Loyola University Chicago
Loyola eCommons

Dissertations | Theses and Dissertations

1985

The Biological Role of Sialosyl Transferase Activity in Rat Brain

Robert Paul Durrie
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

 Part of the Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation

Durrie, Robert Paul, "The Biological Role of Sialosyl Transferase Activity in Rat Brain" (1985). *Dissertations*. 2466.
https://ecommons.luc.edu/luc_diss/2466

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1985 Robert Paul Durrie

THE BIOLOGICAL ROLE OF SIALOSYL TRANSFERASE ACTIVITY IN RAT BRAIN

by

Robert Paul Durrie Jr.

Library--Loyola University Medical Center

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree

of

Doctor of Philosophy

December

1985

ACKNOWLEDGEMENTS

I wish to express my appreciation and deep sense of gratitude to Dr. Abraham Rosenberg, my advisor and friend. He has the remarkable ability to guide the scientific direction of, and instill productivity in, his students, without inhibiting their freedom of thought. In fact, he inspires it.

I gratefully acknowledge all the help, suggestions, encouragement, and friendship I received from the members of the laboratory, and from the faculty and staff of the Department of Biochemistry and Biophysics. Special thanks must go to Dr. Mitsuo Saito for his leadership, to Gesila Kindel for her expertise in brain slice methodologies, and to Dr. Mariko Saito and Michael Hogan for their technical help and companionship.

I was very fortunate to have some excellent technical assistance in the area of electron microscopy. Dwan Taylor of the Department of Biochemistry and Biophysics did beautiful work, and Dr. Raoul Fresco of the Department of Pathology was most generous in helping me in this area.

And finally, I wish to thank my wife Lauren, whose unfaltering love has been responsible for sustaining me physically, mentally, and spiritually, throughout the course of this dissertation.

VITA

Robert Paul Durrie Jr. is the third child and second son of Robert Paul Durrie Sr. and Mary (Stephensen) Durrie. He was born on April 4, 1952, in Lincoln, Nebraska.

His elementary education was obtained in the public schools of Lincoln, Nebraska. He graduated from high school in 1970, fron Lincoln Southeast High School, in his home town.

He briefly attended the University of Nebraska at Lincoln, and then transfered to Iowa State University, where he majored in Animal Science-Pre Vet. After his junior year he was accepted to, and entered the College of Veterinary Medicine, at Iowa State University. In May of 1979, he recieved a Doctor of Veterinary Medicine degree.

Dr. Durrie practiced small animal veterinary medicine and surgery in Franklin Park, IL, for one year. In July of 1980 he accepted a Basic Science Fellowship in the Department of Biochemistry and Biophysics at the Loyola University Medical Center, in Maywood, IL. Here he under took graduate studies in the Ph.D. program, under the directorship of Dr. Abraham Rosenberg.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure page

Figure page

LIST OF ABBREVIATIONS

Abbreviation	Identity
AChE	Acetylcholine esterase
AMP	Adenosine monophosphate
BSA	Bovine serum albumin
Cer	Ceramide
ChAT	Choline acetyltransferase
CMP	Cytidine 5' monophosphate
CNS	Central nervous system
CPM	Counts per minute
CST	Cerebroside sulphotransferase
C/M	Chloroform, methanol solution
DPM	Disintegrations per minute
Gal	Galactose
GalNAc or GalN	N-acetyl-galactoseamine
Glu or Glc	Glucose
GlcNAc or GlcN	N-acetyl-glucosamine
GSL	Glycosphingolipid
g	Force of gravity
HM	Homogenizing medium
LS	Lysing solution
Man	Mannose
ManAc	N-acetyl-mannosamine
NANA, or NeuAc	N-acetyl-neuraminic acid
PBS	Phosphate buffered saline

Abbreviation	Identity
PNS	Peripheral nervous system
rpm	Revolutions per minute
S.E.M.	Standard error of the mean
SPM	Synaptic plasma membrane
STase	Sialosyl transferase
UDP	Uridine 5' diphosphate
vol.	Volume
wt.	Weight

CHAPTER I

REVIEW OF GANGLIOSIDES

Make no mistake about it! The goals and themes that have inspired the writing of this dissertation are to understand the significance and function of the molecular membrane components, called gangliosides. From the dissertation title, one could get the idea that this work is an enzymological study of a peculiar enzyme, sialosyltransferase, located in the brain. This may be a disappointment to the pure enzymologist, but no Km, Vmax, or Lineweaver-Burke plots are found in these pages. Yes, an enzyme is studied here, but this is mainly due to the fact that this enzyme's existence could be very enlightening to the understanding of the function of gangliosides. And "the function of gangliosides" has been a topic of great interest but little substance for half a century. An understanding of the importance of this research will require knowledge of the biochemical, and topographical nature of brain gangliosides.

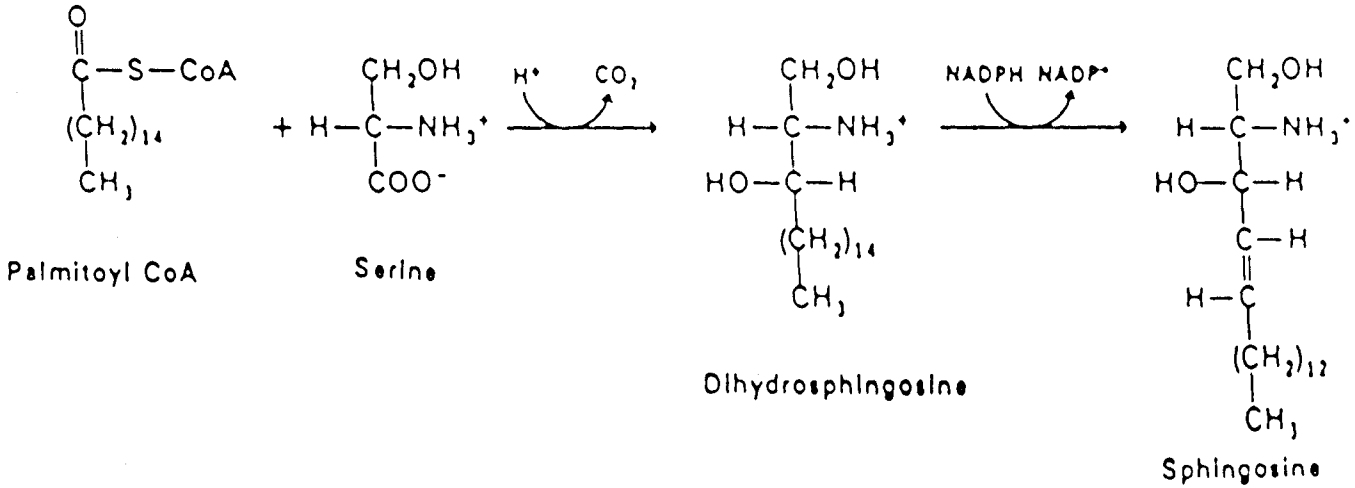
GLYCOSPHINGOLIPIDS

By the simplest definition, gangliosides are sialic acid containing glycosphingolipids. The much broader

group, the glycosphingolipids (GSL), are membrane component molecules made up of a lipophilic moiety, a ceramide, and a straight or branched oligosaccharide chain.

Ceramides are made up of a long chain base (usually sphingosine), which is in amide linkage to a free fatty acid. Sphingosine is the product of condensation-decarboxylation-dehydration reactions of a free fatty acid (usually C_{16}) to the carboxyl group of a serine molecule. A characteristic allylic double bond is formed. The sphingosine (or related long chain base) is N-acylated by a second free fatty acid (C_{14} to C_{26}) to form the ceramides (see figure 1.1). This lipophillic component of GSLs is similar in size, shape, and amphiphilicity to the phosphatidic acid moiety of the phospholipids (see fig. 1.2). Although the ceramides and GSLs tend to form micelles rather than bilayers in aqueous solvents, in a mixture with phospholipids, they co-exist with the phospholipids in lipid bilayers (reviewed by Tettamati, 1976). Thus the ceramide portion of GSL's is thought to be anchored in biological membranes (Steck and Dawson, 1974). Although there is abundant heterogeneity in the fatty acyl moieties of the ceramides, in the classification of GSLs, the ceramides are usually ignored, and the carbohydrate portion is the basis of classification. For a review of ceramide biochemistry, see Kishimoto, 1983.

BIOSYNTHESIS OF THE CERAMIDES



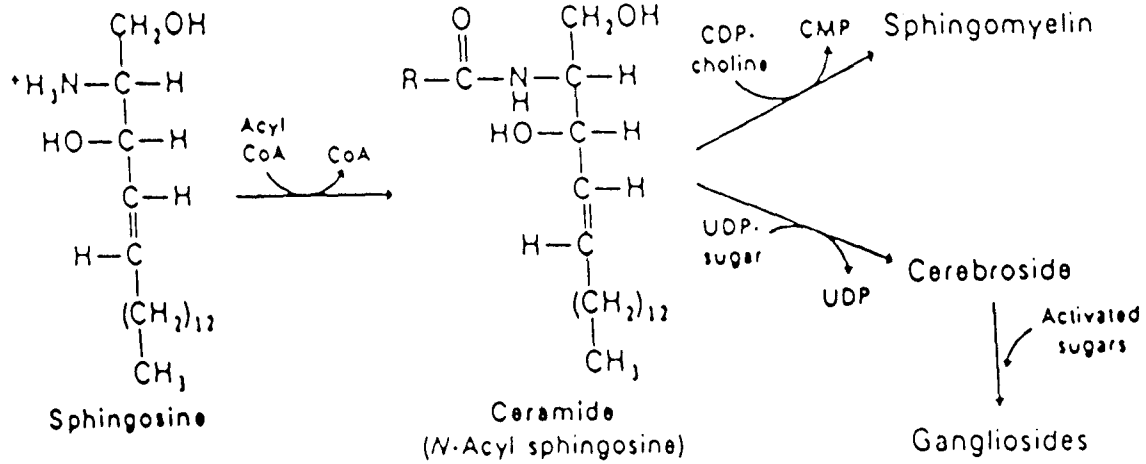


FIG. 1.1

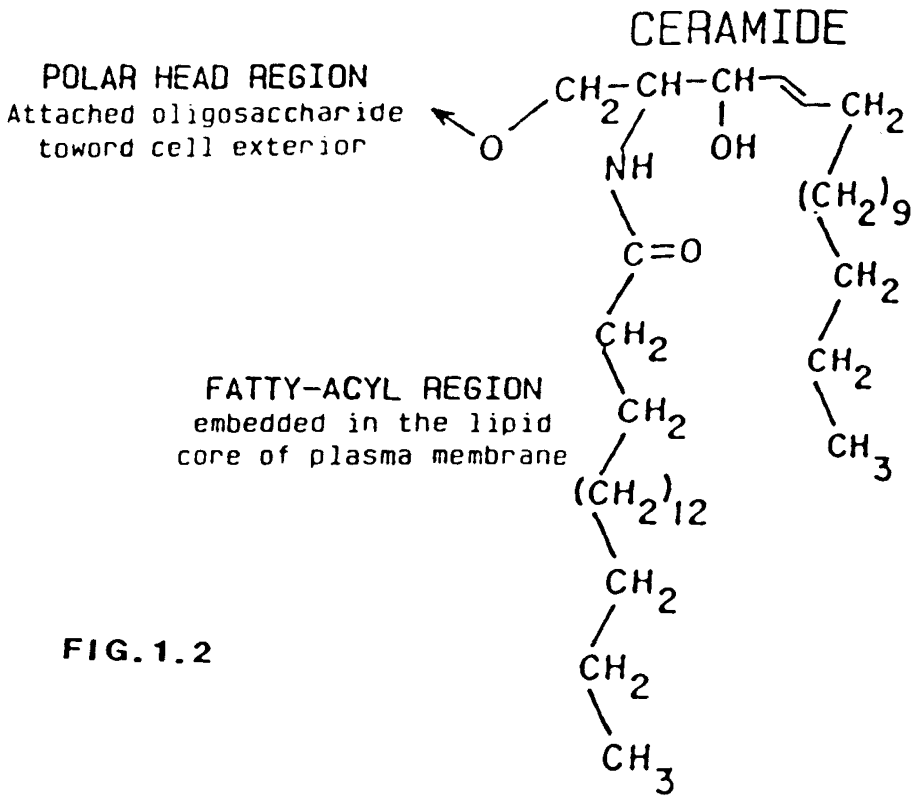
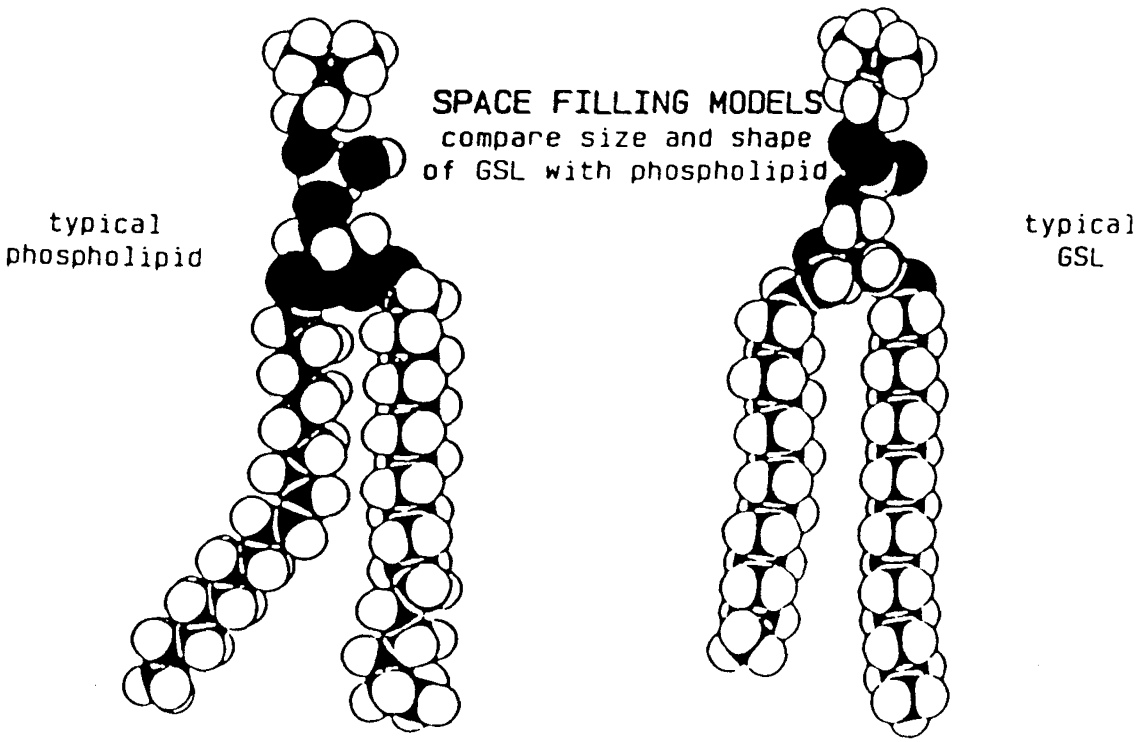

CERAMIDE
POLAR HEAD REGION
Attached oligosaccharide
toword cell exterior
FATTY-ACYL REGION
embedded in the lipid
core of plasma membrane
CH2-CH-CH=CH2
O
NH
OH
C=O
(CH2)9
(CH2)12
CH3

FIG. 1.2


SPACE FILLING MODELS
compare size and shape
of GSL with phospholipid
typical
phospholipid
typical
GSL

In glycosidic linkage to the primary hydroxyl group of ceramide, the GSLs have an oligosaccharide chain of from one to possibly 30 monosaccharides. The most common sugars are: glucose (Glu), galactose (Gal), N-acetyl galactosamine (GalNac or GalN), N-acetyl-glucosamine (GlcNac or GlcN), and the sialic acids. Fucose and mannose are found in GSLs not too uncommonly, and other sugars will spring up in the literature every now and then. Most of the oligosaccharide chains are fairly short (less than 10 glycosidically linked sugars), are straight or branched, and extend outward from the surface of the membrane. There is much evidence that the GSLs exist mainly in the outer leaflet of the lipid bilayer that make up the plasma membrane of cells, (Gahmberg and Hakomori, 1973; Wiegandt, 1972). Thus the carbohydrate chain is exposed to the extracellular environment. The carbohydrate rich glycocalyx that surrounds all mammalian cells, and determines cell surface properties, includes the GSLs.

Sugars, when in the form of complex carbohydrates such as GSL and glycoprotein, exhibit exquisite biologic specificity in mature. A single sugar substitution in even the most complex oligosaccharide chain produces a completely unique complex carbohydrate molecule. The diversity in the oligosaccharide chains has caused much

confusion in the nomenclature of GSLs. The most widely used classification scheme breaks the GSLs down into several "series" groups based on the linkage sequence of the first few sugars to the ceramide moiety. Most GSLs begin with either galactose or glucose linked to ceramide. This forms the basis of the first GSL classification, those based on galactosylceramide, and those on glucosylceramide, the latter being the more extensive group. The sequence of the next 2 or 3 sugars added on, gives rise to the basic GSL "series" groups (e.g. globo-, lacto-, ganglio-, hemato-, etc.). These can be seen in fig. 1.3. By adding specific sugars, in specific glycosidic linkages, to these basic GSL "series" groups, an enormous number of specific GSLs are formed. New ones are isolated from biological sources and are reported frequently. Often a single unusual GSL will only be isolated from one biological source, and no where else.

Where are GSLs found? They are believed to be ubiquitous throughout the animal kingdom. It is generally believed that they display cell type specific patterns and are present in the outer leaflet of the plasma membrane bilayer. There have been some reports of the isolation of GSLs from plants (Laine, 1981). But this has been studied very little. There are no conclusive reports of GSLs in prokaryotes. Possibly the enveloped viruses (e.g.

GLYCOSPHINGOLIPID CLASSIFICATION

SPECIFIC GLYCOSIDIC LINKAGES ARE NOT SHOWN

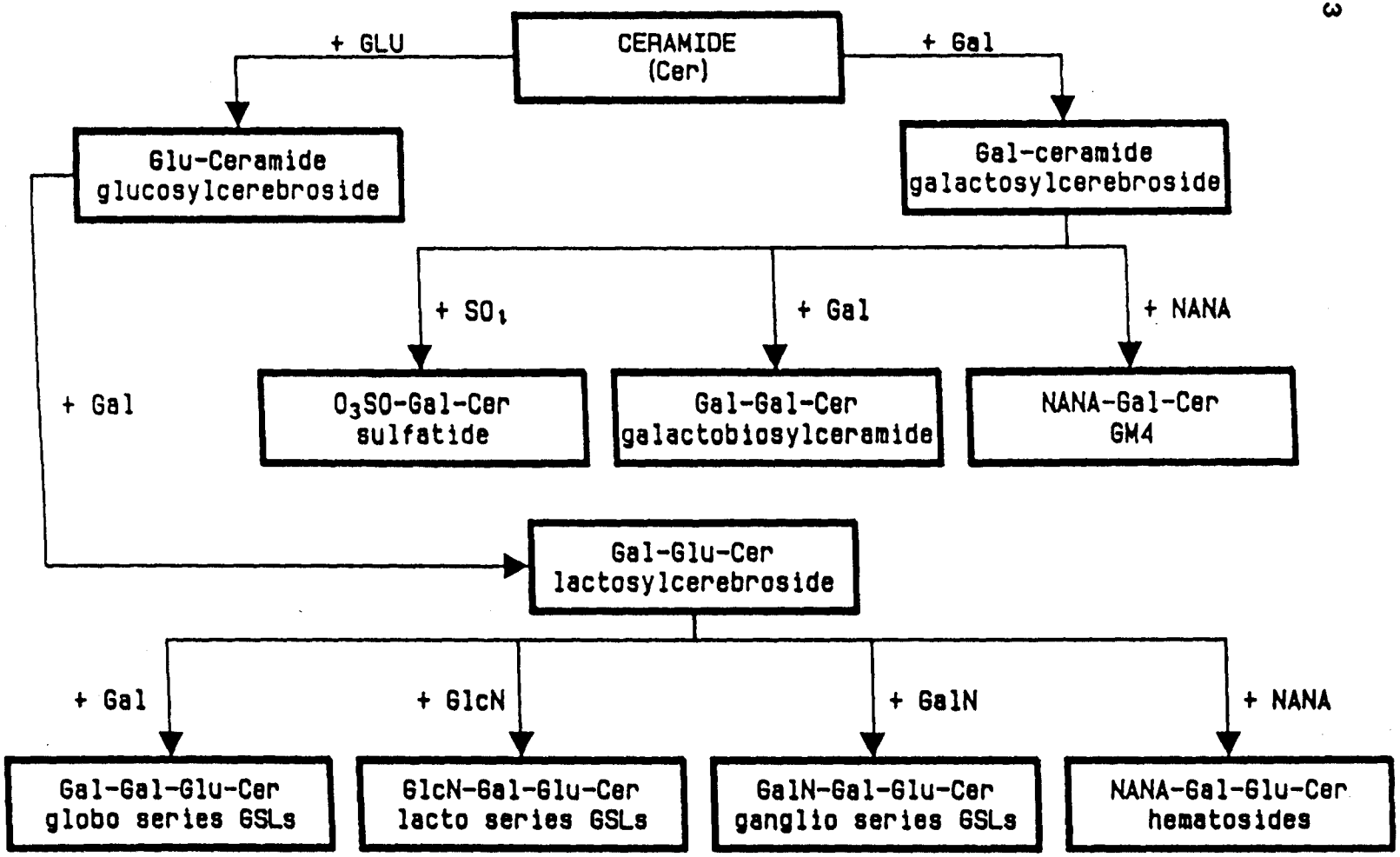


FIG. 1.3

mixoviridae) have GSLs, but these probably originated from the viral host's plasma membrane.

What is the function of GSLs? Well, their isolation and structural analysis has proven to be much easier than demonstrating specific functions for GSLs. It is generally speculated they are involved in cell-cell interactions such as identification, communication, differentiation, adhesion, and other phenomena that happen when two or more cells come in contact. There are many extensive reviews of glycosphingolipids, in which the original references for many of the above statements are cited. See: Yu-Teh Li and Su-Chen Li, 1982; Hakamori, 1981; Brunngraber, 1979.

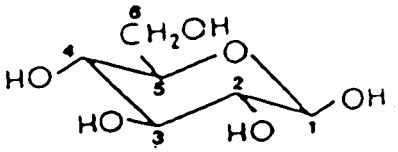
THE SIALIC ACIDS

There are components of the oligosaccharide portion of gangliosides that set them apart from all other GSLs. These are the sialic acids. They comprise a family of derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosomic acid). This nine carbon polyhydroxy keto acid cyclizes to form a pyranose ring structure (see fig. 1.4) similar to the naturally occuring hexoses, i.e. glucose, galactose, and mannose. The amino group is always stabilized in nature, usually by N-acetylation to form, N-acetyl-neuraminic acid (NANA, NeuNac or NeuAc), which is the most common member of the sialic

FIG. 1.4

STRUCTURES OF GLUCOSE AND THE SIALIC ACIDS

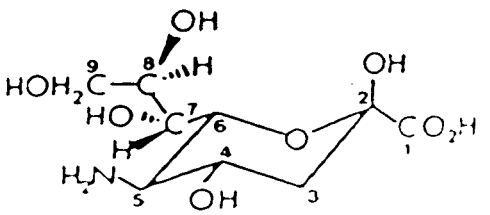
A.



D-Glucose

D-glucopyranose
all hydroxyls are equitorial

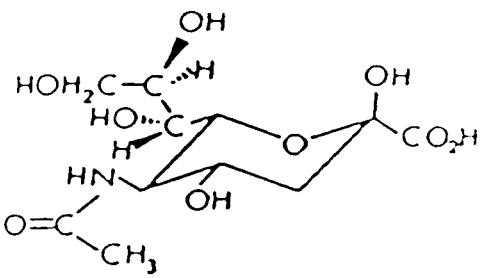
B.



Neuraminic acid

5-Amino-3, 5-dideoxy-D-glycero-
D-galacto-2-nonulopyranomic acid

C.



N-Acetylneuraminic acid

5-Acetamido-3, 5-dideoxy-glycero-
D-galacto-2-nonulopyranomic acid

acids. But the amino group can be N-glycolylated, or the various hydroxyl groups can be O-methylated, O-acetylated, O-lactylated, or phosphorylated giving rise to a diverse group of sialic acids. Each sialic acid is a unique entity, exhibiting great biological specificity. In this dissertation, "NANA" will be used extensively, but unless specified otherwise, will refer to all sialic acids. The sialic acids are an extremely interesting group of biologic compounds. This interest is based upon one reoccurring theme in sialic acid biochemistry, and that is uniqueness.

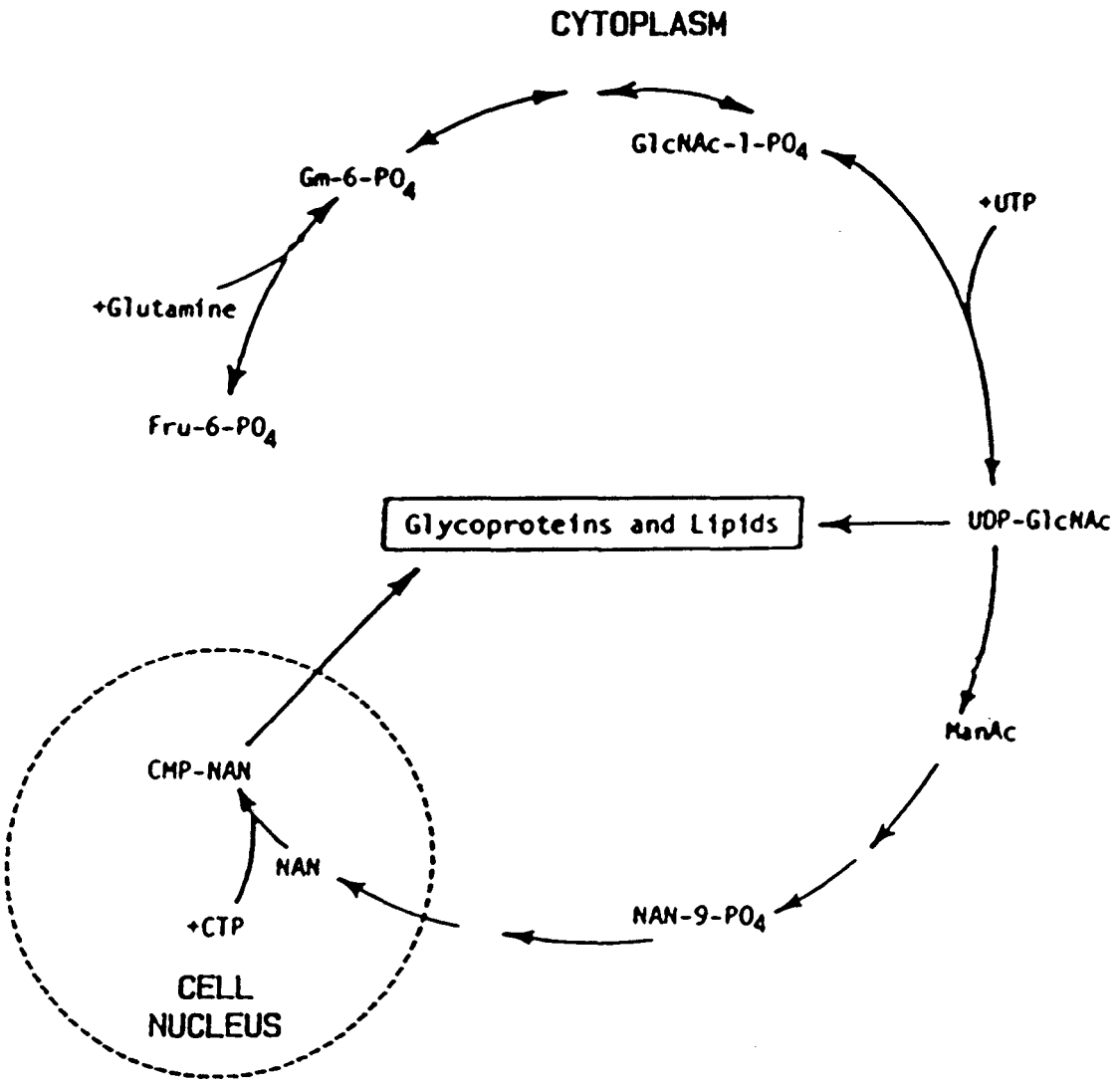
First of all, look at the unique structure of NANA (fig. 1.4). It may not fit the definition of a true sugar. Note the lack of a hydroxyl group at carbon #3 (fig.1.4-B.). Note the three carbon polyhydroxy "tail" at carbon six of the pyranose ring, and of course the carboxyl group at carbon #1. Sialic acids are "L"-sugars, while most other biological sugars are the "D" form. Most sugars form "beta" glycosidic linkages, but sialic acids form "alpha" glycosidic linkages. Why are sialic acids so unique among biologic sugars? This is an interesting question and it seems likely that special biological functions exists for the sialic acids.

The biosynthesis of sialic acids is also totally unique among sugars, and is shown in Fig. 1.5. The synthesis is totally irreversible from the point of N-acetyl-

FIG. 1.5

DE NOVO SYNTHESIS OF SIALIC ACIDS

note that all enzymatic steps from the point of N-acetyl-mannosamine on are irreversible



mannosamine. The activated transfer form of sialic acids is the cytosine-mono-phosphate form (CMP-NANA), while in most of the other sugars, the uridine-diphosphate derivatives (e.g. UDP-glu) are the activated-transfer sugar forms. CMP-NANA activation takes place in the nuclear membrane of the cell, and is irreversible.

Sialic acids are found only in chain terminating positions (or linked to another sialic acid) in cell surface complex carbohydrate oligosaccharides. Thus, when sialic acid is present on a cell, it is at the end of cell surface oligosaccharide chains, and is one of the first molecules that would be encountered when that cell would be approached by another cell or molecule.

Sialic acids are wide spread throughout the animal kingdom. The existence of sialic acids is questionable in the plant kingdom. Sialic acids are present in the prokaryotes, with a direct, positive correlation to animal pathogenicity.

The question still remains: Why is there such a unique sugar; with unique synthesis, activation form, and metabolic compartmentalization; in such a specialized location? And why has this set of metabolic pathways (of sialic acids) been maintained throughout a long evolutionary history? Most people in the sialic acid

field believe that the answer must be that the sialic acids have important, key regulatory roles in the cell and in cell-cell interactions. For a review of the sialic acids, see: Ledeen & Yu, 1976; Warren, 1976; Jeanloz and Codington, 1976; and Corfield & Schauer, 1982.

THE GANGLIOSIDES

The content of one or more sialic acid residues as a constituent carbohydrate distinguishes gangliosides from other GSLs. Thus far, sialic acids have been only found in the "ganglio"-, "hemato"-, and "cerebro"- series of GSLs. The "Ganglio" series is by far the major group of gangliosides. There are over 60 different gangliosides that have been described to date. Again, a convenient system of nomenclature was needed to simplify reading the literature. The system of Lars Svennerholm (1963) will be used in this dissertation, as it is in most of the current literature. Table 1.1 is a partial listing of the nomenclature, of the most common gangliosides.

In fig. 1.6 the structures of the common, mammalian brain gangliosides are shown. These are: GM4, GM3, GM2, GM1, GD3, GD1a, GT1a, GD1b, GT1b, and the GQ1 series. Notice that the sialic acids (or NANA in this case) are always linked to a Gal residue (or to another NANA). In

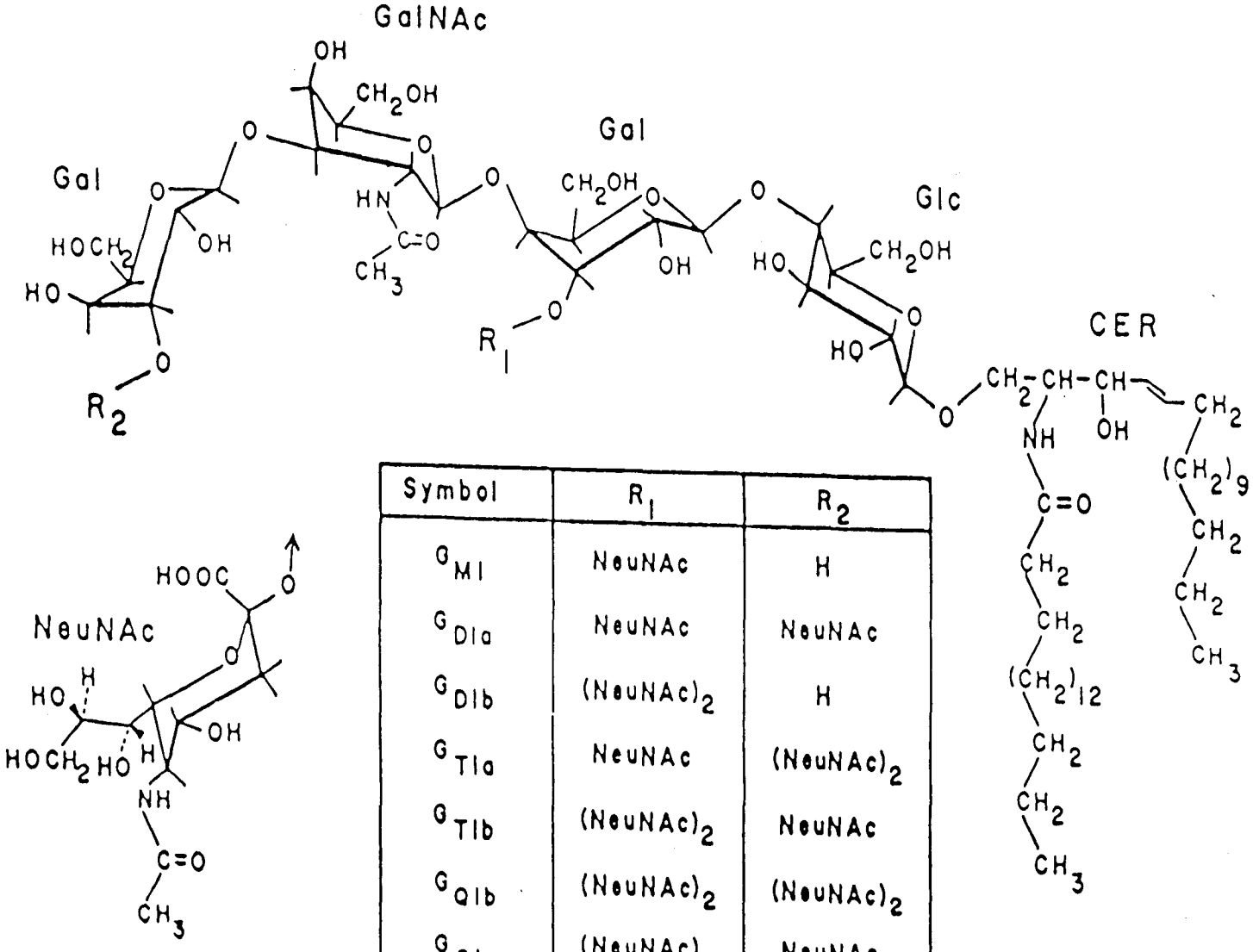
TABLE 1.1

STRUCTURE AND NOMENCLATURE OF GANGLIOSIDES

MOST OF THE COMMON MAMMALIAN BRAIN GANGLIOSIDES ARE SHOWN. AND OTHER MOLECULAR SPECIES DO EXIST

STRUCTURE	IUPAC-IUB	Wiegandt	Svennerholm
Gal-Cer NANA	II^3NeuAc-Gal-Cer	G_{GAL}^1	GM4
Gal-Glu-CER NANA	II^3NeuAc-Lac-Cer	G_{LAC}^1	GM3
Gal-Glu-Cer NANA NANA	II^3NeuAc_2-Lac-Cer	G_{LAC}^2	GD3
GalNac-Gal-Glu-Cer NANA	II^3NeuAC-$GgOse_3$-Cer	G_{Gtrl}^1	GM2
GalNac-Gal-Glu-Cer NANA NANA	II^3NeuAc -$GgOse_3$-Cer	G_{Gtrl}^2	GD2
Gal-GalN-Gal-Glu-Cer NANA	II^3NeuAc -$GgOse_4$-Cer	G_{Gtet}^1	GM1
Gal-GalN-Gal-Glu-Cer NANA NANA	IV^3NeuAC, II^3NeuAc-$GgOse_4$-Cer	G_{Gtet}^{2a}	GD1a
Gal-GalN-Gal-Glu-Cer NANA NANA	$II^3(NeuAc)_2$ -GgOse - Cer	G_{Gtet}^{2b}	GD1b
Gal-GalN-Gal-Glu-Cer NANA NANA NANA	$IV^3(NeuAc)_2$. II^3NeuAc -$GgOse_4$-Cer	G_{Gtet}^{3a}	GT1a
Gal-GalN-Gal-Glu-Cer NANA NANA NANA	IV^3NeuAc. $II^3(NeuAc)_2$ -$GgOse_4$-Cer	G_{Gtet}^{3b}	GT1b
Gal-GalN-Gal-Glu-Cer NANA NANA NANA NANA	$IV^3(NeuAc)_2$. $II^3(NeuAc)_2$ -$GgOse_4$ -Cer	G_{Gtet}^{4b}	GQ1b
Gal-GalN-Gal-Glu-Cer NANA NANA NANA NANA	IV^3NeuAc . $II^3(NeuAc)_3$ -$GgOse_4$-Cer	G_{Gtet}^{4c}	GQ1c

The specific glycosidic linkages are not shown.



