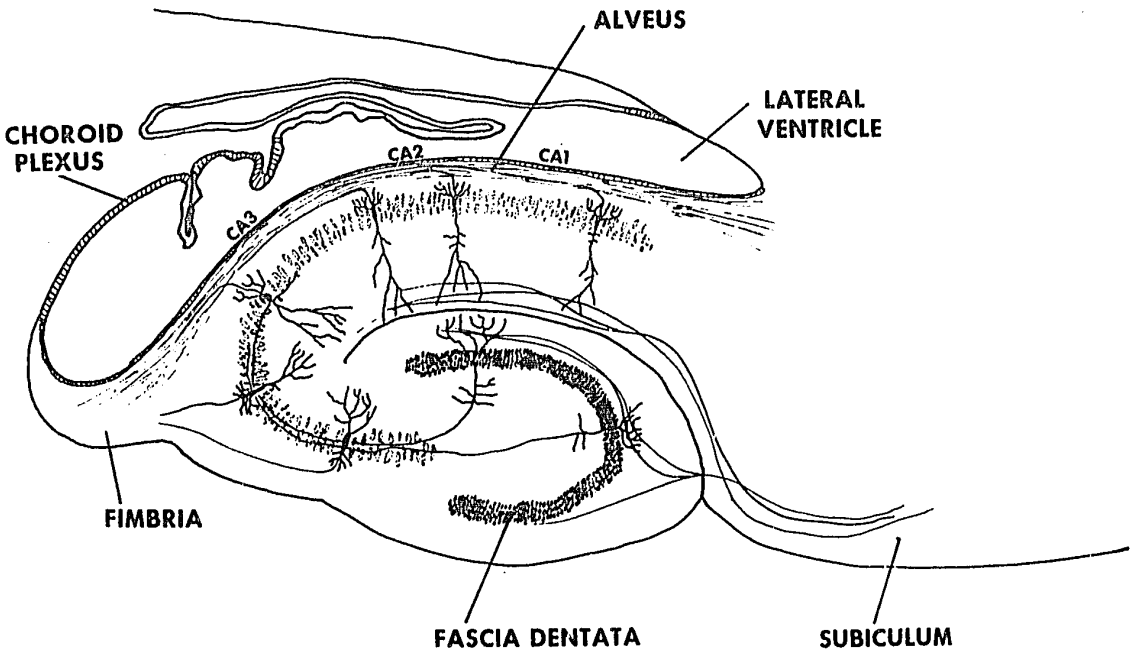
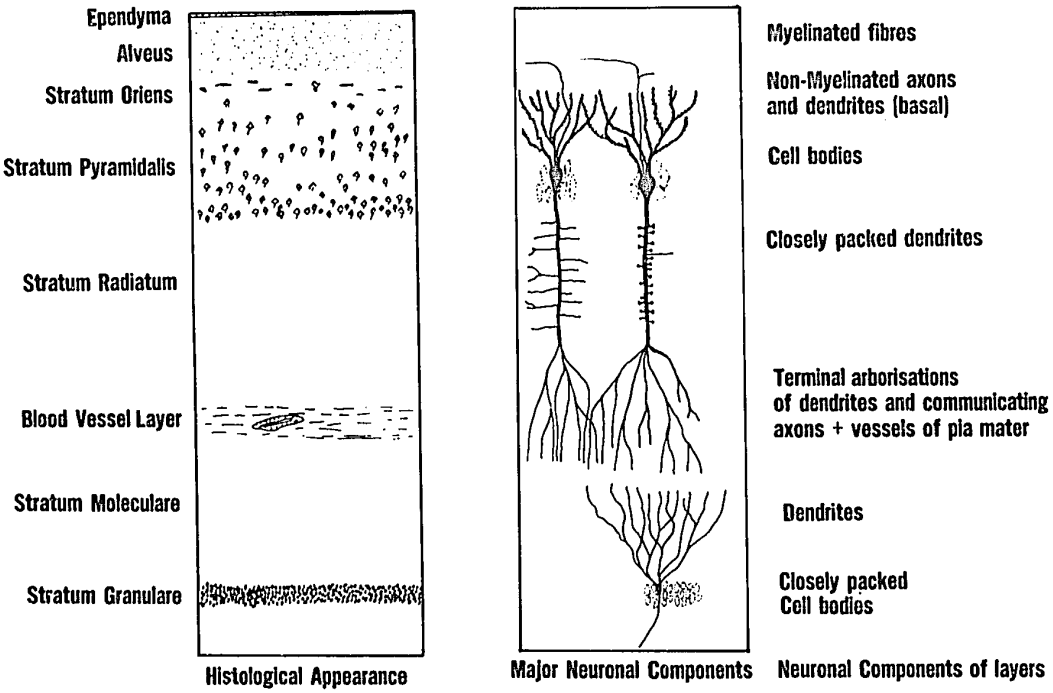


FIGURE 5

Drawing of histological appearance of area CA2 when stained with eosin and methylene blue and when silver stains of Cajal are used. The principal neuronal components of each layer are listed.

DIAGRAM OF AREA CA2 OF THE HIPPOCAMPUS (OX)



temperature of the chamber being too cold (144, 166). Therefore, in spite of the fact that slices were always cut 24 μ thick it was not always possible to obtain all of the slice for analysis.

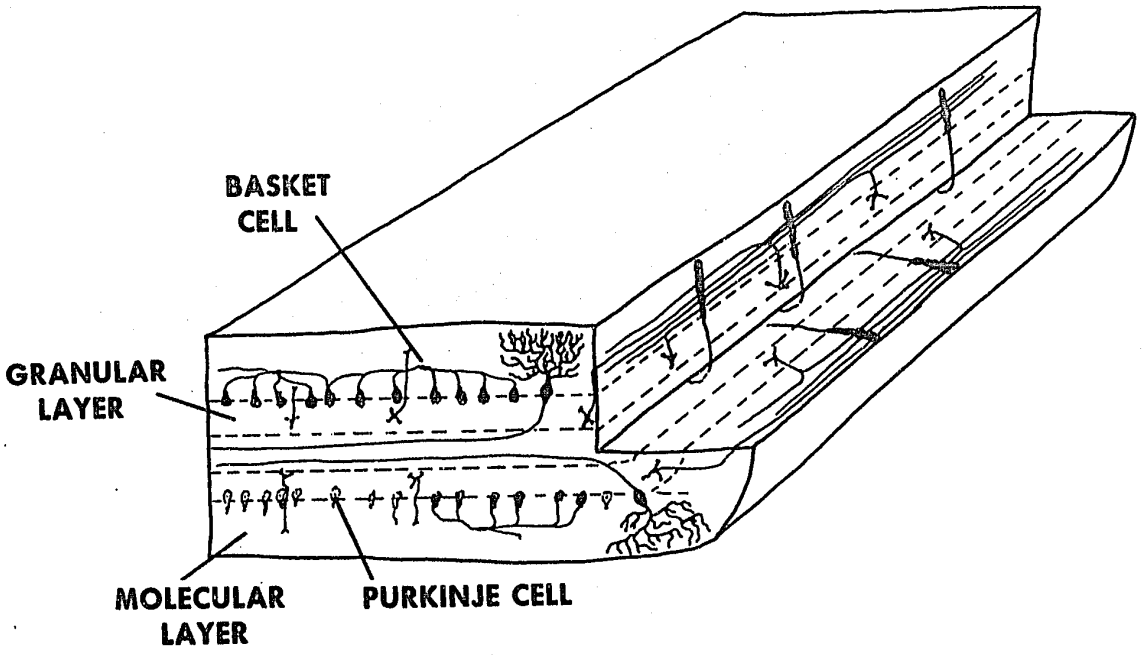
The ox cerebellum is unique in having in about 60% of brains examined one large folium (4 x 8 mm.) occurring bilaterally in an area adjacent to the vermis which is free of microfolia. This allows a flat block of tissue to be cut out of similar surface area to that obtained for the hippocampus. This block of tissue, frozen, was mounted with pial surfaces, top and bottom on the microtome chuck. Figure 6 shows a simplified diagram of the cerebellar folium and the manner in which the block was cut away from the folium, together with the major neuronal components.

At the beginning of this project an attempt was made to weigh the tissue slices on a Mettler semi-micro balance (stated sensitivity $\pm$ 5 μgm). Briefly this involved weighing a small glass tube made from a Pasteur pipette and washed thoroughly prior to placing the slice in the tube. The sections were then cut on a freezing microtome and the wet slice transferred by means of a small glass rod to a small amount of water in the bottom of the tubes. The water was then frozen and the contents of the tube freeze-dried. Finally, the tubes were weighed again and the difference represented the weight of the tissue slice. This procedure was not only cumbersome, but errors in the weighing could never be fully controlled. Approximately six complete analyses of the hippocampus were carried out in this fashion but because of the doubtfulness of the results due to the inaccuracy of the weighing, they are not given in this thesis.

FIGURE 6

Diagram of three dimensional relationship of different neuronal components in the different histological layers of an ox folium from the cerebellum. The molecular layer consists mainly of the branching dendrites of the Purkinje cells and the axons of the granule cells. The granular layer consists mainly of the cell bodies of the granule cells. The centrally placed white matter consists mainly of the myelinated axons.

SIMPLIFIED DIAGRAM OF CEREBELLAR FOLIUM



B. Weighing of Sections

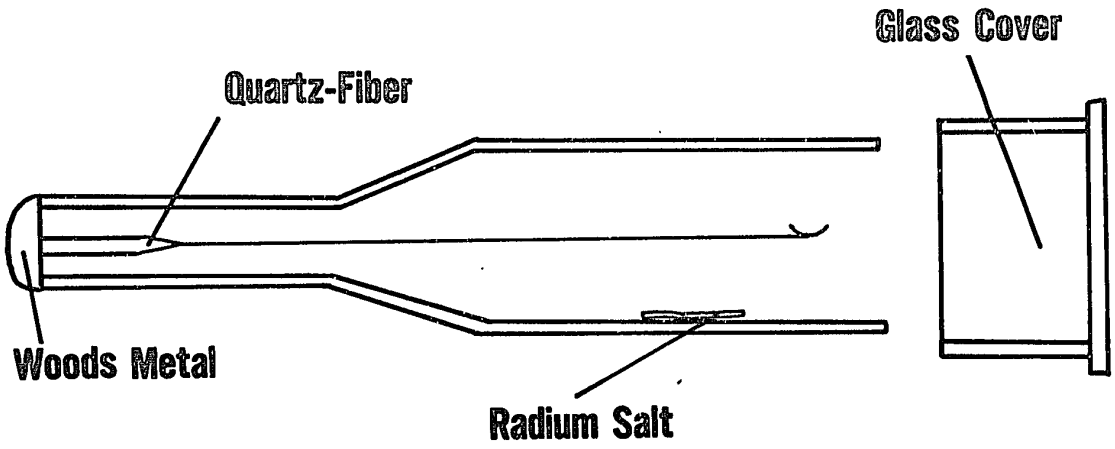
To eliminate the problem of inaccurate weighing a quartz fibre microbalance was constructed. Figure 7 shows a quartz fibre balance diagramatically drawn as constructed in the laboratory for the present study. The glass tube into which the quartz fibre was placed was built by Fisher Scientific. The quartz fibre (vitreosil) was drawn out to the required length and diameter for the particular sensitivity needed. A small piece of quartz tubing (1 mm. bore) about four cms. long was suspended by means of a clamp from a position about six feet off the ground. An oxygen-gas flame was then used to heat the quartz tubing at its midpoint until the quartz piece below the point of heating started to drop towards the floor of its own weight. The flame was then removed. It was found with practice that the desired length and thickness of the tubing could be achieved within five or six attempts. The diameter of the drawn fibre for the first quartz fibre (A) was approximately 15 microns.

As Lowry (144) has mentioned, when weighing slices of more than one microgram, it is necessary to have a small glass pan on the end of the fibre. This was constructed by first taking a Pasteur pipette, sealing the end and then heating it and blowing a bubble until a spectrum of colors could be seen on the bubble. A small piece was cut out with iridectomy scissors and placed onto the end of the quartz fibre at the spot at which some DeKhotinsky cement had been placed. A small amount of heat was applied to the glass side of the pan which weighs less than five micrograms, and this fixed it to the end of the fibre. The other end of the fibre was placed in melted woods metal and upon cooling the woods metal served as a firm base for the quartz fibre. Radium salt painted on a piece of wood

FIGURE 7

Diagram of principal parts of quartz fibre microbalance. Quartz fibre is imbedded in woods metal. Small piece of wood painted with radium salt is placed in chamber to dissipate electrostatic charges.

QUARTZ-FIBER BALANCE



was placed in the chamber to dissipate electrostatic charges. Figure 8 shows the standard line obtained with the quartz fibre balance. The range that could be measured on this particular balance was between 10 and 120 micrograms. The quartz fibre deflections were viewed through an American Optical dissecting microscope with a 2x objective and a 5x eyepiece which contained a graduated microdisc.(see Fig. 9). Readings could be made to the nearest 1 μgm.

For purposes of calibrating the balance a standard line was obtained for trypan red in four ccs of water measured at 505 mμ in the Beckman spectrophotometer (Fig. 10). Small crystals of trypan red were placed on the pan and the deflections as measured in divisions of the microdisc was noted. The crystals were then transferred to 4 ccs of water in a cuvette. The amount of trypan red that was on the balance was easily obtained from the standard curve.

Each section which had been freeze-dried was placed on the quartz fibre balance and its weight calculated from the standard line. Depending upon whether the slice was from white matter or cortex a correction was made for the humidity of the room at the time of weighing using the humidity-weight curves of Lowry (144).

After the slice was weighed it was transferred directly to a microtube (5 mm. x 6 cms.) without any loss of tissue prior to extraction.

C. Extraction of Gangliosides

The gangliosides were extracted from the tissue sections with 0.5 mls of chloroform:methanol:water (16:8:1) (v/v/v) at 60° for one hour in sealed microtubes. The microtubes were opened and the gangliosides partitioned three times into an aqueous upper phase according to the

FIGURE 8

Standard line obtained for quartz fibre microbalance (A). Range of usefulness 10-120 µgms. Plotted as micrograms dry weight versus divisions on microdisc with an accuracy of ± 1 µgm.

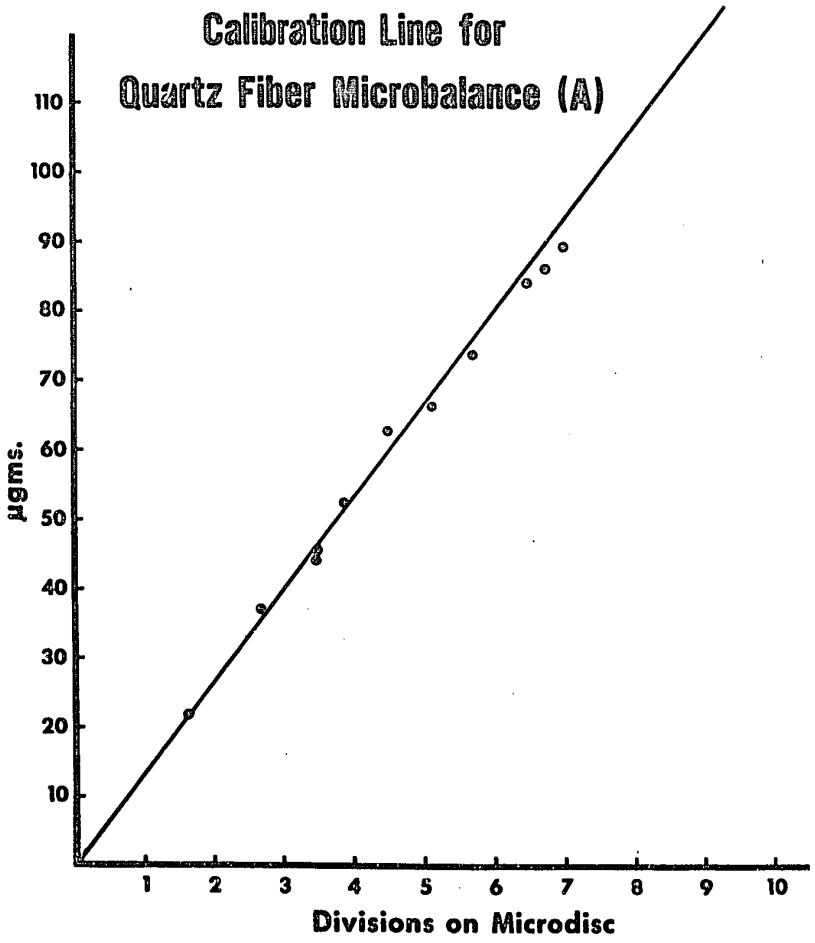

Calibration Line for
Quartz Fiber Microbalance (A)
µgms.
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
Divisions on Microdisc

FIGURE 9

Photograph of dissecting microscope in relationship to quartz fibre microbalance. Glass tube housing the quartz fibre is set in a specially build holder.

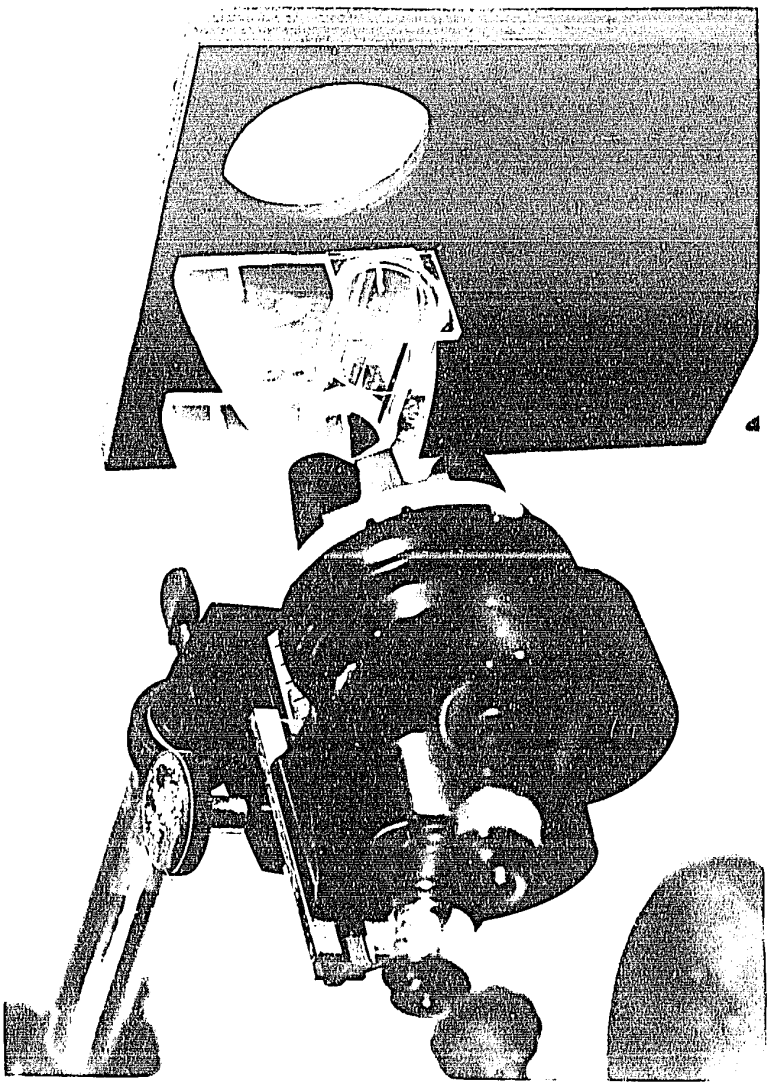
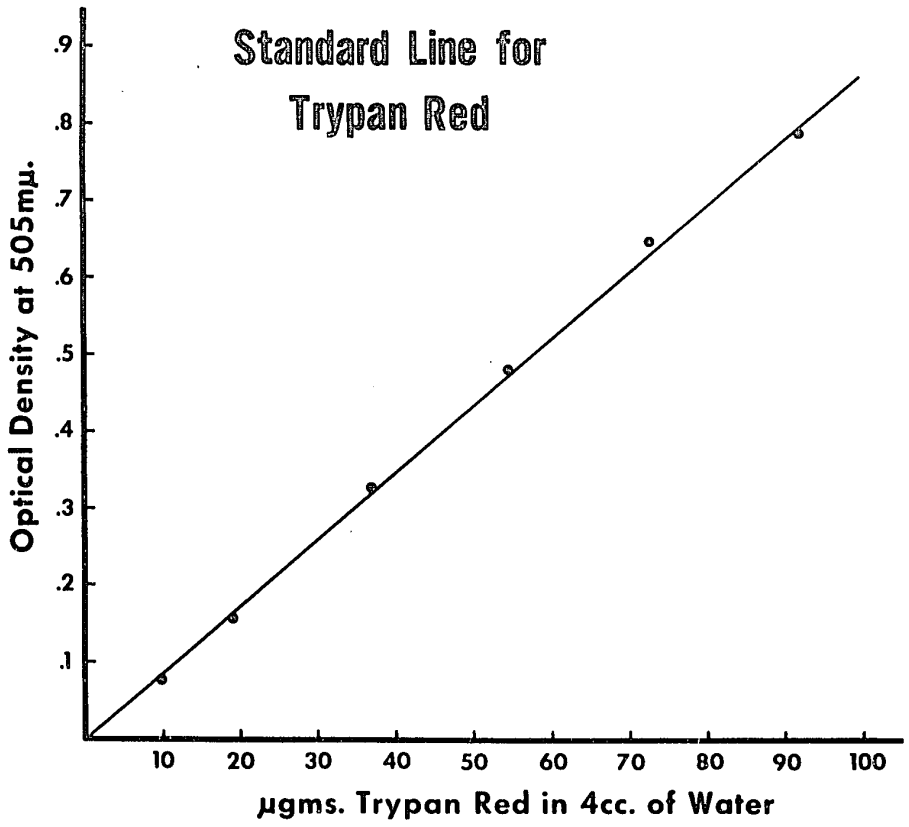


FIGURE 10

Standard line obtained for trypan red in 4 cc of water when read in Beckman Spectrophotometer at 505 mμ. Optical density versus μgms of trypan red in 4 cc of water.


Standard Line for
Trypan Red
Optical Density at 505mμ.
.9
.8
.7
.6
.5
.4
.3
.2
.1
10
20
30
40
50
60
70
80
90
100
μgms. Trypan Red in 4cc. of Water

procedure of Folch, Lees and Sloane-Stanley (51). All of the additions and removals of upper phase were performed with a 100 microliter Hamilton syringe. The combined aqueous phases were placed in a second series of microtubes in a special tube rack, and were dried in a partially evacuated desiccator over sodium-hydroxide. Figure 11 shows a picture of the microtube rack on the left. The buzzer used for mixing is shown in the center. On the right is shown a desiccator containing the microtube rack with microtubes in it. When the volume of the upper phases had been reduced to one-half, full evacuation was applied and the aqueous upper phases taken to dryness.

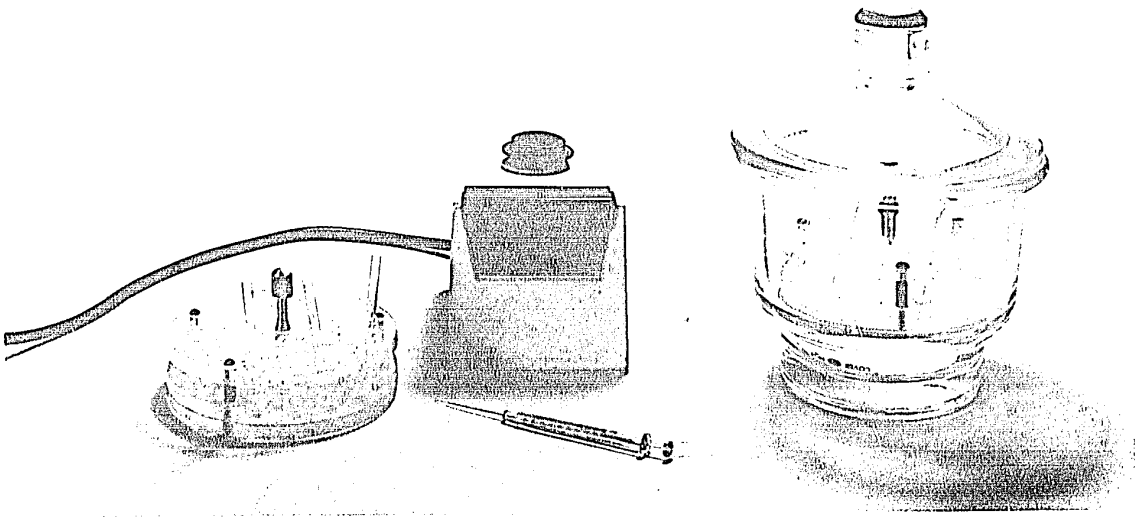
D. Fluorometric Analysis of Gangliosides

Hess and Rolde in 1964 (79) described a technique for the fluorometric method for measuring N-acetylneuraminic acid in free or bound form. It was found that the 2-desoxy sugars and in general aldehydes of the type $R\text{-}CH_2\text{-}CHO$ will react with 3,5-diaminobenzoic acid when heated in mineral acids to yield highly fluorescent quinaldines. A standard line was constructed for the reaction of 3,5-diaminobenzoic acid with purified unfractionated ox ganglioside preparation isolated by the procedure of Trams and Lauter (239). The ganglioside contained 28.5% N-acetylneuraminic acid as determined by the resorcinol method of Svennerholm (221), as modified by Miettinen and Takki-Luukkianen (154). It contained no protein, less than 0.1% phosphorus and no free N-acetylneuraminic acid. Neutral glycolipids were not detected on thin layer chromatography.

The fluorometric technique of Hess and Rolde (79) was followed exactly. A solution of 3,5-diaminobenzoic acid hydrochloride (Aldrich Chemical Company), 0.005 molar, and 0.125 N HCL was made up fresh before each set of analyses according to the method of Hess and Rolde (79).

FIGURE 11

Instruments and equipment used for extraction of gangliosides. On the left is microtube holder. In center, Hamilton syringe and buzzer. On right desiccator with microtube rack in it.



One hundred microliters of this solution was added with a Lamda pipette (microchemical specialties) to each of the microtubes containing the gangliosides in the dried upper phases, buzzed, and the tubes sealed and heated for 16 hours at 100° centigrade. The tubes prior to reopening were centrifuged and then buzzed to avoid condensation errors. Fifty microliters of the reaction solution was diluted in fluorometer tubes with 4 mls of 0.15 N HCL. The tubes were again buzzed and read in a Turner fluorometer, model 110, with the high sensitivity conversion unit (#110-865) attached and interference filters transmitting at 435.8 millimicrons in the primary and 525 millimicrons in the secondary.

The standard line obtained from many separate sets of determinations is shown in Figure 12. The standard error of the estimate for the best calculated line was ± 27 mμgms of ganglioside. However, measurements on the tissue sections were made between 400 and 800 mμgms of ganglioside, a range in which the error of measurements was less than 5%.

Microtubes were made from Pasteur pipettes purchased from Fisher Scientific. Prior to making the microtubes they were cleaned by rinsing 16 times with distilled water, placed in boiling 4 N HCL for one-half hour, rinsed 16 times with distilled water, soaked in acetone and finally the acetone was removed by air suction and heating the tubes in a flame (79). This procedure was found to give consistent low blank values.

The fluorescent spectra of the products formed between the reaction of the standard ganglioside and the 3,5-diaminobenzoic acid (DABA) when heated in 0.125 N HCL for 16 hours at 100° is shown in Figure 13. The spectra was measured in an Aminco Bowman fluorescent spectrophotometer. The activating wave length in the region of 320 mμ which might be due to

FIGURE 12

Standard line obtained in 3,5-diaminobenzoic acid reaction with purified ganglioside. Fluorescent divisions on 10x scale of Turner fluorometer versus millimicrograms of ganglioside. Standard error of the estimate was $\pm$ 27 mμgms.

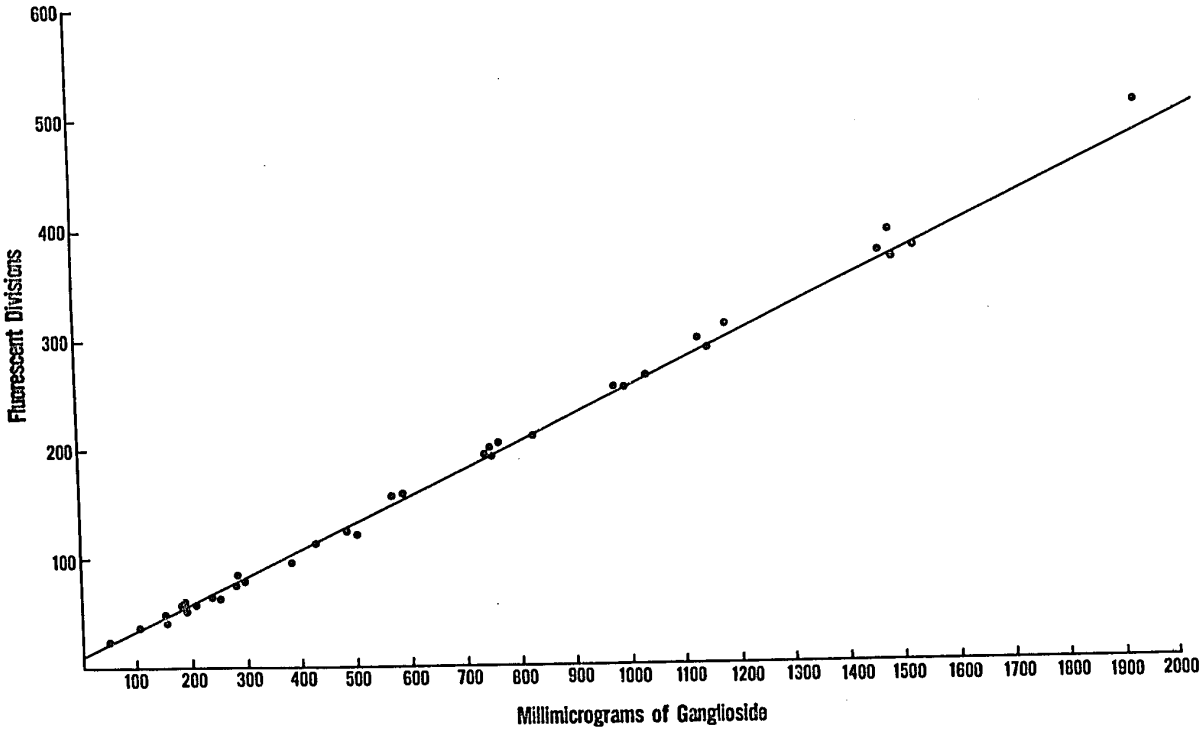
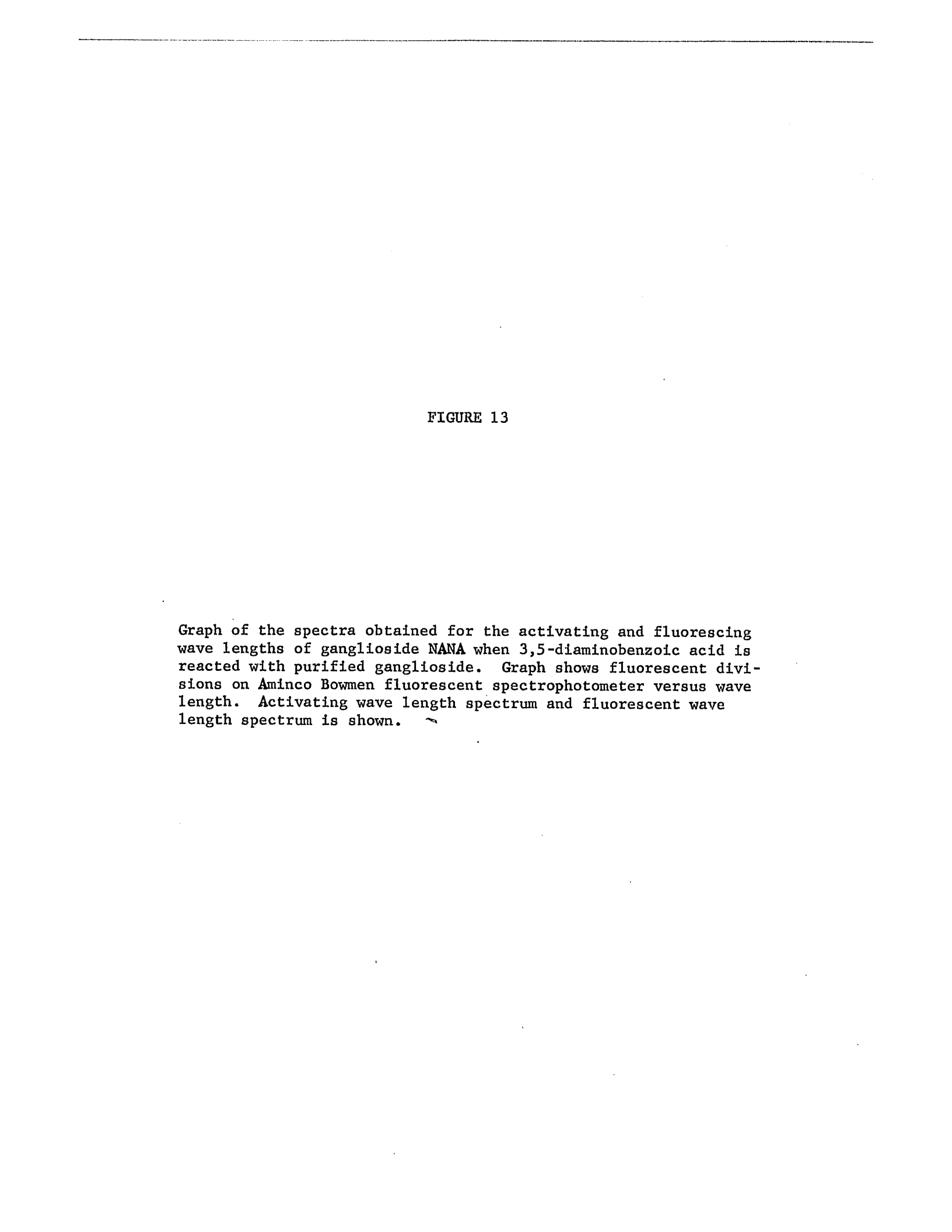
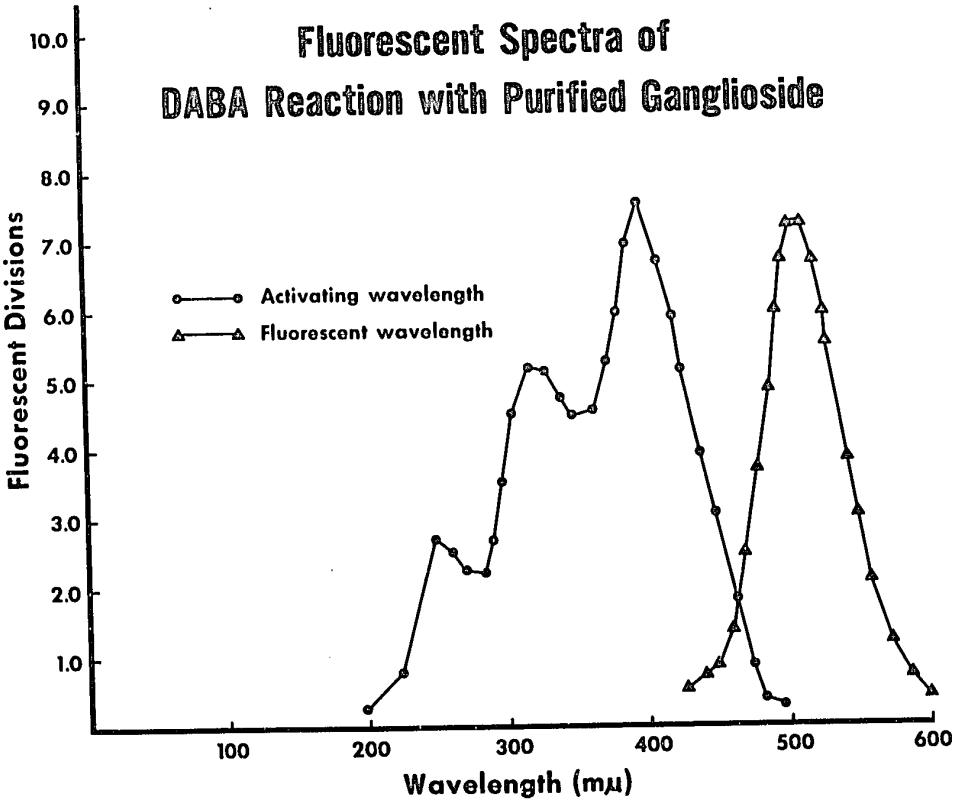

600
500
400
300
200
100
Fluorescent Divisions
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
Millimicrograms of Ganglioside

FIGURE 13



Graph of the spectra obtained for the activating and fluorescing wave lengths of ganglioside NANA when 3,5-diaminobenzoic acid is reacted with purified ganglioside. Graph shows fluorescent divisions on Aminco Bowmen fluorescent spectrophotometer versus wave length. Activating wave length spectrum and fluorescent wave length spectrum is shown.


Fluorescent Spectra of
DABA Reaction with Purified Ganglioside
Activating wavelength
Fluorescent wavelength
Fluorescent Divisions
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
100
200
300
400
500
600
Wavelength (mμ)

the interference from hexsoses mentioned by Hess and Rolde (79). The wave lengths at which maximum activation and maximum fluorescence occurs correspond exactly to those of Hess and Rolde (79).

Hence, gangliosides which had been extracted from each slice were reacted with the DABA reagent and the ganglioside present in the tube could be calculated. From this the ganglioside content of each slice in mgs of ganglioside per gram dry weight was calculated. Each complete run for one block of tissue usually took approximately four weeks.

Chapter IV

GANGLIOSIDE ANALYSES OF SERIAL CRYOSTAT SECTIONS

A. Sections from Area CA2 of Ox Hippocampus Cut Parallel to the Layers

In the ox hippocampus it was possible to cut blocks of tissue 2 x 4 mms in cross-section through to the stratum granulare of the fascia dentata, without any significant mixture of the laminae caused by curvature of the layers. A total of 130 to 150 consecutive 24 microns thick frozen sections were cut through the hippocampus, starting from the ependymal surface of the lateral ventrical in area CA2.