Symbol	R_1	R_2
G_{M1}	NeuNAc	H
G_{D1a}	NeuNAc	NeuNAc
G_{D1b}	$(NeuNAc)_2$	H
G_{T1a}	NeuNAc	$(NeuNAc)_2$
G_{T1b}	$(NeuNAc)_2$	NeuNAc
G_{Q1b}	$(NeuNAc)_2$	$(NeuNAc)_2$
G_{Q1c}	$(NeuNAc)_3$	NeuNAc

FIG. 1.6

the major group of ganglioside there are 2 Gal residues:

Gal - GalNac - Gal - Glu - Cer

The one nearest the ceramide is called the proximal galactose, the other the distal galactose. In the Svennerholm naming system: "G" stands for ganglioside, followed by a uppercase letter, then a numeral. The latter defines the hexose backbone. A "1" denotes four sugars shown above. The middle letter tells you how many NANA residues there are (i.e. M=mono, D=di, T=tri, Q=quatro). For GM1, the single NANA, always is linked to the proximal Gal (fig. 1.6). For GD1, there is a choice; one NANA on each Gal, or two NANA's (a disialosyl linkage) on the proximal Gal. Note that the proximal Gal is always sialosylated first: all gangliosides have a proximal NANA-Gal linkage. The lower case descriptor designates the position of the NANA in instances where there is a choice. GD1a has a NANA on both Gal residues; GD1b has both NANA's on the proximal Gal. GT1b has a similar disialosyl linkage on the proximal Gal, and GT1a has the disialosyl group on the distal Gal. Three NANAs could be linked to the proximal Gal (GT1c) but this is rare. Four NANAs could be linked 2 proximal + 2 distal (GQ1b); 3 proximal + 1 distal (GQ1c) and other confirmers are possible. In this dissertation,

all quatro- sialosyl gangliosides shall be called GQ1 because they are not separated by our chromatography method.

The absence of the distal Gal from the "1" series backbone leaves the backbone for the "2" series gangliosides. GM2 and GD2 are usually present in samples, in very small amounts. Further absence of the GalNac residue describes the "3" series, or the hemodoside series, which are gangliosides based on the structure of lactosylceramide (Gal-Glu-Cer). GM3 is a major ganglioside in many tissues, and GD3 is usually quite small in amount, but there are some exceptions (i.e. embryonic chick muscle and brain, embryonic rat brain, peripheral nerve, etc.). GM4 is found in some tissues (i.e. myelin) and is the result of sialosylation of galactosyl ceramide.

The gangliosides have been found in all tissues, so far analyzed, of the higher animals. They are absent probably in only the lowest animal life such as sponges, and coelenterates. Plants do not have gangliosides, but may have negatively charged GSLs (glycophosphosphingolipids) that may serve a similar function as animal gangliosides. Prokaryotes are not believed to synthesize gangliosides, although they do metabolize other sialic acid containing glyco-conjugates (i.e. colomic acids, glycoproteins, etc.).

Gangliosides have been implicated in many biological processes, which like all GSLs, are involved in cell-cell interactions (reviewed by Hakomori, 1981; Jeanloz and Codington, 1976). An incomplete list of some of the specific functions that gangliosides have been implicated in might be: cellular recognition; cellular adhesion; cellular differentiation; cell growth control; oncogenesis; immune recognition; receptor components; ion complex formation; and many others, including those in the nervous system which will be addressed below.

GANGLIOSIDE BIOSYNTHESIS

The gangliosides (like all GSL's) are believed to be synthesized from ceramide, with stepwise addition of sugars, by membrane bound glycosyltransferases, in the microsomal organelles of cells, probably the golgi complex (Landa et al., 1977). The sugars are transferred from their activated transfer forms (UDP-Glu, CMP-NANA, Etc.). In one common theory, all the transferases needed for a given GSL, are physically bound together into multi-glycosyltransferase complexes (reviewed by Brunngraber, 1979). Thus when ceramide binds to a specific complex, the finished GSL species is the only species released.

These multi enzyme complexes have been isolated and

the individual transferases have been solubilized. The individual transfer steps have been worked out for the major gangliosides. This represents a compilation of the work of many researchers, the details and references of which can be found in any of the following reviews of ganglioside synthesis: Wiegandt, 1982; Brunngraber, 1979; Ledeen and Yu, 1982; or Svennerholm et al., 1980. A composite synthesis flow chart is given in fig. 1.7.

THE GANGLIOSIDE CONNECTION

GANGLIOSIDES AND THE CNS - It is almost "Mystery and intrigue" that has kept investigations into ganglioside metabolism going. This is in the face of little "hard" evidence to answer the basic questions about ganglioside function. This "intrigue" can be likened to the three basic rules of real estate:

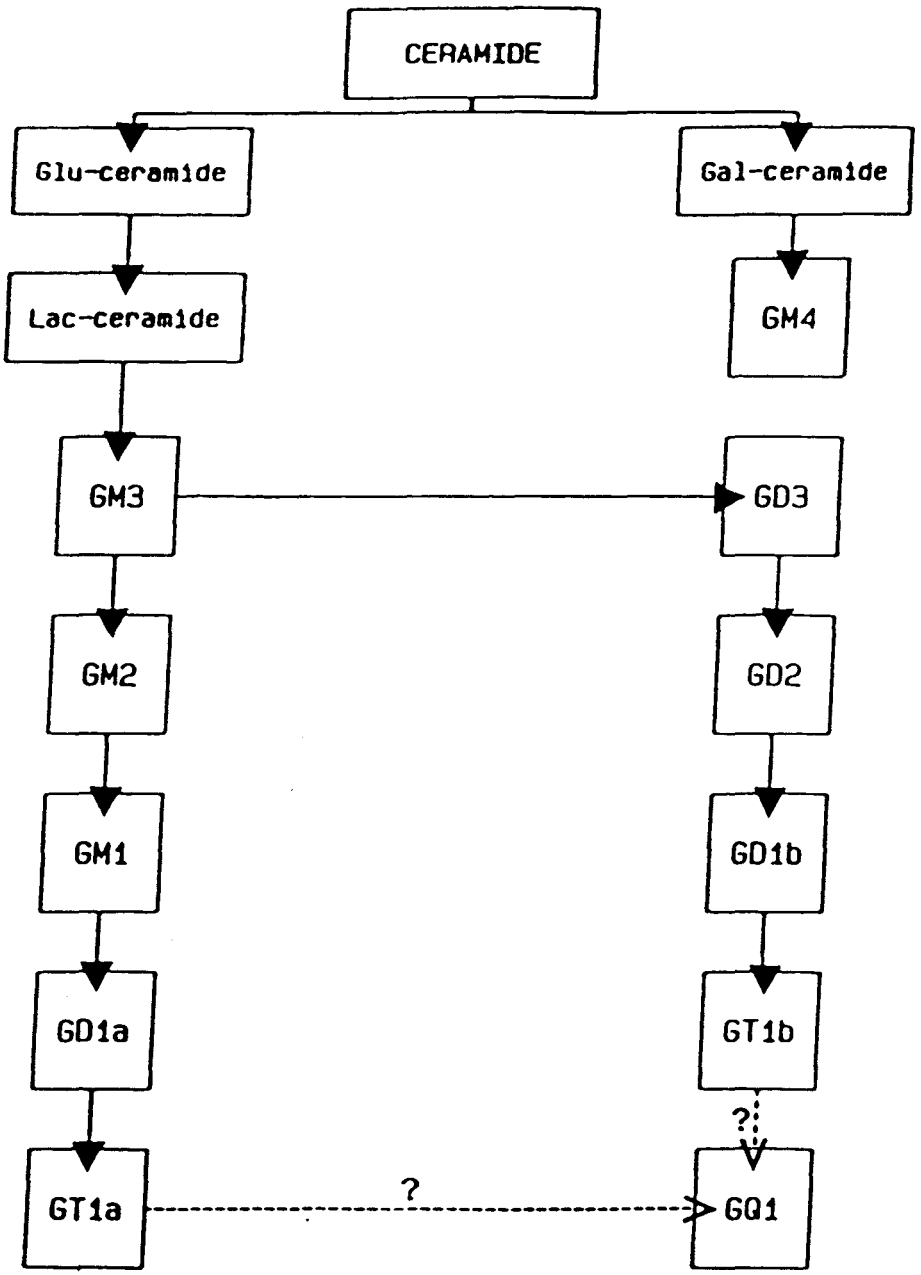
"Location; location; location!"

Gangliosides have been isolated from all mammalian tissues that have been studied so far. But brain may have well over 10 fold higher ganglioside concentration than most non neural tissue (Wiegandt, 1982).

There is an evolutionary correlation between gangliosides and CNS development (Ng and Dain, 1976). The first sialic acid containing GSLs appeared in the anne-

FIG. 1.7

GANGLIOSIDE SYNTHESIS RELATIONSHIPS
THE PATHWAYS SHOWN ARE GENERALLY ACCEPTED FOR SYNTHESIS VIA GOLGI MULTI-GLYCOSYLTRANSFERASES



lids, ecindoderms, and molluscs. This correlates to the nervous system changing from a primitive nerve net, to a system with true central control, or primitive brain (Wiegandt, 1982). There is also a correlation between the appearance of gangliosides and a calcium dependency for neuron function (Veh and Sander, 1981). There are correlations between the level of brain organization, and the level of ganglioside concentration in animal evolution; also a correlation between CNS "complexity" and ganglioside structure complexity (Wiegandt, 1982). For instance, it has been noted that the rays, cartilaginous fishes, and lower animals, have gangliosides with short oligosaccharides (i.e. hemato-series or ganglio-triose-series) while the higher animal brains contain more gangliosides, and these are mainly of the ganglio-tetrose-series.

Further investigations revealed that grey matter is many fold higher in ganglioside concentration than white matter (Suzuki, 1965). One basic difference from white matter is that grey matter is characterized by more neuron cell bodies and more neuron-neuron connections, or synapses. Brain is unique, compared to other tissues, in that most of its total sialic acid is lipid bound, rather than protein bound, (Ledeen, 1984). Mammalian grey matter has over 90% of its gangliosides in the ganglio-tetrose series (e.g. GM1, GD1a, GD1b, GT1a, etc.). White matter

contains more hemato-series gangliosides (Svennerholm, 1963). Also, grey matter has a much higher proportion of the poly-sialosyl ganglioside (di-tri-quatro sialosyl) than does other tissues (Brunngraber, 1979).

Many studies have been done in which the ganglioside content was analyzed in different anatomical regions of the gray matter (reviewed by Brunngraber, 1979). The data suggested that gangliosides were in the highest concentration in areas which are rich in dendrites and axonal plexuses, rather than in areas richer in cell somas and glial cells. Extensive studies of the subcellular fractionation of brain (Derry and Wolfe, 1967; Wiegandt, 1967; Breckenridge et al., 1972; Schengrund and Rosenberg, 1970; Yoe and Rosenberg, 1977; Morgan, et al., 1976; DeRobertis et al., 1976; Burton, 1976; Leskawa and Rosenberg, 1981), have concluded that the membranes richest in gangliosides are the microsomal subfraction and the synaptosomal subfraction (pinched off resealed nerve endings). The latter should be particularly enriched in the synaptic plasma membrane (SPM) itself (a view not shared by all: Ledeen, 1978).

Considering that the microsomal gangliosides are thought to represent a biosynthetic pool (Landa et al., 1977), the major site for functional gangliosides should

then be the SPM. The measured ganglioside to phospholipid ratio in the SPM is 1:10 (Breckenridge, 1972). Gangliosides are reported to be located mainly in the outer leaflet of the SPM bilayer (Hansson et al., 1977, Rosenberg, 1978). Thus on the extra synaptic surface of the SPM, there is possibly one ganglioside per every five phospholipids.

It is said that seeing is believing, therefore the most convincing evidence for ganglioside enrichment in the synaptic region has come from histochemical microscopy studies, utilizing ganglioside specific binding molecules. The bacterial cholera and tetanus toxins have been shown to specifically bind certain gangliosides. These have been used to stain brain tissue sections, and neuronal cell cultures (Fishman, 1982; Dimpfel, 1977; Rodgers and Snyder, 1981). Monoclonal antibodies have been raised to individual gangliosides, and these ultra specific probes have also been used to stain intact neurons (Eisenbarth et al., 1979; Seifert and Fink, 1984; Rafl et al., 1979). These studies have shown that gangliosides are highly enriched in the neuronal plasma membranes involved in synaptic junctions and dendritic networks. Neuronal cell somas, myelin, and glial cells do not stain as well. In histochemical studies of cell cultures, the developing neurites, and the neuropil at the tip of growing axons are

histocytochemical studies of cell cultures, the developing neurites, and the neuropil at the tip of growing axons are the areas of highest ganglioside concentration. Although all neuronal membranes have relatively high ganglioside content compared to most tissue, studies such as these indicate the synapse is enriched in ganglioside content many fold.

THE FUNCTION OF BRAIN GANGLIOSIDES

The human brain is by far the most incredible organ system in the biosphere. Hubel (et al., 1979) has calculated that the brain contains in the neighborhood of 10^{11} nerve cells, a quantity that could be compared to the number of stars in the Milky Way galaxy. Each of these cells makes a functional connection with at least 2 other cells, most having many more connections. Their role is to rapidly receive, conduct, then transmit very specific information from one cell to another.

The neuron receiving information in the synapse must be exactly right or the system will not function. Yet the neurons are all different types, probably no two are exactly alike. Information is transferred chemically, cell to cell, via specialized areas of the plasma membranes of the transmitting and receiving cells. This transmission, over the junction we call the synapse, rep-

resents the ultimate in cell-cell interactions. Also information is conducted the length of the cell, by the progressive local electrical charge depolarization of the neuronal plasma membrane. Together these represent highly specialized membranes which are required for the function of the neuron.

Logically, there should be something physically unique about neuronal membranes, to allow for these specialized functions. For this reason gangliosides, having unique character in neurons, have been speculated to have a functional role in nerve conduction and synaptic transmission for quite some time. The only problem has been the proof.

There is a large volume of literature offering data to support specific functions of gangliosides in the CNS. Much of this evidence seems circumstantial and is subject to interpretation. Therefore, there is no CNS ganglioside dogma. There are many comprehensive reviews of ganglioside function that are well worth reading (Brunngraber, 1979; Wiegandt, 1982). What follows, is a limited survey of ganglioside function in the CNS. My objective will be to point out some of the general areas of research.

An obvious implication for ganglioside involvement in CNS function is the existence of gangliosidosis diseases (reviewed by Tallman and O'Brady, 1976). These

are diseases caused by insufficiencies of specific enzymes involved in the catabolism of gangliosides (e.g. Tay-Sachs disease). These diseases are characterized clinically by neurologic deficits, which implies involvement of gangliosides in neurotransmission. It is noteworthy that no disease states exist which is caused by enzyme insufficiencies in the anabolism of ganglioside. This implies a lethal mutation, and that gangliosides are essential for life.

Cholera and tetanus toxins interfere with neurotransmission, specifically by antagonizing the postsynaptic response to acetylcholine and by blocking acetylcholine release, respectively (reviewed by Fishman, 1982). These toxins have been shown to bind specifically to certain gangliosides, and are concentrated in the brain in regions high in ganglioside content. One conclusion is that gangliosides may be receptors, or be part of the receptors for neurotransmitters.

Receptor functions would be an intuitive assumption for unique, cell surface components located in the synaptic area, (reviewed by Hakomori, 1984). There are many reports to this effect in ganglioside literature. The strongest evidences for ganglioside receptors are cholea, tetanus, and botulinum toxins, (Simpson and Rapport,

1971). The serotonin receptor has received much attention for being a ganglioside (Van Heyningen, 1974; Detle and Weseman, 1978). Gangliosides have been shown to modulate neurotransmitters that activate adenylate cyclase (Dawson and Berry-Kravis, 1984); and with other biogenic amine transmitters (Maggio et al., 1977). Gangliosides have been shown to be involved with neurohormones such as thyrotropin (Lacetti et al., 1984). Usually a true protein receptor has been isolated for the neurotransmitters, but gangliosides apparently affect or modulate their binding, or response.

Gangliosides inhibit or stimulate the effects of neuropharmacologic substances. Among these are chlorpromazine (Janes and Fotherby, 1963); d-turbocurarine (Irwin et al., 1962); organophosphates (Rosengart and Taranova, 1969); tetrahydrocannabinol (Sarker, 1969); and other neurogenic agents (Boegman, 1976).

The ganglioside contents and patterns in areas of the brain have been shown to be altered in vivo in states where the electrical activity of the brain is changed, or is abnormal: in epilepsy; drug induced convulsions; CNS hypoxia; and controlled learning, training, and sensory stimuli experiments in animals (reviewed by Brunngraber, 1979). These experiments suggest a role for gangliosides in neurotransmission.

Considering the structure of gangliosides, the only chemically active functional group present is the anionic carboxyl group of the sialic acid moiety. A logical relationship can be envisioned between the negatively charged, cell surface gangliosides, and ions which are required for neuronal function. Gangliosides and ion fluxes have been studied extensively, and many of these studies are summarized in review articles (Wiegandt, 1982; Brunngraber, 1979). Most noteworthy is the binding affinity of gangliosides to calcium ion (Leskawa and Rosenberg, 1981). Ca^{++} is fundamental for synaptic transmission, and must be present in the intersynaptic space for both pre and post synaptic events. It has been hypothesized that synaptic gangliosides bind Ca^{++}, serving as a reservoir or "calcium buffer", and supply the ion during synaptic transmission (Svennerholm, 1979; Veh and Sander, 1981).

Gangliosides may have the ability to self aggregate by physical forces due to their structure (Yohe et al., 1976). Conceivably, these ganglioside aggregates could form a bilayer spanning, intramembrane micellular structure, which could function as a Ca^{++} pore or ionophore (Tettamanti et al., 1980).

The struggle continues. Specific functions for gangliosides in the nervous system remain a open debate.

Research in this area is saddled by overwhelming obstacles. The brain is a mass of cells; of numerous types, both neuron and non-neuronal; most of which have extensive, interwoven, yet delicate micro-processes. Isolation of pure cell types for careful study is unrealistic. Researchers must remember that their biologic samples, and subcellular fractions are contaminated by all sorts of cells and membranes. Since gangliosides are membrane lipids they cannot be added or removed from their assay systems in a controlled manner. Hopefully, new research tools, such as the ultra specific monoclonal antibody, will bring some significant breakthroughs to our understanding of these complex issues.

As a summary, it is relatively safe to say that gangliosides are cell surface components of neuronal membranes that are responsible for unique neuronal membrane characteristics. The character of these membranes must be maintained within very narrow limits in order for the neuron to function. And gangliosides are involved in the cell-cell, cell-transmitter, and cell-neurogenic agent interactions of the nervous system.

NEUROBIOLOGY OF GANGLIOSIDES; A NEW HORIZON

In the very recent history of ganglioside research, there has been an explosion of new concepts that place CNS

gangliosides in a different light. It has been discovered that gangliosides have neuritogenic and possibly neurotrophic properties which are capable of influencing neuronal differentiation *in vitro* and nerve regeneration *in vivo*. The pace of discovery in this new field is increasing almost logarithmically. This is due to the enormous potential for the clinical treatment of neuronal injuries that have been considered permanent.

The initial observations of the neuritogenic-neurotrophic properties of gangliosides, were from the work of Purpura and co-workers (Purpura and Suzuki, 1976; Purpura and Baker, 1977; Walkley *et al.*, 1981). While studying cultured neurons from cats with GM1 and GM2 gangliosidoses, they observed that mature neurons could produce new processes. Secondary neurites were extended and new synapses were formed. Normal cells do not do this. The growth of the new processes originated from areas of the cells that were laden with stored ganglioside. The behavior was limited to certain cell types. A rough correlation was made between the ability of neuron types to produce new neurites and a naturally high gangloside content in the area of the brain that the neuron type came from. The interpretation was that something in the micro environment of the neuronal plasma membrane (gangliosides) caused a response similar to a developing neuron during

extension of axons and before differentiation.

Since that time, a large number of neuronal cell culture lines, and primary neuronal cell cultures have been studied, for their response to exogenous gangliosides. These results are summarized in a review article by Ledeen, 1984 (this a journal issue totally devoted to the neuritogenic and neurotrophic properties of gangliosides, J. Neuroscience Research vol. 12, no. 2/3, 1984). The cellular response to gangliosides varies with the specific cell type.

Some neuronal types underwent prolific neuritogenesis, some produced mature looking synapses. Other cells failed to respond. As the work continues the concept now is that neuronal growth, development, synatogenesis, and maintenance are complex systems involving very cell type specific humoral neurotrophic factors (e.g. Nerve Growth Factor for dorsal root ganglion cells), and information that the cell "reads" from its environment through cell-cell or cell-surface interactions (as summarized by Cotman and Nieto-Sampredro, 1984). The latter is the role that gangliosides may play.

There is tremendous potential for the use of gangliosides therapeutically in the treatment of degenerative neuronal diseases, and traumatic neuronal injury. Reports

of the use of ganglioside for specific disorders include: recovery from stroke, (Bassi et al., 1984); recovery from neurotoxins, (Jonsson et al., 1984); treatment of diabetic neuropathy (Gorio et al., 1983); effects on Amyotrophic Lateral Sclerosis (Bradley, 1983); and numerous reports on the effects of gangliosides on lesion induced neuronal injury in brain and spinal cord (reviewed by DeFelice and Ellenberg, 1983). This is an obviously exciting field of ganglioside biochemistry, and time will only tell its potential.

SIALIC ACID ENZYMOLOGY AT THE CELL SURFACE

Enzyme activities have been described that remove (sialidase) and add (sialosyltransferase) sialic acid residues on cell surface gangliosides, and glycoproteins. As with most ganglioside concepts, definitive proof is lacking for the existence of these enzymes. If they do exist on the cell surface, they represent a substantial testimonial to the importance of the function of gangliosides. These two activities represent a desialosylation-resialosylation "cycle-system" for control over the cell surface ganglioside character. When such enzymatic cycle-systems exist, they invariably denote an important control point in metabolism.

Sialidase (neuraminidase, N-acetylneuraminosyl gly-

cohydrolase, EC 3.2.1.18) catalyzes the hydrolysis of sialic acid from complex carbohydrates. This reaction was first reported by McCrea (1947) in prokaryotes ("receptor-destroying enzyme"). The early study was confined to prokaryotic sialidase in its relationship to pathogenicity, and as a tool to discover the biological roles of sialic acids. These sialidases can be isolated in pure form, and much of their properties and mechanisms are known.

Warren and Spearing (1960) first reported the existence of mammalian sialidase. Surveys of the literature (e.g. Rosenberg and Schengrund, 1976) reveal that sialidase is broadly distributed in animals and is an integral part of all organs containing sialyl compounds. Although there are reports of soluble sialidases, the weight of evidence shows it mainly to be a membrane component. It has an acidic pH optimum and was believed to be lysosomal. The data now show that distinct lysosomal sialidase does exist, but the majority exists on the cell surface.

In this text, sialidase activity towards gangliosides is the focus. In general, the membrane fractions richest in gangliosides are also enriched in sialidase. Schendgrund and Rosenberg (1970) found sialidase concentrated in synaptosomes of bovine brain, and Ohman (1971)

found this activity to be localized in the SPM. These have been supported by data from many species.

The concept of high concentrations of gangliosides and of sialidase in the outer leaflet of the synaptic membrane has caused a great deal of speculation on a role in neurotransmission for these components: action on acetylcholine esterase (Brodbeck et al., 1973); in the dispersion of cationic neurotransmitters (Rosenberg and Schengrund, 1976); in regulation of ion fluxes (Schengrund and Nelson, 1975); as part of the serotonin receptor (Vaccari et al., 1971); and many more.

There are overwhelming problems in the study of sialidase. Activity is lost when the enzyme is removed from its membrane environment. It has not been studied in pure form. Enzyme preparations are crude membrane fractions, grossly contaminated by a variety of membranes. The substrates (gangliosides) are bound in the same membrane as the enzyme. Neither component can be controlled for enzyme kinetic or mechanism study (reviewed by Corfield et al., 1981). The rates of this type of reaction are very low. So far the concept of a ganglioside-sialidase system in the SPM has persevered for 15 years, in spite of the obstacles.

Sialosyltransferase (STase) has been reported to be

present, and concentrated in the SPM (Den et al., 1975; Preti et al., 1980). Also, it has been reported on the cell surface (an ecto-enzyme) of cultured neurons and retinal cells (Dreyfus et al., 1975). The enzyme catalytically transfers NANA (from CMP-NANA) to gangliosides, and other glycoconjugates. The concept of a synaptic STase ecto-enzyme is still quite controversial (Depert and Walter, 1978; Ng and Dain, 1977). Since STase is known to be a golgi associated enzyme (Keenan et al., 1974), the low levels of this enzyme observed in SPM preparations could easily be due to golgi contamination.

STase has a pH optimum near neutral, quite different from sialidase (4.0 - 5.0). If sialidase and STase coexist in the SPM, the possibility exists of a system to control the sialic acid level on the outer surface ganglioside. Even a convenient system control point is explained (local changes in pH). This is truly exciting because every biologic function proposed for neuronal gangliosides is enhanced by the existence of such a system. The ganglioside character of these membranes (hence their function) would be under tight control. There are several elegant hypotheses that explain how the system possibly functions in synaptic transmission (Veh and Sander, 1981; Schengrund and Nelson, 1975; Tettamanti et.al., 1980).

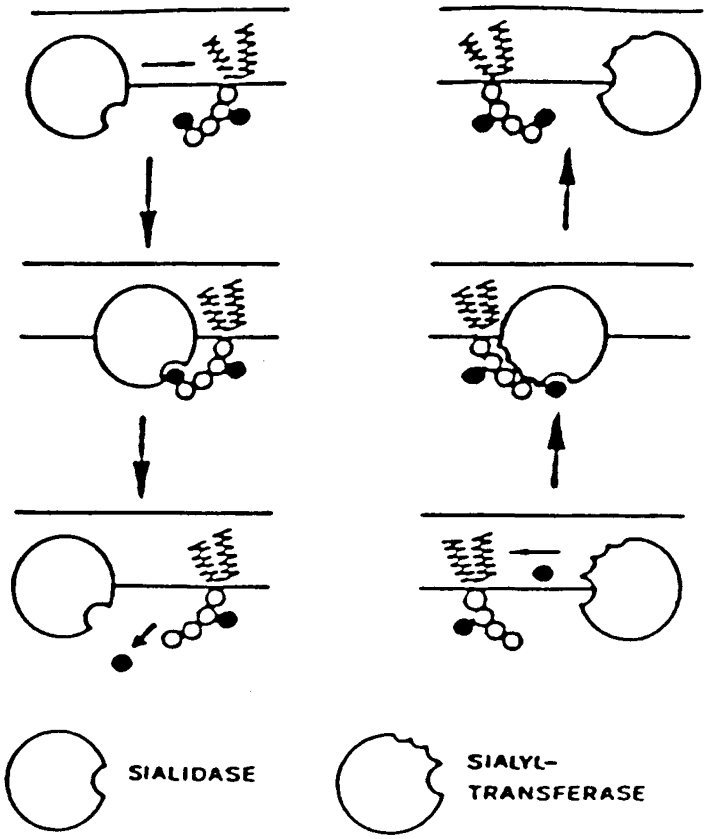
The weakest link in this system is the evidence supporting a STase ecto-enzyme activity in the synaptic plasma membrane. The enormous potential importance of this system to the function of CNS gangliosides has resulted in the undertaking of the present dissertation research.

RESEARCH GOALS

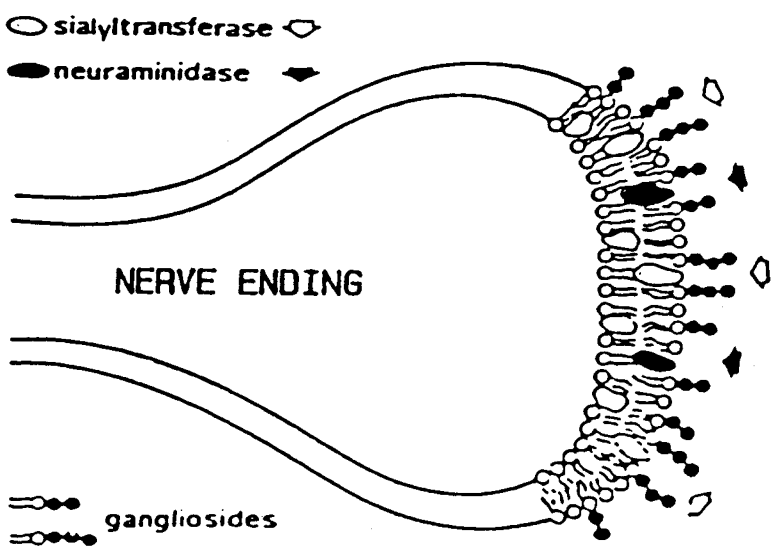
The purpose for these experiments is to obtain new knowledge of the biological character of sialosyltransferase enzyme activity that has been reported in synaptosomal preparations (Den *et al.*, 1975). A major point of conflict is the inherited heterogeneity of membrane subfractions prepared from brain, such as synaptosomes. Measured STase activity could easily be artifactual, coming from golgi contamination of preparations. This work shall approach this problem from a new direction.

Thus far there are no reports describing the ganglioside labeling pattern (substrate-product specificity) of STase from brain. The data have been reported as NANA transfer into the total endogenous ganglioside fraction, or as activity for transfer to exogenously added GSL substrates. These reports also have utilized detergents to increase the STase activity.

FIG. 1.8



DESIALOSYLATION-RESIALOSYLATION CYCLE



According to proposed functions, the STase of golgi complex would be for the biosynthesis of gangliosides *de novo*. They exist in physical multi-glycosyltransferase complexes. On the other hand, synaptic STase would function to modify and interconvert pre-existing membrane gangliosides. It would exist as a single enzyme on the outer face of the SPM.

Since these proposed functions are quite different, the ganglioside labeling patterns also should be different. These experiments will try to identify any of these differences. STase assays will be done under physiologic conditions, with balanced electrolyte incubation media. The media and atmosphere will be saturated with O_2, with enough CO_2 tension to establish a physiologic bicarbonate buffered pH. Glucose in the media will allow the brain samples to respirate, and metabolize energy. These conditions attempt to keep the STases under study, as unperturbed as possible.

The assay system will include no exogenous GSL substrates or detergents. These additions in the previous reports would disrupt the normal architecture of the STase environment, thus affecting both acceptor substrates, and the membrane bound enzyme. In this study, efforts will be made to keep the normal STase-acceptor relationships intact. Rat brain will be used as the enzyme source in the

procedures of this work. In the first part of these experiments, the STase activity in synaptosomal fractions will be studied in detail. After STase assay the synaptosomal gangliosides will be extracted, purified, and separated into individual species by high performance thin layer chromatography. The ganglioside profile and STase labeling pattern will be analyzed.

In the second part of this dissertation, isolation of true golgi complex membranes are attempted with confirmation. The STase assay procedures will be applied to these fractions. The biological character of golgi STase will be compared to the synaptosomal STase. The question of whether the golgi enzyme could be the source of the described synaptosomal activity will be addressed. The procedures and comparisons will also be applied to synaptic plasma membrane preparations.

Finally, the STase assay procedures will be applied to cortical brain slice samples. Here the entire neurons are intact. Methods for selective inhibition of golgi function and cell surface function will be applied to STase activity in the brain slices. The ability to inhibit either of the two putative STases without affecting the other, should lend weight to evidence in support or against synaptic plasma membrane ganglioside STase.

I undertook this work with no preconceived notion. I will report my findings, with three possible conclusions:

a. The data support the existence of a ganglioside sialosyltransferase in the synaptic membrane, and the biochemical role of this enzyme will have been documented.

b. The data support the existence of a contaminating subcellular membrane in synaptosomal preparations that gives rise to the previously reported sialosyltransferase in synaptic membranes.

c. The data from these experiments document the characteristics of this enzyme but neither support nor refute the existence of synaptic plasma membrane sialosyltransferase. More work in the area is required.

CHAPTER II

MATERIALS AND METHODS

All reagents not cited were of the highest purity available from well known biochemical suppliers and solutions were prepared with distilled-deionized water redistilled in the lab on a glass apparatus. All solutions were prepared fresh the day before use. The formulae of all solutions are given in table 2.1. All the homogenization and centrifugation steps in the preparation of the brain subfractions were maintained at 0-4° Centigrade.

ANIMALS AND HUSBANDRY- The twenty eight day old rat pups used in these experiments were produced on the premises, from Sprague-Dawley breeding stock . The original breeding rats were obtained from King Laboratory Animal Supply Co. of Milwaukee, WI. An out breeding schedule was followed as much as possible among the breeding animals. Breeding females were culled after producing four litters of pups and were replaced by one of their progeny. Breeding males were used until one year of age, then were culled and replaced from the progeny. The colony was housed and maintained by the Loyola Department of Lab Animal Resources, in a community rat room, and fed standard, commercially prepared rat diets, and were housed individually, in filter bonnet cages.