In Figure 14 are shown the results of ganglioside analyses expressed as mg ganglioside per gram dry weight in each of the sections. A diagram of the histological appearance of sections immediately adjacent to the block analysed and perpendicular to the layers is shown after staining with eosin and methylene blue. The graphs are arranged to show the exact histological depth at which each analysis was carried out. In the center are shown the results of one complete experiment on one ox brain and on the right is shown a second experiment on a second ox brain.

In Table 4 are presented the same results when analysed for their ganglioside content in each of the histologically defined layers. The results of experiments 1 and 2 (Fig. 14) were combined and mean and range for the ganglioside contents of sections from each histological layer of the hippocampus calculated. For each layer, the approximate thickness, the number of sections and the major neuronal components are given.

It is seen that there are marked differences in the ganglioside content of sections through the various zones. Even adjacent sections can differ widely in ganglioside content. The mean ganglioside content

FIGURE 14

Results of two experiments on ganglioside analyses of serial cryostat sections through area CA2 of ox hippocampus. Histological appearance of a section cut perpendicular to the layers from a block adjacent to the block analysed when stained with eosin and methylene blue is shown. Names of histologically defined layers are given at the appropriate points. The ganglioside content of each individual slice at the respective depths is plotted in terms of mgs ganglioside per gram dry weight.

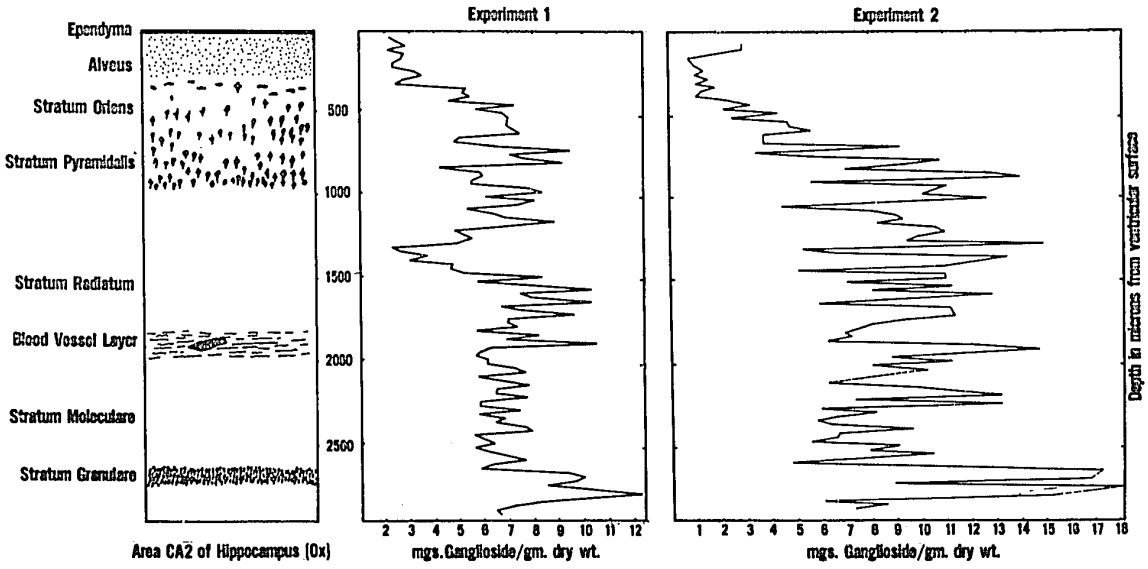

Ependyma
Alveus
Stratum Oriens
Stratum Pyramidalis
Stratum Radiatum
Blood Vessel Layer
Stratum Moleculare
Stratum Granulare
Area CA2 of Hippocampus (Ox)
Experiment 1
500
1000
1500
2000
2500
2 3 4 5 6 7 8 9 10 11 12
mgs.Ganglioside/gm. dry wt.
Experiment 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
mgs. Ganglioside/gm. dry wt.
Depth in microns from vestibular surface

TABLE 4

GANGLIOSIDE CONTENTS IN THE MAJOR ZONES OF THE OX HIPPOCAMPUS

Layer	Approximate Thickness μ	No. of Sections	Ganglioside Content mg/g dry wt. mean	range	Major Neuronal Components
Alveus	350	14	2.23	0.82-3.57	Myelinated axons of pyramidal cells.
Stratum oriens	200	8	5.06	2.60-7.20	Unmyelinated axons, basal dendrites, some pyramidal cells.
Stratum pyramidalis	400	16	7.72	3.56-14.10	Pyramidal cells and basal dendrites.
Stratum radiatum					
1st part	250	10	8.15	4.81-14.90	Major stems of pyramidal cell apical dendrites crossed by myelinated fibres.
2nd part	200	9	5.38	2.31-11.30	
3rd part	450	17	8.82	5.64-14.70	Terminal arborizations of apical dendrites and major synapses with communicating axons.
Stratum moleculare	675	28	7.32	5.57-13.20	Arborizations of apical dendrites of cells of stratum granulare.
Stratum granulare and part of polimorph layer	150	7	12.54	8.22-18.20	Closely packed granular cell bodies and dendrites.

The results of experiments 1 and 2 (Fig. 14) were combined and mean and range for the ganglioside contents of sections from each histological layer of the hippocampus calculated.

of sections through the entire tissue block of experiments 1 and 2 from ependyma to stratum granulare was 6.28 and 7.90 mgs ganglioside per gram dry weight, respectively. The range of ganglioside content in individual sections of experiment 1 was 2.03-11.85 and experiment 2, 0.82-18.20 (see Table 4). Expressed as glycolipid N-acetylneuraminic acid, the overall mean value for the two experiments was 1.98 mgs per gram dry weight (assuming a N-acetylneuraminic acid content of 26% for purified unfractionated gangliosides). Kishimoto and Radin (101) obtained from rat brain a value of 1.13 μmoles of ganglioside per gram wet weight using a gas chromatographic technique to determine brain gangliosides by the determination of ganglioside stearic acid. Lowden and Wolfe (142) used the Bial's orcinol reaction for NANA and obtained a value of 1.1 $\pm$ 0.2 μmoles ganglioside per gram wet weight for rat brain. The mean value for the two experiments on the ox hippocampus was 0.95 μmoles ganglioside per gram wet weight. The slightly lower value obtained in the overall hippocampus could be due to the contribution of the lower values obtained in the alveus (white matter). It can be noted that in all of these cases a Folch procedure was used for extraction of the ganglioside. The higher values for ganglioside content of brain cortex obtained by Suzuki (214) and Svennerholm (221, 222) have now been shown to be due to a prior extraction of the brain tissue with chloroform:methanol (1:2) followed by another extraction with chloroform:methanol (2:1) (214). It has been found that there is a greater extraction of the more polar gangliosides.

Table 4 and Figure 14 indicate that the alveus of both experiments and part of the stratum radiatum of experiment 1 have the lowest ganglioside contents. In the ox hippocampus the stratum oriens and stratum

pyramidalis are not clearly defined. A gradual increase in the ganglioside content in the sections was found extending from the alveus into the stratum oriens and stratum pyramidalis. In experiment 1 there was a marked fall in ganglioside content to values which were the same as the alveus (white matter) in a region of the stratum radiatum. This was not found in experiment 2 and a possible reason for this will be given in the discussion. In order to more clearly define this particular area the stratum radiatum was divided into three parts. The third part of the stratum radiatum, which contains the dendritic arborizations, showed some of the highest values for ganglioside content. In both experiments high values (average 7.32 mg ganglioside per gram dry weight) were seen in the stratum moleculare. The highest average ganglioside contents of all were found in the stratum granulare of the fascia dentata. The values obtained were found to be consistently high through six consecutive sections of the stratum granulare. It is seen that there is some overlapping of the high values obtained which extend into the polimorph layer of the fascia dentata. This could be due to the slices being cut on a plane which was not exactly parallel to the layer and hence spreading the peak values over more slices. If this were so then depending on how exactly the knife blade was orientated with respect to the granular layer the ganglioside content could be even higher in some slices. This could partially account for the fact that the values in the granular layer of experiment 1 are not as high as the values of experiment 2.

The most striking finding in both experiments is the difference in ganglioside content that is seen even in adjacent sections. This phenomenon is more clearly seen in experiment 2. Also, except in the stratum granulare, there is no correspondence of the peak values obtained

in different layers at the same depth in the two experiments. In both experiments high ganglioside values occurred in the stratum pyramidalis and stratum granulare where the nerve cell bodies are located. In both experiments there were high average values for ganglioside content in the third part of the stratum radiatum, a region in which the dendritic arborizations are maximal.

B. Sections from a Folium of an Ox Cerebellum Cut Parallel to the Layers

In the folium of the ox cerebellum, the neuronal components histologically appear to be symmetrically placed around the centrally located white matter. Hence the ganglioside content of slices at each depth from the pial surface should be the same or nearly the same.

Eighty-three, 24 microns thick frozen sections were made from pial to pial surface, parallel to the cellular zones in a folium of an ox cerebellum (see method section) and ganglioside analyses made in a similar fashion to those in the hippocampus. In Figure 15 is shown the ganglioside content in mgs of ganglioside per gram dry weight of each section at each depth. The histological appearance of the section cut perpendicular to the layers when stained with eosin and methylene blue is shown on the left.

Table 5 shows the mean ganglioside content of the different histological layers as well as the range of values found. For each layer, the approximate thickness, the number of sections cut, and the major neuronal components are shown. The results show a nearly symmetrical pattern of ganglioside distribution through the folium but there are still differences in the precise levels of the high values. Again wide variations in ganglioside content in consecutive sections were found,

FIGURE 15

Ganglioside analyses of serial cryostat sections cut parallel to the layers of a single ox folium of the cerebellum. The histological appearance of a section cut perpendicular to the layers from a block immediately adjacent to the block analysed when stained with eosin and methylene blue is shown. The names of the histologically defined layers are placed at the appropriate places. Results of the ganglioside content of each section at each depth are presented as mgs ganglioside per gram dry weight.

SERIAL SECTIONS THROUGH CEREBELLAR FOLIA

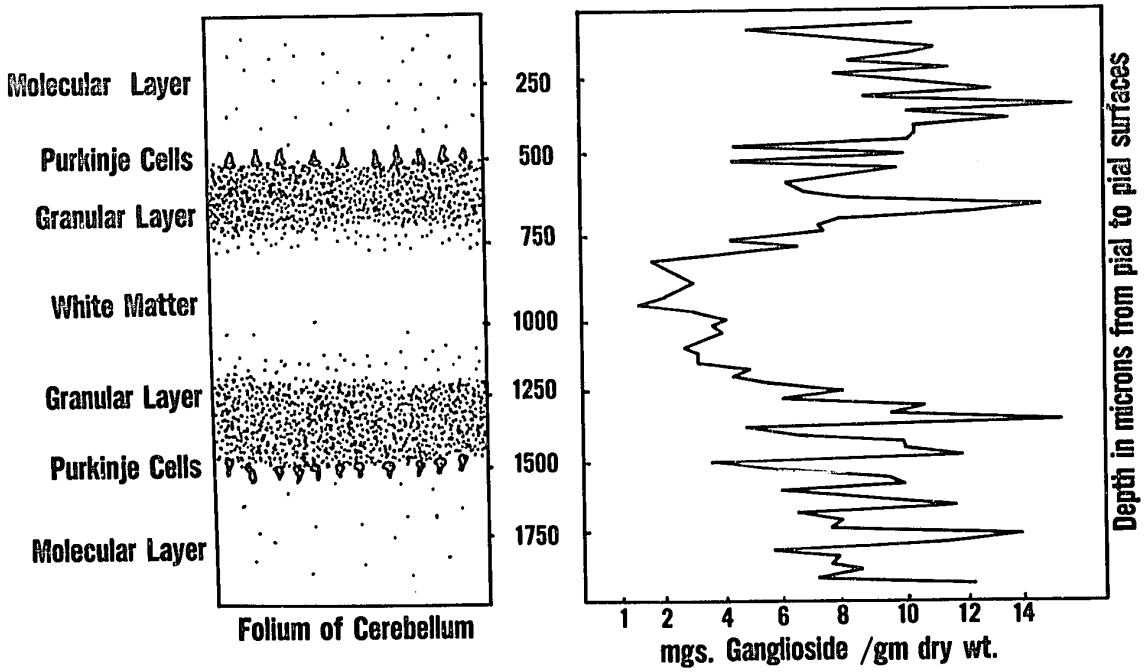


TABLE 5

GANGLIOSIDE CONTENT OF VARIOUS ZONES OF THE OX CEREBELLUM

Layer	Approximate Thickness μ	No. of Sections	Ganglioside Content mg/g dry wt. mean	range	Major Neuronal Components
Molecular	450	18	10.00	5.1-15.1	Apical dendrites of Purkinje cells, axons of granular cells, basket cells, climbing fibres.
Purkinje cell	75	3	6.43	4.71-10.00	Purkinje cell bodies, axon collaterals of basket cells.
Granular	300	12	8.20	4.65-14.1	Granule cell bodies and dendrites, mossy fibres, axons of Purkinje cells, climbing fibres.
Cerebral white matter	450	18	3.50	1.66-5.20	Efferent and afferent myelinated and unmyelinated fibres.
Granular	300	12	8.80	5.05-14.7	as above
Purkinje cell	50	2	3.91	-	" "
Molecular	450	18	8.7	5.70-13.4	" "

particularly in the molecular and granular layers. The centrally placed white matter contained low ganglioside values as did the narrow zone corresponding to the Purkinje cell bodies. The highest values in ganglioside content corresponded to the regions of dendritic branching of the Purkinje cells and the cell bodies of the granular layer.

C. Sections from Area CA2 of the Ox Hippocampus with Sections Cut Perpendicular to the Layers

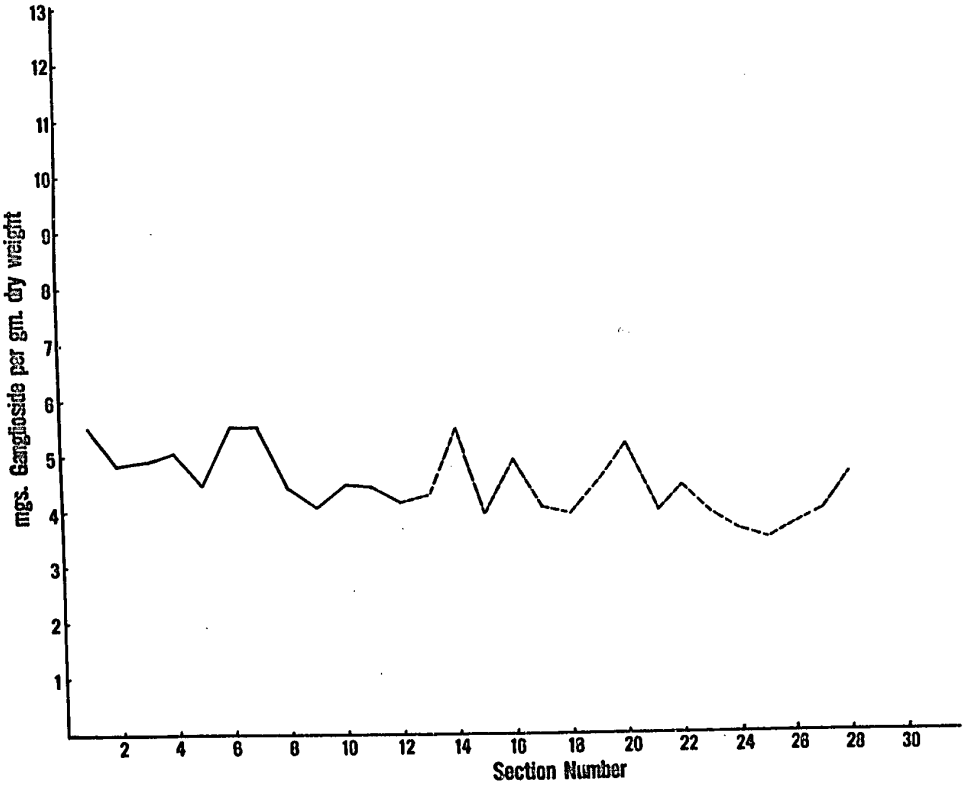
In the hippocampus it is difficult to remove a block of tissue from exactly the same location from different animals or even from the same animal on different sides of the brain. Lorente de Nó (138) has discussed in detail the complexity of the afferent and efferent axonal connections of the different regions along the longitudinal axis of the hippocampus. Therefore error could be introduced if the location of the block excised was not the same with respect to the longitudinal axis. At the same time since area CA1, CA2, and CA3 (138) are not sharply defined it is not possible to be at exactly the same region of the ventricular surface each time a tissue block is prepared.

In order to show that the variability in ganglioside content seen in the serial sections cut parallel to layers was real, another approach was taken. A block was excised from area CA2 in exactly the same manner as before but instead serial and random cryostat sections were cut and analysed in a plane perpendicular to the layers seen histologically. The results are shown in Figure 16. The mean ganglioside content of 28 sections was 4.48 mgs ganglioside per gram dry weight with a range of 3.46 to 5.55. The variations from section to section in these analyses was far less than in sections cut parallel to layers of hippocampus (see Table 4). It should be noted that sections in the perpendicular direction

FIGURE 16

Results of ganglioside analyses of serial and random cryostat sections cut perpendicular to the histological layers of a block cut from area CA2 of the ox hippocampus. The graph is shown as ganglioside content of each slice in mgs of ganglioside per gram dry weight plotted against the section number. The first 12 sections are serial sections and the dotted line indicates random sections through the rest of the block.

SERIAL AND RANDOM 24 MICRON SECTIONS THROUGH AREA CA_2 OF OX HIPPOCAMPUS



still do not assure perfect histological homogeneity of adjacent sections, as the pyramidal cells in the ox are not evenly distributed and sections could be passing through slightly different concentrations of cell bodies. However, the heterogeneity is far less than in slices cut horizontally. The mean ganglioside content of the 28 sections cut in the perpendicular direction was over 70% of the mean content of all the sections cut parallel to the layers.

As well, to confirm that the stratified organization of the neurones was indeed directly related to the ganglioside content in the folium of the ox cerebellum, a series of frozen sections were cut perpendicular to the layers. It was found that in the perpendicular sections the variations in ganglioside content in the consecutive sections was greatly reduced. The highest difference in ganglioside content of these consecutive sections in mgs ganglioside per gram dry weight was 2.1 compared with 10.7 for sections cut parallel to the pial surfaces.