TABLE 2.1A

FORMULARY

	SOLUTION NAME	SOLVENT	COMPONENTS	pH
SUBCELLULAR FRACTIONATION MEDIA	homogenization medium (HM)	aqueous	0.32M sucrose 1.0mM Tris HCl 0.1mM EDTA	7.2
	sucrose density gradient solutions	aqueous	either: 0.4; 0.6; 0.8; 0.9; 1.0; 1.1; or 1.2M sucrose, 1.0mM Tris HCl 0.1mM EDTA	7.2
	Ficoll density gradient solutions	HM	either: 5%; 7%; 9%; 11%; 13%; or 16% Ficoll in HM (% = gm/100ml)	7.2
	50% sucrose	PBS	50 gm sucrose per 100ml PBS	ND
	phosphate buffered saline (PBS)	aqueous	(in gm/L): NaCl, 8.0; $NaH_2PO_4 \cdot 7H_2O$, 2.16; KH_2PO_4, 0.2; KCl, 0.2	7.2
ELECTRON MICROSCOPY SOLUTIONS	glutaraldehyde fixing buffer	PBS	4% glutaralehyde (gm per 100ml PBS)	7.2
	osmium tetroxide buffer	PBS	1% OsO_4 (gm per 100ml PBS)	7.2
SIALOSYL-TRANSFERASE AND SIALIDASE ASSAY MEDIA	Krebs-Henseleit buffer	aqueous	117mM NaCl 4.7mM KCl 0.9mM $MgSO_4$ 1.9mM $CaCl_2$ 1.17mM NaH_2PO_4 1.18mM KH_2PO_4 25.0mM $NaHCO_3$ 11.1mM glucose	7.2
	sialidase assay media	aqueous	o.5 M $NaC_2H_3O_2$	5.5

TABLE 2.1B

FORMULARY

CONTINUED

	SOLUTION NAME	SOLVENT	COMPONENTS	pH
GANGLIOSIDE PURIFICATION SOLVENTS	chloroform-methanol; for lipid extraction	organic	2:1 or 1:2 $CHCl_3$: CH_3OH vol. to vol.	ND
	chloroform-methanol-water for column elution	complex	50:50:15 $CHCl_3$:CH_3OH: H_2O, vol. to vol. to vol.	ND
	alkaline hydrolysis solution	CH_3OH	0.4N KOH	12
TLC DEVELOPING SOLVENTS	normal ganglioside system	complex	(vol. ratio) 50:40:10 $CHCl_3$:CH_3OH: 0.25% $CaCl_2$ (%=gm/100ml H_2O)	ND
	alkaline ganglioside system	complex	50:40:7:3 $CHCl_3$:CH_3OH: conc. NH_4OH: 0.25% $CaCl_2$	ND
	free NANA derivative system	complex	2:2:1 2-propanol: methyl acetate :0.25% $CaCl_2$	ND
TLC STAINING SPRAYS	resorcinol reagent	aqueous	10ml 2% resorcinol (gm/100 ml H_2O), 80ml conc.HCl, 0.25ml 0.1M $CuSO_4$, qs to 100ml w/H_2O	ND
	anthrone reagent	aqueous	66ml 95%H_2SO_4 34ml H_2O, 50mg anthrone, 1.0gm thiourea	ND

TABLE 2.1C

FORMULARY

CONTINUED

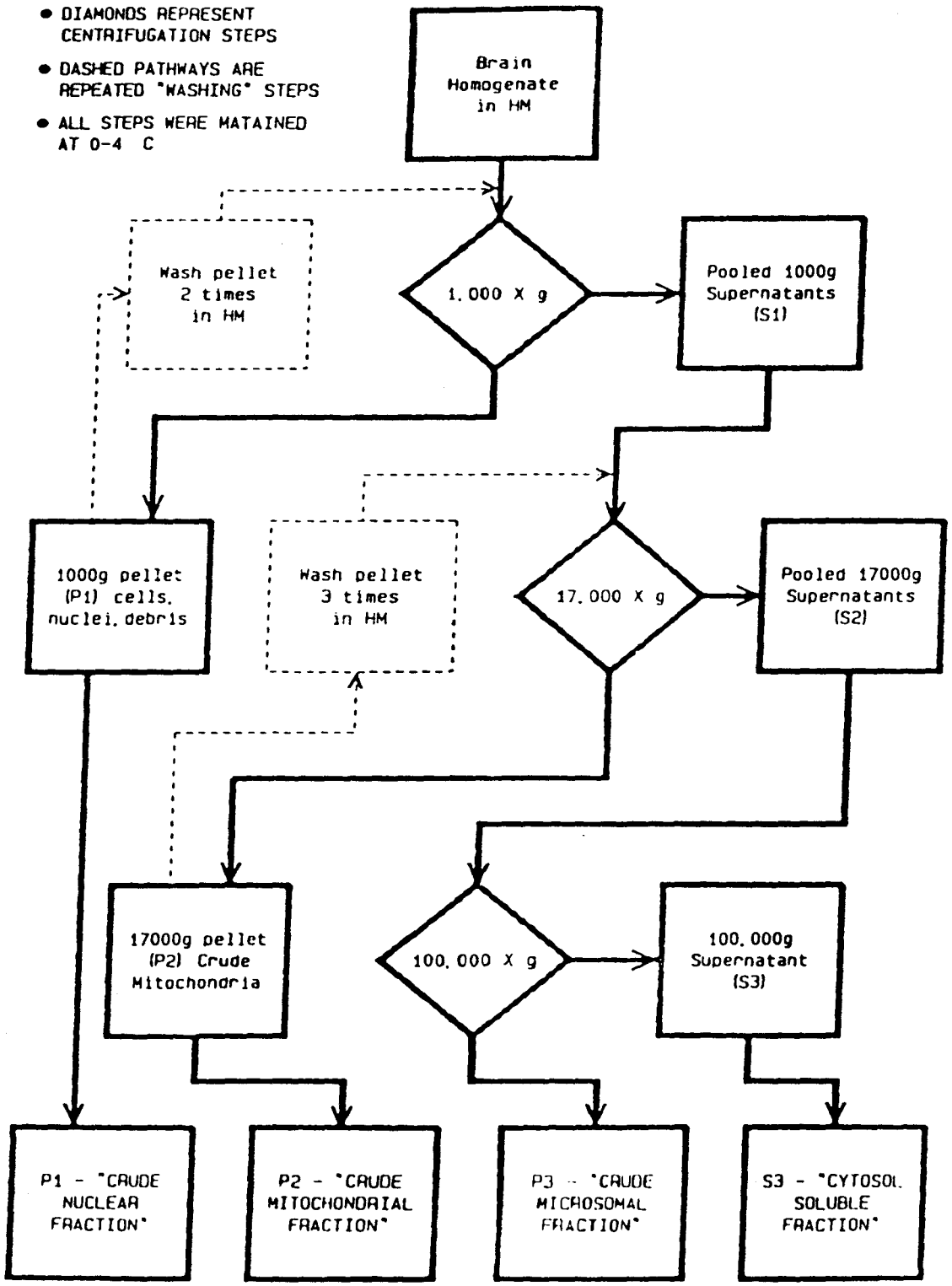
	SOLUTION NAME	SOLVENT	COMPONENTS	pH
PROTEIN ASSAY SOLUTIONS	solution A	aqueous	0.2% $NaNO_3$, 10% Na_2CO_3, 0.5% NaOH	ND
	solution B	aqueous	2% $Na_2C_4H_4O_6$, 1% $CuSO_4$ in 0.1N NaOH	ND
	solution C	aqueous	1 vol. Folin-Ciocalteu's phenol reagent in 2 vol. H_2O	ND
	solution D	aqueous	5% sodium dodesyl sulfate in 0.5N NaOH	ND
MARKER ENZYME ASSAY MEDIA	5'nucleotidase assay medium (0.3ml)	aqueous	45mM Tris HCl 10mM $MgCl_2$ in 0.1% Triton X-100	8.5
	5'nucleotidase stopping media (0.1ml each)	aqueous	0.25M $Ba(OH)_2$	ND
		aqueous	0.25% $ZnSO_4$	ND
	cerebroside sulfotransferase assay medium (0.4ml)	aqueous	50mM imidizole HCl, 12.5mM NaCl, 0.025% deoxycholate, 0.1mg bovine cerebrosides	7.0
	choline acetyl transferase medium (0.25ml)	aqueous	300mM NaCl, 41mM NaH_2PO_4, 0.05% Triton X-100, 10mM choline chloride, 0.1mM phycostigmine salicilate	7.4
	acetylcholine esterase	aqueous	100mM NaH_2PO_4 0.5mM DTNB	8.0

PREPARATION OF THE BASIC MEMBRANE FRACTIONS FROM RAT BRAIN- The method of Gray and Whittaker (1962), was used to prepare the basic membrane fractions: P1, the nuclear pellet containing nuclei, whole cells, large myelin fragments, and other large debris; P2, the mitochondrial pellet consisting of myelin, crude synaptosomes, and mitochondria; P3, the microsomal pellet which is made up of mainly intracellular membranes; and S3, containing soluable components from the homogenized brain. A detailed description of the procedures follow, and a "flow chart" of these steps is shown in fig.2.1.

After light ether anesthesia, the pups were decapitated and the forebrains were quickly removed and placed in an ice cold 0.32 M sucrose, 1mM Tris HCl, .1 mM EDTA solution (pH 7.4), hereafter referred to as homogenizeng medium (HM). The brains were homogenized in four volumes (ml/gm brain) of HM by eight strokes of a motor driven Potter type glass-teflon homogenizer at 800 rpm. The suspension was brought to 10% (wt./vol.) by the addition of HM, and the suspension was centrifuged at 3,000 rpm (1,000 x g) in a SS-34 Sorvall rotor and high speed centrifuge (Sorvall-DuPont Instrument Co., Newtown, CT). The pellet was washed two times by resuspending the pellet in the original volume of HM and recentrifuging, and all supernatants were pooled. This washed pellet is the "P1" fraction. The pooled supernatants (S1) were centrifuged

FIG. 2.1

BASIC FRACTIONATION OF RAT BRAIN



at 11,500 rpm (17,000 X g) in the SS-34 rotor for 30 minutes. The pellet was resuspended in the original volume of HM and recentrifuged three more times. This washed pellet was the "P2" fraction. The pooled supernatants (S2) were centrifuged at 100,000 X g in a Beckman L2-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) at 27,000 rpm for one hour in the SW28 swing out rotor. The resulting pellet was the "P3" fraction and the supernatant was the "S3" fraction.

SUBFRACTIONATION OF THE BASIC BRAIN FRACTIONS- Samples of the fractions described above were subfractionated by discontinuous density gradient centrifugation. This procedure is basically the same for all of the different samples that were subfractionated in the experiments that follow. The density gradient solutions vary with the particular sample being subfractionated. In figure 2.2 a generalized "flow chart" for discontinuous density gradient centrifugation is shown. The detailed conditions for the subfractionation of four specific samples follows, designated I, II, III, and IV.

I. For the preparation of the synaptosomal enriched subfraction, used in the initial experiments of this work, the procedures of Gray and Whittaker (1962) as modified by Booth and Clark (1978) were used. The P2 fraction was suspended in a small amount of HM and layered on the top of tubes containing discontinuous ficoll density grad-

PREPARATION OF BRAIN SUBFRACTIONS
generalized scheme for discontinuous density gradient centrifugation

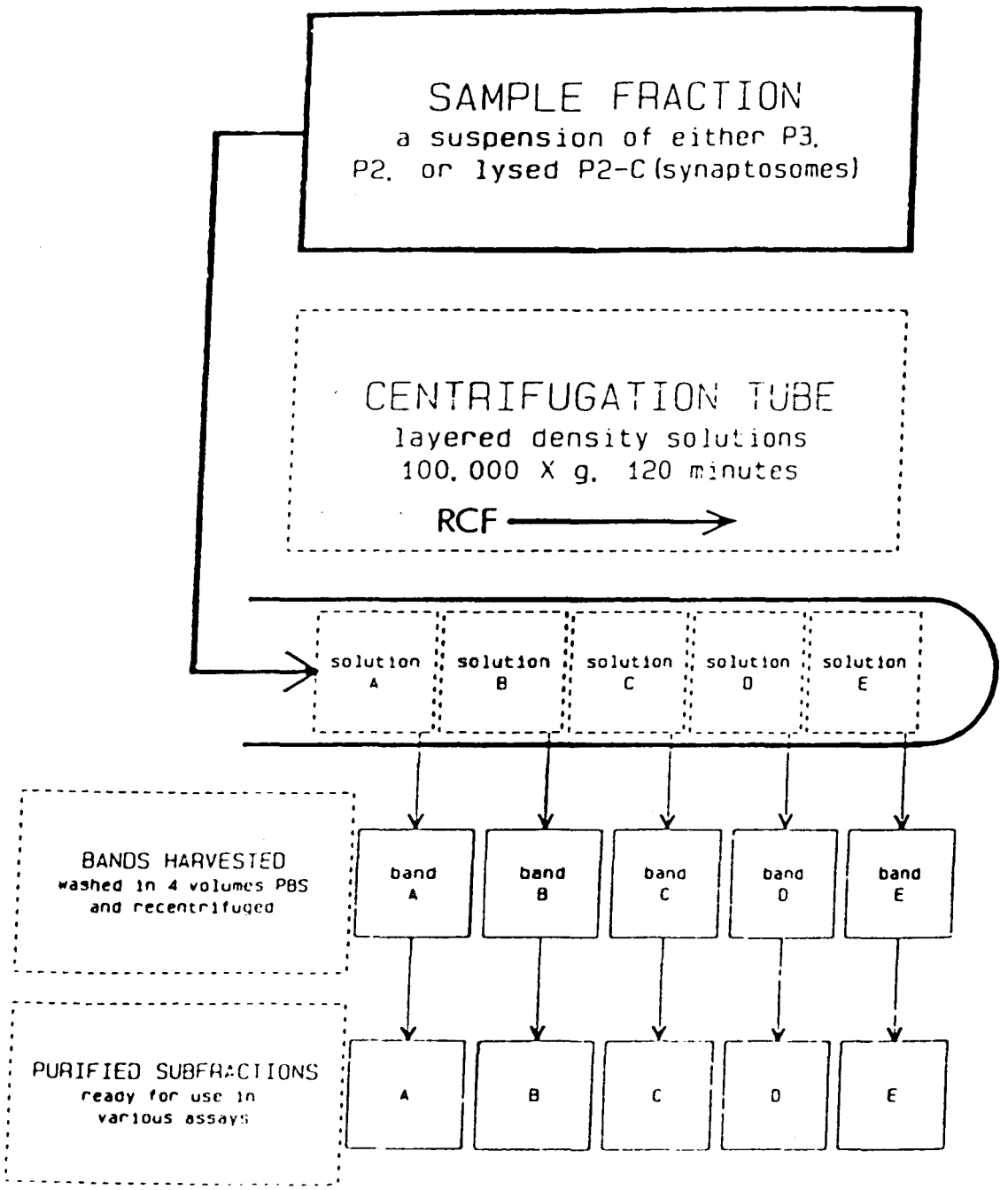


FIG. 2.2

ients. From top to bottom the layers of the gradient were: 15 ml of 9% (gm/100ml HM) ficoll (Pharmacia Fine Chemicals, Piscataway, NJ); 15 ml of 16% ficoll; and 2 ml of 50% sucrose (gm/100 ml H_2O) which acts as a "cushion" for the pellet. The tubes were centrifuged in a SW-28 swing out rotor in an L2-65B Beckman ultracentrifuge at 27,000 rpm (100,000 x g) for two hours. The band of membrane material at the 9%-16% ficoll interface was aspirated out by Pasteur pipet and this fraction was washed by suspending it in three volumes of phosphate buffered saline, pH 7.4 (PBS) and centrifuging at 15,000 rpm in the SS-34 rotor for 30 minutes. The resulting pellet is the "P2-B", synaptosome enriched subfraction of Gray and Whittaker (1962) and was used immediately in the enzyme assay procedures below.

II. The P3 fraction (microsomes) was subfractionated by the use of a discontinuous sucrose density gradient. This gradient was designed after a procedure by Siegrist et al. (1979). The continuous sucrose density gradient that they used (from 0.8 to 1.3 M sucrose), was modified into a discontinuous gradient. The layers were: 0.6; 0.8; 0.9; 1.0; 1.1; and 1.2 M sucrose from top to bottom. The sucrose solutions were also 1.0 mM in Tris HCL and 0.1 mM in EDTA, pH 7.2. Six ml of each solution was gently layered in polyalomar tubes that fit the Beckman SW28 rotor, with 2 ml of 50% sucrose solution at

the bottom of the tube as a cushion. The P3 fraction was suspended in a small amount of HM and layered on the top of the tubes. The gradients were centrifuged in the Beckmen ultracentrifuge at 27,000 rpm (100,000 X g) for two hours. Visable bands formed at the top six gradient interfaces, no material passed through the 1.2 M sucrose layer. The bands were harvested and washed in 4 volumes PBS and recentrifuged in the same rotor at the same speed for 30 minutes. Subfractions were harvested from the following interfaces: HM-0,6; 0.6-0.8; 0.8-0.9;0.9-1.0; 1.0-1.1; and 1.1-1.2 M sucrose, which were given the respective names: M-A; M-B; M-C; M-D; M-E; and M-F. The washed subfractions were used immediately in the enzyme assays below.

III. In latter experiments, the P2 fraction was subfractionated by an expanded discontinuous ficoll density gradient. The 3 layer gradient of Booth and Clark (1979) described above (I.), was subdivided into a 6 layer gradient which resulted in increased subfractionation. The layers of the gradient were: 5%; 7%; 9%; 11%; 13%; and 16% ficoll (gm per 100 ml HM), from top to bottom. The P2 fraction was suspended in a small amount of HM, layered on the gradients, cenrifuged, and the bands harvested and washed as described for P3 subfractionation. Bands formed at every interface and were termed (from top to bottom): P2-a; P2-b; P2-c; P2-d; P2-e; P2-f; and the

pellet P2-MIT. These washed subfractions were used immediately in enzyme assays. A point of clarification is in order. Please note that the subfractions described here are named with a lower case descriptor (i.e. P2-c), which should not be confused with the traditional subfractions of Gray and Whittaker which have a upper case descriptor (P2-C).

IV. Synaptic plasma membranes were prepared and subfractionated as described by Cotman and Mathews (1971). The washed synaptosomal preparation (P2-B) was suspended in 30 ml/gm (of starting brain material) of a hypo-osmotic lysing solution (LS; 0.1 mM Tris HCl, pH 8.0) for one hour at 4°C. At 30 minutes into this period, the suspension was placed in the Potter glass-teflon homogenizer, and given one up and down stroke of the pestle. After the lysing period, this suspension was centrifuged at 12,000 rpm (17,000 X g) in the Sorval SS34 rotor for 15 minutes. The supernatant, containing the lysed synaptosomes was centrifuged at 30,000 rpm (100,000 X g) in a Beckman SW41 swing out rotor for 30 minutes. The pellet was resuspended in one ml of LS and layered on top of the discontinuous sucrose density gradient. The layers, from top to bottom, were: 2 ml each of 0.4; 0.6; 0.8; 1.0; 1.1; 1.2 M sucrose; and a 0.5 ml 50% sucrose cushion. The tubes were centrifuged in the Beckman SW41

rotor at 30,000 rpm (100,000 X g) for 2 hours, and the bands harvested and washed as described before. No bands could be seen at the top two gradient interfaces. The subfractions obtained were: SPM-C; SPM-D; SPM-E; SPM-F; and a pellet, SPM-P. These were found at the 0.6-0.8, 0.8-1.0, 1.0-1.1, 1.1-1.2, and 1.2-50% sucrose interfaces respectivly (sucrose solutions are given in molarity). These washed subfractions were used in the assays to follow right away.

ELECTRON MICROSCOPY- After resuspending the P2-B pellet (synaptosomes) in PBS a small aliquot was added to a centrifuge tube containing 2 ml of a 4% glutaraldehyde solution in PBS and this suspension was kept on ice for two hours. The tube was filled to the brim with ice cold PBS and centrifuged at 30,000 rpm in the SW-41 swing out rotor for 30 minutes. The supernatants were poured off and the pellets were post fixed <u>en bloc</u> by addition of ice cold 1% osmium tetroxide in PBS. After one hour on ice the solution was poured off, the fixed pellets were rinsed and then stored in ice cold PBS. These samples were given to Dwan Taylor, of the Department of Biochemistry and Biophysics at the Loyola Medical School, who stained (uranyl-lead procedure), embedded, microtomed and handled the microscopy of the samples.

In latter experiments, involving the extensive subfractionation of the rat brain fractions, a some what

more drastic fixation method was utilized. This method is very good for the identification of golgi membranes (as demonstrated by Siegrist et al., 1979). It was a goal of these experiments to detect golgi contamination in the subfractions enriched in synaptic structures, so this same fixation method was used in all the subfractions, even though it may have "over-fixed" synaptosomes. The only difference from the above method was that after the glutaraldehyde fixation, and pelleting, the 1% OsO_4 in PBS was added to the pellet and left on ice overnight. The osmium solution was removed and replaced by PBS, and the fixed pellets were turned over to the electron microscopy laboratory of the Department of Pathology, here at the Loyola University Medical Center. This lab handled staining, embedding, and sectioning of the samples. The electron microscopy was done by Dr. Raoule Fresco of the Department of pathology.

SIALOSYLTRANSFERASE INCUBATION- The P2-B fraction was suspended in ice cold Krebs-Henseleit buffer (117 mM NaCl, 4.7 mM KCl, 0.9 mM $MgSO_4$, 1.9 mM $CaCl_2$, 1.17 mM NaH_2PO_4, 1.18 mM KH_2PO_4, 25 mM $NaCO_3$, 11.1 mM D-glucose, pH 7.4) in a ratio of one ml per one gm fresh brain. The Krebs buffer had been been saturated with 95% O_2-5% CO_2 by bubbling on ice for several hours to oxygenate and establish the bicarbonate buffering system of the solution. These conditions for incubation of synaptosomes are those

of Pastuszko et al. (1982). The synaptosomal suspension was divided into 2 ml samples, which were placed in separate, sealable injection vials. As a control, some of the samples were heat denatured by heating to 100° C for ten minutes. One tenth of a microcurie of Cytidine-5'-monophosphate-(^{14}C)-N-acetyl-neuraminic acid (CMP-NANA) from Amersham International, Amersham, U.K. (247 mCi per mmole) was added to each sample. Each vial was sealed and 95% O_2-5% CO_2, from a compressed gas cylinder, was forced into the vials via a hypodermic needle inserted into the injection septum vials, creating the atmosphere over the reaction media. Another needle was inserted into each vial that vented the excess gas into the room atmosphere. Samples were incubated at 37° C in a water bath, slowly shaking. Some samples were kept on ice as controls.

The reactions were stopped in either of two ways. In some experiments, at time points the material in the vials was removed to centrifuge tubes, cooled to 0°C, and centrifuged at 15,000 rpm for 15 minutes in the Sorvall SS-34 rotor. The supernatants were removed and one ml of chloroform-methanol solution, two to one vol./vol. (C/M-2:1) was added to the pellets and the samples were kept at -20°C until the next day. In the other experiments, the total reaction mixtures were quick frozen, and then lyophilized. To the freeze dried residue the C/M 2:1 solution

was added. A schematic representation of these STase incubation procedures is given in figure 2.3.

GANGLIOSIDE EXTRACTION- A "flow chart" of the extraction scheme is given in figure 2.4. The procedures used were based on the partitioning method of Folch et al. (1951) as modified by Suzuki (1964) and Irwin and Irwin (1979). The brain samples in C/M-2:1 at room temperature were vortexed , sonicated briefly, and after one hour at 23°C were centrifuged in a table top clinical centrifoge for 10 minutes. The C/M supernatants were removed, and kept in separate test tubes. This extraction procedure was repeated on the insoluble residue, with C/M-1:2 and again with C/M-2:1. All C/M extracts were pooled and dried under a stream of N_2 gas. The dry extracts were dissolved in one ml of C/M-2:1 and applied on indivual 0.5 cc beds of Unisil (a 100-200 mesh activated silica gel, Clarkson Co., Williamsport, PA) in a Pasteur pipet. Neutral lipids and non-ganglioside glycosphingolipids were washed from the columns with two ml of C/M-2:1, this fraction was not studied. The gangliosides were eluted with two ml of C/M/W(water)-50:50:15, and dried under a N_2 gas stream.

The gangliosides thus extracted were purified of alkaline labile phospholipids by the method of Saito and Rosenberg (1982). The dry ganglioside residues were dissolved in 1 ml of 0.4 N KOH in methanol and allowed to

SIALOSYLTRANSFERASE ASSAY

- Reaction medium is Krebs-Henseleit buffer which was presaturated with 95% O_2 - 5% CO_2 . pH 7.2.
- Reaction was started by addition of the substrate. CMP-(14C) NANA.
- Reaction was stopped by quick freezing and then lyophilization.

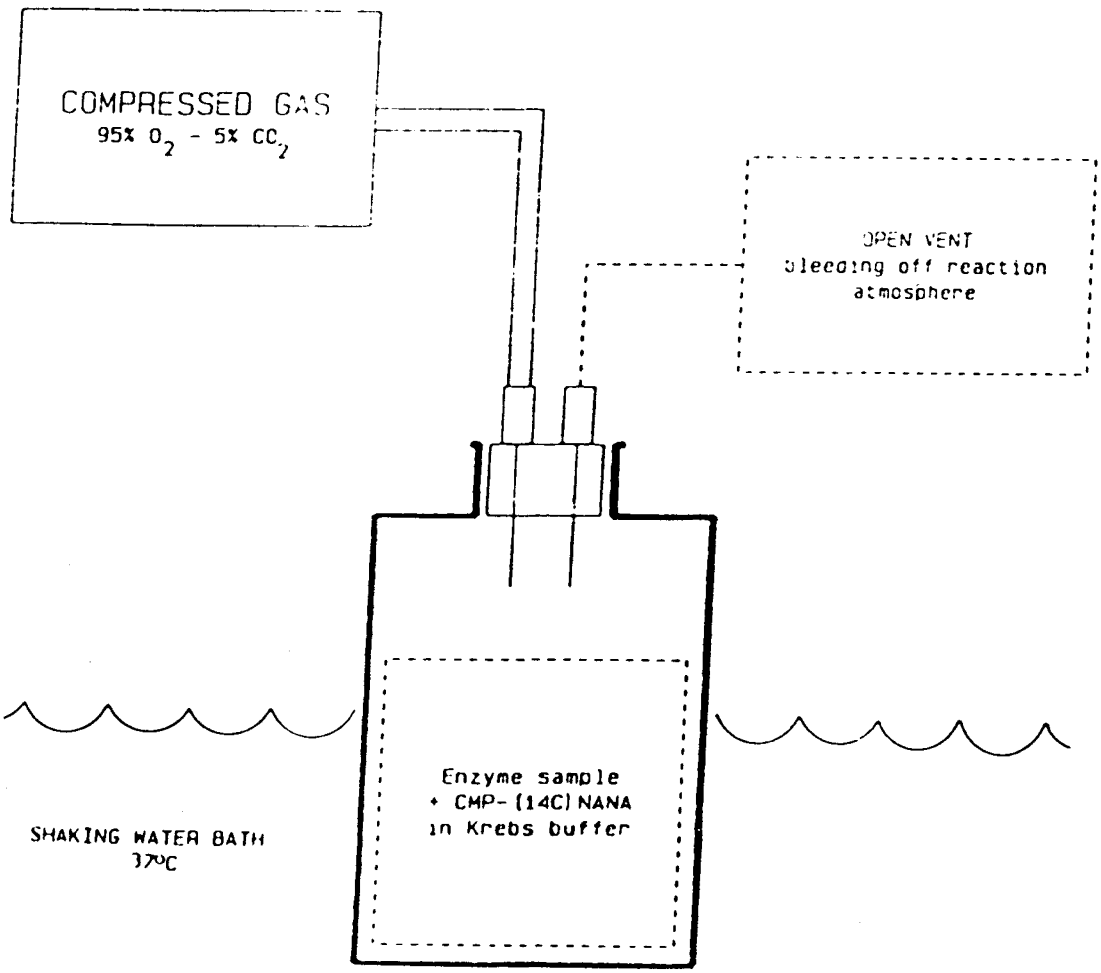
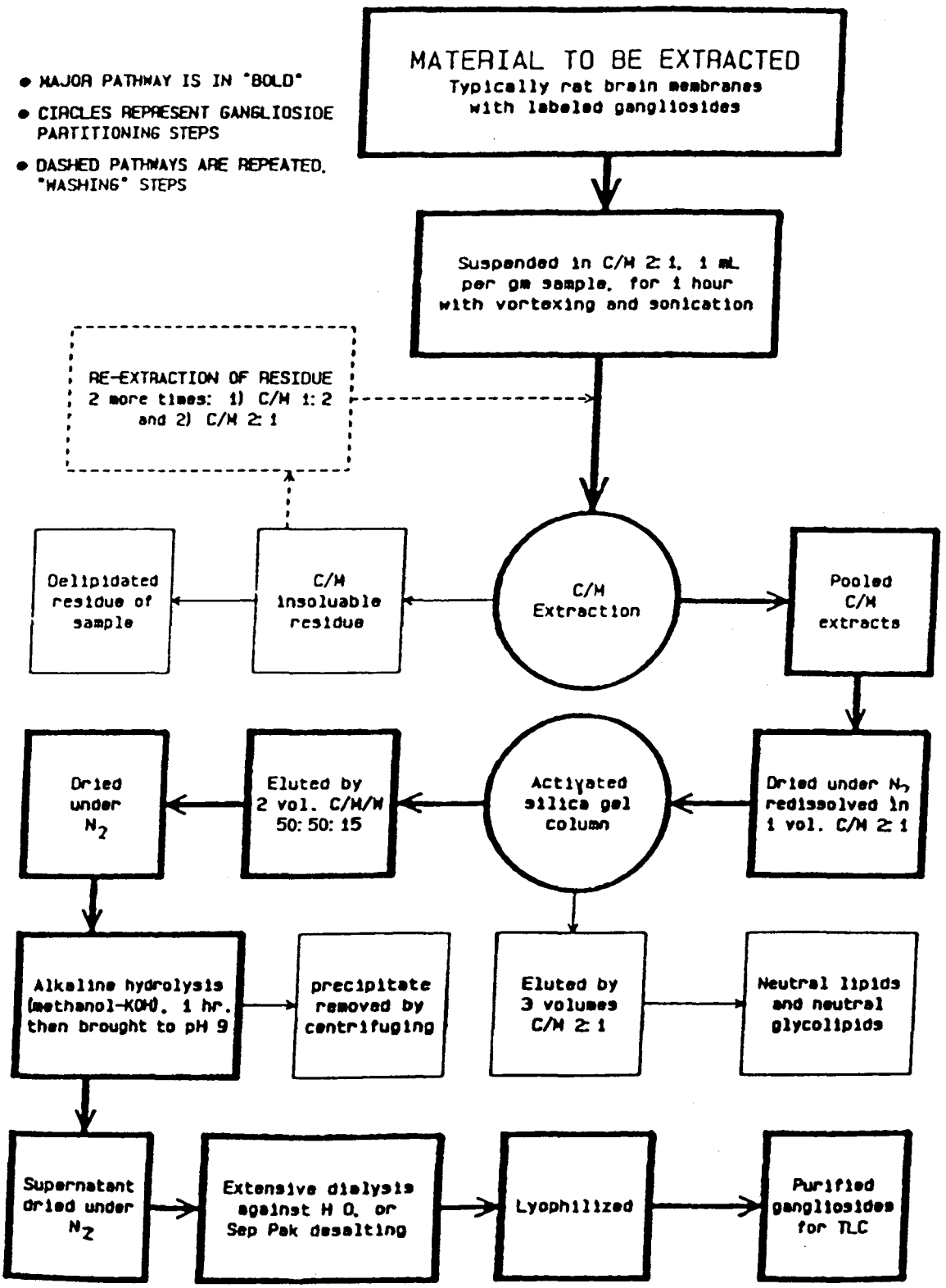


FIG. 2.3

FIG. 2.4 EXTRACTION OF GANGLIOSIDES



hydrolize for one hour at 23°C. The samples were neut ralized with concentrated HCl to pH 9.0 and centrifuged in a clinical centrifuge to sediment the KCl precipitate. The supernatants were removed and dried under N_2.

The samples were further "cleaned up" in either of two ways. In most of the experiments the dried samples were dissolved in 100 microliters of H_2O, and dialysed for 3 days against several changes of distilled water. The samples were then lyophilized. Later on, the dialization step was replaced by a desalting column chromatography step. This procedure was published by Williams and McCluer (1980), and is a great time saver. The method utilizes Sep Pak C_{18} (reversed phase) cartridges obtained from Waters Associates, Milford, MA. Briefly, the cartridges were fitted to three way stopcocks and were washed alternately with 10 ml methanol and 20 ml C/M 2:1, three times, using a glass syringe with gentle pressure. The cartridges were then washed once more with 30 ml methanol and the equilabrated with 10 ml of aqueous 0.1 M KCL. The dried ganglioside samples were dissolved in 5 ml H_2O and gently forced through the cartridge. The effluent was reserved and reapplied. The cartridges were then washed with 15 ml H_2O, then the gangliosides were eluted with 15 mI of C/M 2:1, and this eluate was dried on a rotary evaporator. The dry gangliosides were then dissolved in H_2O and lyophilized. The Sep Pak cartridges could be used

up to ten times with washing and re-equilibration.

THIN-LAYER CHROMATOGRAPHY(TLC)- The purified ganglioside samples were taken up in 5 microliters of C/M 2:1 and spotted in one cm streaks on 10x20 cm high performance silica gel 60 TLC plates (0.2 mm thickness, E. Merk Co., Darnstadt, West Germany). Please note that in references to "TLC" in these experiments, the plates used were always "high performance thin layer chromatography" plates that are sometimes referred to as "HPTLC" in scientific literature. A well characterized mix of ganglioside standards (bovine brain gangliosides, Calibiochem-Behring, San Diego, CA) was co-spotted on each plate for purposes of identification of the sample ganglioside species by Rf.

A 10 microgram sample of GM3, which was prepared as discribed previously (Saito and Rosenberg, 1982), was also spotted on each plate for quantitation purposes. The normal developing solvent system was C/M/0.25% aqueous $CaCl_2$-50:40:10 (vol./vol./vol.). The solvent front reached the top of the plate in about one hour.

After drying, gangliosides were visualized by spraying the plates with Svennerholm's resorcinol reagent (1957) and baking the plates at 120^{o}C for 15 minutes. The bands on the plates containing NANA (the gangliosides) give a purple, resorcinol positive, color. The rest of the plate remains white, except for occasional yellow

artifacts. These may be sucrose, Ficoll, or a lipid species. At any rate, these are not resorcinol positive (no NANA present in these bands).

QUANTITATION OF INDIVIDUAL GANGLIOSIDES- The individual gangliosides after TLC separation were quantitated by the direct densitometric method of Smid and Reinisova (1973) using a SD3000 spectrodensitometer (Kratos, Schoeffel Instrument Corp., NJ), measuring reflected light at wavelenght of 585 nm, which is specific for the resorcinol positive color reaction. The densitometer scans the length of each lane of the TLC plate, and the reflectance signal was recorded on a strip-chart as reflectance per mm distance scanned (i.e. Rf). Each band on the plate can be associated with a peak on the densitometer tracing. The peaks were integrated by a Hewlett-Packard 3390A computing integrater, which reported the results as the area under each peak in arbitrary area units, and as the percent of the total area of all the peaks in a given TLC lane.

The areas of the peaks corresponding to each ganglioside, identified by comparison to the standards, were converted to microgram amounts by division by the area of the standard GM3 peak, which is a known quantity, after correction for the number of NANA residues per ganglioside species of that peak.

Another method was used to quantitate gangliosides. Here the total amount of NANA in the total ganglioside

extract was determined by the method of Svennerholm (1957), which is described below. Then this amount of NANA was integrated with the densitometic analysis data of the gangliosides, separated by TLC. The total NANA was divided by the percent densitometric area for each ganglioside peak. This results in the amount of NANA in a given ganglioside band. Using the number of NANA residues in a given ganglioside species, the molecular weight of NANA, and the molecular weight of that ganglioside species, this result can be converted into micrograms of each species of ganglioside.

TOTAL GANGLIOSIDE SIALOSYLTRANSFERASE ACTIVITY- The protein concentration of the original subfraction samples were determined by the method of Hess et al. (1978) which will be described below. An aliquot of the purified gangliosides from the STase assays (dissolved in H_2O) was placed in 15 ml scintillation vials along with ten ml of Aquasol (New England Nuclear, Boston, MA) as the fluor. The samples were counted on a Beckman LS-1800 scintillation spectrophotometer (Beckman Instuments Inc., Palo Alto, CA) which converted the counts to disintegrations per minute (DPM) by the means of an onboard quench curve program and a set of increasingly quenched CMP-(^{14}C)NANA standards (5 quench levels, each in duplicate). By using the specific radioactivity of the substrate, the specific

activity of sialosyltransferase was calculated and the units used were picomoles of NANA transferred into the ganglioside fraction per miligram subfraction protein per hour.

GANGLIOSIDE LABELING PATTERNS OF SIALOSYLTRANSFERASE- The specific activity of the STase for individual ganglioside species was determined as follows. The developed and densitometrically analyzed TLC plates were placed on a light source and mm ruled tracing paper was overlain, and the ganglioside bands were traced, making a spacial record. Sequential 1.0 or 2.0 mm sections Of the silica gel, of each lane of the TLC plate, were scraped into individual scintillation vials. One ml of H_2O was added to each vial and the vials were sonicated to elute the gangliosides off of the silica gell. Ten ml of Aquasol was added, vortexed, and the samples were counted. The DPM per sequential segment of the lane was plotted on a bar graph in order of the distance of each segment from the origin. This graph was aligned with the tracing of the TLC lane, which allowed the assignment of DPM to the correspounding ganglioside band. After quantitation of the gangliosides in a given band by densitometric analysis (described above), the DPM incorporated into each nanomole ganglioside species per hour was calculated.

PREPARATIVE TLC- The gangliosides from 15 day old chick embryo brains were extracted as above. The chick

brain gangliosides, or labled rat brain ganglioside (by sialosyltransferase as above), were spotted in wide streaks (15 cm) across the TLC plate, one cm from the bottom. A one cm streak of the standard bovine brain ganglioside was spotted at either end of the sample streak. The chromatogram was developed as above. Using a glass cutter, the standards plus one cm of the sample streak were removed from both ends of the plate, and were stained by the resorcinol procedure. The middle sections of the plates were exposed to iodine vapors which will reversibly stain all the lipids on the plate. Realignment of the ends of the plate to the middle section allows identification of the gangliosides on the preparative streak which were circled in pencil, and the desired bands were scraped off the plates. The ganglioside was eluted from each silica gel scraping by sonication in 5 ml C/M/W-50:50:15 and after one hour sedimenting the silica gel in a clinical centrifuge, reserving the supernantant. This elution step was repeated twice on each scraping and the pooled supernatants were dryed by rotary evaporation. The residues were dissolved in 2 ml H_2O, and lyophilized.