D. Discussion

Wide variability was found in ganglioside content of serial tissue sections obtained parallel to the major areas of structural differentiation as defined by cytoarchitectonic studies in Ammon's horn and fascia dentata of the hippocampus, and the cortex of the cerebellum. The analysis of area CA2 of the ox hippocampus shows that a considerable variability exists in ganglioside content of the sections at corresponding depths in the hippocampus in different animals. The ganglioside analyses of one ox cerebellar folium also showed intracortical variability. These studies clearly indicate that repeated experiments in the hippocampus from different animals would produce only further unique patterns. Thus averaging values at a particular arbitrarily defined intra-hippocampal or

intra-cerebellar depth in different animals would obscure completely the differences between levels in the tissue. These differences are related to the unique cytoarchitectonics in each tissue block. Also, even within a particular cortical area, such as the stratum pyramidalis and stratum radiatum of area CA2 of the hippocampus, or the molecular and granular layers of the cerebellar cortex, there are wide differences of ganglioside content in adjacent 24 micron sections. These differences do not appear to be artifacts since they disappear when sections are cut at right angles so as to include all the structural areas in one section.

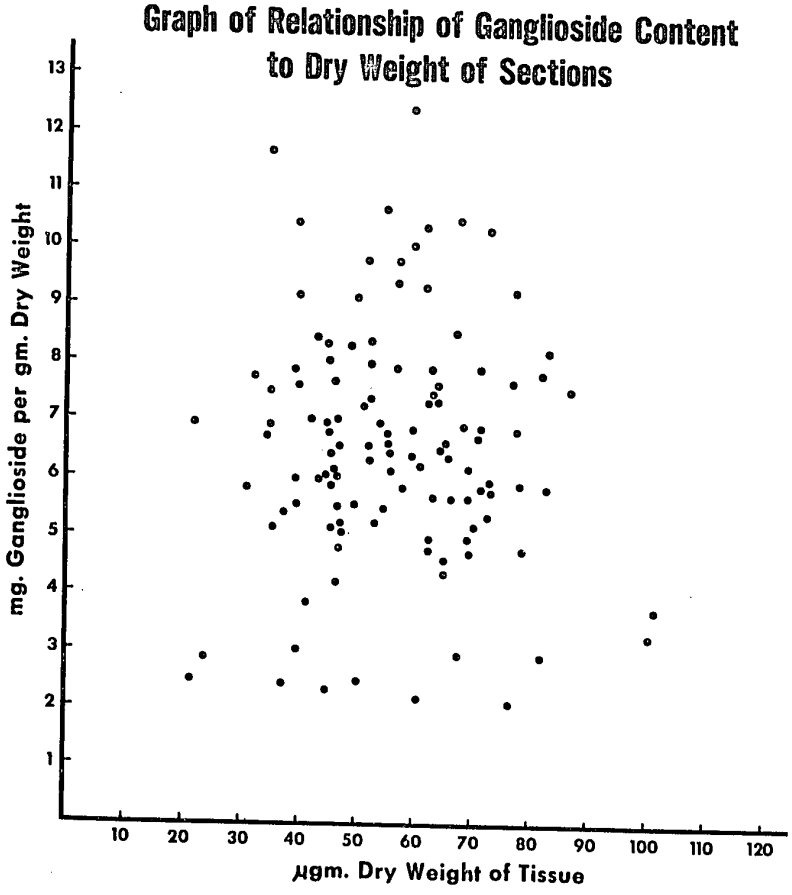
In Figure 17 the ganglioside content in mgs of ganglioside per gram dry weight was plotted against the dry weight of the slice. A completely random distribution was obtained indicating that there was no correlation between the weight of the slice and its ganglioside content. The variability in slice weight was partly due to some fragmentation of slices during sampling. It was discovered that the fragmentation was most likely due to the temperature of the cryostat chamber being too low (-20 to -25°C). Pope (161) and Lowry (144) have found that higher temperatures (-12°C) prevent fragmentation. We also found that per unit volume the total weight was almost twice as high in the alveus as in the stratum pyramidalis as was found by Lowry and Hyden (147).

The difficulties involved in evaluating chemical analyses in relation to the cytoarchitectonics of the localized tissue area have been discussed by Pope (166); Hess and Pope (75); Pope, Hess, Ware and Thomson (174); and Hess and Thalheimer (80).

Pope, Hess, Ware and Thomson (174) found when studying cytochrome oxidase activity in the rat cortex that the numerical values showed considerable variability at corresponding intracortical depths from animal to animal. The reproducibility of their microchemical procedures was $\pm$ 3%.

FIGURE 17

Graph of the ganglioside content of sections cut through area CA2 of ox hippocampus presented as mgs of ganglioside per gram dry weight versus the weight of the sections analysed. The graph shows the random distribution of the points indicating that there is no relationship between ganglioside content and dry weight of the sections.


Graph of Relationship of Ganglioside Content to Dry Weight of Sections
mg. Ganglioside per gm. Dry Weight
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
110
120
µgm. Dry Weight of Tissue

There are a number of reasons which can account for the large standard errors obtained when the numerical values of enzyme activities or chemical constituents at a particular depth in the tissue are averaged from different animals.

(1) There may be differences in the mean cortical enzyme activities from animal to animal.

(2) The sampling procedures are not exactly reproducible. This may be due to:

  (a) Difficulty in reproducing exactly similar anatomical locations from animal to animal;

  (b) Difficulty in cutting out the tissue block exactly perpendicular to the pial surface so as to avoid intermixture of layers during sectioning;

  (c) A variability among different animals in the thickness of the layers;

  (d) A variability in the anatomical composition of the different layers, such as having more cells or dendrites in a particular layer of one animal which may not be present in quantitatively similar amounts in identical regions of another animal.

(3) Correlation of the quantitative chemical results to particular morphological features is often difficult.

  (a) There are no methods available for quantitative determination of the mass or surface area of dendrites, axons, or neuroglial processes.

  (b) Quantitative measurements of synaptic end-knobs or terminal boutons are inaccurate because of the generally incomplete impregnation found by all types of metallic stains.

(c) The dry weight of sections of uniform volume often differs from layer to layer and tends to be higher in those layers with myelinated fibres. A most striking example of weight difference in sections of uniform volume is seen between the alveus and stratum pyramidalis. The dry weight of sections of uniform volume from the alveus is almost twice the dry weight of sections from the stratum pyramidalis. This was also found by Lowry and Hyden (147).

(4) Differences in the methods of sampling.

(a) In Pope's laboratory (166, 75, 174, 80, 168, 172, 72, 73) serial cryostat sections are cut in groups of four. The first section is used for fixation, staining and histological study. The second and fourth are used for chemical analysis or enzyme study and the third is dried and weighed on a quartz fibre balance. Considerable error could be introduced if there was a change in weight per volume between the section that is weighed and the section analysed.

(b) In the work presented in this thesis serial cryostat sections were each weighed and analysed. The histological control was obtained from the blocks of tissue adjacent to the block analysed.

(5) Differences in methods of presenting results.

(a) Pope (166), Hess and Pope (75) and Pope, Hess, Ware and Thomson (174) have grouped results from different animals on the basis of subpial depth, regardless of their histological assignment. It was felt that this type of analysis allowed for greater objectivity in interpreting the data.

It is not based upon the presupposition that division of the cortex into six layers of neuronal cell bodies has unique significance in relation to the distribution of biochemical constituents. Also this method is independent of the subjective judgement of the investigator regarding the assignment of the sample to the cortical layers and sublayers (75).

(b) Hess and Pope (75, 76) have also analysed their results in terms of the cytoarchitectonics of layers or sublayers as identified by the histological control material. Lewin and Hess (134) when presenting the results of analysis of sodium-potassium activated adenosinetriphosphatase activity in rat cortex only presented the results in terms of subpial depth. It was felt that because the volume of neurone perikarya constituted such a small part of the cortical volume and because the classical layers were not homogeneous histologically that the significance of biochemical studies with respect to the layers would be difficult to interpret.

(c) The results presented in this thesis are a complete series of analyses running through the whole block. The advantages of this technique are presented below.

(i) The differences between adjacent sections are revealed.

(ii) The variability within all histologically defined layers in one tissue block is readily apparent.

(iii) Intra-animal differences are readily shown (see Fig. 14). Further experiments on blocks of tissue from different animals it would seem, would not reveal any more information as to the mean values within a particular histological layer. They would only illustrate further differences which can occur within the same layers.

(iv) Narrowly defined layers such as the stratum granulare could be almost completely missed if only every fourth section were being analysed. It would be quite possible for only one section to be analysed through this layer. The same could be said for the two low values which occur in the folium of the ox cerebellum at the site of the Purkinje cells.

(v) In this thesis the sections which were weighed were analysed. Errors due to variability in the weight of the sections are reduced.

(vi) As Lewin and Hess (134) have pointed out, composite curves made up of the mean of many measurements of the Na-K adenosinetriphosphatase actively in rat cortex tend to even out differences when compared with the curves drawn from sections through individual cylinders of the tissue. The chemical characteristics within a single layer of an individual block of tissue are obscured by this procedure.

The results of ganglioside analyses of serial sections through the hippocampus demonstrates that the amount of biochemical variability between animals is quite great. The most conspicuous difference between experiment 1 and experiment 2 as shown in Figure 14 is that, in the stratum radiatum of experiment 1, there is a marked fall in ganglioside contents close to the values found in white matter. This does not occur in experiment 2. Mossy fibres from the granule cells of the fascia dentata make up a bundle of myelinated fibres which synapse with the apical dendritic stems of the pyramidal cells of area CA3 in the stratum radiatum (see Fig. 4) (12). Because the exact delineation of the boundaries of area CA1, CA2 and CA3 is not possible (138) it appears very likely that the block of experiment 1 was closer to area CA3 than in experiment 2. Thus sections in the stratum radiatum would contain this projection of myelinated fibres and consequently have low ganglioside contents.

In the hippocampus the alveus consists mainly of myelinated efferent axons and in this region there are low values with little variation in ganglioside content (see Table 4).

The stratum oriens and pyramidalis are not well differentiated in the ox. The neuronal structures in this region are the basal dendrites of the pyramidal cells and pyramidal cell neuronal perikarya (138) (Fig. 14 & 5). The electron microscopic studies of Hamlyn (67) have shown that axo-somatic synapses on the pyramidal perikarya are large and do not cover the whole perikarya. Large areas of the perikaryal membrane are in contact with glial processes. The highly branched basal dendrites however, are richly covered with small synapses. The variations in ganglioside content in sections through these regions may be related to a difference in quantities of dendritic surface membranes, axo-dendritic

synapses or both. The first part of the stratum radiatum contains the main trunks of the apical dendrites of the pyramidal cells and a few axo-dendritic synapses (67). As has been mentioned above, in this region the ganglioside content of the section was low for 8-9 consecutive sections in one of the tissue blocks studied. The rise in ganglioside content and the marked variations from section to section which occur in the deeper regions of the stratum radiatum and the stratum moleculare appears related to the breakup of the pyramidal cell apical dendrites into smaller and smaller dendrites, with great numbers of small synaptic contacts sometimes occurring in "cuffs" over short lengths of the dendritic branches (67). This region is penetrated by numerous unmyelinated afferent fibres of the perforant path from the entorhinal cortex which synapses with the terminal arborizations of the pyramidal cells (138). There appears to be three possible explanations for the differences in ganglioside content from section to section. Firstly, the differences could represent different numbers of synaptic endings in each section. Secondly, it could represent differences in the total amount of dendritic plasma membrane, whether derived from cell bodies, dendrites, endings or unmyelinated fibres. Thirdly, the possibility that at least part of the variation is due to the variation in the relative distribution of the different ganglioside types, i.e., trisialoganglioside as opposed to monosialoganglioside. The study of Suzuki (214) demonstrated that the linear increase in ganglioside content in rat brain between 0 and 20 days (176, 207, 208) does not represent what is happening to the individual ganglioside types. The marked increase in the faster moving disialoganglioside (G_3) and decrease in value of the trisialoganglioside (G_1) suggests that possibly disialoganglioside (G_3) is localized in a certain anatomical structure which is proliferating between day 0 and

day 10. The decrease in the trisialoganglioside (G_1) may represent the fact that the specific anatomical structure to which G_1 is attached may have already reached a point of adult differentiation. Consequently, it is possible that the small tissue sections taken in this study are uncovering the aggregation of anatomical structures to which different types of gangliosides could be attached.

That gangliosides are probably not restricted to the dendritic membrane is indicated by the finding of very high ganglioside contents in sections through the granular layer which consist of small neurones tightly packed together. It is interesting to note that Altman and Das (2) found the granular layer of the dentate gyrus of rats and cats to be essentially devoid of the nuclei of glial cells. However, Blackstad and Dahl (10) found that one-third of the surface of some of the neurones in the granular layer of the fascia dentata of the rat were covered with astrocytic processes. But, in spite of this, in this particular layer cell bodies and the axo-somatic synapses must be by far the predominant anatomical structure.

The ganglioside analyses of sections through the laminae of the cerebellum also showed high peaks in the molecular layers suggesting again an association of gangliosides with the orientation and/or concentration of dendritic branches and their synapses. A drop in ganglioside content occurred at the level of the Purkinje cells. As in the hippocampus, sections through the densely packed small neurones of the granular cell layer had relatively high average ganglioside contents.

Recent studies have indicated that the overall function of the cerebellum probably is predominantly inhibitory, the Purkinje cells inhibiting in Deiter's and intra-cerebellar nuclei monosynaptically (90,

91, 92). The basket, stellate, and Golgi cells are believed to play inhibitory roles within the cerebellum. The basket cells of the molecular layer make numerous inhibitory synapses on the basal region of the somata of the Purkinje cells. The superficial stellate cells of the molecular layer form inhibitory synapses on the dendrites of Purkinje cells. The Golgi cells found in the superficial parts of the granule cell layer make inhibitory synapses on the dendrites of the granule cells. Afferent excitatory fibres reach the cerebellum via the climbing and mossy fibres, which excite the dendrites of the Purkinje and granule cells, respectively.

Kuriyama, Haber, Sisken and Roberts (124) studied the amounts of gamma-aminobutyric acid (GABA) in the cerebellum layers, because of the interest that has been focused on GABA as an inhibitory substance in the central nervous system (178). They found their highest values for GABA (5.42 ugm per mg protein) to be in the layer containing the Purkinje cells. The molecular layer and granular layer contained 3.24 and 2.22 µgm GABA per mg protein, respectively. The white matter contained the lowest amounts of GABA (1.26 µgms/mg protein).

Hirsh and Robins (83) found essentially the same distribution of GABA in the cerebellar cortex of the monkey and the rat but they did not analyse the Purkinje cells. Except for the Purkinje cells the distribution of gangliosides that was found in the ox cerebellum parallels the distribution of GABA. As mentioned in Chapter 1 tetanus toxin, which is known to affect inhibitory synapses (40) combines readily with ganglioside (241, 242, 243). These facts support the evidence that gangliosides might be related to inhibitory synapses of the central nervous system.

The present results suggest that gangliosides are not uniformly distributed throughout the plasma membrane of the neurones but occur in local concentrations, possibly in regions densely supplied with synaptic

endings. However, it is also possible that the variation in ganglioside content observed may be due to differing amounts of total membrane surface area included in the section resulting from the directional changes in the dendrites and/or terminal branches of incoming fibres. As has been discussed by Pope, Hess, Ware and Thomson (137) quantitative estimates of synaptic distribution and surface membrane area in the various regions of the hippocampus and cerebellum are not obtainable at this time. Mosaics of electron micrographs are needed to correlate the variations in a chemical constituent, such as gangliosides, with a particular morphological structure. However, this information is difficult and tedious to obtain and so far is not available.

Some of the possible approaches to the problem of clarifying the neuronal localization are:

(1) Quantitative laminar analysis of gangliosides in the cerebral cortex.

(2) Quantitative laminar analysis of the layers of the retina.

(3) Isolation of pure neurones and glial cells for ganglioside analysis. This could be achieved by:

  (a) method of Hyden (86, 87, 88).

  (b) method of Roots and Johnston (187, 188).

  (c) isolation of pure elements from tissue cultures.

The method of Hyden for isolating pure neurones and glial cells offers the most direct approach to obtain conclusive evidence for the neuronal localization of gangliosides and results obtained after the development of this technique are given in the next two chapters.

Chapter V

ANALYSIS OF GANGLIOSIDES IN ISOLATED NEURONES AND GLIA:METHODS

A. Introduction

The quantitative pattern of distribution of gangliosides in various regions of the nervous system strongly suggests that gangliosides are located specifically in neurones, either in the cell bodies, dendrites, or both (214, 141, 127). Further support of such a localization is given by the following findings: (1) in pathological conditions the histological demonstration of the loss of neurones without a corresponding loss of glial cells was associated with a decrease in the amount of gangliosides that could be extracted from the cerebral cortex (140); (2) the absence of gangliosides in glial tumors (141, 140); (3) the presence of gangliosides in all layers of the frontal lobe of the cerebral cortex and the demonstration of greater amounts in the superficial layers which are known to be rich in dendritic processes (from cell bodies which lie in deeper layers of the cerebral cortex) (141); (4) the inability to detect antiganglioside antibodies in sera from rabbits immunized with optic or sciatic nerve (205); (5) the demonstration of ganglioside in high concentrations in sections cut through layers of closely packed neurones and dendrites of area CA2 of the ox hippocampus and folium of the ox cerebellum.

Because gangliosides have been found in low concentrations in pure white matter such as the centrum semiovale it has been postulated that gangliosides also occur in axons (100). However, all the evidence for the occurrence of gangliosides being only in neuronal structures of the brain

is indirect. To obtain more direct information of the distribution of gangliosides in neuronal structures, techniques for the isolation of individual neurones and glial cells similar to those of Hyden (86, 87, 88) were developed.

B. Adaption of Fluorometric Technique

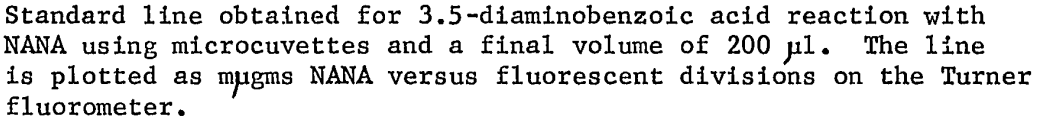
In order to be able to analyse gangliosides in groups of neurones and glial cells isolated from the brain stem by the method of Hyden, it was necessary to scale down the fluorometric method and construct a more sensitive quartz fibre balance for weighing.

A five times recrystallized preparation of N-acetylneuraminic acid prepared from human meconium (258) was used as a standard. A comparison of this preparation with that of Sigma Chemical Company N-acetylneuraminic acid (batch A11B-216) showed no difference in the molecular extinction coefficient in the resorcinol reaction. Figure 18 shows the standard line obtained using the fluorometric technique of Hess and Rolde (79) when the volumes for analysis were decreased by twenty times, using the microcell attachment to the Turner fluorometer (#110-866). Each determination was done in 200 µl of diluent. The new standard line shows that the accurate determination of NANA can be made down to between 10 and 70 mµgms of NANA.

C. Construction of a More Sensitive Quartz Fibre Microbalance

A new quartz fibre microbalance was constructed using the same techniques described in Chapter III, but it was found unnecessary to place a pan on the end of the quartz fibre as the small pieces of tissue stuck to the end of the fibre quite easily (144). The new quartz fibre balance enabled the measurement of samples of dry tissue in the region of one to ten ugm to the nearest 0.1 µgm (Fig. 19).

FIGURE 18



Standard line obtained for 3.5-diaminobenzoic acid reaction with NANA using microcuvettes and a final volume of 200 µl. The line is plotted as mµgms NANA versus fluorescent divisions on the Turner fluorometer.