NEURAMINIDASE DIGESTION OF GANGLIOSIDES- Type X Clostridium perfringes neuraminidase was obtained from Sigma Chemical Co., Saint Louis, MO. Purified ganglioside samples containing approximately 50 micrograms of NANA were disolved in 25 microliters of .05 M sodium acetate

buffer (pH 5.5) in separate microtubes. To each was added another 25 microliters of the same buffer containing .01 unit of the bacterial neuraminidase. Some of the samples received heat denatured neuraminidase as a control. The samples were incubated at 37°C for various time intervals, and the reaction was stopped by rapid addition of 100 microliters of ice cold methanol and the tubes were cooled on ice. The tubes were centrifuged at high speed on a Beckman/Spinco 120 Microfuge (Beckman Instrument Corp., Palo Alto,CA) for five minutes to pellet the protein precipitate. The supernatants were removed and spotted directly in 1 cm streaks on TLC plates with the bovine brain ganglioside standards. The chromatograms were developed, then analyzed densitometrically and for radioactivity, as described above.

TLC OF FREE NANA AND IT'S DERIVATIVES- The techniques used here are essentially the same as for the TLC of gangliosides, except the difference in developing solvent. Samples of reagent standards of NANA (Sigma Inc., Saint Louis, MO), CMP-NANA (Sigma Inc.), and 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (NADNA, from Boehringer Mannheim Biochemicals, Indianapolis, IN), were spotted on the same TLC plates, in the same manner as above. These were spotted alone or as a mixture, and at times a radio-labeled biological sample was "over-spotted" along with these standards. The developing solvent used

was a mixture of 2-propanol/methyl acetate/0.25% aqueous $CaCL_2$, in the vol. to vol. to vol. ratio of 2:2:1. The techniques for the development, staining, and analysis of the plates were the same as those described above.

PROTEIN DETERMINATIONS- The method that has been most widely used in biomedical research is the method of Lowry et al. (1951), due to the linearity of the results among proteins of varying amino acid composition. The method was improved by Hartree (1972), who increased the linearity of the results in the higher protein concentation range by modifying the technical composition of the reagents. These methods remain somewhat unreliable in the study of membrane bound proteins, because the reagents used to solubilize membrane proteins cause artifactually increased protein concentrations in the results. In this dissertation, I have used a further modifacation of the Lowry method, as described by Hess et al. (1978), which produces acceptably linear results for membranous proteins, yet is reasonable in time and difficulty factors.

The basis of the modification is the solubilization of all samples in a solution of 5% SDS (5 gm sodium dodesyl sulfate per 100 ml volume) in 0.5 N NaOH, which is referred to as solution "D". For the protein standard, a stock solution of 50 mg BSA (bovine serum albumin, Sigma Chemical Co., Saint Louis, MO), in 50 ml "D" was made and

was divided into small portions, and was stored at -20°C in individual sealed vials. At the time of each assay, one of these standards was warmed to 28°C in a water bath and serially diluted with "D" to form a set of standards that contained from 2 to 70 micrograms BSA in 150 microliters of volume, in triplicate. Aliquots of the subfraction samples were taken on the day they were prepared and were kept at -70°C until the day of a protein assay, which were thawed and serial dilutions of the samples were made in "D". The samples were incubated at 28°C until the solution was transparent. Then 150 microliters of each dilution, of each sample was placed in a test tube.

To all tubes, containing standards and samples, 180 microliters of solution "A" (0.2% $NaNO_3$ (gm/100 ml), 10% Na_2CO in 0.55 N NaOH) was added, then vortexed, and incubated for 30 minutes at 28°C. Twenty microliters of "B" (2% sodium tartrate, 1% $CuSO_4 \cdot 5H_2O$, in 0.1 N NaOH) was added, then vortexed, and left for 30 minutes at 28°C. Then 0.6 ml of "C" (Folin-Ciocalteu reagent, Sigma, in H_2O, 1:2 vol./vol.) was forcfully pippetted into the test tubes with constant vortexing, and the tubes vere left standing for one hour at room temperature.

The standards and the samples were placed in glass cuvettes and the absorbance of monochromatic light at a wavefength of 650 nm was read on a Beckman DU7 spectrophotometer, which had been zeroed to control

samples containing no protein. A linear standard curve was made by regression analysis of the readings of the standards. From the standard curve the protein concentrations of the diluted samples were calculated and the protein concentrations of the original subfractions were determined.

DETERMINATION OF TOTAL BOUND SIALIC ACID- The method used was that of Svennerholm (1957). The samples that were measured were the total purified ganglioside extracts of the rat brain subfractions. A set of standards of reagent grade NANA (Sigma), containing from 10 to 50 micrograms in 2.0 ml of H_2O, was setup in 15 ml glass test tubes. The lyophilized ganglioside samples were disolved in 0.5 ml H_2O, and 100 microliters of each sample was placed in test tubes and 1.9 ml of H_2O was added to the samples. Two ml of resorcinol reagent (10 ml of a 2% aqueous recorcinol solution, 80 ml of concentrated HCl, 0.25 ml of 0.1 M $CuSO_4$, brought to 100 ml with H_2O) was added with vortexing, and the tubes were heated for 15 minutes at 110°C in a heating block. After heating, the tubes were cooled in running water.

To each tube, 5.0 ml of 2-pentanol was added, then vortexed. Then the tubes were placed in a ice water bath for 15 minutes, and then centrifuged in a table top clinical centrifuge for one minute. Three ml of the upper layer was pipeted off and placed into glass cuvettes, and

the absorbance of monochromatic light at a wavelength of 480 nm was read on the Beckman DU7 spectrophotometer which had been zeroed on control samples containing no NANA. A standard curve was calculated by regression analysis of the readings from the NANA standard sets. The readings from the ganglioside samples was converted to microgram amounts by linear regression using the standard curve. Using the dilution factors, the NANA content of the original lyophilized ganglioside extracts were determined.

MARKER ENZYME ANALYSIS OF THE RAT BRAIN MEMBRANE SUBFRACTIONS- Marker enzyme assays are used in biochemical research to evaluate the identity and purity of subfractionated biological samples by assessing the enrichment in certain enyme activities in those samples, that are reported to be specific for certain organelles or membranes. After the subfraction described above were harvested and washed, several 0.3 ml aliquots of each sample (in PBS) were taken and stored at -70°C. These samples were used for the marker enzyme asays described below (I, II, III, and IV), which were done within 2 months of the preparation of the subfraction.

I. As a general plasma membrane marker, 5' nucleotidase was assayed as described by Rome et al. (1979). The frozen subfraction samples were thawed, and .05 ml of each subfraction was placed in 4, 1 ml centrifuge tubes (n=4). To each tube 0.3 ml of the incubation medium was

added. The media composition was: 10 mM $MgCl_2$; 45 mM Tris HCl; 0.1% Triton X-100 all adjusted to pH 8.5. The reaction was started by the addition of .05 ml of substrate solution which contained a total of 0.45 micromoles of adenosine monophosphate (AMP) which contained .03 microcuries of ^{3}H-AMP (New England Nuclear, Boston, MA). This resulted in a substrate that had a specific radioactivity of 14,666 DPM per micromole AMP, and 6600 DPM per assay. The tubes were incubated at 30°C for 20 minutes, then were stopped by the addition of 0.1 ml of 0.25 M $Ba(OH)_2$ and 0.1 ml of 0.25 M $ZnSO_4$. This formed a white precipitate which traps the unhydrolyzed AMP and the precipitate is pelleted by centrifugation of the tubes at 15,000 rpm in the Sorvall high speed centrifuge and SS34 rotor. The supernatants were removed and placed in scintilation vials, 5 ml Aquasol were added as the fluor, vortexed, and the radioactivity was measured by the Beckman LS1800 scintillation spectrometer which reported the results as DPM (via a quench curve program on board). The DPM oberved was converted to units of 5' nucleotidase (in micromole AMP hydrolized per mg protein per hour) by the following equation:

$$\frac{(DPM)}{(20\ \text{min. SAMPLE})} \times \frac{(1\ \text{umole AMP})}{(14666\ \text{DPM})} \times \frac{3(20\ \text{min})}{\text{hour}} \times \frac{(\text{SAMPLE})}{(X\ \text{mg pro})}$$

= 5' nucleotidase units

II. The marker enzyme assay for golgi membranes was cerebroside sulphotransferase (CST). The method used was that of Seigrist *et al.*, 1977. The stored subfraction samples were thawed and .05 ml of each sample was placed in a glass test tube (in quadruplicate). To each was added: .05 ml of .02% deoxy cholate; 100 micrograms of bovine cerebrosides (Sigma) in 0.1 ml 0.9 M NaCl; and 0.2 ml of 100 nM imidizole/HCl buffer, pH 7.0. The reactions were started by the addition of the substrate: 10 pmol of 3'-phosphoadenosine-5'-phospho(^{35}S)sulfate (PAPS from New England Neuclear) at a specific radioactivity of 4,500 DPM per pmole. Incubation was carried out at 37^{o}C for one hour. The reaction was stopped the addition of 5 ml of C/M 2:1. To help partition the labeled cerebrosides between the organic and aqueous phases, 1.2 ml of 0.74% aqueous KCl was added, vortexed, and the tubes were centrifuged for one minute on a clinical centrifuge. The upper phase was removed to scintilation vials and the samples were dried under a N_2 gas stream. Aquasol was added as the fluor, and the samples were counted as before. By the use of the protein concentration of the sample and the substrate specific radioactivity, the CST specific activities were calculated in units of pmol sulfate transferred per mg protein per hour:

$$\frac{(\text{DPM})}{\text{SAMPLE}} \times \frac{(1 \text{ pmole PAPS})}{(4500 \text{ DPM})} \times \frac{\text{SAMPLE}}{(\text{X mg protein})} = \text{CST UNITS}$$

III. Choline acetyl transferase (ChAT) was used as a marker enzyme for synaptic plasma membranes, and was assayed by the method of Fonnum, 1975. The assay is based upon the disappearance of acetyl-thio-coenzyme A (Acetyl-SCoA) which has an absorbance of monochromatic light at 232 nm. The stored subfractions were thawed one at a time and .05 ml of the samples were added to quartz cuvettes, in quadruplicate. To the samples, 0.2 ml of the reaction medium was added, which consisted of: 300 mM NaCl; 41 mM NaH_2PO_4; 0.1 mM physostigmine salicalate (ACE inhibitor); 10 mM EDTA; 10 mM choline chloride; and .05% Triton X-100, at pH 7.4. The reaction mixtures were allowed to equilibrate for 5 minutes. At this time the cuvettes were placed in the Beckman DU7 specrophotometer, set to read absorbance at 232 nm, and the instrument was zeroed. Then 0.1 ml of the substrate solution was added and quickly mixed, which made the final incubation mixture 0.2 mM in Acetyl-SCoA. After 15 seconds an absorbance reading was taken. After 30 minutes of incubation at room temperature, the cuvettes were read again. The difference of the two readings represents the loss of absorbance units per 30 minutes, which was converted to the change in absorbance per minute. The molar extinction coefficient of Acetyl-SCoA is 4.5×10^3 cm^{-1}. Dividing the absorbance change by this factor results in micromoles Acetyl-SCoA hyrolyzed per minute, per sample. This result was norm-

alized by the protein content of the sample to produce the final result in units of micromoles acetate transferred per mg protein per minute.

IV. Acetylcholine esterase (AChE) is a marker enzyme for neuronal plasma membranes as well as neuronal microsomal membranes. The method of Ellman et al., 1961 was used. In disposable cuvettes, .05 ml of a subfraction, 2.5 ml of 0.1 M NaH_2PO_4 (pH 8.0), and 0.1 ml of .01 M dithiobisnitrobenzene (DTNB) were added, mixed, and allowed to equillibrate for five minutes. The cuvettes were placed in the Beckman DU7 spectrophotometer, and the instrument was zeroed, at the absorbance of 412 nm. Then .02 ml of the acetyl-thiocholine iodide (21.67 mg /ml PBS) was added and the samples were incubated for 10 minutes at room temperature. At this time the samples were measured for absorbance.

In this coupled reaction the thiocholine group produced from the hydrolysis of Acetyl-S-choline reacts with DTNB to form a dithio-choline-nitrobenzene adduct, plus the yellow anion of 5-thio-2-nitro-benzoic acid. Since the molar extinction coefficient of the anion is known, the rates of AChE can be converted to absolute units, viz.:

$$\text{rate(moles/l. per min.)} = \frac{\text{change in absorbance/min.}}{13600}$$

CHAPTER III

RESULTS AND DISCUSSION

PART 1.- GLYCOLIPID SIALOSYLTRANSFERASE OF TRADITIONALLY PREPARED SYNAPTOSOMES.

SYNAPTOSOMAL PREPARATION: Figure 3.1 is a photograph of tubes containing the discontinuous ficoll density gradient used to make the synaptosomal enriched preparation (P2-B), after centrifugation at 100,000 x g for two hours. The procedures are given under method I., of the subfractionation section, in chapter II., Materials and Methods. A dense, white, cohesive band formed at the 9%-16% ficoll interface. This band was removed, washed and termed the P2-B or the crude synaptosomal fraction. An unbiased low power electron micrograph (110,000 X magnification) of this fraction is shown in figure 3.2. The picture exemplifies the character of the fraction. Figure 3.3 is a biased electron micrograph at higher magnification (300,000 X) showing detail of the most numerous stuctures in the fraction. The large cystic structures containing mitochondria and many neurosecretory vesicles are typical of the pinched off, resealed nerve endings known as synaptosomes.

TOTAL GANGLIOSIDE SIALOTRANSFERASE ACTIVITY OF RAT BRAIN SYNAPTOSOMES: In figure 3.4 the specific activity

SYNAPTOSOMAL PREPARATION
DENSITY GRADIENT CENTRIFUGATION

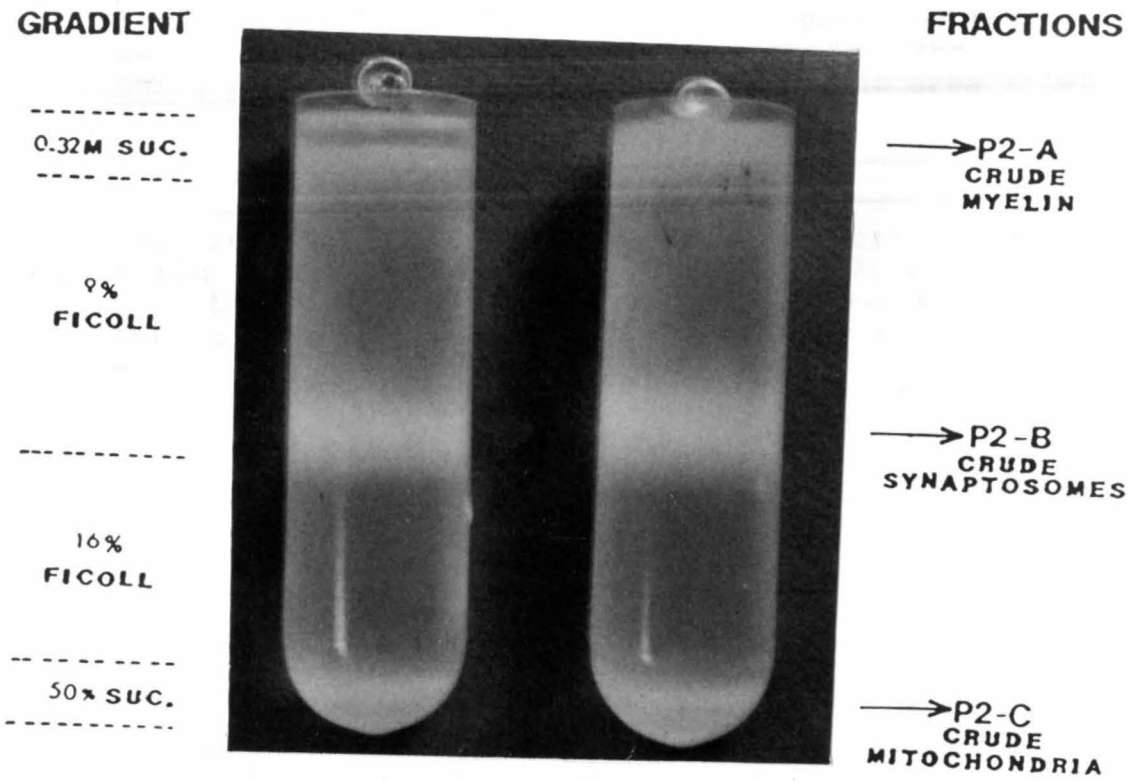


FIG.3.1

Fig.3.1: synaptosomal preparation. Photograph of the discontinuous ficoll density gradient after centrifugation of the "P2" fraction of 28 day old rat brain at 100,000 X g for 120 minutes at 4°C. The "P2-B" fraction was pipetted out and washed in PBS and pelletted by centrifugation, and used for STase assays.

Fig.3.2: electron micrograph of an unbiased area of the "P2-B" or synaptosomal subfraction as prepared by the procedure described in materials and methods. The structures are magnified 110,000 times and the area shown is representative of the entire fraction.

Fig.3.3: electron micrograph of the P2-B fraction showing higher magnification (300,000 X) of typical synaptosome stuctures that were observed throughout the sample, and were the most numerous structures identifiable in the sample.

ELECTRON MICROGRAPH OF SYNAPTOSOMAL PREPARATION (P2 -B)

110,000 X MAGNIFICATION

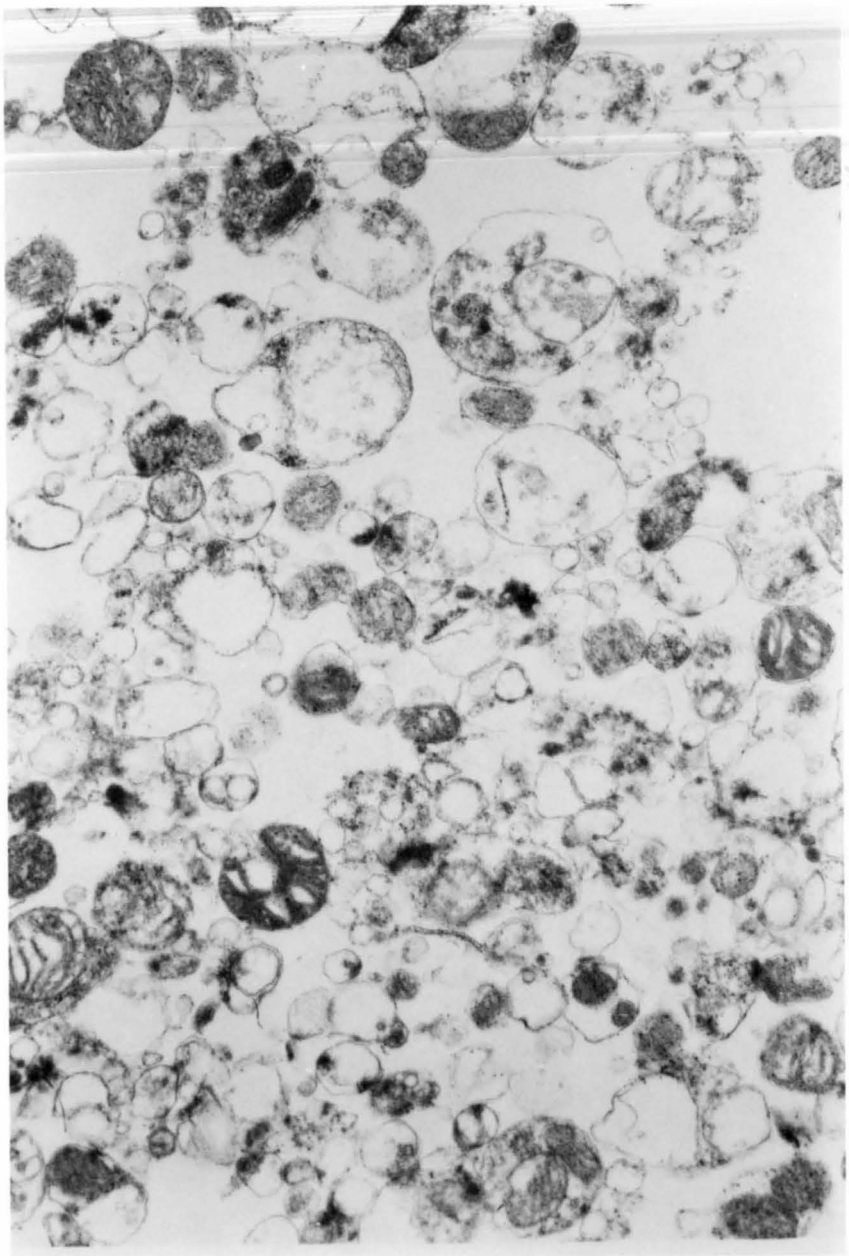


FIG. 3.2

ELECTRON MICROGRAPH OF SYNAPTOSOMAL PREPARATION (P2-B)

300,000 × MAGNIFICATION

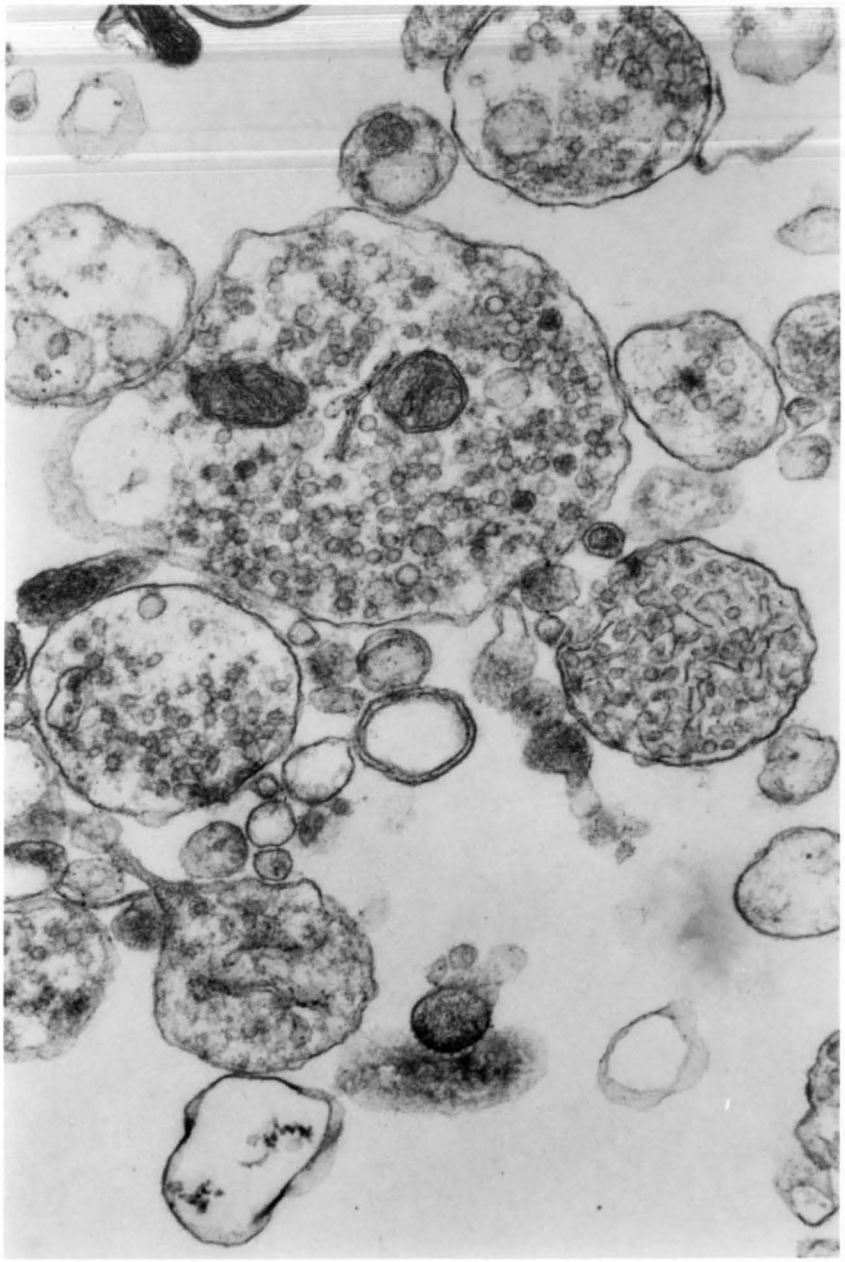


FIG.3.3

of the ganglioside-sialosyltransferase (STase) is shown over a six hour time course. The DPM incorporated into the synaptosomal gangliosides were converted to picomoles NANA transferred by means of the specific radioactivity of the substrate and then normalized by the protein concentration of the synaptosomal fraction. The activity seems minute at first but this is an enzyme that is thought to be membrane bound and its acceptor substrates are lipid membrane components located presumably within the same membrane. No detergent activators, or exogenous acceptors have been added. Classical enzyme kinetic parameters are over shadowed by factors such as the mobility of all members within the membrane. The activity we observed was 0.84 pmole NANA transferred / mg synaptosomal protein / hour (mean of 4 experiments , standard error of the mean = 0.03).

The specific activities measured here can be compared with the values noted by Ng and Dain (1977) who observed a STase of 0.26 nmol NANA transferred/mg protein/ 30 min., which is a higher activity by aproximately 50 fold. These researchers used fractions collected from a continuous sucrose density gradient. Their total synaptosome fractions were less concentrated in protein and possibly more purified in true synaptosomes. These factors may result in their higher activity. Also, our incubation media contained Ca^{++} which is known to form ionic com-

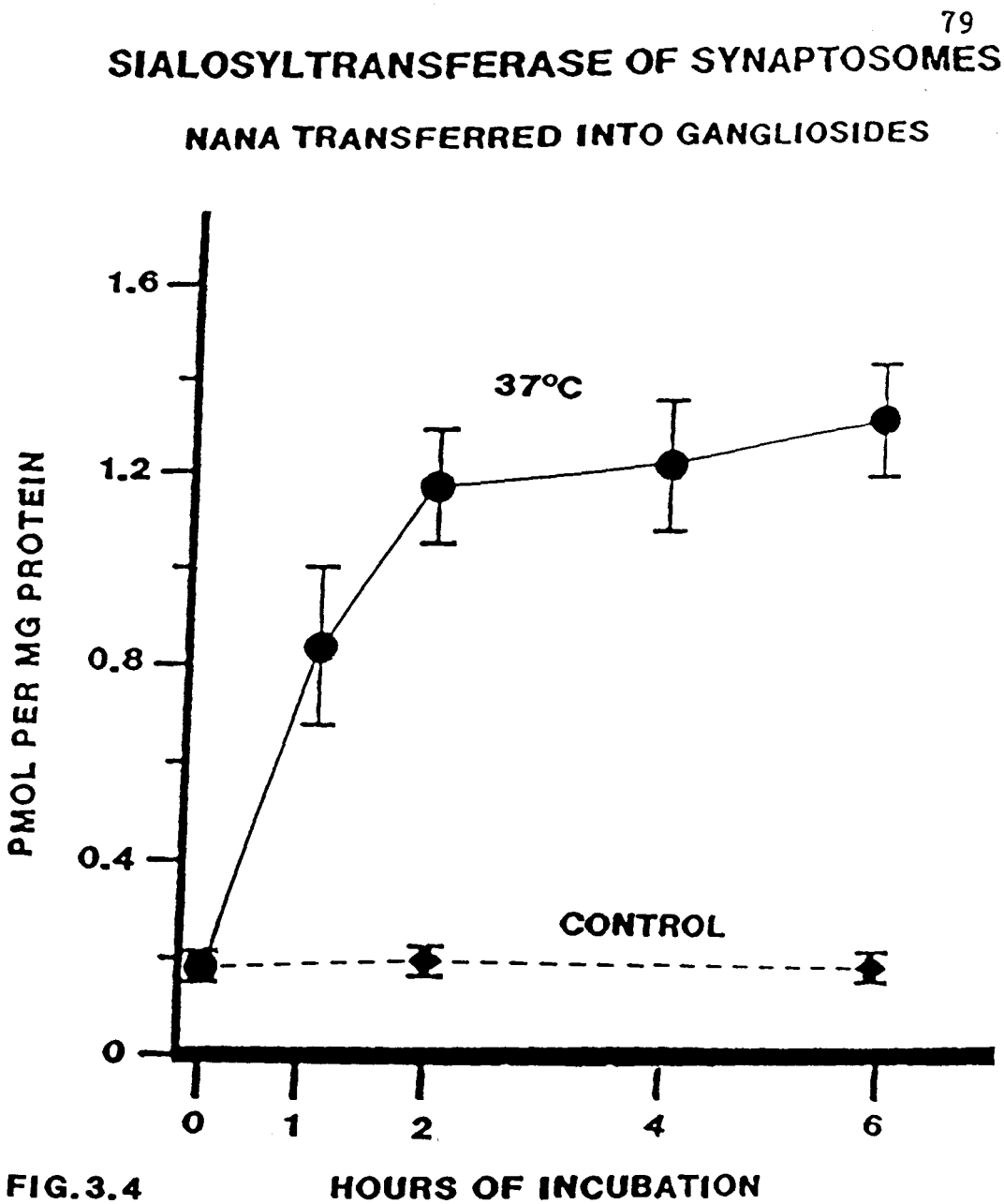


FIG.3.4

Fig.3.4: synaptosomal ganglioside sialotransferase time course assay. The STase assay procedures are described under materials and methods. The points on the solid line represent the mean pmoles of NANA incorporated into the total endogenous gangliosides extracted from the incubated synaptosomes, which has been normalized by the protein content of the samples (mean of 4 samples +/- S.E.M.). The dotted line is the combined data from the controls incubated at 4°C and those denatured by heat (100°C, 10 min.) prior to incubation.

plexes with gangliosides and inhibit enzymes involved in the metabolism of ganglioside sialic acid such as sialidase (Leskawa and Rosenberg, 1980). Other reports on STase have reported the STase activity in units of radioactivity (CPM) transfered / mg protein / hour, which makes it difficult to make comparisons (Preti et al., 1980; Landa et al.,1977; Den et al.,1975). Qualitativly, our result is comparable with these. In general, glycolipid glycosyl transferases have exhibited specific activities in the pmole to nmole / mg protein / hour rang (Gasa and Makita, 1982). Thus our STase activity measured in the synaptosomal fraction is low but reasonable for this type of reaction.

SYNAPTOSOMAL GANGLIOSIDES: Analysis of the synaptosomal ganglioside species profile proved interesting. The key to identificication of ganglioside species on TLC under the solvent system used here is shown in figure 3.5. The pattern is consistent with reviews on this subject (Ledeen and Yu, 1982). It should be noted that there was a variable, usually quite faint resorcinol positive band or bands running near the origin and below GQ1. This band appears to be positive under iodine vapor, but gives an uninterpretable response to ninhydrin reagent. Thus it remains unclear whether this band represents a polysialosyl glycolipid, or something else like a sialic acid containing glycopeptide (Schengrund and Repman, 1982). In

FIG.3.5 RAT BRAIN GANGLIOSIDES

KEY TO IDENTIFICATION BY Rf ON TLC

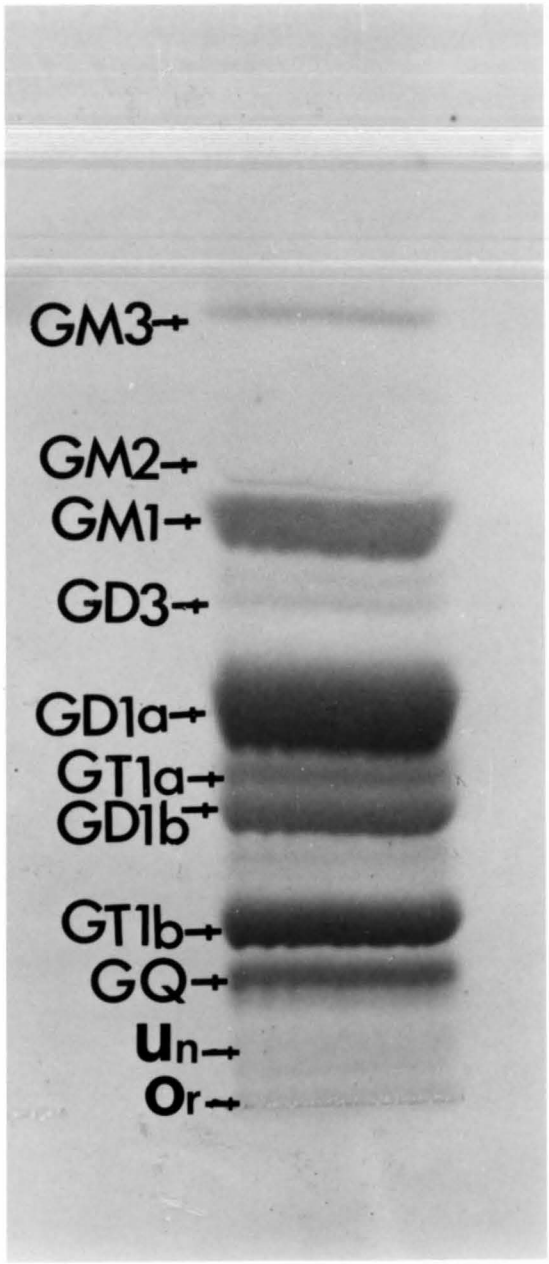


Fig.3.5: key to identification of ganglioside species on TLC plates. Each plate was spotted with a well characterized bovine brain ganglioside standard. The major band can easily be located which is GD1a. Above it the next heavy band is GM1. Two major bands can be seen below GD1a which are GD1b, and below it GT1b. The minor bands can be named by their relationship to the major bands.

further discussion this band is termed UNK.

Figure 3.6-A is a photograph of a typical TLC of the gangliosides from a time course STase assay, of the P2-B subfraction. Figure 3.6-B is the TLC of the experimental controls. The ganglioside profiles remain stable throughout the time course, with no visible change in any species. A typical densitometric tracing of a lane of a TLC of the STase assay gangliosides is shown in figure 3.7. By integration of the peaks, and correcting the areas of each peak for the number of NANA moieties in the ganglioside species of that peak, the percent of the total gangliosides for each species was determined. Figure 3.8 shows the relative percent distribution of the ganglioside profiles. The means of six experiments are shown, all time points and controls are included (n=32 for each value). Again no change in the relative amounts of individual gangliosides is noted in the STase time course as detectable by the sensitivity of the resorcinol-densitometry method of analysis. The percent distribution we obtained were highly compatible with other reports as reviewed by Ledeen (1979).

SYNAPTOSOMAL SIALOSYLTRANSFERASE GANGLIOSIDE PRODUCT SPECIFICITY: Raw data showing the CPM measured in each sequential one mm segment of a typical TLC lane of the P2-B STase assay is reproduced in figure 3.9. Just below, is the tracing of the same lane that matches up, mm for mm,

Fig.3.6: TLC of gangliosides from synaptosomes in a time course STase assay: A) time course samples with bovine brain standards and 10 ug GM3 co-spotted; B) the experimental controls. The HPTLC silica gel 60 plates were developed in C/M/0.25 aqueous $CaCl_2$-50:40:10 (v/v/v) and the gangliosides were visualized by the resorcinol reagent spray .

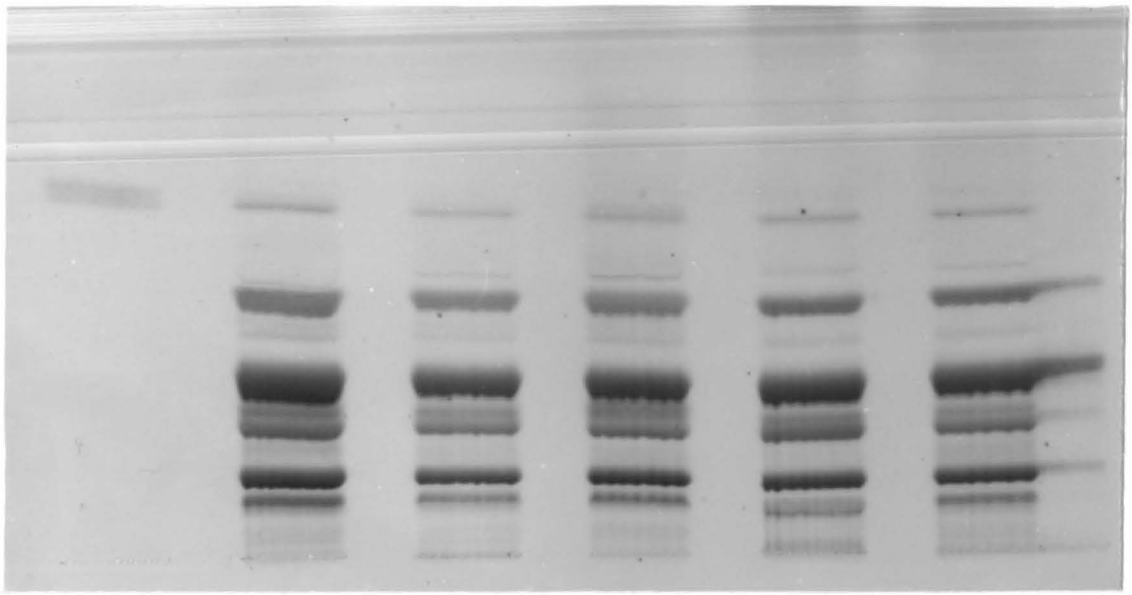
Fig.3.7: typical densitometric tracing of a lane of a TLC of the gangliosides extracted from the synaptosomal preparation. The areas under the peaks represent the reflectance of light at 585 nm as the densitometer scans the length of the lane. Below is a tracing of the actual resorcinol positive bands on the lane.

Fig.3.8: pattern of the endogenous ganglioside species extracted from the synaptosomal samples. The areas under the densitometer peaks corresponding to specific ganglioside bands on TLC, were normalized by the number of NANA residues on the specific ganglioside, and integrated into the total area of all peaks on that TLC lane. The wedges represent the mean percent of the total gangliosides for each species. The values are the means of 36 lanes from the controls and all time point of STase assays. No increase of any ganglioside species was detectable by densitometry durring STase assays.

TYPICAL TLC OF SYNAPTOSOMAL GANGLIOSIDES

SIALOSYLTRANSFERASE TIME COURSE ASSAY

A.



B.

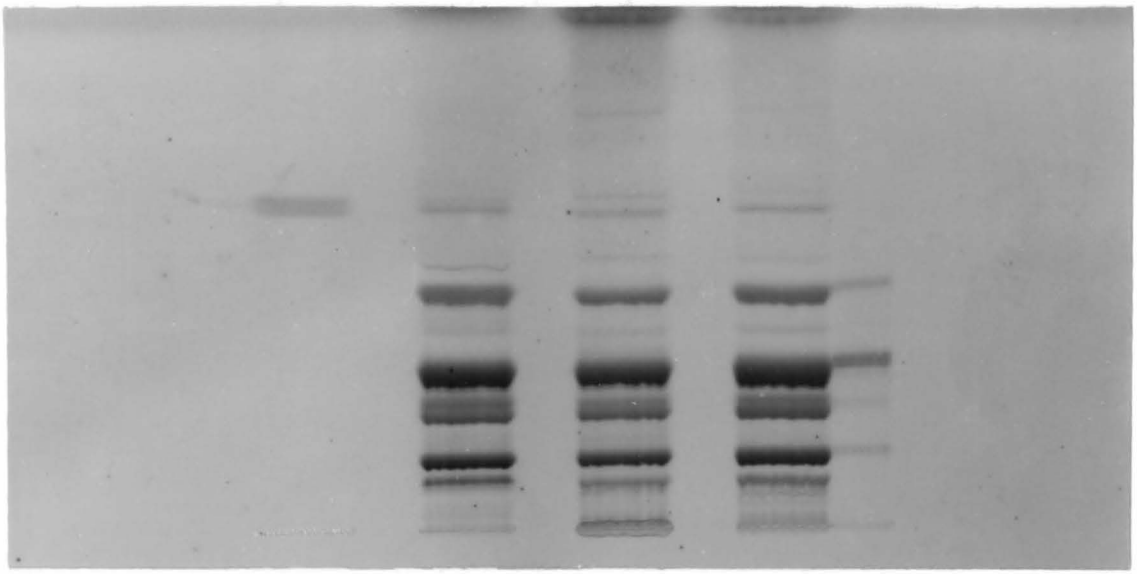


FIG.3.6

DENSITOMETER ANAYSIS OF TLC PLATES

ABSCRBANCE AT 585 NM REPRESENTS GANGLIOSIDE BOUND NANA

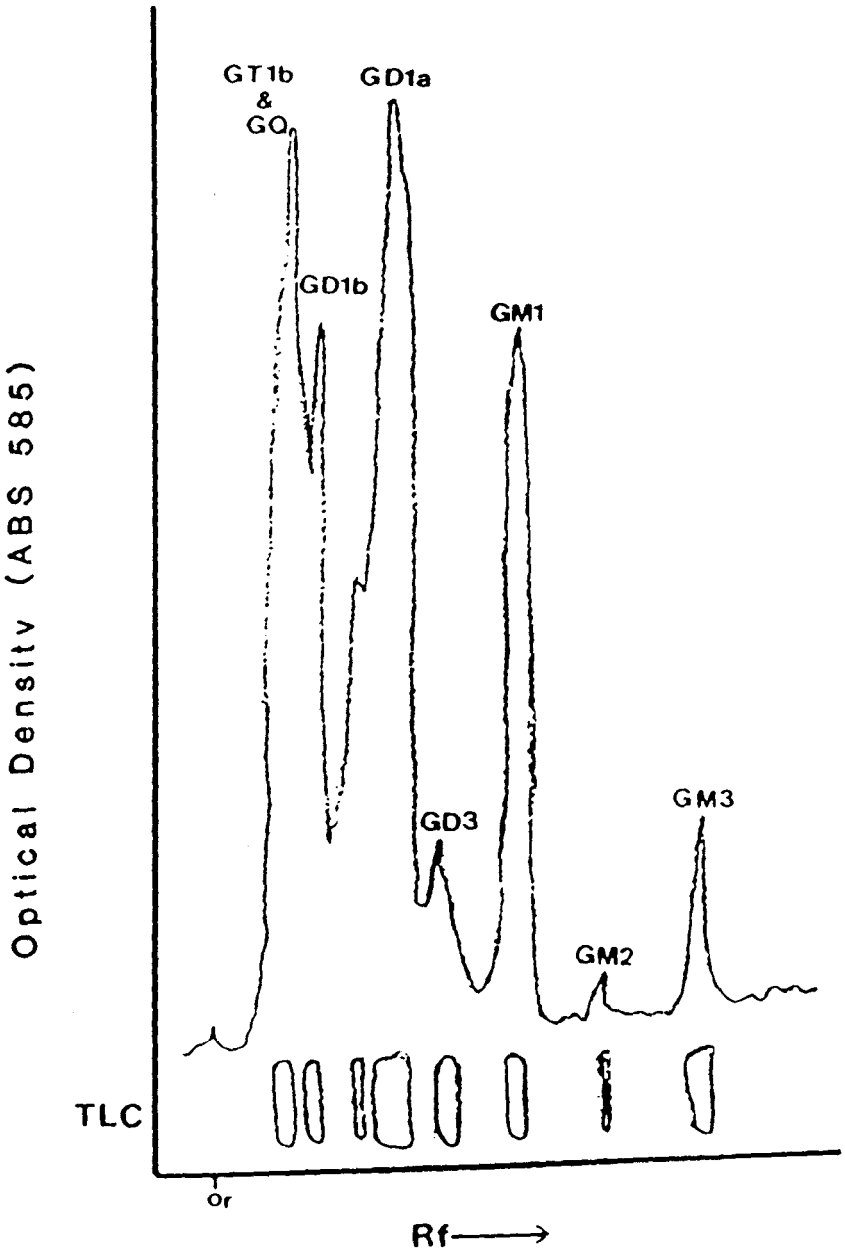
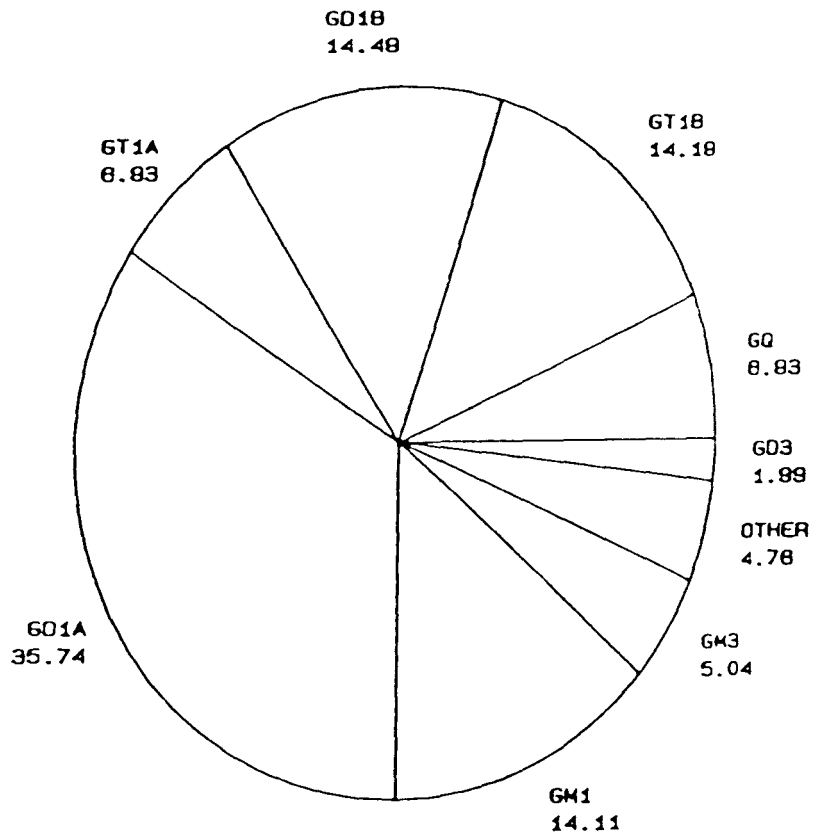


FIG.3.7

GANGLIOSIDE CONTENT OF SYNAPTOSOMES

RESULTS OF THE INTEGRATION OF DENSITOMETER TRACINGS OF TLC

WEDGES REPRESENT THE MEAN % OF THE TOTAL AREA FOR EACH PEAK



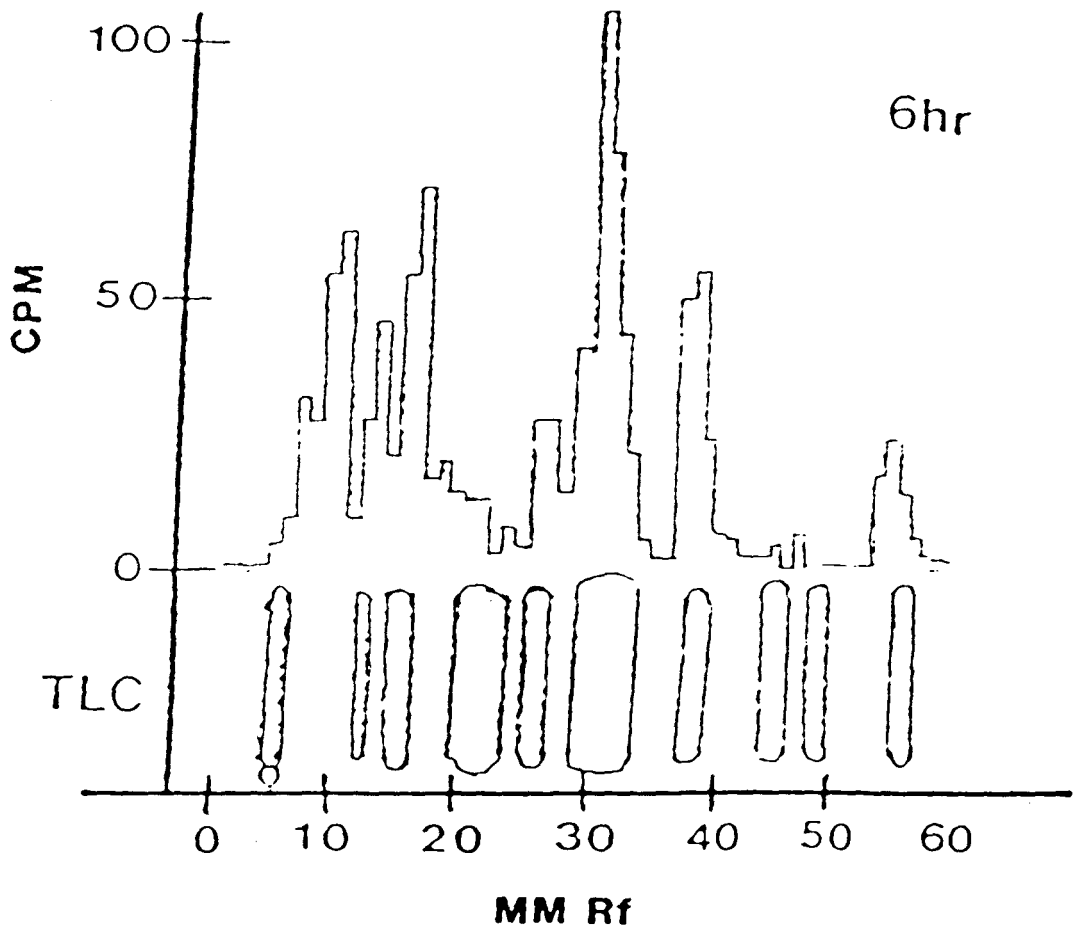
DATA FROM 9 EXPERIMENTS($n=36$). STD. ERROR IS INSIGNIFICANT

FIG.3.8

FIG.3.9 SIALOSYLTRANSFERASE ACTIVITY

TLC OF GANGLIOSIDES AFTER INCUBATION

OF BRAIN HOMOGENATE WITH CMP—(14C)NANA



COMPARISON OF THE LOCATION OF RADIOACTIVITY

WITH THE RESORCINOL POSITIVE BANDS

Fig.3.9: method of assignment of radioactivity on TLC to a ganglioside species. Gangliosides from the STase assays were separated on TLC and the lanes were scraped and counted as described. The merged bars represent the DPM found in each consecutive mm of the lane, and were plotted on 1 mm ruled tracing paper. Below is a tracing of the actual TLC lane showing ganglioside bands. All counts could be easily assigned to the proper species.

with the radioactivity counts. It should be noted that all the radioactivity found on the lane corresponds to a resorcinol positive band on the lane. In this way the DPM can be assigned to a specific ganglioside species identified by Rf with the bovine brain standards. Figure 3.10 is a composite of several of these raw data graphics, showing the incorporation of radioactivity per mm Rf with time, for one typical synaptosomal incubation.

The radioactivity of each ganglioside was converted to a relative percent value by expression as the percent contribution of a specific ganglioside DPM, to the total DPM of all the gangliosides on a given lane. This allowed for easy comparison of the data from separate experiments. In figure 3.11 the mean and standard error of the percent distribution of DPM per ganglioside is shown for the 4 hour time point of 6 P2-B experiments.

The two gangliosides that incorporate the most radioactivity were GD1a (26%) and GD3 (33%). There was very little transfer of NANA into GD1b and GM3. Although these are major gangliosides quantitatively, the lack of label is a reasonable finding. GD1b is believed to be synthesized by the N-acetyl-galactosaminylation and then galactosylation of GD3, and GM1 by the same glycosylation of GM3 (for reviews of ganglioside biosynthesis see: Kishimoto, 1983; Wiegant, 1976; Brunngraber, 1979). Since the UDP-activated transfer forms of galactose and galact-

osamine were not included in the incubations, it would be reasonable to expect that GD1b and GM1 would not be labeled in the STase assay.

GD1a is quantitatively the major ganglioside and therefore would be expected to be a major location of DPM on the TLC. The most striking finding is the incorporation of large amounts of label into the GD3 band which is a very minor band quantitatively. The densitometric analysis data shows that GD1a is roughly 20 fold more abundant than GD3, yet they both incorporate about the same high level of radioactivity.

In figure 3.12 a bar graph of the DPM found in consecutive 2 mm segments of a TLC lane, is superimposed over the densitometer tracing of that lane. This data is from one representative 4 hour time point of a P2-B STase assay and a reproduction of the TLC lane of the gangliosides is shown below.. At about 23 mm Rf, which is the GD3 band, the radioactivity bar extends far above the densitometer scan line. This is the only point at which this happens, and this is a consistent observation.

By utilizing the known amount of GM3 standard that was cospotted on each TLC plate the gangliosides can be converted to nmole amounts by integration of the densitometer peak areas. In figure 3.13 the DPM incorporated into a given ganglioside by STase has been normalized by the quantitative amount of ganglioside. Here the specifi-

SIALOSYLTRANSFERASE TIME COURSE
SYNAPTOSOMAL FRACTION

TLC OF GANGLIOSIDES VS. RADIOACTIVITY IN CONSECUTIVE 2MM SEGMENTS OF THE LANE

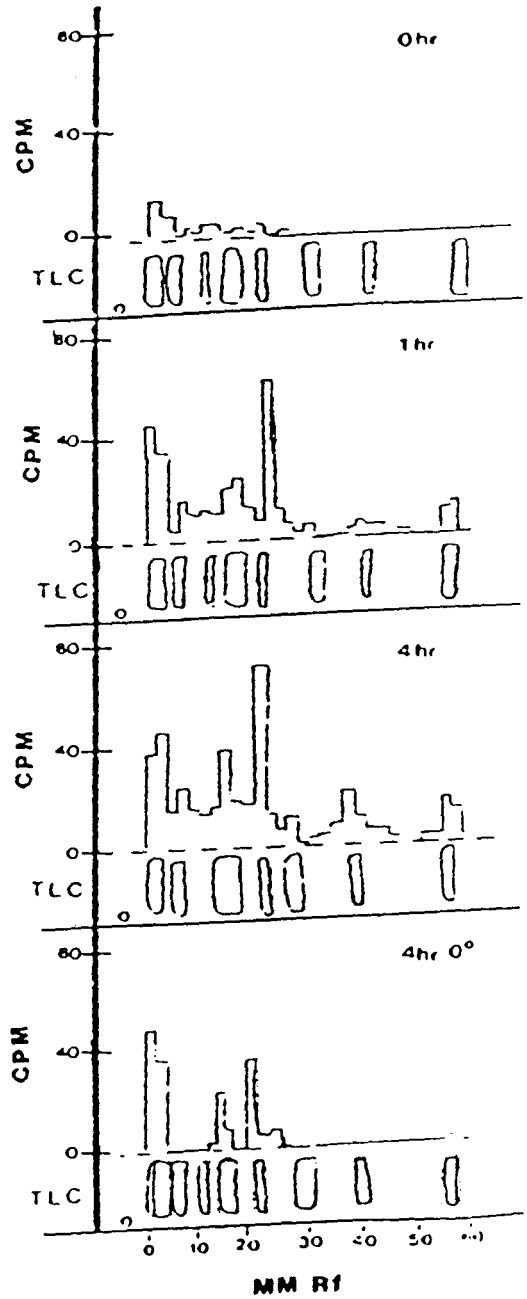


FIG.3.10

Fig.3.10: sialosyltransferase time course. The bars show the location of DPM in relation to ganglioside bands on TLC. DPM were assigned to ganglioside as described.

FIG.3.11

SYNAPTOSOMAL SIALOSYLTRANSFERASE

DISTRIBUTION OF RADIOACTIVITY AMONG GANGLIOSIDES AS PERCENT OF TOTAL DPM, 4 HR. SAMPLES

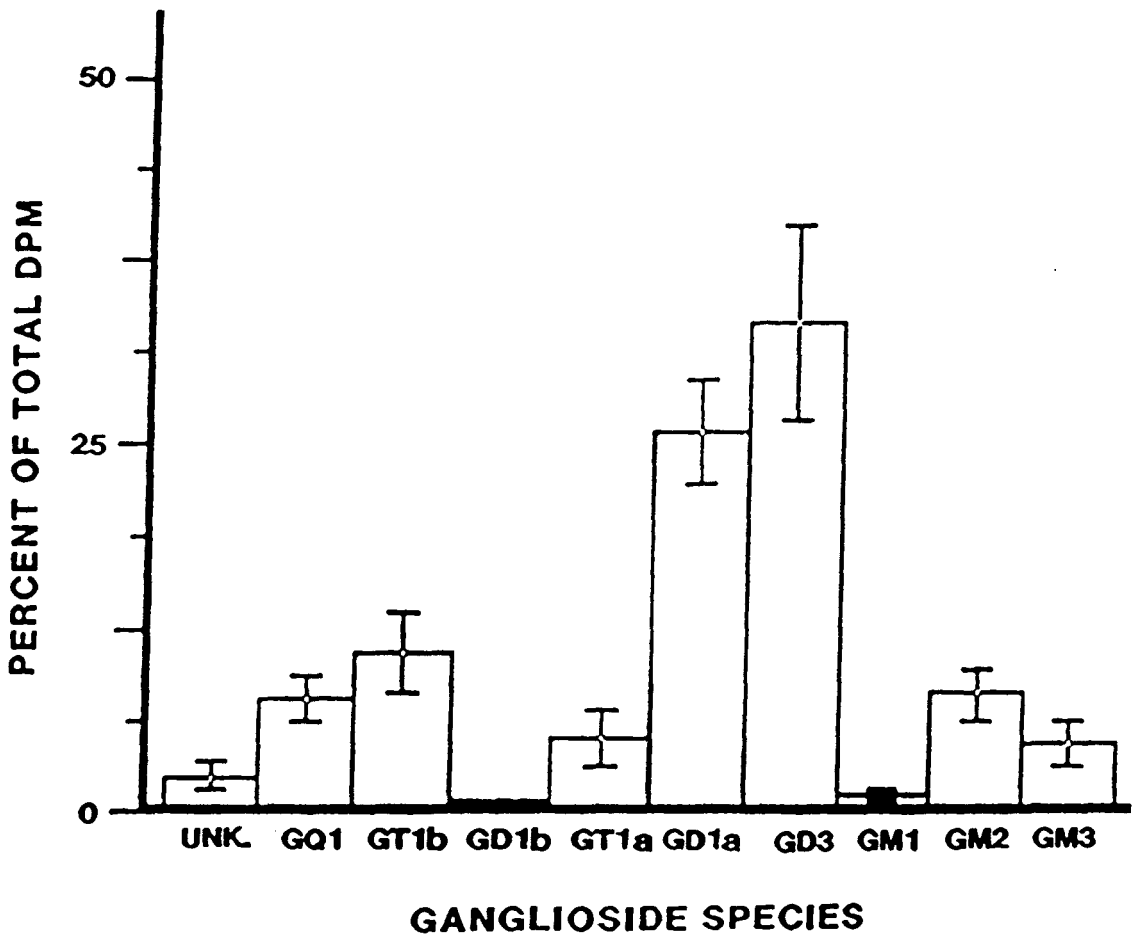
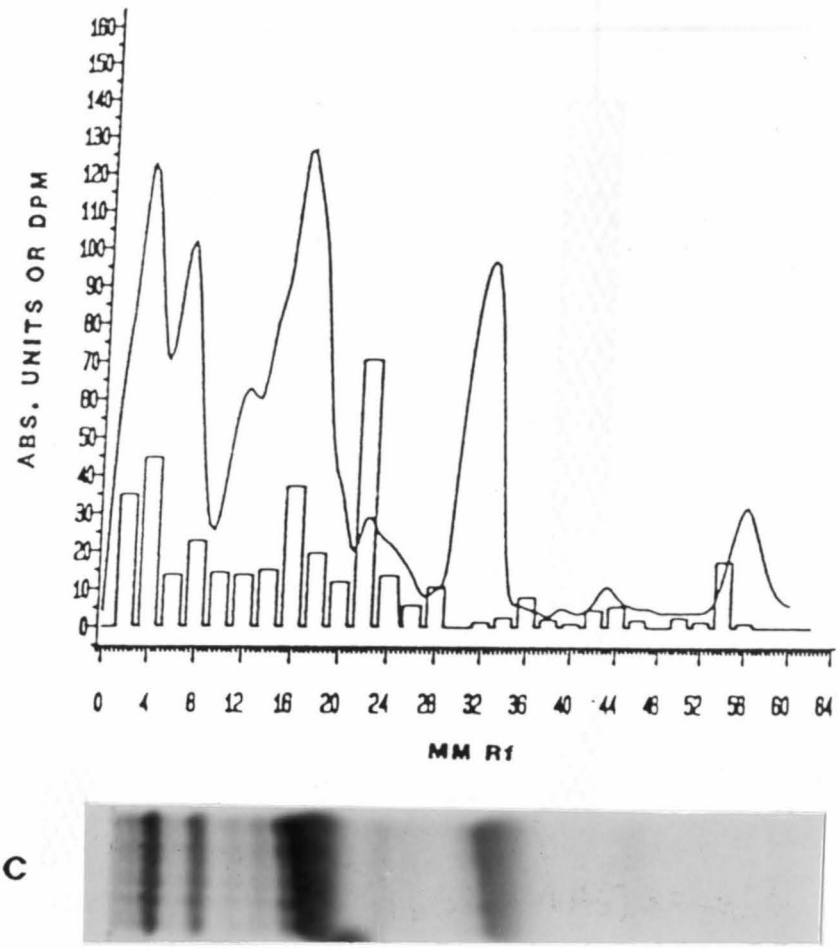


Fig.3.11: percent distribution of radioactivity transferred to gangliosides by synaptosomal STase. Bars represent the mean DPM of a ganglioside species as the percent of the total DPM of all gangliosides in that sample. The means are of six samples after 4 hours of incubation and the standard error of the mean is shown.

SYNAPTOSOMAL GANGLIOSIDES LABELED BY SIALOSYLTRANSFERASE FOR 2 HOURS

OVERLAY OF TLC DATA:

DENSITOMETER TRACING AND RADIOACTIVITY



LINE- DENSITOMETRIC TRACING OF TLC

FIG.3.12 BARS- RADIOACTIVITY PER 1 mm SECTION OF TLC

Fig.3.12: overlay of the densitometer tracing, from a TLC of synaptosomal gangliosides, with the DPM found in consecutive 2mm segments of the lane. Below is a photograph a the actual TLC lane. The sample is the 4 hour time point of a STase assay. Note the band, bar, and densitometric tracing at about 23 mm Rf which is GD3.

SPECIFIC RADIOACTIVITY OF GANGLIOSIDES LABELED BY SYNAPTOSOMAL SIALOSYLTRANSFERASE

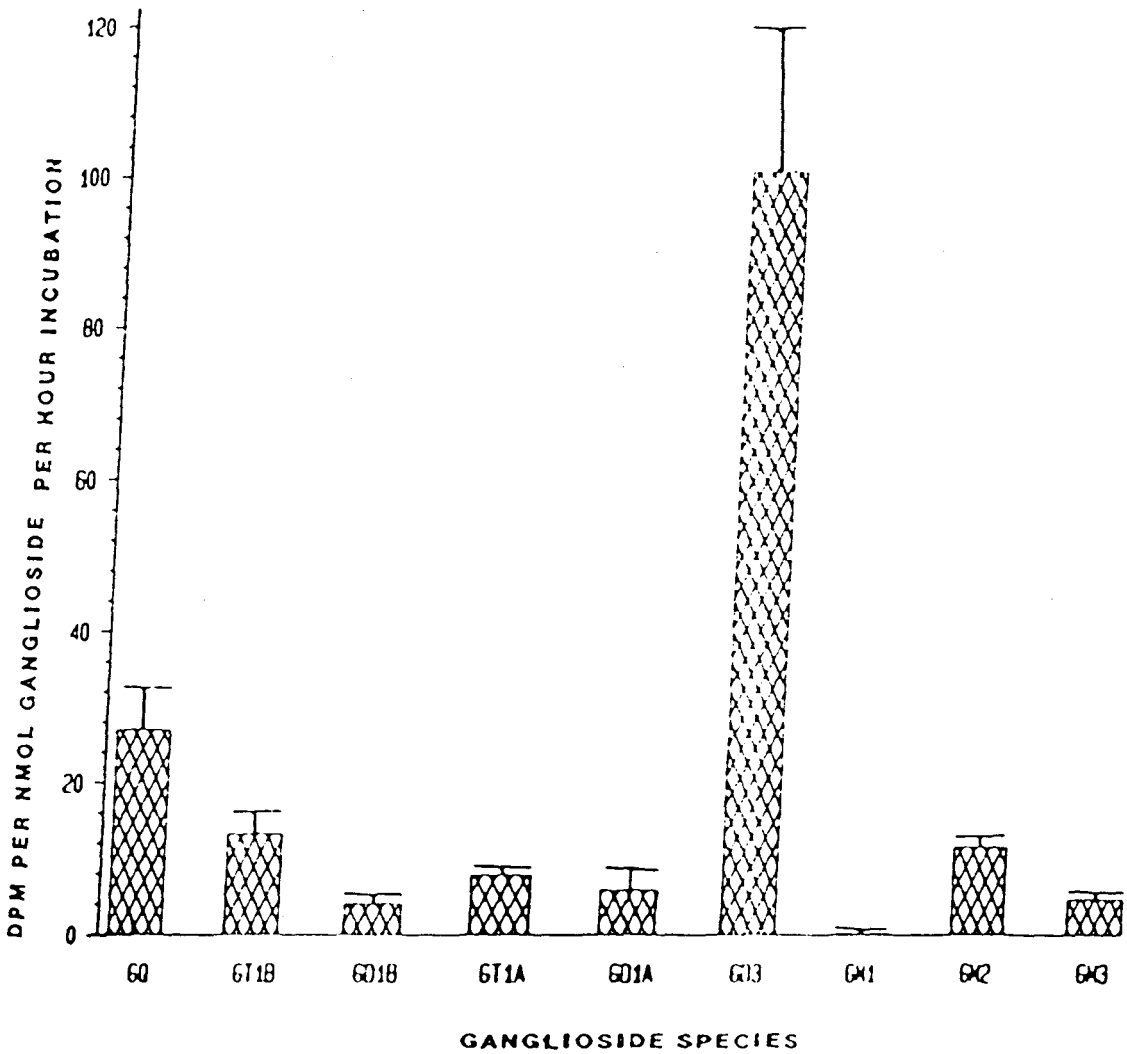


Fig.3.13: specific radioactivity of the products from the STase assay. Using the 4 hour time points, the ganglioside species extracted from the synaptosomal samples were quantitated from the TLC data as described under materials and methods. The bars represent the mean DPM per nmole product formed per hour by synaptosomal STase (n=6, std. error of the mean shown).

city of STase of rat synaptosomes to prefer GD3 as a product is unmistakably brought out. The specific radioactivity of GD3 is aproximately 15 fold higher than GD1a and 4 fold higher than the next highest, GQ1. The data from six identical, synaptosomal (P2-B) ganglioside STase assay experiments is summarized in tabular form, in table 3.1.

The first explanation of these results is that there exists great specificity in the STase to label GD3. Atempts were made to test this, which are represented by the next set of experiments below. One other explanation deserves discussion. By studying figures 3.11 and 3.13, one can see that a large percentage of the radioactivity is found in the gangliosides that contain a di-sialosyl linkage on the proximal galactose. combining the average data from gangliosides GD3, GT1b, GQ1, and GD1b, the gangliosides with this linkage accounts for only 24% of the total gangliosides (or 9.26 nmoles lipid bound NANA/mg protein), but carry 53% of the radioactivity.

On a specific radioactivity basis these species have 146.7 DPM per nmole ganglioside, while the rest of the gangliosides have a combined specific radioactivity of only 35.9 DPM per nmole ganglioside. This proximal di-sialosyl linkage specificity may be even more enhanced when GDIb and GD2 are considered. As mentioned before, GD1b would not be expected to become labeled in this assay system because it is reported to be synthesised by the

glycosylation of GD3 and not by sialosylation of GM1. Also GD2 is a minor gangloside that co-migrates with GD1a in these TLC conditions. Part of the DPM assigned to GD1a could be due to GD2. Both of these factors could make the STase specificity for the proximal di-sialosyl bond even more pronounced. Whichever the case, the highly specific labeling of GD3 remains the most significant finding of these initial experiments.

ANALYSIS OF THE SPECIFICALLY LABELED GD3 OF SYNAPTOSOMAL SIALOSYLTRANSFERASE- A question that must be addressed is the possibility that the high radioactivity we see in the GD3 area is an artifact due to another molecule that is very highly radioactive, that also co-migrates in this area of the TLC. Some likely candidates would be the free derivitives of NANA that could have escaped removal during the ganglioside extraction and purification steps in trace amounts.

Figure 3.14 is a photograph of the gangliosides fron a synaptosomal STase incubation. Standards of NANA (Sigma Chemical Co., Saint Louis, MO) and N-acetyl-2,3-didehydro-2-deoxyneuraminic acid (NADNA, Boehringer Mannheim Biochemicals, Indianapolis, IN) were cospotted on the plate. NADNA is a derivative of NANA that has been shown to be present in small quantities in mammalian brain (Saito and Rosenberg, 1984), and is an inhibitor of sialidase (Veh and Sander, 1981). Free NANA chromato-

graphs between GD1b and GD1a which is an area of low DPM on the plate. NADNA appears between GD1a and GD3, which is close to the area in question but seems to be clearly separable.

In figure 3.15, synaptosomal gangliosides labeled by STase, were streaked on TLC plates. The origins of three such lanes were spiked with minute quantities of very highly labled CPM-(^{14}C)NANA, or (^{14}C)NANA (New England Nuclear, Boston, MA), or (^{14}C)NADNA (as prepared by Saito and Rosenberg, 1984). Figure 3.15.A shows that CMP-NANA forms a sharp band between GQ and GT1b. Free NANA forms a broad band engulfing the area including GT1b through GD1b which is shown in fig. 3.15.B. Figure 3.15.C shows that NADNA is located at the leading edge of the GD1a band, but the normal labeling band we had associated with GD3 is clearly distinguishable. Therefore, the labeling of GD3 does not appear to be an artifact of contamination from trace amounts of nonlipid compounds derived from CMP-NANA.

The highly specific labeling of GD3 is so dramatic that some simple experiments were designed to gain more evidence that this observation is true. When gangliosides are chromatographed in an alkaline solvent system the Rf pattern changes slightly (Ledeen and Yu, 1982). The theory being that the alkaline conditions ionize the hydroxyl groups on all the sugars, so that the separation

SYNAPTOSOMAL GANGLIOSIDE SIALOSYLTRANSFERASE

P2-B SUBFRACTION INCUBATION; STATISTICAL DATA FROM THE 4 HOUR SAMPLES OF 6 EXPERIMENTS

TABLE 3.1

	GANGLIOSIDE DISTRIBUTION % OF TOTAL GANGL.	RADIOACTIVITY DISTRIBUTION % OF TOTAL DPM	SPECIFIC GANGLIOSIDE CONCENTRATION nmole GANGL./mg PRO.	GANGLIOSIDE RADIOACTIVITY DPM /nmole GANGL.
TOTAL GANGL.	100	100	25.54 (2.79)	18.3 (3.7)
UNK.	2.45 (0.45)	2.5 (0.9)	0.75 (0.30)	5.1 (2.0)
GQ1	9.38 (0.90)	7.8 (1.5)	2.04 (0.25)	28.8 (7.5)
GT1b	18.40 (1.20)	11.1 (2.7)	3.81 (0.28)	11.9 (3.8)
GD1b	14.88 (2.00)	0.7 (0.1)	3.00 (0.50)	4.9 (2.0)
GT1a	6.30 (0.49)	5.1 (3.3)	1.17 (0.24)	8.1 (1.7)
GD1a	28.63 (1.71)	25.8 (3.4)	6.32 (0.45)	7.2 (2.7)
GD3	1.68 (0.31)	33.2 (6.8)	0.41 (0.05)	101.1 (17.4)
GM1	11.53 (1.59)	1.3 (0.3)	3.90 (0.79)	0.9 (0.3)
GM2	0.83 (0.40)	8.0 (1.8)	0.82 (0.44)	11.8 (2.7)
GM3	4.71 (1.62)	4.7 (1.6)	1.63 (0.43)	7.9 (1.9)

-Values are the means of 6 samples (+/- S.E.M.)