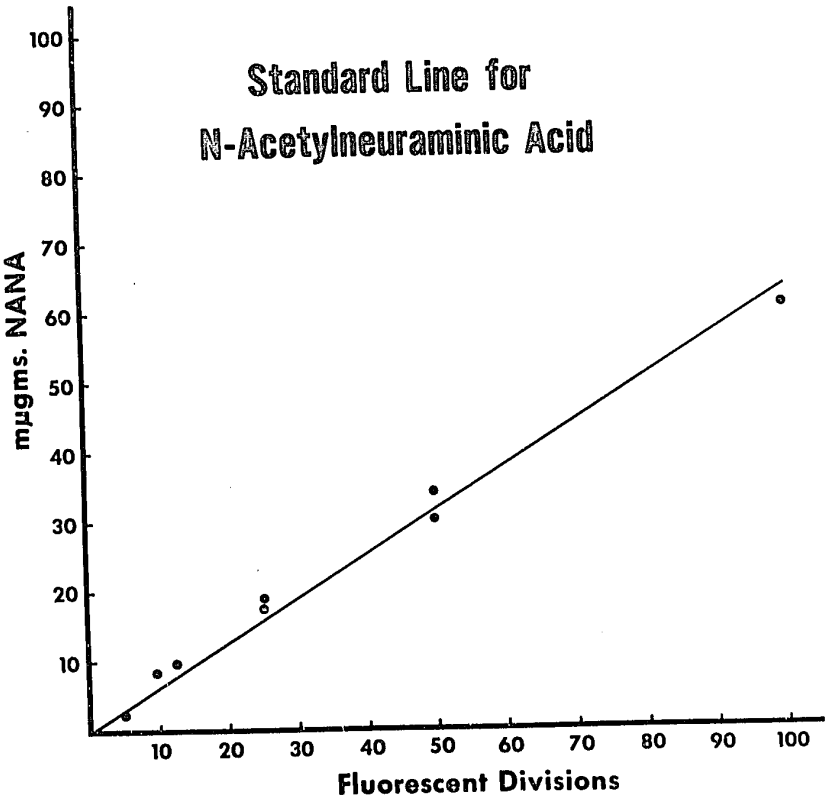
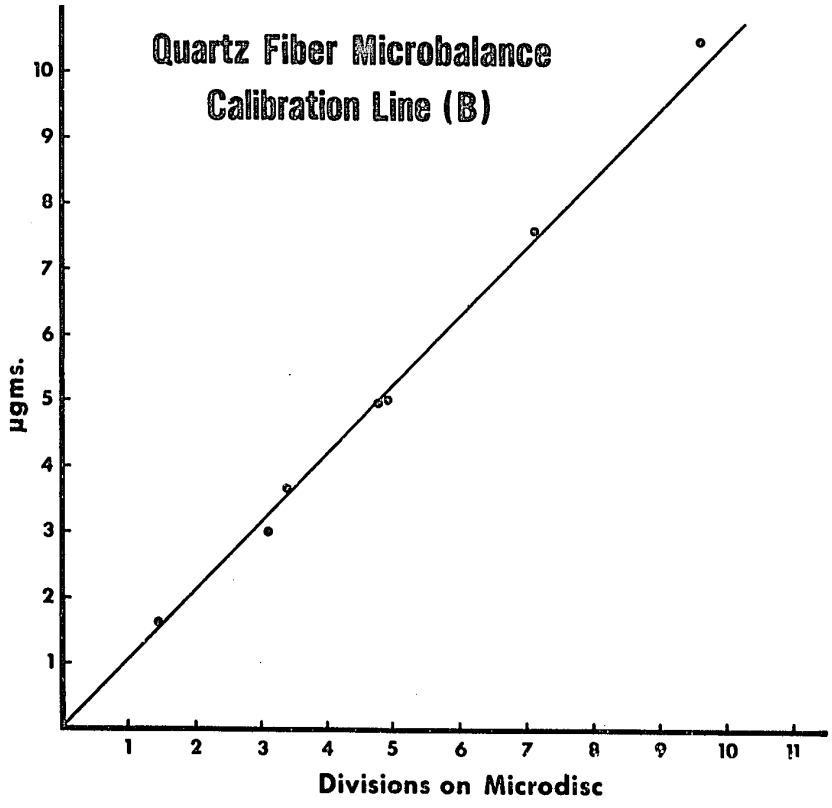

Standard Line for
N-Acetylneuraminic Acid
mμgms. NANA
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
Fluorescent Divisions

FIGURE 19

Standard line of quartz fibre micro-balance (B). Micrograms are plotted against divisions on the microdisc using the 3x scale of the optics of the microscope.


Quartz Fiber Microbalance
Calibration Line (B)
µgms.
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
Divisions on Microdisc

Because the American Optical dissecting microscope has three magnifications 1x, 2x and 3x it is possible to extend or narrow the range which can be weighed as well as to increase or decrease the sensitivity of the balance. Because the new balance only needed to be able to weigh between one and ten ugms accurately it was possible to use the first quartz fibre balance to calibrate the second. The range of weight which can be weighed on each balance overlaps. By weighing a minute cotton thread on the first quartz fibre balance at the highest magnification (3x) the thread could then be transferred to the new quartz fibre balance and viewed at the lowest magnification (1x), the deflection noted, and its weight recorded.

D. Source of Neurones and Glia

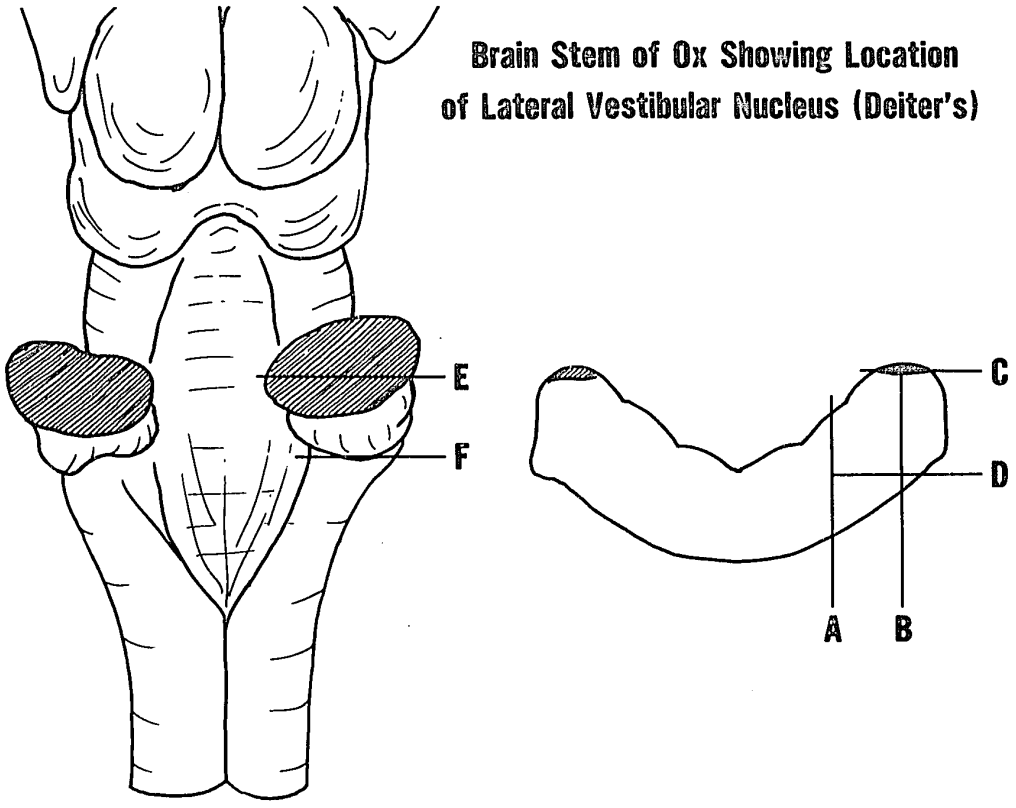
Ox brain (kosher killed) were obtained at the slaughter house and brought to the laboratory in plastic bags on crushed ice. In the ox brain stem the lateral vestibular nuclei lie in the medulla on the floor of the fourth ventrical and extend dorsally to lie on the inner border of the inferior cerebellar peduncles (see Fig. 20) (187). Thin slices of tissue were cut through the lateral vestibular nuclei using a Stadie blade and the slice placed in Krebs-Ringer solution without glucose.

E. Dissection Technique

Using a dissecting microscope at 100 times magnification, neurones were picked out of the lateral vestibular nucleus without the use of any stains. It has been found that dilute methylene blue used by Hyden to locate neurones severely interfered with the 3,5-diaminobenzoic acid reaction. In all of the samples collected no staining material was used. The neurone with its surrounding glia was removed with a small stainless

FIGURE 20

Diagram showing location of lateral vestibular nucleus (Deiter's) in the ox brain stem. Lines A and B outline the length and C and D the width of the nucleus. Lines E and F show anterior-posterior limits of the nucleus.



Brain Stem of Ox Showing Location of Lateral Vestibular Nucleus (Deiter's)

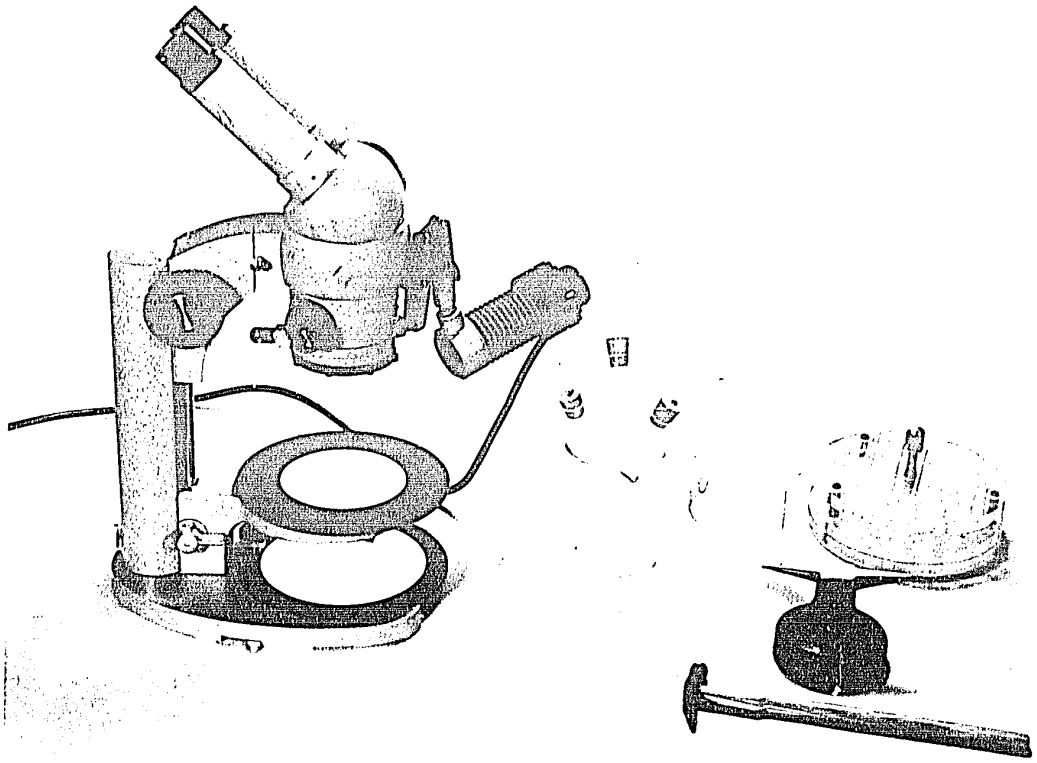
steel wire (15 microns in diameter) which had been made into a spatula by means of cold forging using a watchmaker's anvil and hammer (Fig. 21). The neurone in its surrounding glia was placed in a small drop of Krebs-Ringer solution on a glass slide. The surrounding glia was carefully dissected away from the neurone and then the neurone and glia were collected in separate piles. To obtain a dry weight of the collected neurones it was necessary to place the neurones briefly in distilled water just prior to freeze-drying since crystals of salt from the Krebs-Ringer would contribute to errors of the dry weight. The neurones were collected together in distilled water and then the water removed grossly from around them. The water was removed from around the neurones by sucking it up into progressively finer capillaries. After as much water had been removed as was possible without loss of neurones, the slide was placed on a block of dry ice and the small clump of neurones frozen. The slide was then transferred to a Virtus flask. After approximately six hours the slide was removed and the small clump of freeze-dried neurones was then weighed on the quartz fibre balance. The weight of an individual neurone was calculated by dividing the total dry weight by the number of neurones dissected.

F. Microscopy of Isolated Neurones and Glial Cells

During the development of the techniques for isolation of neurones, the Carl Zeiss Company very kindly provided us with the Nomarski differential interference contrast system (hereafter referred to as the Nomarski system) (8). For purposes of determining the purity of the neurones and glial masses after dissection and for ascertaining the morphological characteristics of the glia and neurones, samples were photographed with a phase contrast system and also the Nomarski system.

FIGURE 21

Instruments and apparatus used for isolation of neurones. On the left a dissecting microscope. In the center are steel threads mounted in glass tubes. On the right are the watchmaker's anvil and hammer used for cold forging the steel threads to make spatulas and knives.



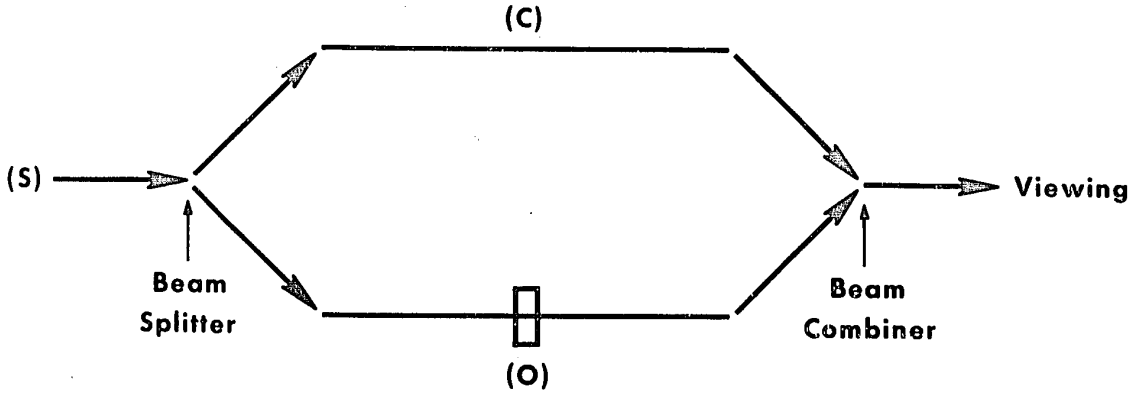
For a review of the interference microscopic systems see David (35), Ross (195), Bajer and Allen (8) and Davies (36). The general principle upon which the design of any double beam interference microscope is based may be made clear by briefly considering a purely schematic version (see Fig. 22). A ray of light from the source (S) is split into two parts of equal intensity by a device known as a beam splitter. One ray passes through the specimen (O) and another, the comparison ray (C), some distance to the side of the specimen. These rays are then recombined by another device, the beam combiner. Since the two combined rays have arisen from a point source (S), any phase difference between them remains constant; that is, they are coherent and hence can interfere to give light or dark. A refractile object in one beam causes a retardation (optical path difference). The main difference between the various forms of interference microscopes lies in the way in which the beam splitting and combining are carried out. The measuring beam is shifted in phase in relation to the reference beam by an amount proportionate to the optical path difference between the object and the immersion medium.

Interference microscopes have two main advantages over phase contrast microscopes which greatly extend their scope for making refractive index measurements on living cells. Firstly, the optical artifacts, the "halo" and "shading off" effects, that are inevitable with a phase contrast system, are absent. Secondly, interference microscopes enable the phase changes of the different regions of an object to be measured directly, by passing fringes in the order of the Newtonian series of interference colors across the field, and measuring the displacement in color at the fringe in the object compared to that of the field in its immediate vicinity (195).

FIGURE 22

Schematic diagram of principals of interference microscopes. The source light (S) is split by a beam splitter into two beams of light. Beam (O) passes through the specimen and the beam (C) passes through the neighbouring medium. The light is recombined by a beam combiner.

Schematic Drawing of Principals of Interference Microscopy



An important characteristic of the Nomarski system is the relative freedom from phase disturbances of structures above and below the plain of focus. At high working apertures, the field is quite shallow (8).

Chapter VI

MORPHOLOGY AND GANGLIOSIDE ANALYSES OF ISOLATED NEURONES AND NEUROGLIAL CELLS

A. Microscopy of Isolated Neurones and Glial Cells

Figures 23A and 23B show the appearance of a neurone imbedded in a birefringent neuropil, slightly stained with methylene blue, immediately after dissection from the brain stem. It is from microsamples such as these that the neurones and glial cells are isolated by teasing away the glial cells. Since with experience it was possible to dissect out single neurones with their surrounding glial cells without staining, all the other photomicrographs in Figures 23 to 26 are from unstained preparations.

Glial cells were not clearly distinguishable within the glial clump. The appearance of glial cells when isolated in isotonic sucrose (Fig. 24A) is quite different from glial cells isolated in Krebs-Ringer solution. Krebs-Ringer solution appears to preserve glial cells in a more physiological state than sucrose solutions.

A few observations were made of the affect of solutions of different composition on the morphology of isolated neurones observed in the Nomarski system. Johnston and Roots (98) found that the addition of gangliosides to the media in which the cells were isolated had a pronounced effect on preserving the electron microscopic image of the cell body. In the study presented here no change in the neuronal membrane could be detected even in solutions containing gangliosides.

It was found that when neurones were isolated in Krebs-Ringer or Elliott's solution containing high concentrations of calcium (5-10 mM) that separation of the glial cell mass away from the cell body was

FIGURE 23

Photographs of neurones and glial cells with the Nomarski interference system. At A is shown the neurone imbedded in glial cells when stained with dilute methylene blue (X175). Picture B is a close-up (X380) of same neurone. Pictures C and D show neurones partially cleaned of surrounding glial material.