FIG.3.14

TLC OF RAT BRAIN GANGLIOSIDES
SIALOSYLTRANSFERASE TIME COURSE ASSAY

STANDARDS SHOW Rf OF NANA AND NADNA

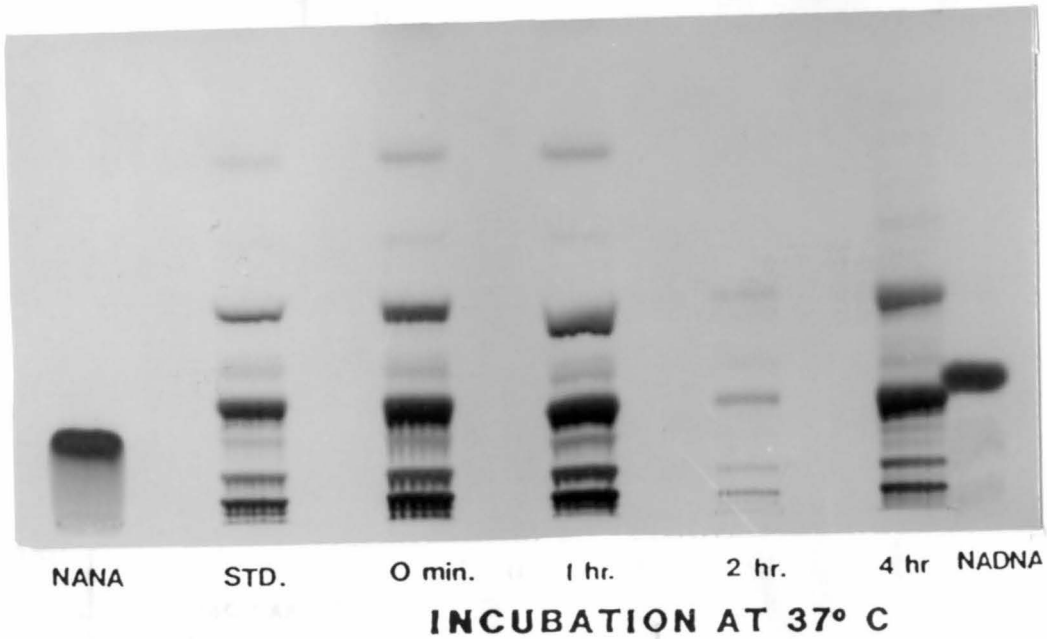


Fig.3.14: photograph of a TLC plate showing the Rf of free NANA and NADNA in relation to the synaptosomal gangliosides of a STase time course assay.

Fig.3.15: location of possible radioactive artifacts for synaptosomal STase assay. The origins of TLC lanes of STase labeled gangliosides were over spotted with a small amount of highly labeled derivatives of the reaction precurser before development: A) CMP-(^{14}C)NANA; B) (^{14}C)NANA; C) (^{14}C)NADNA.

LOCATION OF CMP NANA, NANA AND NADNA ON TLC LANES OF LABELED BRAIN GANGLIOSIDES

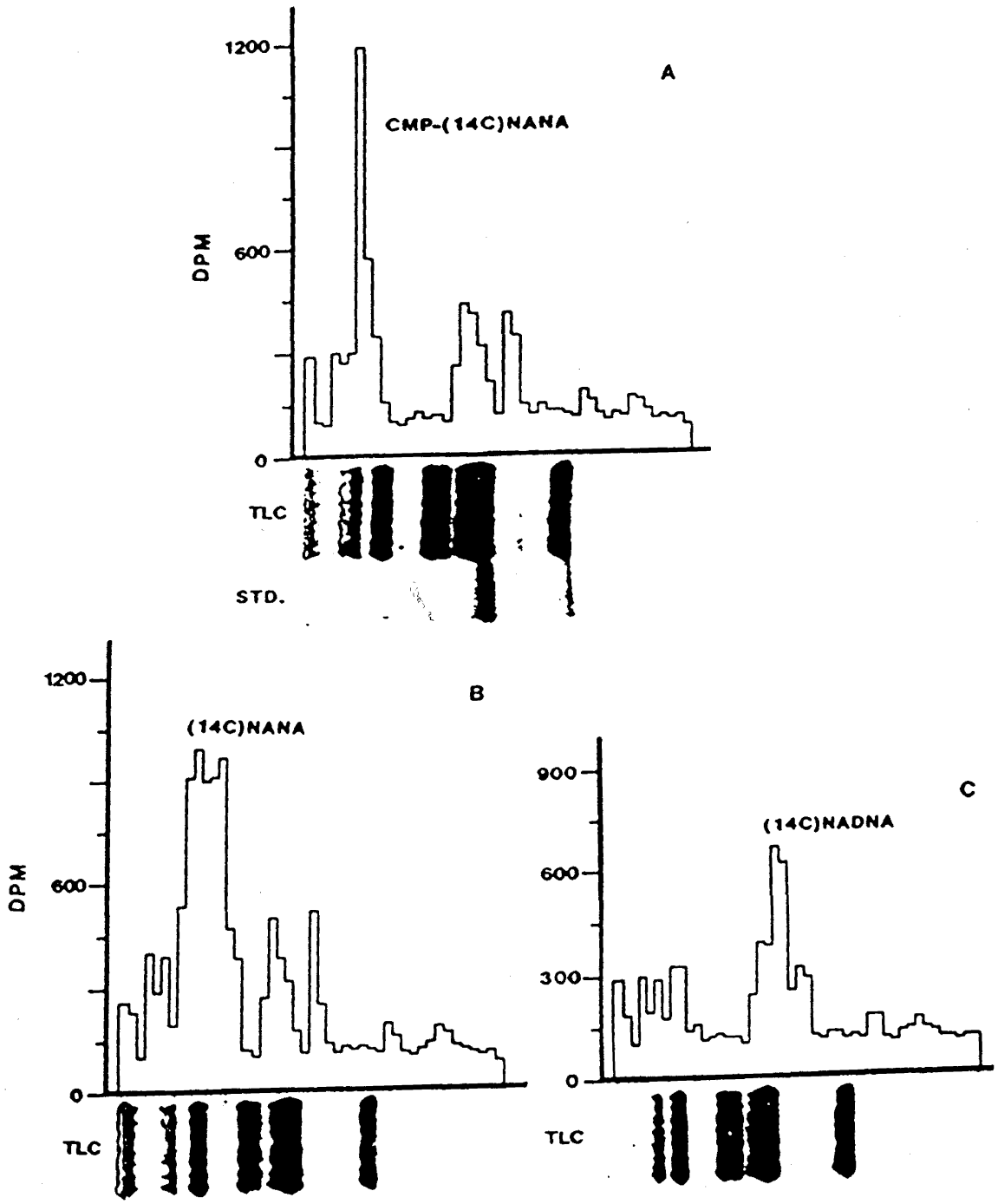


FIG.3.15

by TLC is now based more on the oligosaccharide chain length than on the number of charged NANA residues on the ganglioside. The Rf order is almost the same as for the normal solvent except that GD3 runs ahead of GM1, which is very usful for our purposes. As a standard, some GD3 was prepared by preparative TLC from embryonic chick brain, where GD3 is by far the most abundant ganglioside species (Dreyfus et al.,1975).

Figure 3.16.A is a photograph of a TLC developed with our normal solvent. The band we recognize as GD3 in our labeled rat brain gangliosides chromatographs to the same Rf as the standard GD3 from chick brain, which seems to be a triplet band. Multiple bands are common for GD3, probably are due to differences in fatty acyl composition.

In fig.3.16.B the same samples were developed in the alkaline solvent system, C/M/conc. NH_4OH/0.25%aqueous $CaCl_2$-50:40:7:3. As can be seen in the bovine brain standards, GD3 has indeed switched places with GM1 which is now just above GDIa. Again the chick brain GD3 standard behaves like the GD3 labeled by rat synaptosomal STase. Here the rat brain GD3 also appears to be in the form of three very faint bands.

The lanes containing the labeled rat synaptosomal gangliosides were scraped and counted as before to localized the DPM. In fig.3.17 the radioactivity has been represented as DPM / mg ganglioside which will "flag" GD3

FIG.3.16 TLC OF RAT BRAIN GANGLIOSIDES LABELED BY SYNAPTOSOMAL SIALOSYLTRANSFERASE

COMPARISON OF TWO DEVELOPING SOLVENT SYSTEMS

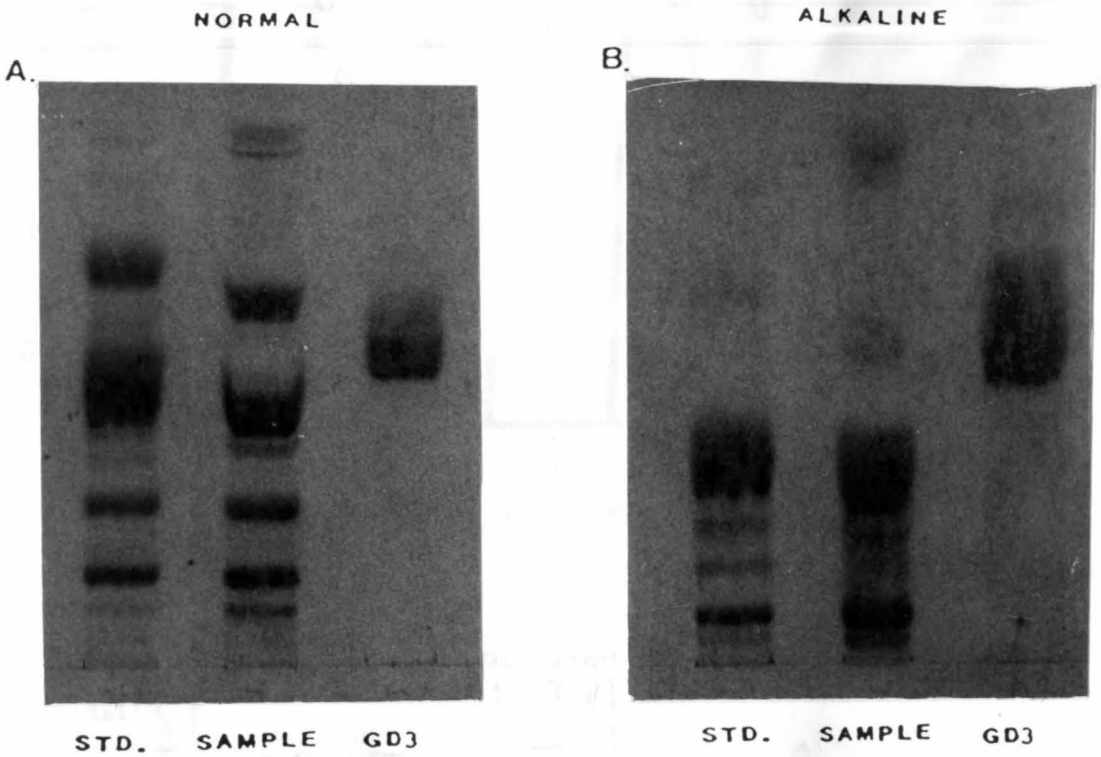
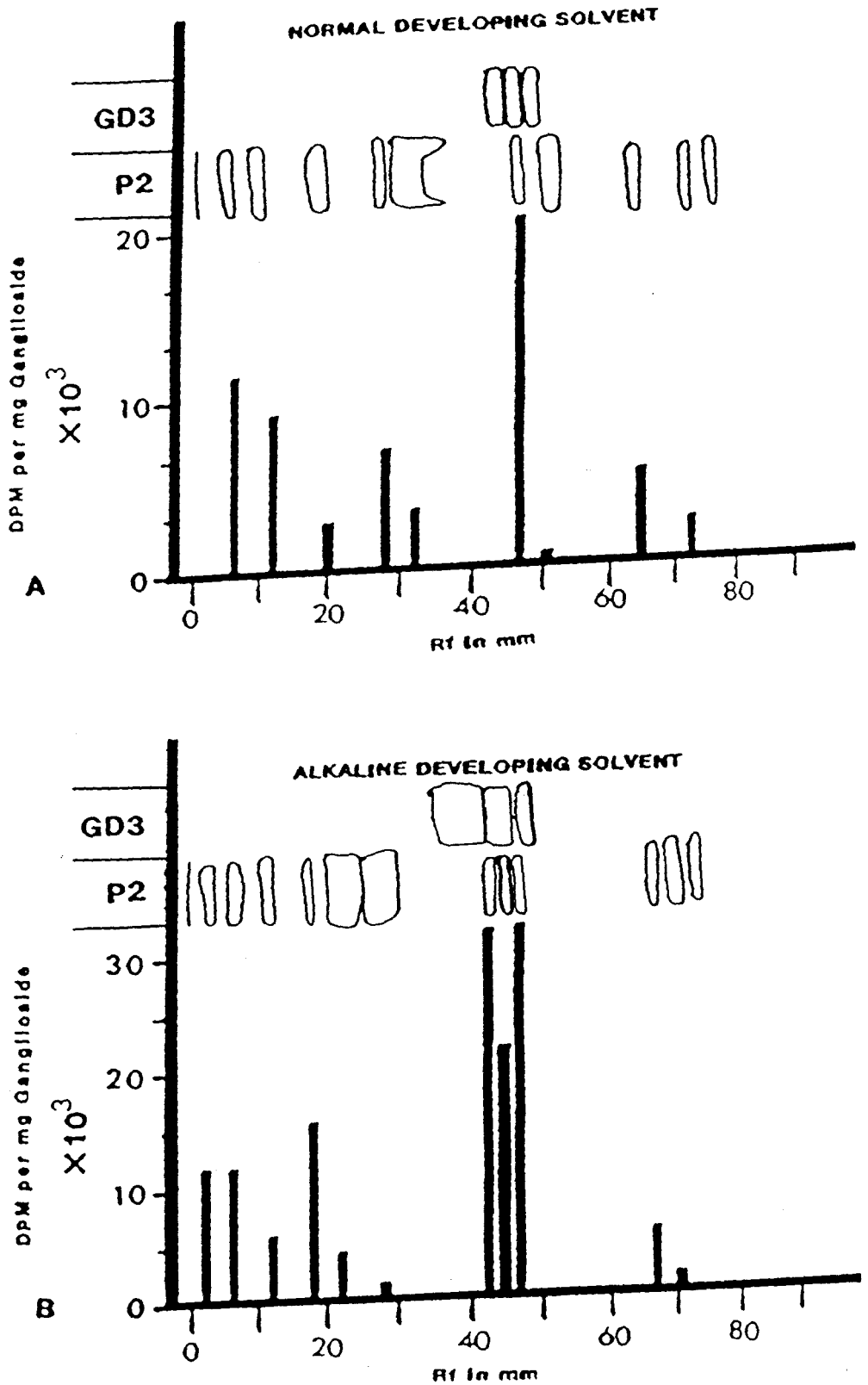


Fig.3.16: TLC plates of synaptosomal STase labeled gangliosides with GD3 standards. GD3 was isolated by preparative TLC from 15 day old chick brains as described under materials and methods: A) normal developing solvent, C/M/0.25%aqueous $CaCl_2$-50:40:10; B) alkaline developing solvent, C/M/NH_4OH/0.25%$CaCl_2$-50:40:7:3.

Fig.3.17: radioactive data from fig.3.16. The lanes with the STase labeled gangliosides were scanned densitometrically and then scraped and counted as before. The gangliosides were quantitated as described, from the densitometer data. The bars represent the DPM per mg ganglioside. Above the bar graphs are tracings of the TLC lanes of the sample gangliosides and standard GD3. A) normal developing solvent; B) alkaline developing solvent.

FIG. 3.17 TLC BEHAVIOR OF GANGLIOSIDES
COMPARISON OF TWO SOLVENT SYSTEMS



as the tallest bars on the graphs. Under both solvent systems the high specific radioactivity is associated with the GD3 bands in the samples and standards (fig.3.17-.A and .B).

To further analyze the nature of the compounds labeled by STase, the ganglioside extracts were subjected to sialidase treatment and the reaction products were analyzed. Figure 3.18 is a direct photocopy of the TLC plate on which the sialidase reaction products were chromatographed. Ignoring the brown, contaminating band marked "X", it can be seen that at 6 hours incubation all of the gangliosides have been desialosylated except GM1, which is known to be resistant to sialidase (Corfield et al., 1981). All the resorcinol positive material has migrated to the Rf of free NANA. The sialidase lability is also shown in fig.3.19.A and .B, where all the radioactivity in the ganglioside samples was released by sialidase and was found in the free NANA band (fig.3.19.B).

An attempt was made to purify the GD3 labeled by rat synaptosomal STase by preparative TLC as described. The fact that GD3 is such a minor ganglioside in amount in rat brain, and that the yield from preparative TLC of gangliosides is highly inefficient (Ledeen and Yu, 1982), the resulting GD3 product was both low in quantity and contaminated by GD1a. For fear of loss of more of the precious labeled GD3, this preparation was used for a neura-

minidase hydrolysis analysis without further purification. The lanes of the TLC shown in figure 3.20 represent the reaction products of a time course digestion of this semi-purified GD3 sample by Clostridium perfringens neuraminidase. It should be noted that the GD1a present was rapidly desialosylated to GM1 and was practically gone by two minutes of incubation as shown graphically in fig.3.21.A by the densitometer analysis. This was accompanied by a rapid rise in free NANA over the first two minutes as shown in fig.3.21.B.

The GM1 level rose in this time interval, then remained constant as it is resistant to sialidase. The GD3 showed a slower rate of desialosylation (fig.3.21.A). The hydrolysis product of GD3 is GM3 which is also labile to the sialidase. Lactosyl ceramide is the desialosylation product of GM3, and a standard of lactosyl ceramide (Calbiochem-Behringer, La Jolla, CA) was cospotted on the plate and visualized by anthrone reagent (Yamakawa et al.,1960) to establish it's Rf. GM3 showed an increase until fifteen minutes incubation, followed by a decrease (fig.3.21.A). Lactosyl ceramide exhibited a general increase over the time course (fig.3.21.C).

The analysis of radioactivity of the sialidase products is shown in figure 3.22, the results of which were consistent with the densitometer data. GD3 of high specifis radioactivity, showed a slow, constant decrease in DPM

NEURAMINIDASE DIGESTION OF BRAIN GANGLIOSIDES

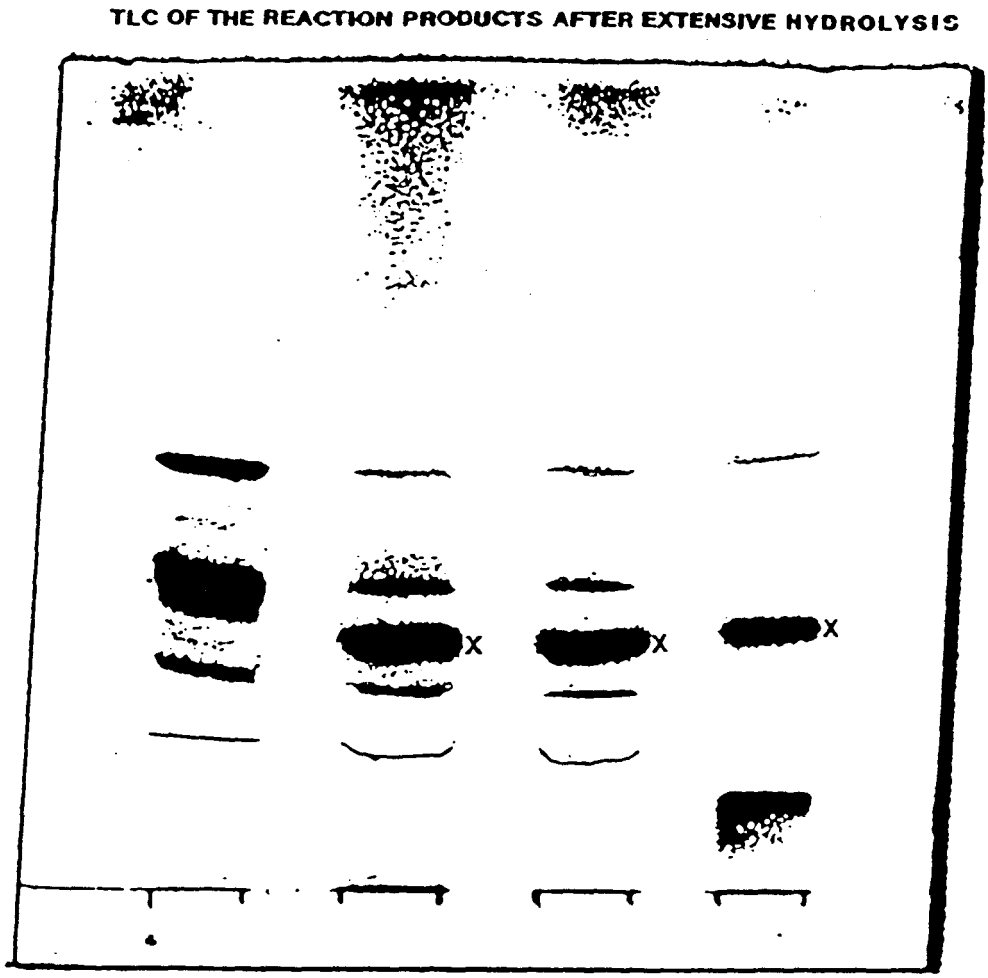


FIG.3.18

Fig.3.18: TLC of the products of a sialidase digestion of the gangliosides labeled by synaptosomal STase. Clostridium perfringens neuraminidase (0.01 unit) was incubated with sample gangliosides from a STase assay. The total reaction mixture was spotted on TLC plates along with standards, a time zero control, and a heat denatured control. There was a large yellow, resorcinol negative band present, and is marked "X".

Fig.3.19: radioactivity data from fig.16. Lanes were scraped and counted as described before. The bars represent the DPM found in consecutive 5 mm segments of the lane (mean +/- S.E.M. of 2 samples). A) location of DPM before incubation; B) location of DPM after sialidase incubation.

NEURAMINIDASE DIGESTION OF BRAIN GANGLIOSIDES

FATE OF THE GANGLIOSIDE ASSOCIATED RADIOACTIVITY

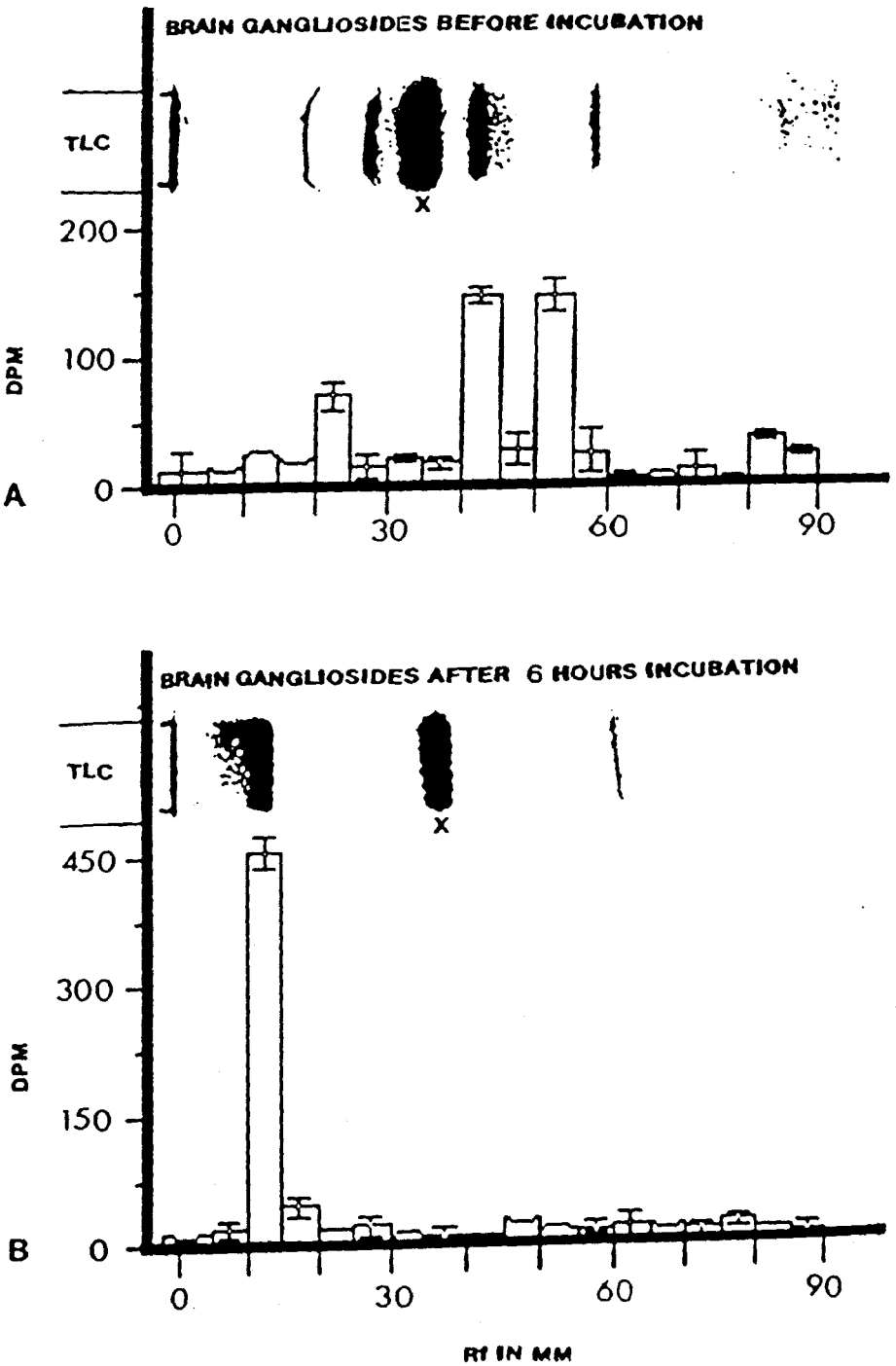


FIG.3.19

FIG.3.20
NEURAMINIDASE DIGESTION OF CRUDE GD3 LABELED BY SYNAPTOSOMAL SIALOSYLTRANSFERASE

TLC OF THE REACTION PRODUCTS

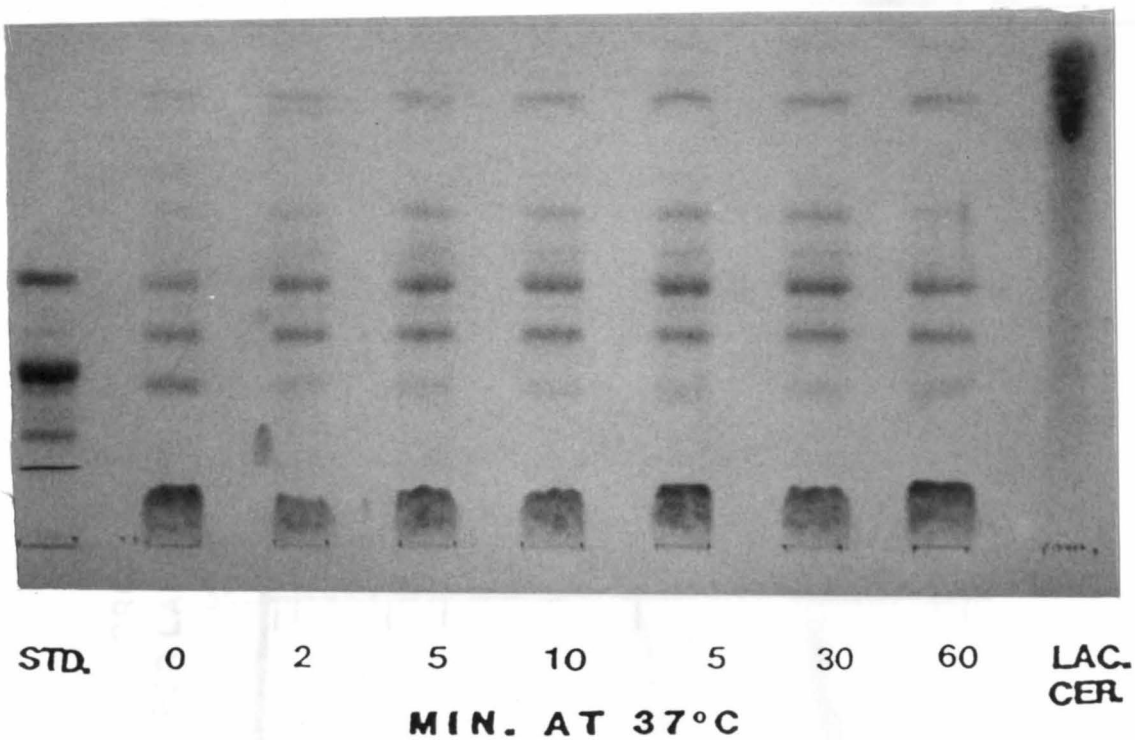


Fig.3.20: TLC of the digestion products of a purified, synaptosomal STase labeled, GD3 by sialidase. The GD3 was the result of preparative TLC of the gangliosides labeled by synaptosomal STase, as described under materials and methods. The lanes represent minutes of incubation of the purified GD3 with C. perfringens neuraminidase as described. The standards of bovine brain gangliosides and lactosyl ceramide were included.

Fig.3.21: densitometric analysis of the TLC plate shown in fig.3.20. The areas under a given peak are expressed as a percent of the total area of all peaks on that lane, except lactosyl ceramide which is expressed as arbitrary area units. A) ■,GD3; ●, GD1a; ◇, GM1; ○, GM3. B) free NANA. C) lactosyl ceramide.

TLC DENSITOMETER ANALYSIS OF NEURAMINIDASE PRODUCTS

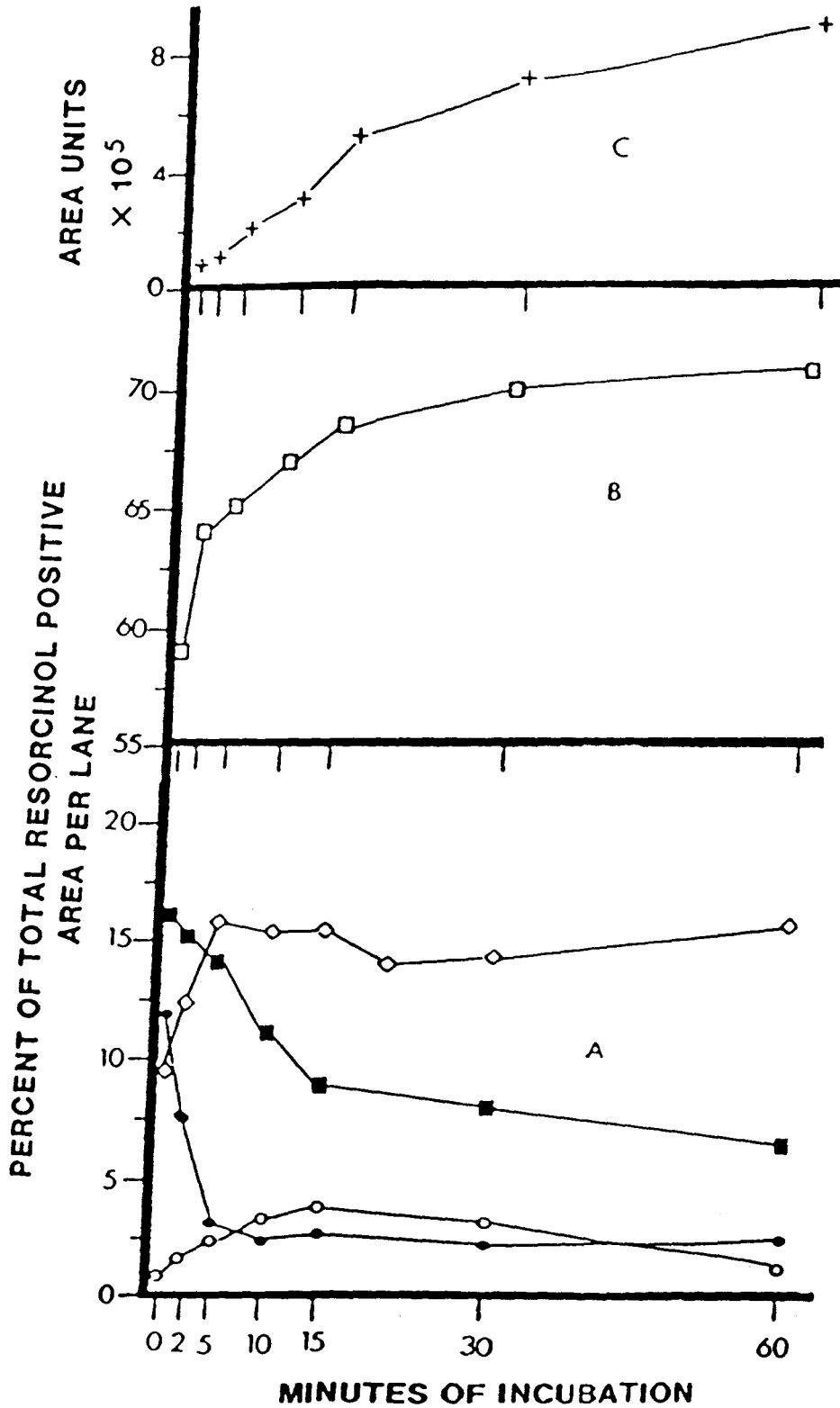


FIG.3.21

FIG.3.22 TLC RADIOACTIVITY ANALYSIS OF NEURAMINIDASE PRODUCTS

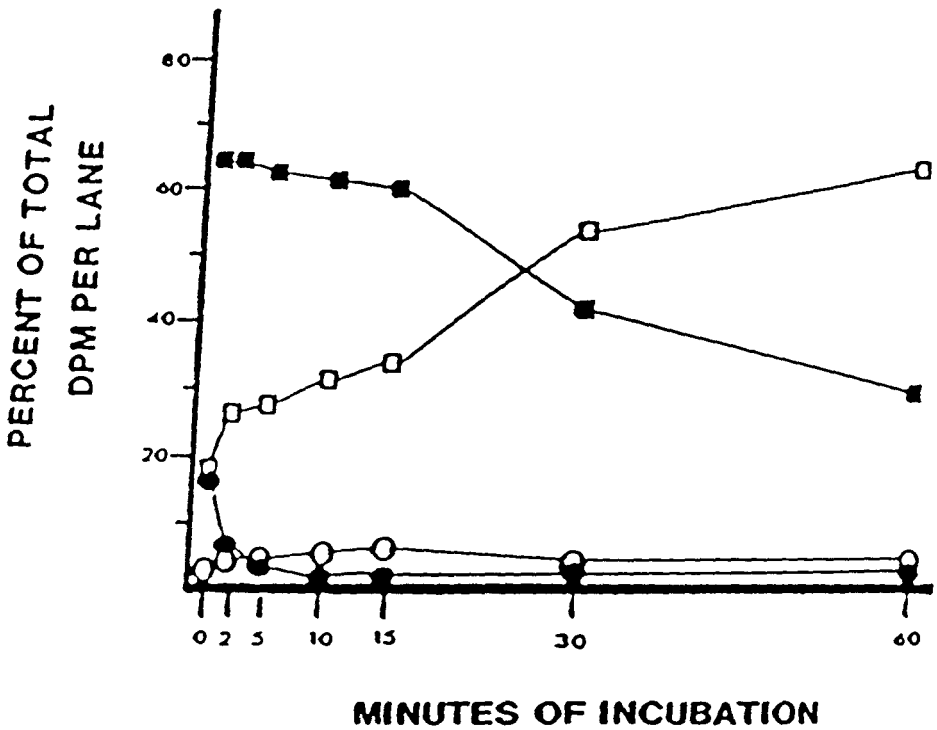


Fig.3.22: the radioactivity analysis of the TLC shown in fig.3.20. The lanes were scraped and counted as before, and the DPM found were assigned to the appropriate ganglioside or NANA band. The DPM are represented as percent of the total DPM in the given TLC lane: ■,GD3; □, NANA; ●, GD1a; o, GM3. There was no DPM associated with GM1 or the lactosyl ceramide bands.

over the time course. GD1a of a low specific radioactivity, showed a rapid decrease and was nearly undetectable at ten minutes. Free NANA had a rapid increase in DPM for the first two minutes, presumably from GD1a hydrolysis, then was steadily increasing which followed the rates of desialosylation of GD3 and GM3. DPM in the location of GM3 rose until fifteen minutes incubation, then decreased. No radioactivity was found in the locations of GM1 or lactosyl ceramide.

The interpretation of these data is that the highly specific labeling ganglioside product of rat synaptosomal STase is GD3 by analysis of it's neuraminidase hydrolysis, by it's chromatographic behavior under different developing solvent system conditions, and by ruling out the artifactual overlap of the GD3 band by possible derivetives of the labeled substrate on the TLC.

ANALYSIS OF INCUBATION MEDIUM- The purpose of this procedure was to determine the fate of the labeled substrate (CMP-NANA) and to see if the substrate became a limiting factor to the STase under the assay conditions. Figure 3.23 shows the separation of possible fates of the CMP-NANA by TLC under the developing conditions described in the methods chapter. It can be seen that standards of CMP-NANA, free NANA, and NADNA are easily separable and identifiable. The post incubation media from a STase assay was spotted on plates and chromatographed as

described above. Each lane was over spotted with a mixture of the three NANA derivative standards. The origin and a spot above NADNA produced a resorcinol negative brown color, and are contaminates in these crude samples. These contaminants are marked by an "X" in fig.3.23.

The lanes of these TLCs were scraped and counted for radioactivity as usual. The radioactivity in the media from a time course STase assay is shown in fig.3.25. CMP-NANA radioactivity decreased steadily over the time course (+), while remaining fairly constant in the heat denatured and 0°C synaptosomal control samples (--+--). This decrease in CMP-NANA was accompanied by a nearly quantitative increase in the presence of free NANA (□). The free NANA did not increase in the controls (--□--). NADNA was very minor in amount, increasing only slightly during the time course (•), in both the controls and the samples.