NEURONES AND GLIAL CELLS

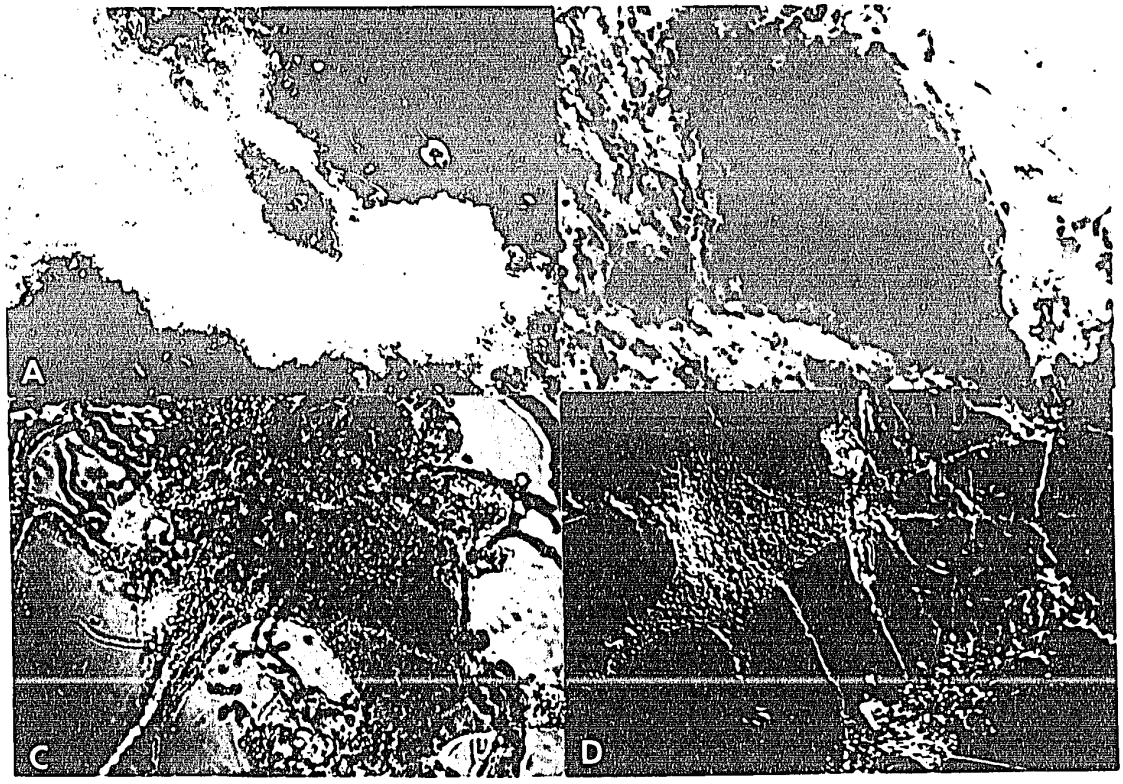


FIGURE 24

Photographs of glial cell clumps with the Nomarski interference system. The glial clump in A (X300) was isolated in 0.32 M sucrose. Glial clumps at B and C were isolated in Krebs-Ringer solutions without glucose. Picture B shows a close-up (X375) of a glial clump. At C is seen a small dendrite extruding from center of the glial cell clump.

GLIAL CELLS

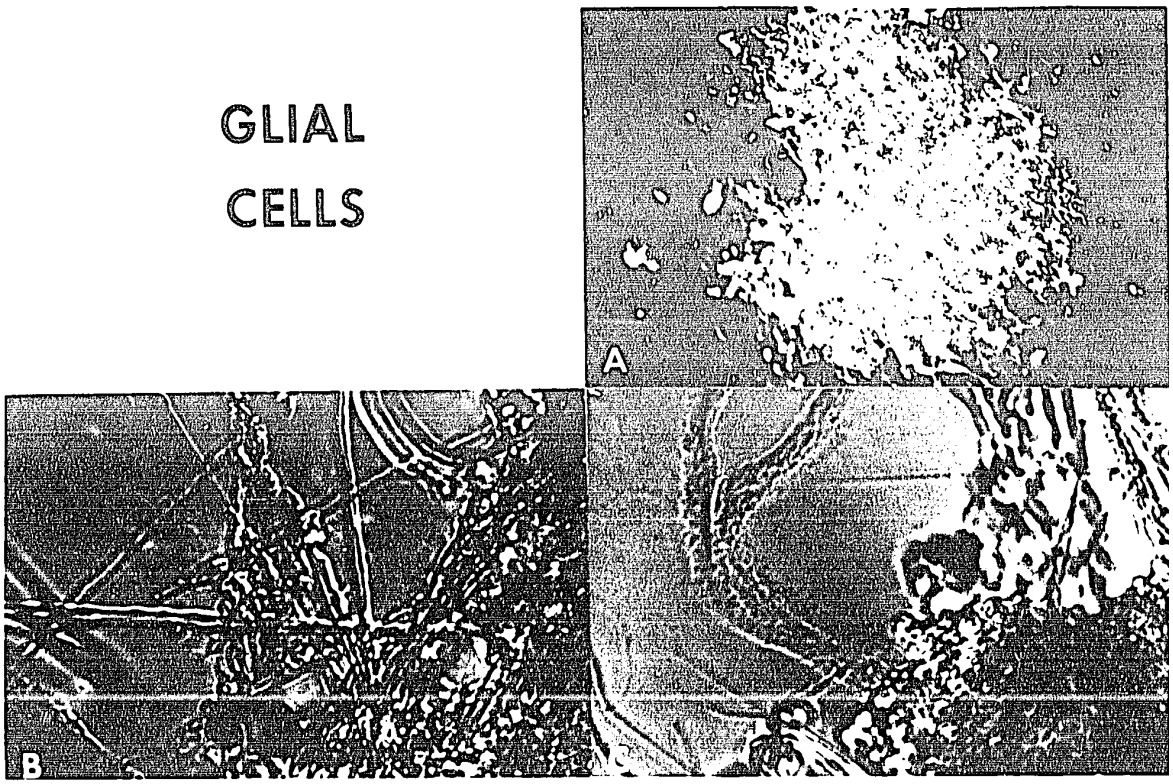


FIGURE 25

A group of six different neurones photographed with the Nomarski system. All magnifications are between 300X and 400X. The prominence of the lipochrome pigment is seen in pictures C, D and E.

NEURONES

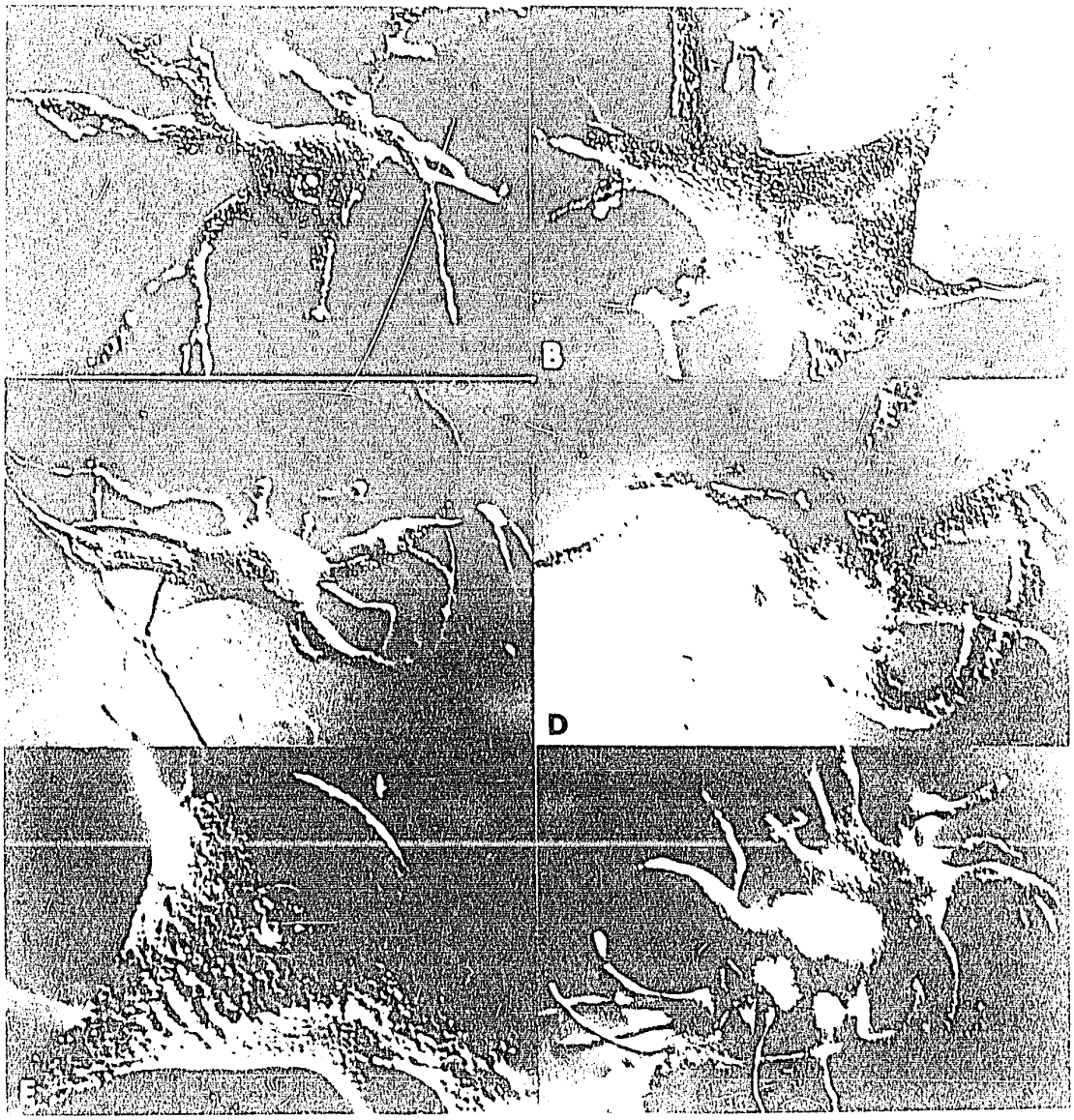
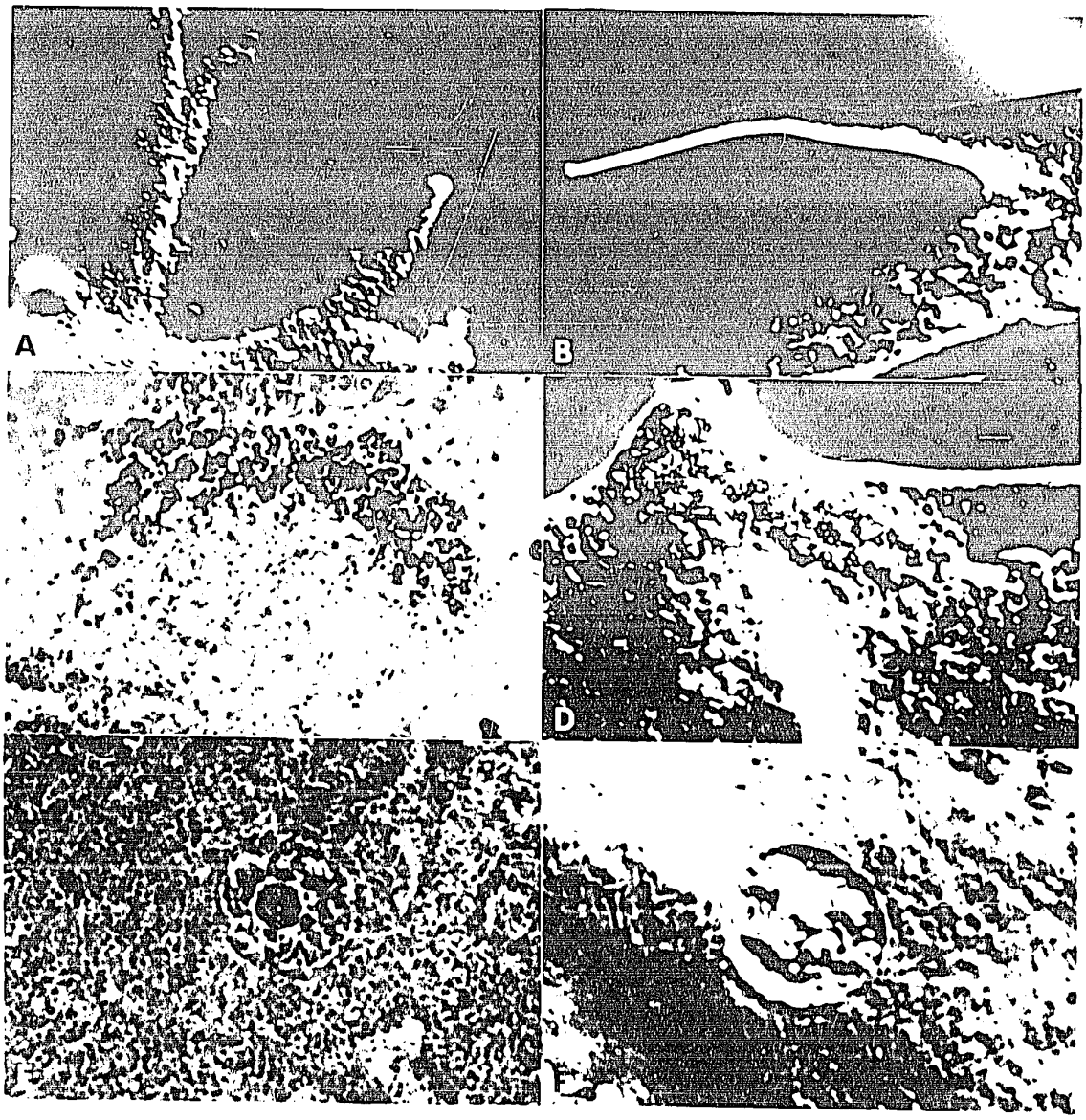


FIGURE 26

Photographs of neurones at 500X magnification to show detailed structural characteristics of neurones in phase contrast and Nomarski system. At A is shown the rough appearance of the surface of a dendrite. At B is shown the smooth appearance of the axon and axon hillock. C and D are photographs with phase contrast and Nomarski system of the lipochrome pigment respectively. At E and F are shown a nucleus of a cell photographed with phase contrast and Nomarski system respectively.

PARTS OF NEURONES



extremely difficult. Separation of the glial cells from the neuronal cell body was easiest in sucrose, more difficult in Krebs-Ringer and the most difficult in Krebs-Ringer with the high concentrations of calcium. Photomicrographs of the glial cell clumps shown in Figures 24B and 24C reveal that the mass contains, besides glial cells, many strands which probably represent pieces of myelinated and unmyelinated axons, many fine nerve terminals and pieces of fine dendrites. It was not possible to remove all contaminating dendritic branches and terminal axons without totally disintegrating the glial cell clumps.

The morphological characteristics of the neurones isolated from Deiter's nucleus of the ox are beautifully demonstrated in Figure 25. There appears to be wide variation in numbers of the main dendritic branches. The dendrites are covered by small knobs or excrescences which give the surface membrane a rough appearance (see Fig. 26A). The surface membrane of the axon is smooth (Fig. 26B).

There is a great difference in the appearance of isolated neurones when viewed through a phase contrast and the Nomarski system (Figs. 26C & D and 26E & F).

B. Results of Analyses of Groups of Neurones and Glial Cell Clumps

Table 6 shows the results of ganglioside determinations expressed as glycolipid NANA of isolated neurones and glial cells of the ox brain. Three different types of samples were analysed.

(1) Clean neurones isolated from Deiter's nucleus uncontaminated with glial cells (Table 6, Sample A).

TABLE 6

ANALYSES OF GANGLIOSIDES IN ISOLATED NEURONES AND GLIAL CELLS OF OX BRAIN

	Number Isolated	Total dry wt. µgm	Average wt. of neurone mµgm	mg NANA per gm dry wt.	mg ganglioside/gm dry wt.
A. Neurones					
Sample 1	60	1.91	32	3.41	12.2
2	51	1.52	30	2.96	10.6
3	64	2.10	33	4.75	16.9
4	68	2.80	41	2.15	7.7
Average					11.8
B. Glial Cell Clumps (trimmed)					
Sample 1	60	3.33	-	3.90	13.9
2	51	1.71	-	1.75	6.3
3	64	2.10	-	4.65	16.6
Average					12.2
C. Neurones & Glial Cell Clumps (not trimmed)					
Sample 1	50	17.0	-	1.62	5.8
2	50	19.5	-	0.95	3.5
Average					4.6

(2) Neuropil tissue removed from the region immediately surrounding the isolated neurones and not including the greater mass of glial cells (Table 6, Sample B).

(3) Small blocks of tissue each containing one neurone together with its surrounding glial cells and neuropil as dissected from Deiter's nucleus (Table 6, Sample C). The neurones and glial cells were not separated in this group (Sample C). The term "not trimmed" means that in these samples more glial cell mass was included. That is, each tissue sample would contain a single neurone but much more glial mass from the neuropil which was not immediately surrounding the neurone cell body. It should be noted that each of these samples therefore contains many more glial cells than in the other samples (see Table 6, Sample B).

Approximately 50-70 neurones were isolated per sample, (Table 6). The weight of individual neurones was calculated by taking the total weight of the sample and dividing by the number of neurones in the sample. The results agree well with those of Hyden (86) and Lowry (145).

Much to our surprise it was found that the concentration of ganglioside on a dry weight basis was almost the same in the clean neurone samples as in the samples of glial cells and neuropil from immediately surrounding the neurone cell body (Table 6, Samples A and B). However, the ganglioside content of the samples containing the same number of neurones but more glial cell mass was considerably lower than either of the other two samples (Table 6, Sample C). Thus, increased glial cell content decreased the ganglioside content when expressed on a dry weight basis.

Hyden and Pigon (89) have mentioned that contamination of the glial cell mass can be avoided by removal of the large dendrites. It was possible for us to remove large dendrites contaminating the glial mass as seen in Figure 24. However, there is no doubt that there is still considerable contamination with smaller dendrites and particularly the fine terminal axons and nerve endings pulled off from the neurones during separation of the surrounding glial cells. The unequivocal demonstration of the morphological characteristics of the glial cell mass will await electron microscopic studies. The techniques to carry out such a study are now being worked out in this laboratory.

According to Hyden (86, 87, 88) approximately 80% of the glial cell mass, if removed from the area immediately around the neurones, is made up of oligodendroglia. There is considerable evidence that neuroglial cells do not contain gangliosides (see Chapter I). If 80% of the glial cell mass surrounding the neurone is oligodendroglia, then the concentration of ganglioside in the non-oligodendroglial elements can be calculated to be a maximum of 60 mgs ganglioside per gram dry weight (Sample B, Table 6). A minimum value for the ganglioside content of the non-oligodendroglial elements can be calculated from Sample C (Table 6) to be 23 mg ganglioside per gram dry weight. Both these values are much higher than the ganglioside content of the isolated intact neurone. Thus, it appears that the highest concentration, on a dry weight basis, of gangliosides occurs in axon terminals and endings on dendrites in the neuropil immediately surrounding the neurone. The ganglioside content of pontine tissue and medulla oblongata when analysed on a gross block of tissue is 2-3 mgs ganglioside per gram dry weight (141). It should be noted that the ganglioside content of clean isolated neurones is five

times greater than the value obtained for the total anatomical region. Also, Sample C (Table 6) which includes more glial mass has a ganglioside content which approaches the value obtained for this anatomical region. From the results obtained on Sample B (Table 6), assuming 80% glial cell content, then these elements would contain about 6% ganglioside on a dry weight basis. This figure is close to the value (7-9%) obtained for the ganglioside content of a membrane fraction obtained by Spence and Wolfe (209) from baby rat brains. Lapetina, Sota and De Robertis (129) and Spence and Wolfe (209) have shown that gangliosides do not occur in synaptic vesicles but are constituents of synaptosome membrane ghosts.

The most notable feature found by Roots and Johnston (187) in cells from Deiter's nucleus of the ox was the absence of a plasma membrane. Roots and Johnston isolated cells by both a homogenization technique and by the method of Hyden and found no difference in the light microscopic and electron microscopic appearance. The plasma membrane appeared to be missing from the greater part of the soma and dendrites. The typical electron micrographic image of the surface membrane was seen only where terminal boutons were present. Organelles in the synaptic boutons were less well preserved but were still recognizable. The synaptic thickening could be seen, and structures corresponding to mitochondria and synaptic vesicles were present in the presynaptic bag (187).

The pictures obtained in this study with the Nomarski system of light microscopy do not clearly indicate whether or not the cell membrane is intact. The overall impression is that the soma of the cell is relatively smooth and that the dendrites are rough. By rough is meant covered with numerous small protuberances which could be either spines or nerve endings on dendrites or both. Morphologically if our neurones

are the same as Roots and Johnston (187) then in the cell bodies the gangliosides are most likely located in the post-synaptic membrane or in the pre-synaptic endings.

In the light of these findings that gangliosides are most likely specifically localized in the fine terminal axons and synaptic boutons, the earlier results of the ganglioside analysis of cryostat sections is further clarified. The wide differences in ganglioside content most likely represent either differences in quantities of synaptic endings or changes in the number of axon terminals entering a particular area of the tissue. Obviously further study is necessary, but these preliminary results of ganglioside analyses on isolated cells have given new direction to the further experimentation on ganglioside location in neurones and may provide clues to the function of these important acidic glycolipids in the brain.

Appendix

OBSERVATIONS ON LATE INFANTILE SYSTEMIC LIPIDOSIS

1. Relationship of Gangliosides to Neurological Diseases

A. Tay-Sach's Disease

In recent years several laboratories have applied modern analytical biochemical techniques to the study of degenerative disease, and several symposia have been devoted to this subject (31, 244, 46, 47, 7). An excellent monograph on Tay-Sach's disease has been compiled by Volk (245). The majority of cases of amaurotic family idiocy are generally categorized either as the infantile form (Tay-Sach's disease) or as juvenile lipidosis (Spielmeyer-Vogt disease). In the former instance the clinical symptoms commence before the age of one year, while in the latter the onset of illness occurs between six and ten years. The rare cases in which the onset of the disease takes place between one and four years of age have been classified as late infantile type (Jansky-Bielschowsky). It is not known yet whether such cases (late infantile type, Jansky-Bielschowsky) constitute a definite group different from the infantile and juvenile form, or are a different manifestation of infantile amaurotic idiocies beginning at a late age, or are cases of juvenile lipidoses commencing unusually early.

Clinically Tay-Sach's disease in the infantile form is a genetically determined recessive disease beginning in the first few months of life. Muscular weakness and blindness develop, and mental development stops or regresses, death occurring at one to five years of age. The head and brain often increase in size, the increase being especially in white matter of the cerebral hemispheres.

Formerly, the emphasis in chemical studies of the lipidoses was on the measurement of the total ganglioside content of the brain tissue obtained from patients who had died from these diseases. But with the purification of several molecular types through the improvement of separation techniques, it has become possible to describe the chemical features of these diseases more precisely. Thus, in Tay-Sach's disease the enormous increase in total brain gangliosides is known to be caused by proliferation of a single ganglioside whose composition has been shown to consist of equimolar amounts of glucose, galactose, N-acetyl-galactosamine, N-acetylneuraminic acid, sphingosine, and a fatty acid (224), (see Table 2). The ganglioside which accumulates in Tay-Sach's disease (G_5) occurs in normal brains in a very small percentage (less than 5%), while the other gangliosides which normally occur in brain tissue are only present in very low amounts (32, 232). The structure of this ganglioside as originally postulated by Svennerholm (224) has been confirmed by several other workers (97, 132).

Electron microscopic pictures of the ballooned neurones in Tay-Sach's disease has revealed small concentric bodies called membranous cytoplasmic bodies (MCB) which are 90% lipid and 10% protein. The ganglioside content of these membranous cytoplasmic bodies is 30% to 40% of their dry weight (201, 202).

Recently, (236) membranous cytoplasmic bodies (MCB) have been formed by the <u>in vitro</u> combination of gangliosides, phospholipids, cholesterol, cerebrosides, amino acid and peptides. In these experiments, bovine gangliosides were used (202, 236). Aside from the membranous cytoplasmic bodies found in Tay-Sach's disease a variety of membranous and granular cytosomes in neurones and glia have been described in

cerebral tissues from children with lipidosis. The structures have been termed membrano-vesicular bodies in juvenile lipidosis (60), pleomorphic lipid bodies in late infantile amaurotic idiocy (246) and zebra-bodies in Hurler's disease (1). Although one "variety" of cytosome may predominate in a specific lipidosis or in a specific case, all forms have been found to some degree in most of these diseases.

Wallace, Schneck, Kaplan and Volk (248) have suggested that the basic disorder in Tay-Sach's disease involved the cell digestive mechanism and its inability to digest the lipids accumulating in the cell, with the consequent formation of large numbers of residual bodies. They have gone on to suggest that the lipid granules found in the neurones were lysosomes containing the accumulated gangliosides. Jatzkewitz and Sandhoff (95) have suggested a block in the metabolic breakdown of gangliosides as the cause of the accumulation of Tay-Sach's ganglioside.

Jatzkewitz, et al (95) and Svennerholm (224) have stated that the storage of Tay-Sach's ganglioside is accompanied by an accumulation of the neuraminic acid-free ceramide trisaccharide. Gatt and Berman (55, 56) have isolated two glycolipids from brain tissue of Tay-Sach's disease. One of the glycolipids contained sphingosine, fatty acid and galactose; and the other equimolar ratios of sphingosine, fatty acid, glucose, galactose and galactosamine. These glycolipids only occurred in 1/12th the amount of gangliosides. These types of glycolipids, more commonly called asialoganglioside, whose structure is similar to gangliosides but with the sialic acid removed, have been found in normal brain tissue in small amounts (114, 232, 34). However, Gatt and Berman (55, 56) could not detect in either infant or adult normal human brain their two glycolipids found by them in Tay-Sach's brain.

B. Tay-Sach's Disease with Visceral Involvement

Although the biochemical observations in the brain of infantile amaurotic idiocy or Tay-Sach's disease have been well documented as noted above, the information on the other types of amaurotic idiocy is scanty and controversial. Until recently there have been very few cases of Tay-Sach's disease with visceral involvement reported in the literature. Davidson and Jacobson (37) describe the pathology of a nineteen month-old Jewish boy with no radiological bone changes in which the viscera contained foam cells in the spleen, liver and kidneys. Brouwer (23) found foam cells in the spleen but none of the other organs were examined nor were X-ray changes mentioned. Marburg (149) described a case briefly with blindness and mental retardation in which a child had the onset of the disease at four months and died at 12 months of age. The only finding mentioned is the presence of foam cells in liver and spleen. Turban (1944), as quoted by Norman (155), described a non-Jewish child who died at the age of two years after a progressive neurological illness. The characteristic cherry-red spot at the macula had been noticed. Analysis of the cerebral cortex by Klenk showed a five-fold increase in gangliosides, and histologically the brain was typical of Tay-Sach's disease. Empty vacuoles were a conspicuous feature of the cells of the liver and renal tubules, and foam cells were present in the pulmonary alveoli and their walls. Norman, et al (156) describe a case of Tay-Sach's disease with visceral involvement who died at 17 months of age after an illness characterized by progressive mental, visual and auditory failure. Foam cells containing empty vacuoles were numerous in the liver, spleen, lymph glands, thymus, bone-marrow, adrenals, lungs and intestines. Chemical examination of the brain proved this to be a case of Tay-Sach's disease, there being a large

increase of ganglioside in grey and white matter. Chemical analyses of the liver and spleen revealed a large excess of hexosamine. There were no radiological bone changes in this case. Craig, Clarke, and Banker (30) described a case of a young girl, who died at the age of 3-1/2 months, whom they have called "a variant of Hurler's syndrome" in which hepatosplenomegaly was a prominent feature. There were radiological bone changes of the dorsal vertebrae. The reticuloendothelial cells were swollen and a striking feature was the swelling of the cytoplasm of the glomerular epithelium in the kidney. San Filippo, Yanis and Worthen (203) have described a patient in whom the clinical features of the Hunter-Hurler syndrome were present but there were normal levels of urinary acid mucopolysaccharides and the presence of numerous foam cells in biopsy specimens of bone marrow, lymph nodes, and liver. There were radiological bone changes compatible with Hurler's syndrome.

More recently Landing, et al (125) have described eight patients with a disease which had the following features: (1) a clinical picture of severe progressive cerebral degeneration leading to death in the first two years of life; (2) the accumulation of a glycolipid in neurones, in histiocytes of the liver and spleen, and in the renal glomerular epithelium; and (3) a presence of skeletal deformities resembling Hurler's disease. They proposed the name "familial neurovisceral lipidosis".

In 1965, O'Brien, et al (160) described a case of a six month old white male which met the criteria established by Landing, et al (125). They found ganglioside accumulation occurring in the brain, liver and spleen. Therefore, the term "generalized gangliosidosis" was used for this disease. The stored ganglioside comprised somewhat greater than

84% of the brain ganglioside and was the only ganglioside detected in the liver and spleen. Thin layer chromatography demonstrated that the stored ganglioside had the same migration rate as G_4, the major normal monosialoganglioside.

In 1965, Gonatas and Gonatas (59) gave the electron microscopy and biochemical analyses in a case of systemic infantile lipidosis and in a case of gargoylism. In the case of the systemic late infantile lipidosis, brain gangliosides were elevated, and thin-layer chromatography and analytical studies revealed a predominance of the G_4 fraction corresponding to the major monosialogangliosides of normal brain tissue. That this ganglioside was of the same structure and type as the major monosialoganglioside of normal human brain was confirmed by Leeden, et al (132). The child, of Puerto Rican origin, demonstrated retardation of psychomotor development at the age of 18 months and died at the age of 25 months. There were no radiological bone changes. Foam cells were seen in spleen and lymph nodes, liver, lungs and bone marrow but the viscera were not chemically analysed. Jatzkewitz and Sandhoff (94) reported a "biochemically special form of infantile amaurotic idiocy", where they found G_4 (the major monosialoganglioside) to be the only predominant ganglioside in the brain preserved in formalin for 26 years. However, Suzuki (213) has reported that any normal brain, after more than four to five years in formalin, will have only G_4.

In 1964 Norman (155) described a case of a non-Jewish child who died at the age of two years after a progressive neurological illness beginning with severe hypotonia. Neuropathologically the changes were typical of infantile amaurotic family idiocy. Chemical analysis of the brain tissue, which had been fixed in 10% formol saline for 14 months,

showed that ganglioside (as estimated from the neuraminic acid content) was increased three and one-half times the normal in the cerebral cortex and ten times in the white matter. Examination of the visceral organs showed foam cells in the alveolar septa and vacuolation of the cells of the liver and renal tubules. The thin layer chromatogram revealed that the ganglioside accumulating in their case appeared to occupy the position of the monosialoganglioside (G_5) described in Tay-Sach's disease by Svennerholm (227). No X-ray abnormalities were noted in this case.

Volk, et al (246, 204) described the accumulation of ganglioside GD1A (disialoganglioside, G_3) in grey matter obtained by surgical biopsy from a patient with late infantile amaurotic idiocy of the Jansky-Bielschowsky type. This disease may be another form of gangliosidosis, although the autopsy in this particular case has not been reported yet. In a recent article Borri, Hooghwinkel and Edgar (20) found no evidence of storage of any single ganglioside in late infantile amaurotic idiocy. This was also found by Svennerholm (227) and Borri and Hooghwinkle (19).

2. Report of a Case of Late Infantile Systemic Lipidosis

F.J., Born June 17, 1960.

The mother's pregnancy and delivery were normal. The baby weighed 9 lbs. 4 oz. at birth. He smiled at 1-1/2 to 2 months, sat up at 7 months and walked at 11 months. He was saying single words e.g. "papa" and "mama" at that time. The child became somewhat unresponsive around 11 to 12 months of age and he stopped walking at 14 months of age. The parents noted that he lost his ability to control his hands and his arms. He developed progressive stiffness of his arms and legs. By two years of age he was unable to speak. There were no seizures. The child was admitted to hospital for the last time at 4 years of age.

Consanguinity was suspected because the grandfathers of the mother and father of the child were married to wives with the same maiden name. Mother, father and three siblings were normal. A fourth sibling developed normally until about the age of 13 months when development stopped. In a similar manner to the case presented this child deteriorated neurologically till death at 4 years of age.

At four years of age he measured 37 inches from crown to heel and weighed 10.7 kilograms. His legs were extended with feet internally rotated. His eyes wandered and he would not fixate, even on a light, with either eye. Pupils were regular and equal and reacted to light directly and consensually. The optic discs appeared slightly pale. Both maculae appeared normal. There was no visceromegaly.

On neurological examination a generalized hyper-reflexia with considerable increase in tone was noted.

X-rays of the skull, chest and bones revealed no abnormality or sign to indicate a lesion such as Hurler's syndrome or pseudo-Hurler's syndrome.

Pneumoencephalogram was suggestive of a generalized cerebral atrophy with slight to moderate dilatation of the ventricular system. The electroencephalogram was grossly abnormal and epileptiform. It suggested a diffuse neuronal disease with maximal involvement in the left frontal region.

The cerebrospinal fluid was clear and colourless and the Pandy test was negative. Protein was 24 mg%, sugar 103 mg% and chloride 121 mg%. Urinary amino acids appeared normal on paper chromatography. Urinary sediment was stained with toluidine blue and alcian blue and

showed a faintly positive reaction on one of two occasions indicating the equivocal presence of small amounts of mucopolysaccharides.

A urinalysis was normal. Hemoglobin was 12.4 grams. Total white blood count was 9100. Urinary sediment was negative for metachromatic granules on three occasions. He died at the age of 4 years, 4 months of pneumonia.

At autopsy, marked cerebral atrophy was present and microscopically there was a marked neuronal lipidosis. Large vacuolated histiocytes were seen to be present in the bone marrow, spleen, liver, and alveoli of the lung. It was noted that the glomerular epithelium showed scanty instances of foam cells. Foam cells were also present in the distal convoluted tubules.

3. The Analysis of Brain and Spleen Gangliosides

Samples of brain (frontal lobe) and spleen were kept frozen until the time of analysis. Gangliosides were extracted from the brain and spleen by the modified Folch procedure of Suzuki (213). This procedure involves the extraction of brain with chloroform:methanol (1:2) (v/v) followed by chloroform:methanol (2:1) (v/v) at room temperature. After this the final combined extracts are adjusted to a ratio of chloroform: methanol (2:1) (v/v). The water soluble gangliosides are partitioned into an aqueous upper phase. This involves partitioning once with 0.1 N KCl, once with ideal upper phase containing KCl and once with ideal upper phase having distilled water in place of the 0.1 N KCl. Extraction of a sample of spleen from this case was carried out in the same manner.

The results of the analysis of the total brain ganglioside are shown in Table 7. The amount of NANA per gram wet weight of cortex was approximately double that which is normally found. The number of mgs of

TABLE 7

	μg NANA/gm Cortex	mg Ganglioside/gm Cortex	μmoles NANA/mg Ganglioside
NORMAL (7)	529 ± 25	1.8-2.3	1.01 ± 0.05
F.J.	1170	8.5	0.45

ganglioside per gram wet weight of cortex was about four times normal. The fact that the amount of NANA found per mg of ganglioside was about half that found in the total normal ganglioside mixture suggested that the ganglioside was a monosialoganglioside.

In order to separate the different types of ganglioside, a modification of the descending thin layer chromatography of Korey and Gonatas (115) was developed, using propanol water and ammonium hydroxide. The thin layer was carried out at 5°C for 24 hours using propanol:water: concentrated ammonium hydroxide (140:44:4.8) (v/v/v). A picture of the normal distribution of the different types of gangliosides seen when chromatographed in this system for 24 hours is shown in Figure 27. The numbering system of Korey and Gonatas (115) is used. At the top is the trisialoganglioside or G_1 and the two major bands seen are G_2 and G_3 corresponding to the two disialogangliosides. The major band at the bottom is G_4 corresponding to the major monosialoganglioside and two unidentified bands occur between G_3 and G_4 one of which probably corresponds to the G_{3a} of Ledeen (130). Tay-Sach's ganglioside and hematoside run below or faster than the major monosialoganglioside.

The descending thin layer pattern seen in standard ganglioside mixtures, Tay-Sach's ganglioside G_5 and ganglioside from the case of late infantile systemic lipidosis is shown in Figure 28. It is clear that the ganglioside in our case consists almost entirely of the major monosialoganglioside. Therefore, in a similar manner to the ganglioside accumulation in Tay-Sach's disease it is found that in this particular case there is an accumulation of the major ganglioside (G_4).

It should be noted that because the spot showing the major monosialoganglioside of this case is so overloaded the rf has been decreased considerably. Repeated thin layers with decreasing quantities

FIGURE 27

Descending thin layer chromatogram of normal ox brain gangliosides. Solvent system used was propanol:water:concentrated ammonium hydroxide (140:55:4.8) (v/v/v). Spray used was 50% sulphuric acid. Samples 1, 2 and 3 all contain standard ganglioside of 200 µgm, 150 µgm and 100 µgm respectively.