The conclusions of this is that there is an active CMP-NANA hydrolase in the synaptosomal fractions which is heat labile and inactive at 0°C. This activity has been reported (Brunngraber, 1979). This activity is much more active than the STase activity which are competing reactions. In any case, there appears to be sufficient CMP-NANA remaining at the end of the STase assay conditions that are used in these experiments so that subtrate availability does not limit the STase results.

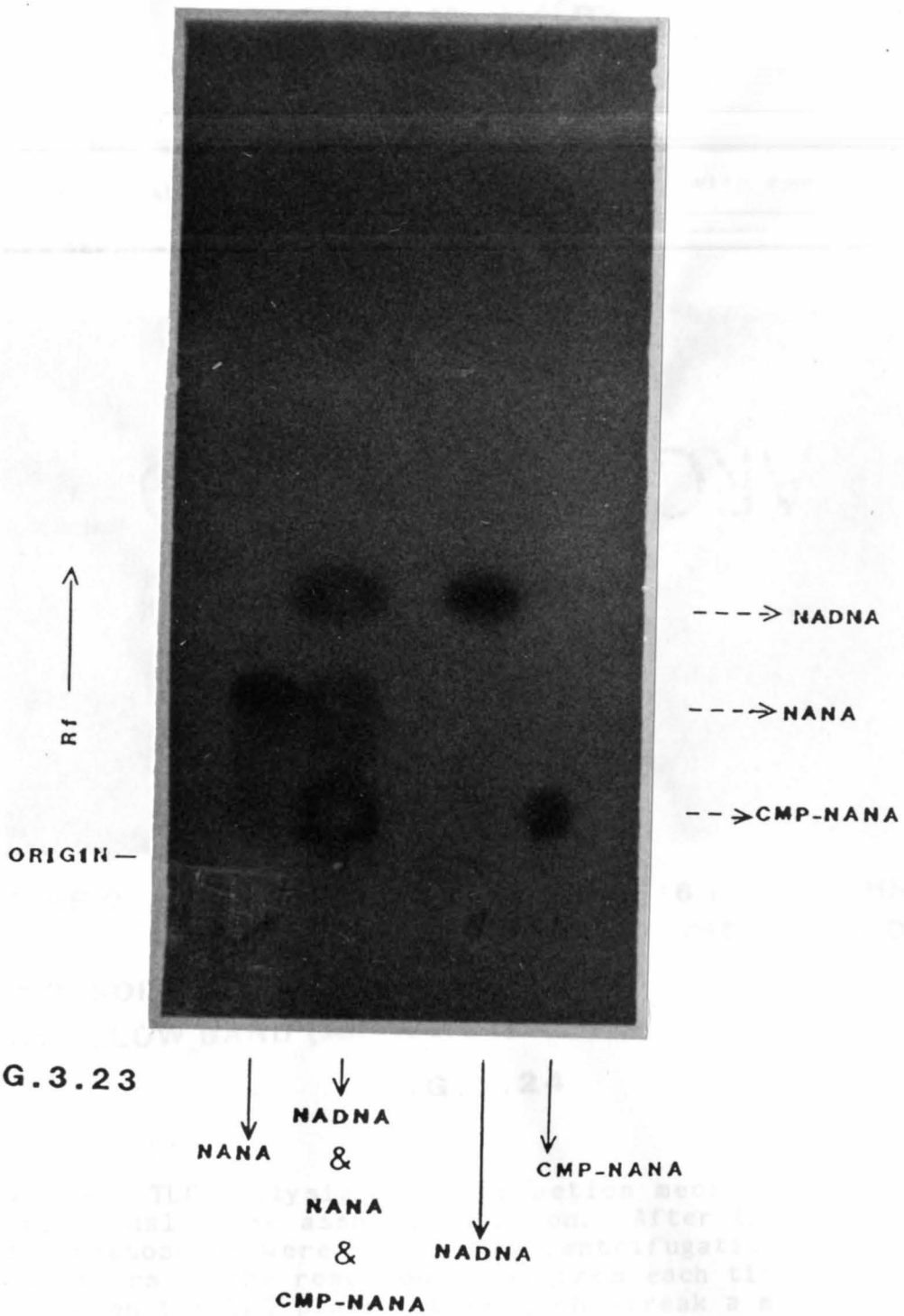
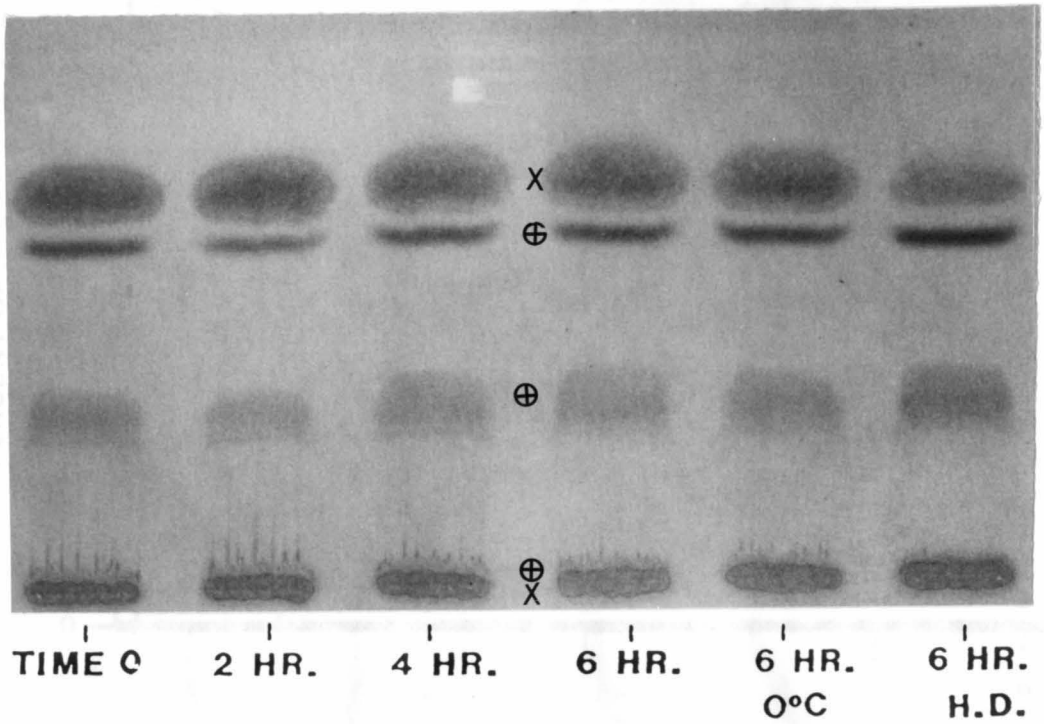


FIG.3.23

Fig.3.23: TLC analysis of sialic acid derivatives. Reagent standards of NANA, CMP-NANA, and NADNA were chromatographed alone and as a mixture and can be easily separated and identified.

ANALYSIS OF SIALOSYLTRANSFERASE REACTION MEDIA BY TLC: THE FATE CMP-(14C)NANA

SYNAPTOSOMAL PREPARATIONS WERE INCUBATED WITH THE SUBSTRATE THEN SEPARATED BY CENTRIFUGATION ;MEDIA SPOTTED ON TLC PLATE



⊕ =RESORCINOL POSITIVE BANDS

X =YELLOW BAND (ARTIFACT)

FIG.3.24

Fig.3.24: TLC analysis of the reaction media from a synaptosomal STase assay incubation. After incubation, the synaptosomes were removed by centrifugation. Ten microliters of the reaction media from each time point was spotted on the TLC plate. Over each streak a mixture of unlabeled NANA, NADNA and CMP-NANA (reagent standards) was co-spotted. After development, the three standards could be identified as resorcinol positive bands marked by two large contaminants, giving yellow bands, are marked with an "X".

SIALOSYLTRANSFERASE REACTION MEDIA

ANALYSIS OF CMP-NANA METABOLITES BY TLC

SYNAPTOSOMES WERE REMOVED BY CENTRIFUGATION,
MEDIA WAS SPOTTED,
THE POSITION OF DPM AGAINST STANDARDS ANALYZED

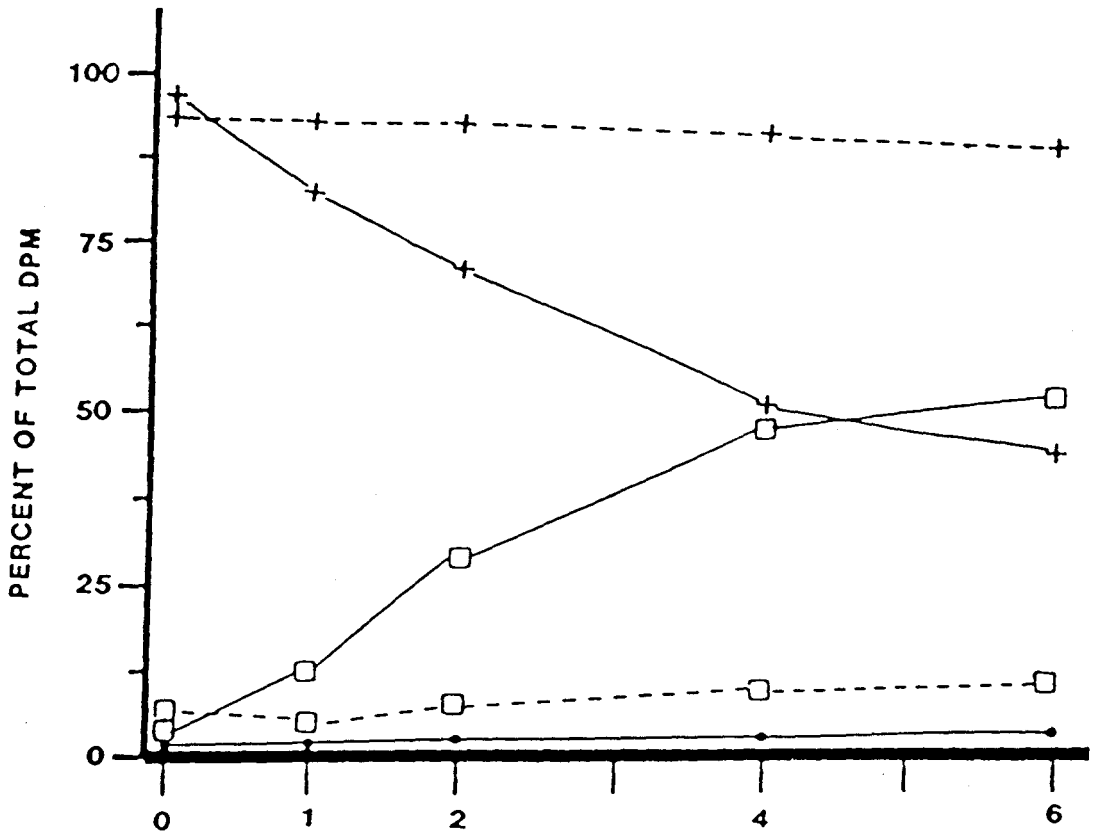


FIG.3.25

Fig.3.25: analysis of the radioactivity from the TLC plate of the STase incubation media shown in fig.3.24. The lanes were scraped and counted as described in materials and methods. All DPM were associated with the areas corresponding to bands of the standards. At 37°C the CMP-NANA band showed a steady decrease with a corresponding increase in free NANA. The controls represent samples incubated at 0°C and heat denatured synaptosomes, combined.

PART 2-GLYCOLIPID SIALOSYLTRANSFERASE OF VARIOUS MEMBRANE SUBFRACTIONS FROM RAT BRAIN

INITIAL OBSERVATIONS - At an early point in my research, I applied the STase assay procedures described in part 1 to the crude P2 fraction (before separation of synaptosomes). The raw data, showing the radioactivity observed on the TLC plates of gangliosides from this fraction is shown in fig. 3.26. The graphs of time course samples, and the CPM per 1mm consecutive scrapings of the lanes, are shown in comparison to the tracing of the ganglioside spots.

There are some differences in STase specificity between this crude fraction and the synaptosomal data. First there is more CPM incorporated into the higher gangliosides (GD1a, GT1b, GQ1, and GT1a) although the GD3 specificity is still present. In addition there is a huge, linearly increasing incorporation into an area located near the origin, but below the GQ1 peak. This is the location of the UN (unknown) spot I have described previously.

In figure 3.27 a sample of each intermediate fraction, during the preparation of synaptosomes (as described above) was placed on a continuous ficol density

Fig.3.26: STase assay of the crude P2 fraction (before purification of synaptosomes). Samples of the crude P2 fraction were assayed as described for synaptosomes. The gangliosides were extracted, separated by TLC, and the plates were scraped, counted, and analyzed as before. The vertical bars show the location of the radioactivity on a given TLC lane, and the radioactivity can be assigned to specific ganglioside species as described in materials and methods. Qualitatively, it can be seen that this cruder preparation incorporates somwhat less NANA into the GD3 band and more into GD1a and the higher sialosyl gangliosides than does purified synaptosomes. Total STase activity was higher. Note the very high specific labeling of the area of the lanes between the origin and GQ. This area corresponds to the band(s) I have designated UNK, as described above.

SIALOSYLTRANSFERASE TIME COURSE

CRUDE HOMOGENATE FRACTION

TLC OF GANGLIOSIDES VS. RADIOACTIVITY IN CONSECUTIVE 1MM SEGMENTS OF THE LANE

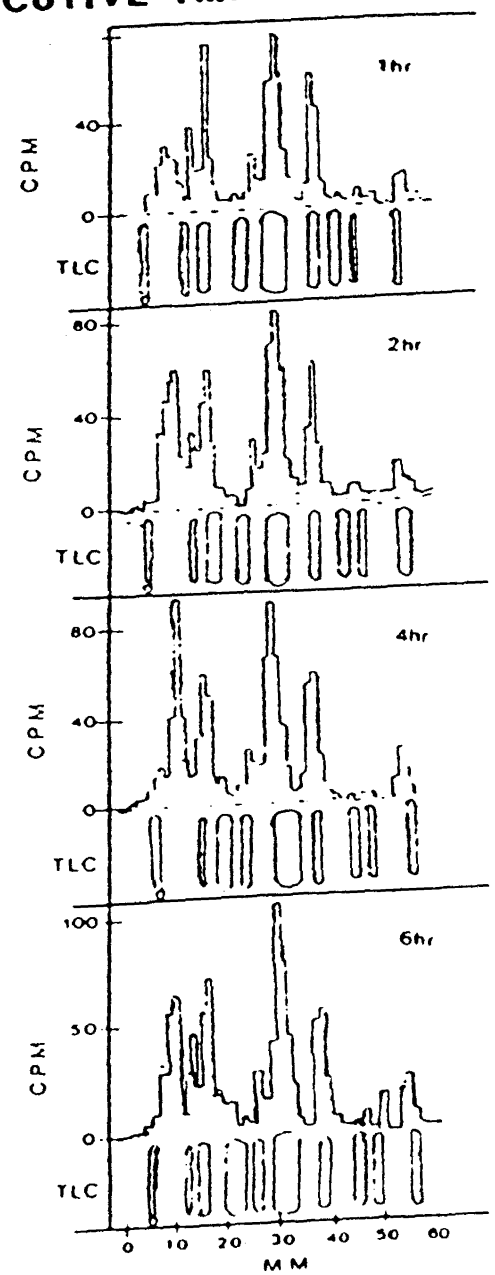
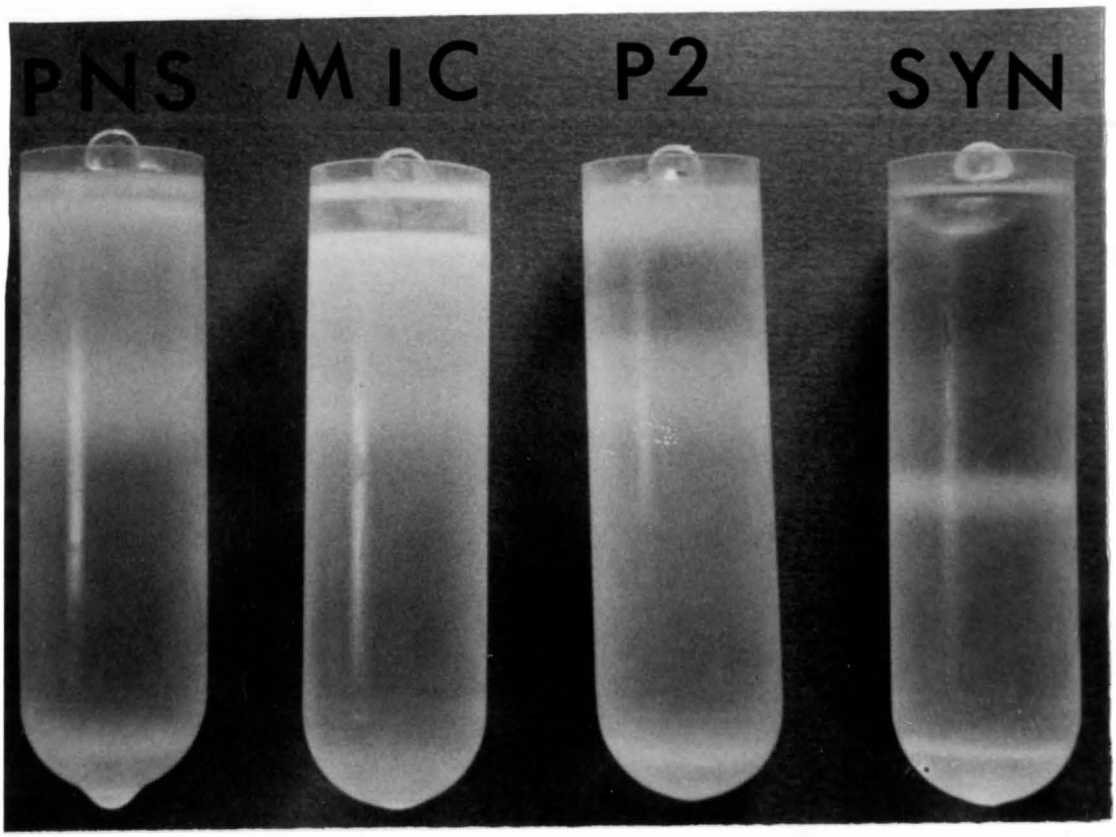


FIG.3.26

COMPARISON OF MEMBRANE PREPS

CONTINUOUS FICOLL DENSITY GRADIENTS



CENTRIFUGATION AT 100,000 × g 120 MIN.

FIG.3.27

Fig.3.27: following the purification scheme of synaptosome preparation described in materials and methods, a partial sample of each sequential fraction was loaded on top of a continuous ficoll density gradient (5% to 16% ficoll in HM), and the tubes were centrifuged at 100,000 x g for 2 hours. The tube at the far left is the post nuclear supernatant (PNS), which contains both microsomal elements and synaptic elements, plus myelin and mitochondria. The next tube to the right (MIC) is the microsomal fraction or P3. The third tube to the right is the P2 fraction (PNS minus MIC) containing myelin, synaptosomes and mitochondrial elements. The tube on the far right (SYN) is a greatly enriched, purified preparation of synaptosomes, or P2-C.

gradient (5% to 16% ficol in HM), and the gradients were centrifuged at 100,000 x g for 2 hours in the Beckman L2-65B ultracentrifuge and SW-28 rotor. The tube marked "PNS" (post nuclear supernatant) is the basic homogenate of rat brain after sedimentation of the large debris. The second tube from the left is the microsomal (MIC) or P3 fraction. The "P2" tube represents what is left from the "PNS" fraction after removal of "MIC". On the far right is the prepared synaptosome or P2-C subfraction (SYN) which is "P2" minus myelin and mitochondria.

Notice the difference between "MIC" and "P2". MIC is missing the heavy myelin layer at the top, but is enriched in numerous small membrane bands in the top 1/3 of the tube. Some of these small membrane bands are also found in the P2 tube. The SYN tube is devoid of all membranes except a band near the middle of the tube, which is also present in the P2 tube, but barely noticeable. This is because the SYN tube represents a large amount of starting material, while the others are a small aliquot of the preparation.

The P3 fraction (or MIC) is obtained from the PNS fraction by sedimenting out the P2 fraction at a low centrifical force (17,000 x g) in a isotonic solution (HM). This separation is based on "sedimentation" rates and spin velocities. On the other hand, the further

subfractionation of P2 to obtain SYN (P2-C) is an "isopycnic buoyant density" procedure, done through increasingly dense solutions at high g forces (100,000g) for long spin times. Thus, the membranes of the MIC fraction are of smaller size, which sediment at a much slower rate than do intact synaptosomes. Sedimentation-type and isopycnic buoyant density-type subfractionation of brain homogenates has been reviewed in detail by Cotman (1972).

It has been established that the major site for de novo biosynthesis of brain gangliosides is in the golgi membranes (Wiegandt, 1982; Landa et al., 1977), which will be present in the "MIC" or P3 fraction. Membranes of similar character are also carried over into the P2 fraction (fig. 3.27) and may be a source of contamination of the synaptosomal preparation. The contamination of synaptosomes by golgi has been the major argument against the existence of a synaptic STase (Depert and Walter, 1978; Ng and Dain, 1977).

Golgi STase should be contained in the multiglycosyl-transferase complexes, and should be involved in the biosynthesis of new gangliosides from simpler precursor molecules (see Brunngraber 1979). If a synaptic STase exists, it should function in a desialosylation-resialosylation system, by sialosylation of pre-existing gang-

liosides. It would be only natural to expect that the labeling patterns and precursor-product relationships of the golgi STase and synaptic STase would be quite different.

Such a difference may be shown in fig. 3.26 (for crude P2 STase) when compared to the labeling pattern obtained from purified synaptosomes (e.g. fig. 3.10). These labeling pattern differences could be used as evidence for the existence of a true synaptic plasma membrane STase. The results to follow represent experimental investigations, in this vein.

SUBFRACTIONATION OF MICROSOMES - A typical discontinuous sucrose gradient after centrifugation of the P3 fraction is shown in fig. 3.28 (procedures in methods chapter). Membranes were harvested at each gradient interface, except at the highest densities, and the subfractions were named as shown. Electron micrographs of these subfractions are shown in figures 3.29-3.31. The characteristic dumbbell shaped, closed vesicular morphology of the Golgi apparatus can be seen in fractions M-C through M-F. Subfraction M-E seem, to be nearly pure golgi structures, and M-D is also greatly enriched.

The subfractions were analyzed for the membrane marker enzyme activities as described in the methods chap-

SUBFRACTIONATION OF MICROSOMES

DENSITY GRADIENT CENTRIFUGATION

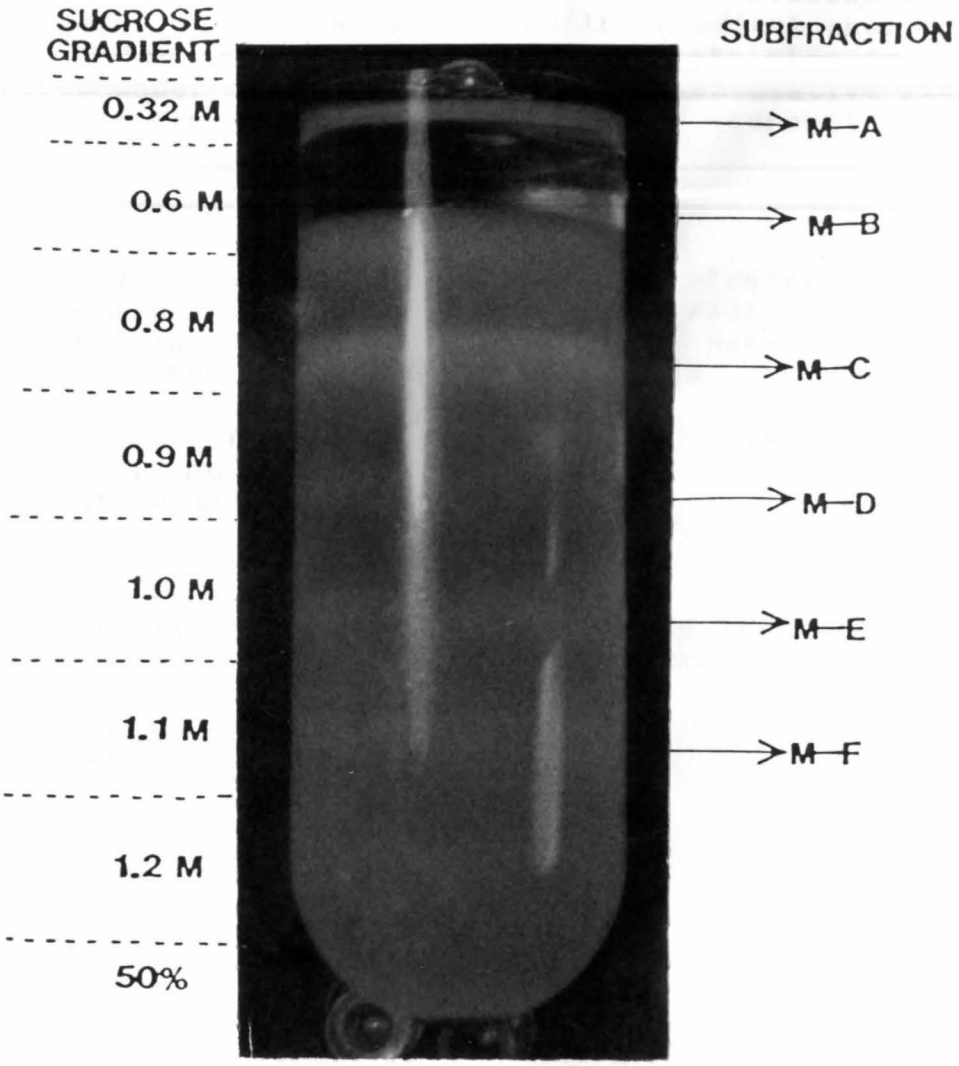


FIG. 3.28 100,000 X g 120 MINUTES

Fig.3.28: the microsomal fraction (P3) from rat brain was subfractionated by discontinuous sucrose density gradient centrifugation. The photograph above shows the gradient after 2 hours at 100,000 x g. On the left is the molarity of the sucrose layers of the gradient and on the right are the resultant membrane bands harvested with the names they were given.

Fig.3.29: electron micrographs of the subfractions of the microsomal fraction obtained from the gradient shown in fig.3.28. The procedure for fixing and staining is described under methods. These are representative fields of each fraction at low magnification (1 micrometer X 48,000).

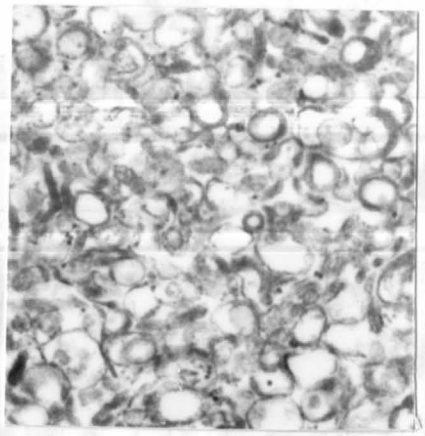
Fig.3.30: electron micrograph of the subfractions of the rat brain microsomal fraction. The subfractions shown are the three least dense fractions, and the name of each fraction is shown on the left. The fields shown describe the character of the entire fraction at a high magnification (1 micrometer X 310,000). The morphological detail shown are of the most numerous and significant structures in the subfraction.

Fig.3.31: electron micrographic detail of the three most dense microsomal subfractions at high magnification (1.0 micrometer X 310,000). Fractions M-E and M-F are highly enriched in structures morphologically identifiable as golgi membranes.

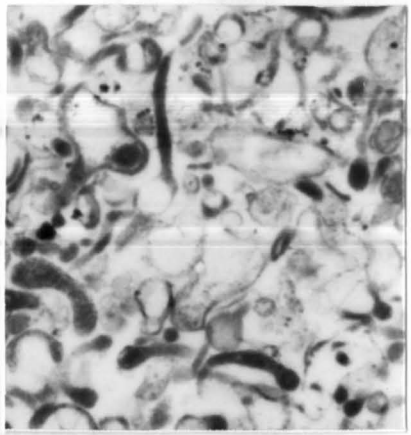
FIG.3.29–MICROSOMAL SUBFRACTIONS[124]

EM MORPHOLOGY; LOW MAGNIFICATION

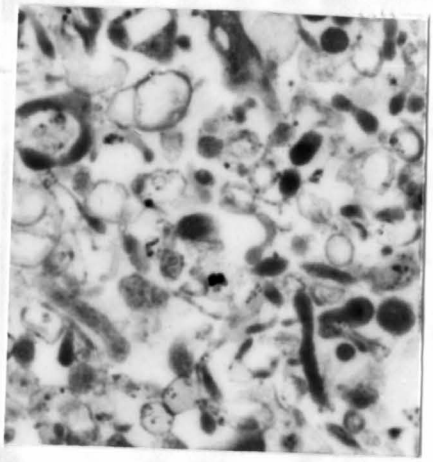
UNBIASED FIELDS OF EACH FRACTION



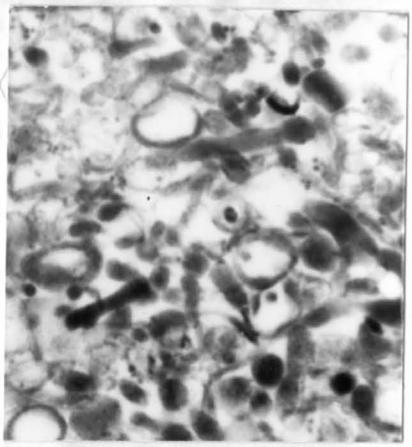
M-A



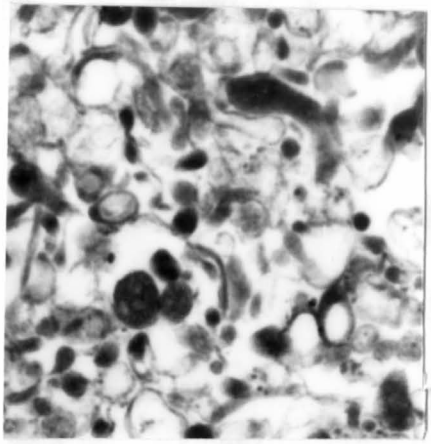
M-B



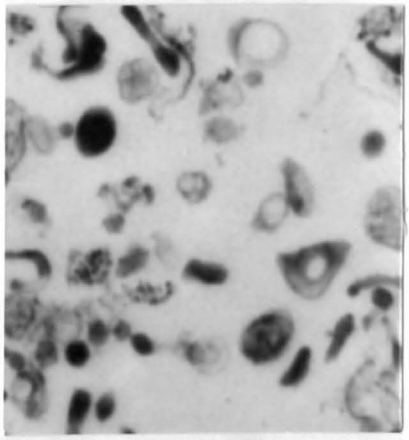
M-C



M-D



M-E



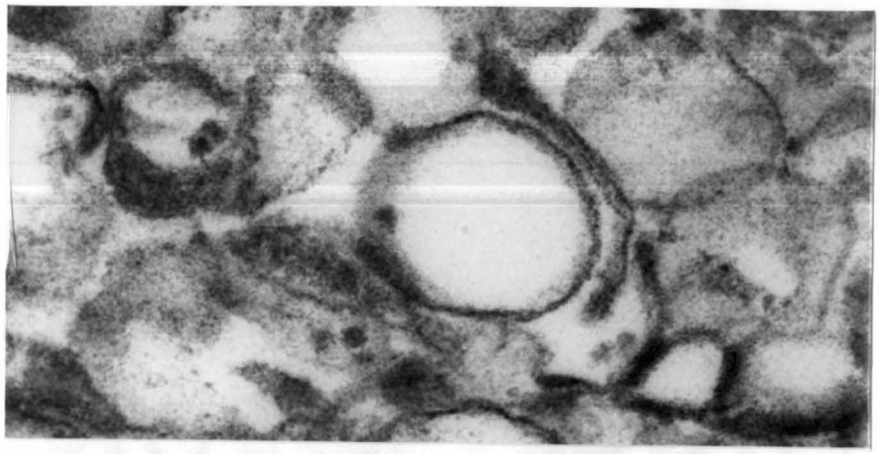
M-F

FIG.3.30–MICROSOMAL SUBFRACTIONS

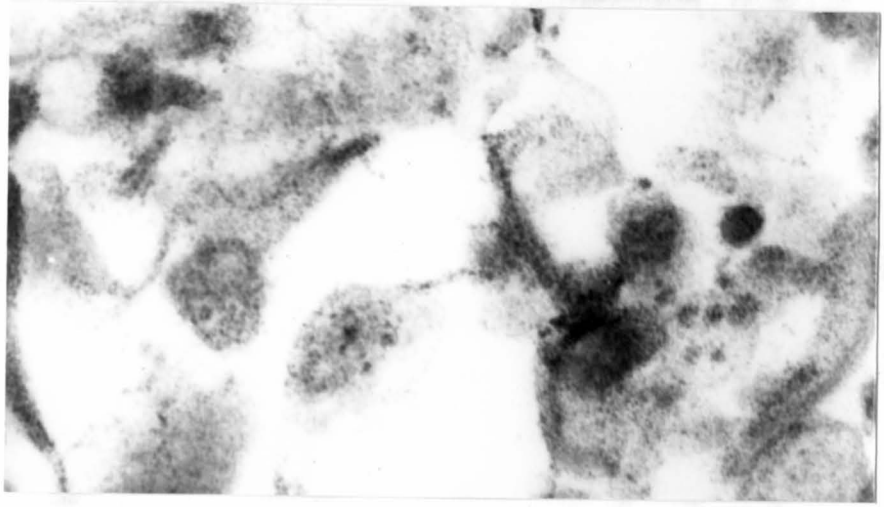
EM MORPHOLOGY–HIGH MAGNIFICATION

SIGNIFICANT STRUCTURAL DETAILS

M–A



M–B



M–C

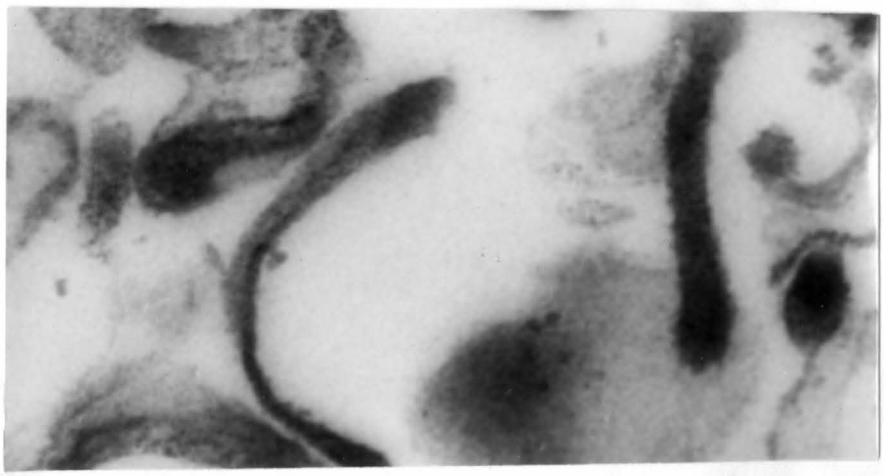
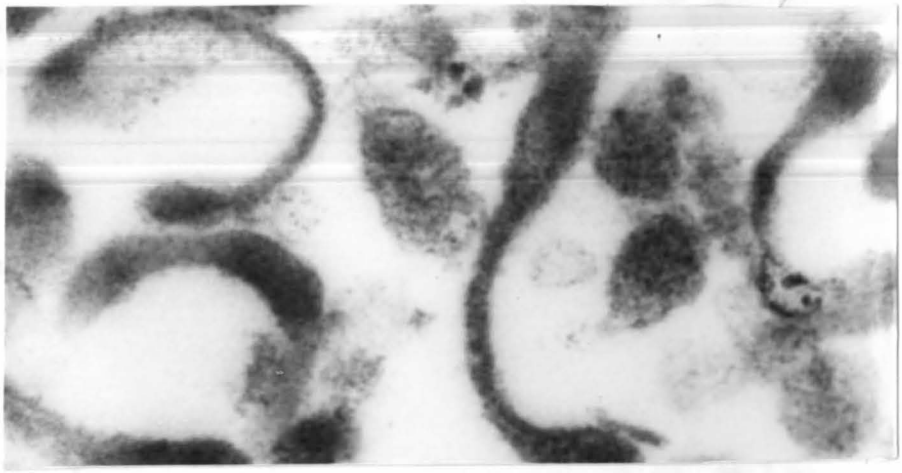