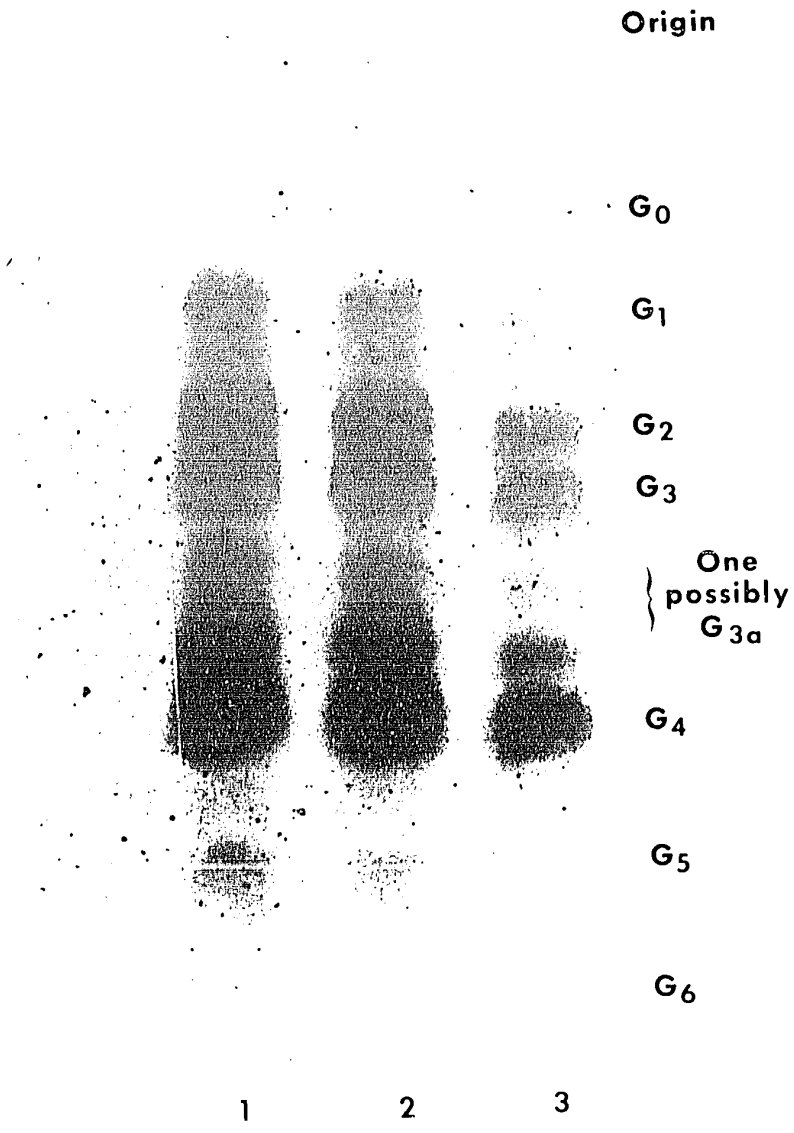
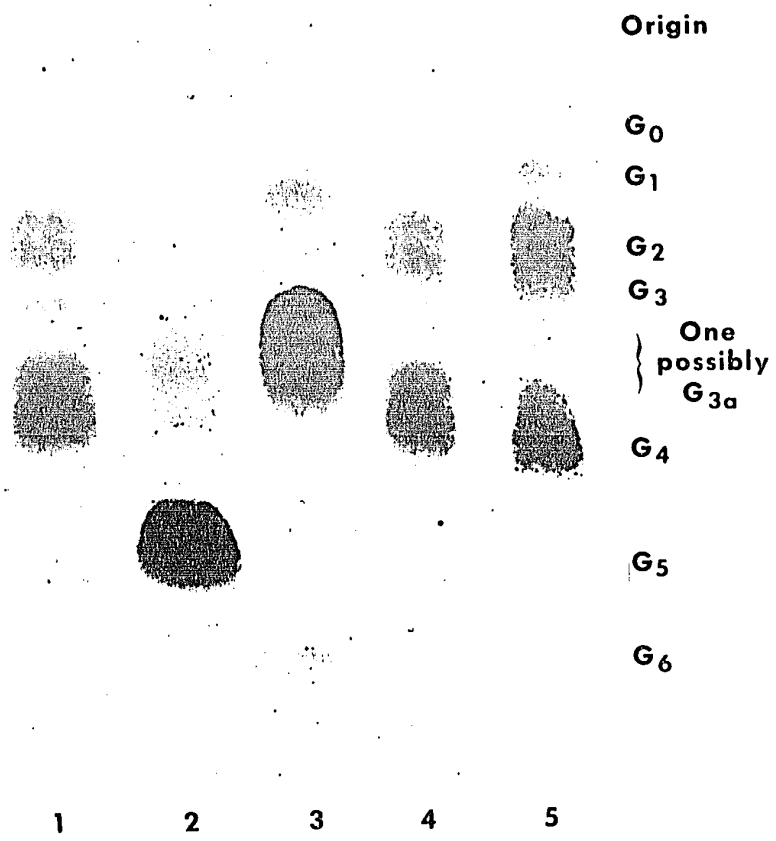

Origin
G_0
G_1
G_2
G_3
One possibly G_{3a}
G_4
G_5
G_6
1
2
3

FIGURE 28

Descending thin layer chromatography of normal brain gangliosides, Tay-Sach's ganglioside and the case of Late Infantile Systemic Lipidosis studied here. Sprayed with 50% H_2SO_4. Samples 1, 4, 5 all contain standard ganglioside preparations. Samples 1 and 5 were prepared by method of Trams and Lauter and column 4 by method of Suzuki. Sample 2 is Tay-Sach's ganglioside and Sample 3 is from the case analysed here.


Origin
G_0
G_1
G_2
G_3
One possibly G_{3a}
G_4
G_5
G_6
1
2
3
4
5

of ganglioside showed the rf to be the same as the major monosialoganglioside when the quantities spotted were the same. Analysis of the sample of spleen revealed that there was 32 μgms of NANA per gram wet weight. The concentration of glycolipid NANA obtained from the analysis of the spleen of our normal control patient was 15.7 μgms NANA per gram wet weight.

Phillapart, Rosenstein and Menkes (165) found that the normal concentration of NANA in the spleen in their case was 7.1 μgm NANA per gram wet weight. Consequently the value we obtained of glycolipid NANA in the spleen was twice what we found to be normal and four times that which Phillapart, Rosenstein and Menkes (165) found to be normal.

O'Brien, Stern, Landing, O'Brien and Donnell (160) found in their case of generalized gangliosidosis a value for spleen ganglioside of 2.3% of splenic lipids. Also the thin layer pattern revealed that all of the stored ganglioside in the spleen was the major monosialoganglioside (G_4). The theoretical percentage weight of the major monosialoganglioside made up by the NANA residue is 21%. The data of O'Brien, et al (160) therefore give a value of 240 μgms NANA per gram wet weight. On the other hand O'Brien, et al (160) were unable to detect any ganglioside in the liver and spleen of normal subjects.

The ganglioside from the spleen in the case of Late Infantile Systemic Lipidosis almost all ran with an rf that was the same as hematoside (G_6), the normal ganglioside found in spleen. A trace of ganglioside could be seen at the rf corresponding to the major monosialoganglioside.

4. Discussion

An analysis of the history, physical findings, pathology and biochemical findings of all cases of Tay-Sach's disease with visceral

involvement and related clinical entities has revealed that each case appears to fall into one of two clinical types (see Table 8). Table 8 lists the principal findings in the history, physical examination, laboratory data and ganglioside chemistry of the cases described in the literature.

In the clinical entity described by Landing, et al (125) and O'Brien, et al (160), which has been called here Type I, (see Table 8), the outstanding features are early onset of neurological deterioration, visceromegaly and radiological bone changes which are similar to Hurler's syndrome. Whereas the clinical entity (Type II) seen in the case described here and in that reported by Gonatas and Gonatas (59) have a later onset (10-15 months) of neurological impairment, no visceromegaly and no radiological bone changes.

Biochemically, the major change found in Type I (160) is an increase of the major monosialoganglioside (G_4) in brain, spleen and liver. The normal ganglioside pattern in brain completely disappears. In Type II the amount of brain ganglioside accumulating is approximately the same. That is, there is a four times increase in ganglioside content of the brain and almost all of this is due to an increase of the major monosialoganglioside. Although a small increase was found in the glycolipid NANA of the spleen, in the case presented in this thesis this was not very striking and the slight increase was not due to an increase of the major monosialoganglioside but of hematoside. The viscera in the case reported by Gonatas and Gonatas (59) have not been examined.

It is not possible at this time to interpret whether or not Type I and Type II Late Infantile Systemic Lipidosis are different manifestations of the same disease or are completely different diseases.

TABLE 8

PRINCIPAL FINDINGS IN LATE INFANT

		HISTORY			PHYSICAL FINDINGS				LA
		Onset Mos	Death Mos	Seiz.	Head	Macula	Liver	Spleen	X-r Spi
TYPE I									
Landing(125) et al	#1	Birth	3.5	-	-	-	↓	↓	+
	#2	4	16	-	N	C.R.S.	↓2cms	↓N	+
	#3	Birth	4	-	-	N	↓6cms	↓3cms	+
	#4	Birth	7	-	N	N			+
	#5	Birth	4.5	-	-	N		-	+
	#6	3	10	-	-	N	↓5cms	-	+
	#7	5	15	yes	-	C.R.S.		-	+
O'Brien(160)	#8	6	21	-	-	-			+
O'Brien(160) et al		Birth	8	-	L	-		-	+
TYPE II									
Gonatas(59) et al		10	25	yes	N	N	↓2cms	N	N
Davison(37) et al		5	25	yes	L	C.R.S.	↓1cm	N	N
Norman(155) et al		12	24	no	-	AbN	N	N	N
Turban		-	24	-	-	C.R.S.	-	-	-
Norman(156) et al		9	17	-	L	C.R.S.	↓6cms	N	N
Brouwer(23)		12	31	yes	-	C.R.S.	-	-	-
Present Study		12	48	yes	N	N	N	N	N

C.R.S. = Cherry red spot. N = Normal. F.Ce

TABLE 8

IN LATE INFANTILE SYSTEMIC LIPIDOSIS

	LABORATORY FINDINGS		AUTOPSY				GANGLIOSIDE ANALYSIS		
Spleen	X-ray Spine	Bone Marrow	CNS	Liver	Spleen	Bone Marrow	CNS	Viscera	Type
↓	+	F.Cells	+	+	+	+	-	-	-
↓N	+	-ve	+	+	+	-	-	-	-
↓3cms	+	F.Cells	-	-	-	-	-	-	-
	+	-	-	+	+	+	-	-	-
-	+	-	N	N	-	+	-	-	-
-	+	-	+	+	+	+	-	-	-
-	+	F.Cells	+	+	+	+	-	-	-
	+	F.Cells	-	-	-	-	-	-	-
-	+	F.Cells	+	+	+	-	4xN		G_4
N	N	F.Cells	+	+	+	+	4xN	-	G_4
N	N	-	+	+	+	-	-	-	-
N	N	-	+	+	+	+	4xN	-	G_5
-	-	-	+	+	+	-	5xN	-	-
N	N	F.Cells	+	+	+	+	4xN	-	-
-	-	-	+	-	+	-	-	-	-
N	N	F.Cells	+	+	+	+	4xN	N	G_4

F.Cells = Foam Cells.

"-" = not done or not mentioned.

Neither of these types appear to be related biochemically either to Tay-Sach's disease of the infantile form in which the ganglioside accumulating is of different structure, or to the late infantile type (Jansky-Bielschowsky) in which no ganglioside abnormality has been reported (19, 20). The case reported by Volk, et al (246, 204) with late infantile (Jansky-Bielschowsky) form of amaurotic family idiocy showed an accumulation of the disialoganglioside. It is possible that due to overloading of their thin layer chromatogram the rf of the unknown ganglioside became more difficult to interpret. As there is no confirmation yet of visceral involvement it is difficult to classify this case.

The case reported by Norman, Tingey, Newman and Ward (155) is also difficult to interpret in terms of our classification. The brain had been preserved in 10% formal saline for 14 months. Also the thin layer chromatogram had again been overloaded making the rf of the unknown ganglioside difficult to interpret. It is a possibility that what they have called accumulation of the Tay-Sach's ganglioside (G_5) is in fact the major monosialoganglioside (G_4). Consequently this case has been listed in Table 8 as Type II Late Infantile Systemic Lipidosis.

Jatzkewitz and Sandhoff (95) found an accumulation of the major monosialoganglioside in their case of "a biochemically special form of infantile amaurotic idiocy". They postulated that the deposition of the ganglioside was due to a block in the degradation of the ganglioside. Figure 29 shows the scheme proposed to account for both the accumulation of Tay-Sach's ganglioside and the major monosialoganglioside.

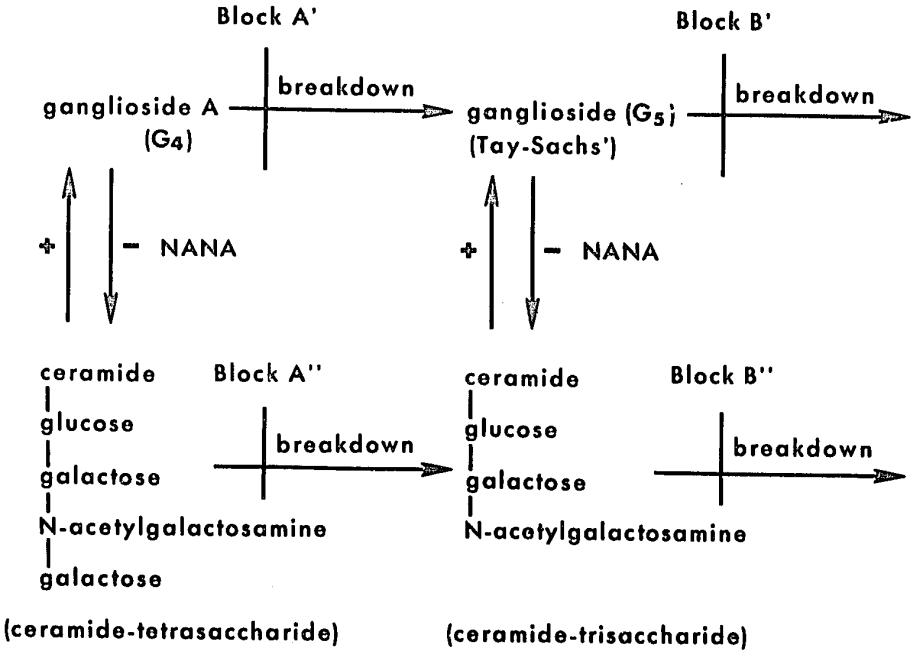
Svennerholm (224) and Jatzkewitz and Sandhoff (95) have found a concommitant increase in the ceramide-trisaccharide in cases of Tay-Sach's disease and an increase in a ceramide-tetrasaccharide in cases where the major monosialoganglioside is accumulating.

A sample of the brain from our case of Late Systemic Infantile Lipidosis was analysed for ceramide-tetrasaccharide by K. Suzuki of the Saul Korey Department of Neurology, Albert Einstein College of Medicine. He confirmed our finding of an accumulation of the major monosialoganglioside (G_4) and also found a concomitant increase of the ceramide-tetrasaccharide. The scheme proposed in Figure 29 shows that the enzymatic defects in Tay-Sach's disease would be a decrease in the enzyme which hydrolyses Tay-Sach's ganglioside (Block B') and a decrease in the enzyme which hydrolyses the ceramide-trisaccharide (Block B''). Similarly the enzymatic defects in Late Infantile Systemic Lipidosis Type I and Type II are a decrease in the enzyme which hydrolyses the major monosialoganglioside (Block A') and a decrease in the enzyme which hydrolyses the ceramide-tetrasaccharide (Block A'').

FIGURE 29

Proposed scheme to explain enzymatic defects in Tay-Sach's disease and Late Infantile Systemic Lipidosis. Blocks A' and A'' are defects in the enzymes which cleave the terminal galactose from the major monosialoganglioside and the ceramide-tetrasaccharide respectively. Blocks B' and B'' are defects in the enzymes which cleave the terminal N-acetylgalactosamine from Tay-Sach's ganglioside and the ceramide-trisaccharide respectively.

Proposed Scheme for Accumulation of Ganglioside in Tay-Sachs' Disease and Late Infantile Systemic Lipidosis



SUMMARY

Using a fluorometric technique for the analysis of N-acetyl-neuraminic acid in gangliosides the distribution of gangliosides in the neurone has been studied. A block of tissue from area CA2 of the ox hippocampus was cut into 24 μ sections parallel to the layers. Each slice was extracted and ganglioside content determined. Ganglioside content in mg ganglioside per gram dry weight was found to be high in the stratum granulare and stratum pyramidalis suggesting that gangliosides are found in cell bodies. The high values found in the stratum radiation and stratum moleculare, where there are no cell bodies, suggest gangliosides are found in dendrites as well.

Analysis of serial 24 μ sections through the folium of the ox cerebellum from pial to pial surface showed that again gangliosides were concentrated at sites rich in cell bodies (granular layer) and as well in the molecular layers where branching dendrites of Purkinje cells occur. One of the most striking findings was the wide differences in ganglioside content within a histologically defined layer. These findings were interpreted as meaning that the variability in ganglioside contents even in adjacent sections probably reflected changes in the number of clusters of the structures in which gangliosides are found, such as synaptic terminals. To show that these variations in ganglioside content were not due to technical artifacts, sections were cut and analysed from similar blocks from the hippocampus and cerebellum perpendicular to the histologically defined layers. Each section cut in this way would contain structures from all layers and would have the same morphological features. The results showed a marked decrease in variability of ganglioside content.

Single neurones and glial cell clumps were isolated from Deiter's nucleus of the ox brain stem. The sample containing pure neurones had a high concentration of ganglioside. The sample of neuropil tissue from immediately around the nerve cell bodies also contained high concentrations of ganglioside on a dry weight basis. Two samples of glial cells and neurones analysed together, and in which more glial mass was included, showed a much lower ganglioside content. These results together with the results found from the analyses of the hippocampus and cerebellar folium are interpreted as strongly suggesting that gangliosides are concentrated in post-synaptic membranes and pre-synaptic nerve endings. A new interference microscope system (Nomarski) of the Carl Zeiss Company was used to reveal details of structure of the neurones and glial cell clumps.

Ganglioside analyses were made on a sample of brain and spleen from a patient who had died with Late Infantile Systemic Lipidosis. The ganglioside content of brain was four times normal and in the spleen was twice normal. Thin layer chromatography revealed that almost all the ganglioside present was the major monosialoganglioside. From the cases reported in the literature, Late Infantile Systemic Lipidosis can be divided into two types on the basis of clinical, radiological and biochemical findings.

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CLAIMS TO ORIGINAL RESEARCH

1. A fluorescent method for the analysis of N-acetylneuraminic acid in the bound or free form has been used to analyse ganglioside as total glycolipid NANA. A standard line using a purified unfractionated ganglioside preparation was made with a standard error of the estimate of $\pm 27 m\mu$ gm ganglioside.

2. A method has been described for analysing the ganglioside content of serial cryostat sections through area CA2 of the ox hippocampus and a folium of the ox cerebellum. The results of ganglioside analyses of serial cryostat sections have been presented. Histological control was carried out by taking histological sections perpendicular to the layers of the respective areas analysed.

3. Results of analyses of ganglioside content as mgs of ganglioside per gram dry weight for each section of all the layers of area CA2 of the ox hippocampus through to the granular layer of the fascia dentata have been presented. Similarly, analyses of sections through a folium of ox cerebellum are presented from pial to pial surface. The results indicate that gangliosides occur in the cell body and as well in regions of dendritic branching. Wide differences in ganglioside content were found in adjacent sections in both the cerebellar folium and ox hippocampus. This is interpreted as caused by variations in the amounts of anatomical structures such as synaptic terminations on dendrites or cell bodies which are rich in gangliosides.

4. To show that the variability of ganglioside content in the layers was not due to technical artifacts, sections of a block from area CA2 were cut perpendicular to the layers and a marked decrease in the

variability resulted. Similar results were obtained when sections were cut perpendicular to the layers of the folium of the ox cerebellum.

5. Single cells and glial cell clumps were isolated from the Deiter's nucleus of the ox brain stem. The ganglioside analysis was carried out on samples of pure neurones, samples of glial cells from immediately around the neurones and samples of neurones and glia together with a much larger contribution of glia than neurones. Results show conclusively that gangliosides are in neurones. However, the finding that gangliosides were present in equal concentration in neuropil samples taken from immediately surrounding the isolated neurones is interpreted as meaning that gangliosides are concentrated in the synaptic terminals on cell bodies and dendrites.

6. The recently developed Nomarski interference contrast optics of Carl Zeiss Co. was used to photograph the isolated neurones and glial cells.

7. A descending thin layer chromatographic system is described using propanol, water and concentrated ammonium hydroxide to separate gangliosides.

8. The brain and spleen of a child with Late Infantile Systemic Lipidosis were analysed for the amounts and types of gangliosides present. It was found that the major monosialoganglioside was accumulating in the neurones in this disease. A slight increase in ganglioside content in the spleen was found.