FIG.3.31–MICROSOMAL SUBFRACTIONS

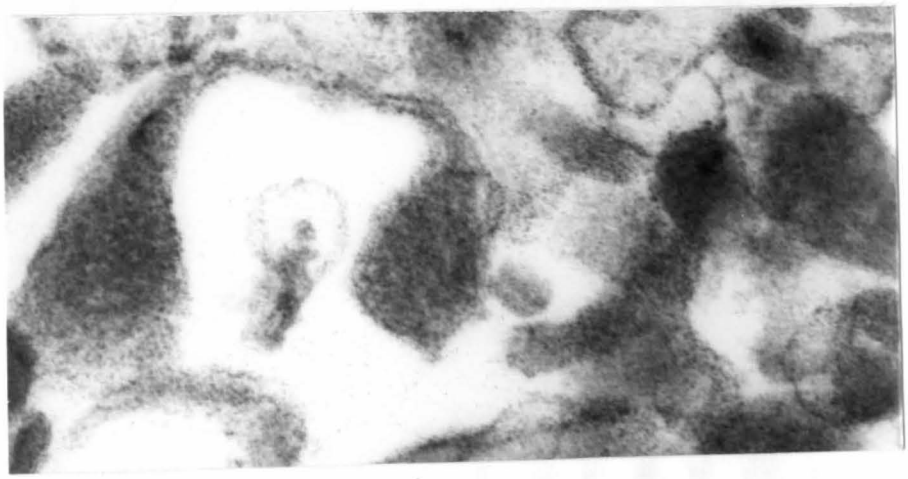
EM MORPHOLOGY–HIGH MAGNIFICATION

SIGNIFICANT STRUCTURAL DETAILS

M–D



M–E



M–F

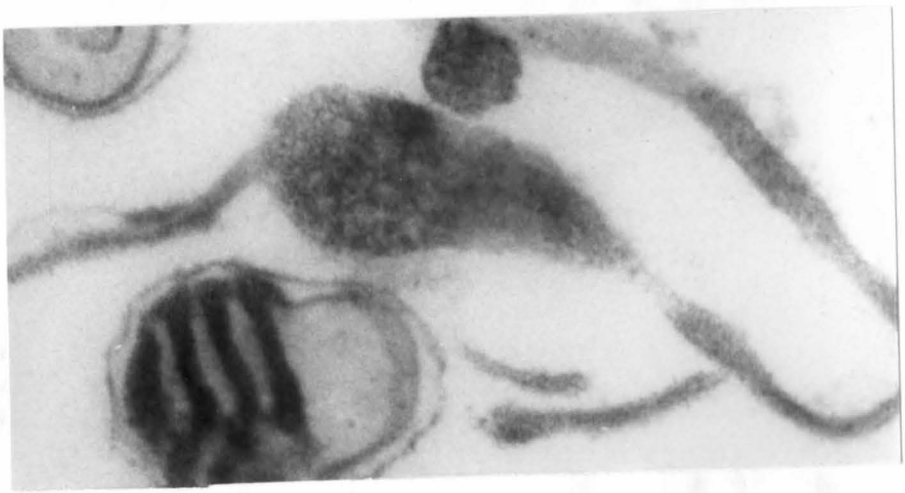


FIG.3.32 SUBFRACTIONATION OF MICROSOMES

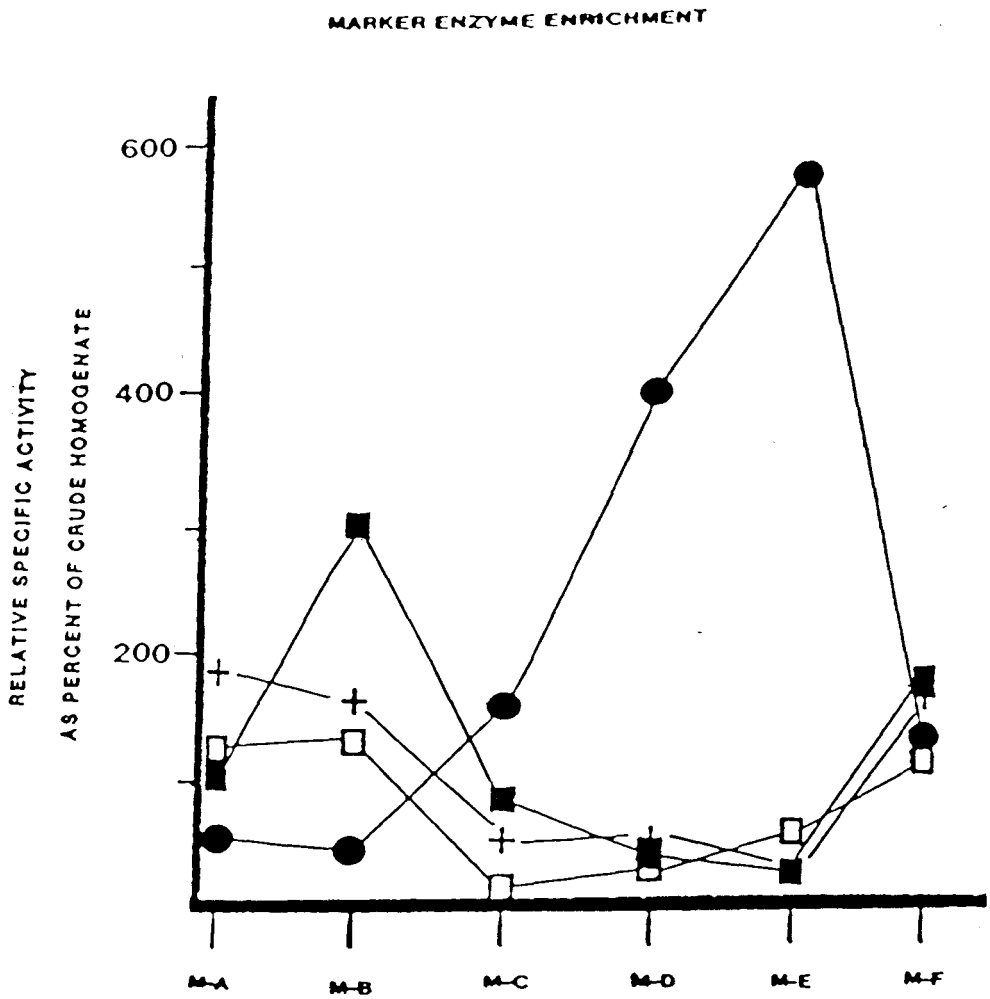


Fig.3.32: marker enzyme analysis of the subfractions obtained from the rat brain microsomal fraction (P3). The units are represented as the percent of each enzyme's specific activity, to the specific activity of the enzyme in rat brain crude homogenate. Scores above 100 represent an enrichment in activity, below 100 means that the enzyme is not enriched in that subfraction. The subfractions are listed along the bottom. The enzymes assayed were: ■,5' nucleotidase; +, acetyl-choline esterase; □, choline acetyl transferase; ●, cerebroside sulpho-transferase. The assay procedures are described under materials and methods.

ter and the data is shown in figure 3.32. The fraction M-E is clearly the most enriched in CST (cerebroside-sulfo-transferase, a golgi specific marker) and least enriched in 5'nucleotidase and ChAT (choline acetyl transferase) which are markers for plasma membrane and synaptic plasma membranes respectively. M-D is also enriched in golgi marker. Plasma membrane markers were enriched to the greatest extent in M-B and somewhat in M-F.

Each subfraction was assayed for total STase activity. The golgi enriched subfraction, M-E, had a total ganglioside activity of 2.64 pmole NANA transferred/mg protein/hour (+ or - S.E.M. of 0.21, n = 4). This activity was 3 fold greater than that observed for the synaptosomal experiments (0.84 units). The total STase activity profile of all the microsomal subfractions with the specific ganglioside content of these membrane fractions is shown in fig. 3.33. M-E and M-D are the most enriched in both of these values.

Note that the lighter subfractions (M-A through M-C) also have total STase activities near or greater than that observed for synaptosomes. These fractions, in general, lack a great deal of morphological detail. The membrane fragments are small, many are spherical closed vesicles. Presumably, these are intracellular membranes, such as endoplasmic reticulum, and plasma membrane fragments. The

most dense subfraction, M-F, has obvious mitochondria and densely staining closed vesicle, possibly lysosomes. Extensive marker enzyme analysis to ascertain the identity of all the subfractions was beyond the scope of these experiments. The enzymatic analysis data from the microsomal subfractions is summarized in table 3.3.2.

After the STase incubation, the gangliosides were extracted, purified, and separated as usual. A photocopy of typical TLC of the gangliosides from the microsomal subfractions is shown in fig. 3.34. Since the subfractions varied greatly in ganglioside concentration, some of the lanes were under loaded with ganglioside (e.g. M-F), while others were overloaded with sample (e.g. M-E). This resulted in chromatograms that were not "picture perfect", but they were satisfactory and were used in this form to retain experimental continuity.

In the golgi enriched fraction, M-E, there were numerous extra resorcinol positive, minor bands, especially around the areas of GD3, GM1, GM2, and GM3. Also there was quantitatively more gangliosides in these areas, as compared to the ganglioside profiles of synaptosomal gangliosides. This could reflect that nature of golgi gangliosides as a "biosynthetic pool", rather than a "functional pool" of gangliosides, as would be assumed for

FIG.3.33 MICROSOMAL SUBFRACTIONS

TOTAL SIALOSYLTRANSFERASE ACTIVITY
AND SPECIFIC GANGLIOSIDE CONCENTRATION

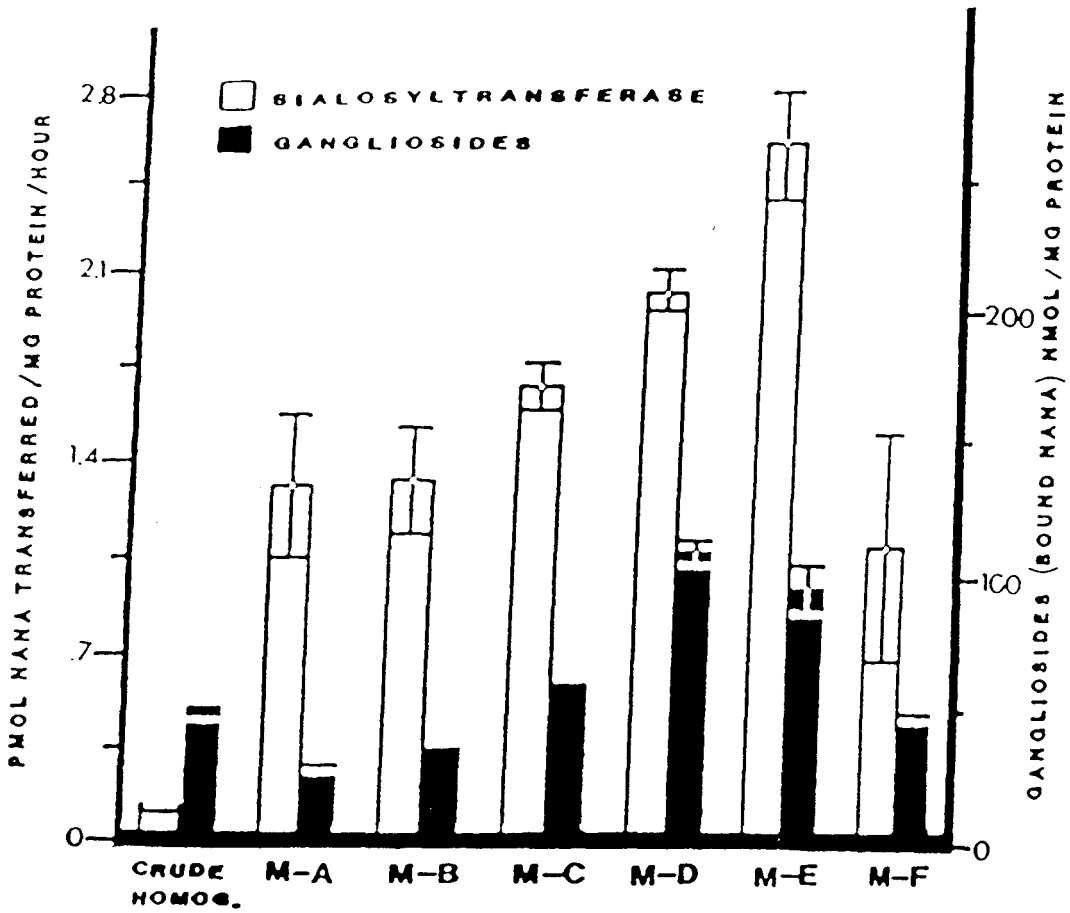


Fig.3.33: total STase specific activity and specific ganglioside content of the microsomal subfractions of rat brain. STase activity was measured as the pmoles NANA transferred into the total gangliosides extracted from the subfraction per mg protein in the subfraction, per hour. The specific ganglioside concentration was calculated by determining the NANA content of the total gangliosides extracted from the subfraction, by the resorcinol method and normalizing the result by the protein content of the subfraction. The procedures are described under materials and methods. The bar height represents the mean specific activity or specific concentration of four samples (+ or - S.E.M.). The subfractions are listed along the bottom.

TABLE 3.2

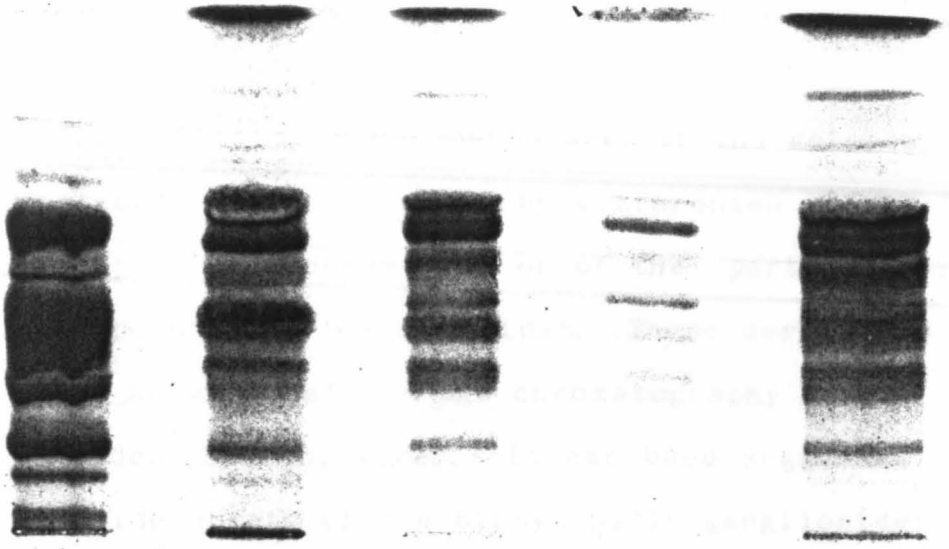
SUMMARY OF DATA FROM THE MICROSOMAL SUBFRACTIONS

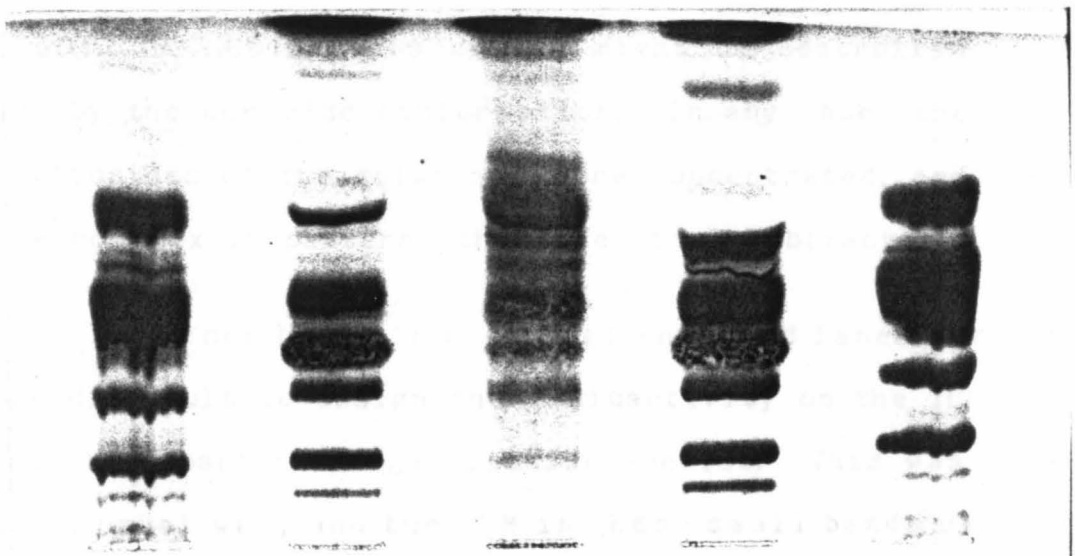
The values given are the means of 4 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each assay are: 5'-nucleotidase (5'-NT), micromoles of AMP hydrolyzed per mg protein per hour; choline acetyl transferase (ChAT), micromoles choline transferred per mg protein per minute; cerebroside sulpho transferase (CST), picomoles sulfate transfered per mg protein per hour; total sialosyltransferase (STase), picomoles NANA transferred per mg protein per hour; ganglioside specific concentration (Sp. Gang. Comc.), nanomoles lipid bound NANA per mg protein per hour.

	M-A	M-B	M-C	M-D	M-E	M-F
5'-NTase	0.69 (0.05)	2.33 (0.24)	0.59 (0.03)	0.16 (0.01)	0.14 (0.02)	1.25 (0.03)
ChAT	0.11 (0.01)	0.27 (0.03)	0.03 (0.01)	0.06 (0.03)	0.01 0.01)	0.23 (0.03)
AChE	3.25 (0.07)	2.84 (0.03)	0.83 (0.07)	0.89 (0.03	0.40 (0.07)	2.85 (0.10)
CST	0.84 (0.03)	0.60 (0.01)	2.36 (0.05)	6.39 (0.07)	7.17 (1.92)	1.83 (0.16)
STase	1.14 (0.27)	1.34 (0.20)	1.71 (0.09)	2.05 (0.21)	2.64 (0.22)	1.10 (0.42)
Sp.Gang. Conc.	27.0 (.8)	26.7 (8.8)	62.7 (1.2)	107.1 (4.8)	94.2 (8.4)	46.7 (0.8)

Fig.3.34: TLC of the gangliosides extracted from the microsomal subfractions of rat brain. The subfractions were assayed for STase activity by incubating the subfractions with CMP-(^{14}C)NANA under the same conditions described previously, for two hours. The gangliosides were extracted, spotted on TLC plates, developed and visualized by resorcinol reagent. This is a photograph of two TLC's. There is a granular appearing, brown artifact band present, near the GD1a band, which is regretable, and is probably sucrose. Bovine brain ganglioside standards and all of the microsomal subfractions are marked beneath their respective origins.

FIG.3.34—MICROSOMAL SUBFRACTIONS
TLC OF GANGLIOSIDES





the synaptosomal membrane.

The multiple, minor bands seen in the golgi enriched subfractions, could be caused by differences in the fatty acyl groups of the ceramides, or of the particular sphingosine type base of the ceramides. These ceramide variations are known to effect the chromatography of the lower gangliosides (Ledeen, 1982). It has been suggested that the ceramide moiety of the biosynthetic gangliosides might be an important factor in determining the synthetic route, or end stage ganglioside product (Ando and Yu, 1984). In other words, when there is a choice: e.g. of going the route of GM2 and on to GM1 from GM3, or to go on from GM3 to GD3...GD2...GD1b; this choice might be controlled in part by the ceramide conformation. In any case, the gangliosides of the golgi are more concentrated, and are more complex in pattern, than the other subfractions.

The minor bands in the golgi enriched lanes made it more difficult to assign the radioactivity on the TLC plate to a particular ganglioside species. This was done in the usual way, and the DPM in these small bands were tallied into the counts of the nearest major band identified by RF comparison with the bovine standards. Any errors in this method should not be significant due to the fact that a minor portion of the radioactivity was found in these areas. The mean percent distribution of DPM

among the gangliosides, as transferred by STase, is given in figure 3.35, for all of the microsomal subfractions (A = M-A, B = M-B,...). Each horizontal bar represents the mean of four experiments, the error bars represent the S.E.M.

In the M-A, M-B, and M-C subfractions, the radioactivity was fairly high in all ganglioside species. These levels were significant as the total STase activity was substantial in these subfractions (see fig. 3.33). In subfraction M-A, GM3 incorporated the highest percentage of label. M-B may show specificity for GD1a, athough all ganglioside incorporated substantial label. In subfraction M-C, GT1b has the highest specific STase labeling. The significance, if any, of these specificities is unknown.

The subfractions M-D and M-E, which are the golgi enriched fractions, show a unique pattern. The unknown band (UN) incorporates by far the most labeled NANA. This is even more specific because this band is in trace amounts. The band is so small that the computing integrator does not distinguish it as a peak on densitometer analysis of the TLC lane. The identity and significance of the UN band are unknown. Further investigation of this area of the ganglioside profile is prudent and vital, but

FIG.3.35

MICROSOMAL SUBFRACTIONS

Product Specificity of Sialosyltransferase

Bars Represent Percent of Total DPM

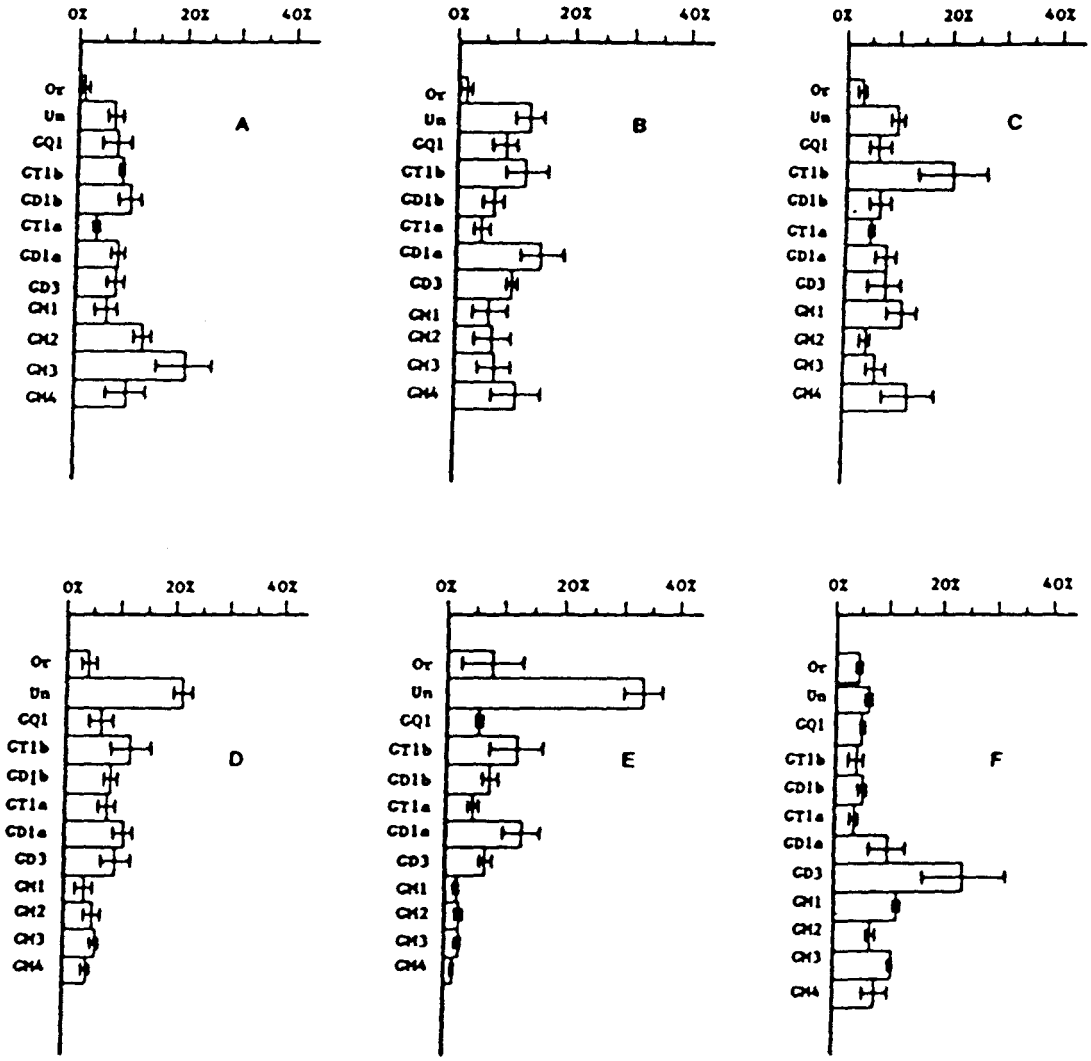


Fig.3.35: labeling pattern of STase of microsomal subfractions. The TLC lanes were scraped and counted and the DPM were assigned to ganglioside species as before. DPM for each ganglioside is represented as a percent of the total DPM in all gangliosides in that sample. The bar lenght is the mean of four samples (+/- S.E.M.). There is a separate graph for each microsomal subfraction.

is beyond the scope of the specific aims of this dissertation.

Ignoring the UN band, if possible, the labeling pattern of the golgi enriched subfractions, roughly correlated to the number of sialic acid residues on the particular ganglioside species. The more NANA residues per ganglioside, the higher the DPM per nmole ganglioside. This could be expected for a ganglioside biosynthesizing organelle.

A "relative specific radioactivity" value (RSRA) has been described (Brunngraber, 1979). RSRA is defined as the %DPM of the total radioactivty divided by the % distribution among the amount of gangliosides for a given ganglioside species (RSRA=%DPM/%DIST). A value of 1.0 means that there is no transfer specificity. The larger the number the greater the product specificity. The data relating to the STase specificity for individuals is tabulated in table 3.3.

Clearly, the labeling pattern of golgi associated STase is different than the labeling pattern observed for synaptosomes previously described.

The M-F subfraction shows a specificity for incorporation of labeled NANA into the GD3 band. The total STase activity of this fraction is low (fig. 3.33). The low power electron micrograph of the M-F subfraction

TABLE 3.3

SUMMARY OF GANGLIOSIDE DATA
FROM THE MICROSOMAL SUBFRACTIONS

The values given are the means of 4 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each ganglioside analysis value are: percent ganglioside distribution (% DIST.), the percent ot the total resorcinol positive area for a given ganglioside species as measured by densitometric integration of TLC lanes; percent distribution of radiolabel by STase (% DPM), expressed as a percent of the total radiolabel present in the total ganglioside sample; and the relative specific radioactivity of each ganglioside (RSRA), which is %DIST / %DPM. (ND = not determined)

		SUBFRACTIONS					
		M-A	M-B	M-C	M-D	M-E	M-F
UNK	%DIS	ND	ND	ND	ND	ND	ND
	%DPM	6.9 (1.4)	12.5 (2.5)	9.7 (1.4)	21.3 (1.7)	33.4 (3.4)	6.2 (0.5)
	RSRA	ND	ND	ND	ND	ND	ND
GQ1	%DIST	5.75 (0.6)	7.9 (1.1)	5.35 (1.3)	7.6 (1.4)	7.2 (1.1)	7.53 (1.0)
	%DPM	7.3 (2.5)	20.2 (5.2)	6.3 (1.9)	9.0 (1.1)	7.98 (1.9)	5.15 (0.40)
	RSRA.	1.4 (0.65)	1.03 (0.46)	1.16 (0.14)	1.30 (0.22)	1.07 (0.84)	0.73 (0.13)
GT1b	%DIST	9.80 (1.08)	8.98 (2.61)	10.28 (0.90)	10.30 (0.89)	10.23 (1.97)	11.75 (1.57)
	%DPM	6.93 (1.63)	11.98 (3.63)	20.2 (6.46)	12.0 (3.62)	14.35 (4.53)	4.18 (1.18)
	RSRA	0.74 0.24.9	1.46 (0.48)	2.09 (0.78)	1.26 (0.47)	1.41 (0.47)	0.38 (0.13)
GD1b	%DIST	6.85 (1.19)	6.23 (1.98)	10.0 (1.40)	5.20 (1.16)	6.33 (0.33)	12.03 (2.31)
	%DPM	9.95 (1.99)	6.45 (1.75)	6.48 (1.85)	8.43 (1.29)	7.58 (1.35)	5.20 (0.69)
	RSRA	1.55 (0.25)	1.22 (0.45)	0.62 (0.15)	2.05 (0.65)	1.19 (0.19)	0.48 (0.12)

TABLE 3.3 CONTINUED

		SUBFRACTIONS					
		M-A	M-B	M-C	M-D	M-E	M-F
GT1a	%DIST	5.73	8.13	5.98	5.53	5.75	7.40
		(0.75)	(1.97)	(1.49)	(0.21)	(0.59)	(1.24)
	%DPM	3.70	4.48	5.05	7.78	4.80	3.80
		(0.59)	(1.31)	(0.72)	(1.46)	(0.96)	(0.48)
	RSRA	0.65	0.69	0.95	1.43	0.89	0.59
		(0.07)	(0.30)	(0.23)	(0.29)	(0.22)	(0.15)
GD1a	%DIST	12.13	11.18	11.65	11.60	14.28	20.05
		(0.97)	(1.81)	(2.47)	(2.30)	(1.07)	(2.34)
	%DPM	7.85	14.78	7.83	10.9	13.23	10.80
		(1.30)	(3.66)	(2.00)	(1.75)	(3.07)	(2.83)
	RSRA	0.67	1.31	0.67	1.00	0.92	0.56
		(0.11)	(0.18)	(0.10)	(0.19)	(0.19)	(0.19)
GD3	%DIST	11.9	9.78	10.03	10.03	12.18	6.83
		(0.53)	(1.11)	(1.47)	(1.09)	(1.60)	(2.31)
	%DPM	7.50	9.48	7.70	6.98	7.03	24.08
		(1.50)	(0.93)	(2.90)	(1.38)	(0.49)	(7.67)
	RSRA	0.63	0.99	0.71	0.68	0.64	4.53
		(0.12)	(0.22)	(0.15)	(0.09)	(0.19)	(1.34)
GM1	%DIST	14.25	21.45	29.43	30.43	18.8	18.6
		(0.839	(0.73)	(2.04)	(2.14)	(2.25)	(1.53)
	%DPM	11.35	6.08	11.03	5.03	2.10	10.03
		(1.97)	(3.02)	(2.74)	(0.82)	(0.43)	(2.07)
	RSRA	0.82	0.28	0.38	0.18	0.12	0.57
		(0.17)	(0.13)	(0.09)	(0.04)	(0.04)	(0.14)
GM2	%DIST	9.65	7.90	13.00	13.00	12.53	4.95
		(1.18)	(0.90)	(1.08)	(1.08)	(0.42)	(1.19)
	%DPM	11.98	6.55	4.30	5.40	2.53	7.03
		(1.60)	(3.14)	(1.06)	(1.61)	(0.57)	(0.76)
	RSRA	1.30	0.70	0.34	0.44	0.21	1.73
		(0.22)	(0.27)	(0.09)	(0.15)	(0.05)	(0.46)
GM3	%DIST	9.80	9.88	4.60	4.56	13.13	8.45
		(1.08)	(1.00)	(0.63)	(0.52)	(1.18)	(1.93)
	%DPM	20.28	6.80	6.35	5.88	2.45	10.85
		(5.18)	(2.77)	(1.65)	(0.77)	(0.48)	(0.51)
	RSRA	2.04	0.64	1.37	1.42	0.20	1.52
		(0.45)	(0.23)	(0.25)	(0.40)	(0.05)	(0.34)

TABLE 3.3 CONTINUED

SUBFRACTIONS

		M-A	M-B	M-C	M-D	M-E	M-F
GM4	%DIST	5.55	5.00	3.38	3.75	7.18	2.38
		(0.76)	(0.61)	(0.54)	(1.21)	(1.08)	(0.68)
	%DPM	10.40	10.76	10.08	4.28	1.50	7.95
		(3.01)	(4.07)	(3.33)	(0.83)	(0.27)	(2.29)
	RSRA	1.83	2.04	2.90	1.27	0.24	3.40
		(0.43)	((0.71)	(0.91)	(0.14)	(0.08)	(1.00)

(fig. 3.29) reveals some large, spherical structures, filled with granular type material which are not unlike synaptosomes. It is possible that small synaptosomal material in this fraction contributes to the high GD3 labeling of this fraction. But the total labeling patterns are still quite different.

In summary of the P3 (microsomal) subfractionation analysis of STase activity, fractions M-E and M-D are highly enriched in golgi membranes, as judged by EM morphology and marker enzyme analysis. Both have increased total ganglioside STase activity, and show an enrichment in ganglioside content. The ganglioside species labeling pattern is very different than that previously shown for synaptosomes (part 1). The golgi STase labeling pattern is highly specific for a uncharacterized band on the TLC ganglioside separation plates, running near the origin and below GQ1. Otherwise the incorporation of labeled NANA into the gangliosides roughly corresponds to the number of NANA residues per ganglioside species.

SUBFRACTIONATION OF THE P2 FRACTION - The tube resulting from the expanded discontinuous ficoll density gradient ultracentrifugation of P2 is shown in fig. 3.36. The density solutions and subfractions obtained are labeled. The membrane morphology of these subfractions is

FIG.3.36

SUBFRACTIONATION OF THE P2 PELLET

DISCONTINUOUS DENSITY GRADIENT CENTRIFUGATION

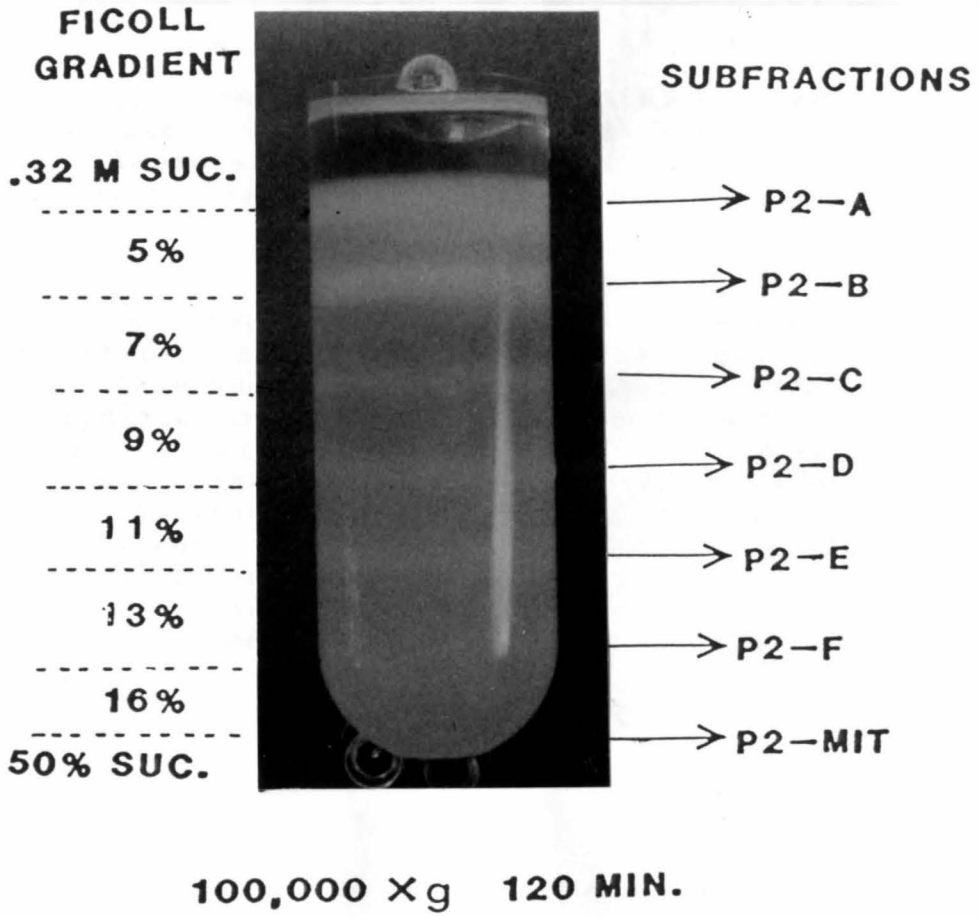


Fig.3.36: subfractionation of the "P2" fraction on a multilayer ficoll discontinuous density gradient. Extensively washed P2 pellets were resuspended in a small amount of HM and were layered on top of the discontinuous ficoll gradient shown on the left of the photograph shown above. The percents given are gm ficoll per 100 ml HM. After centrifugation at 100,000 x g for two hours at 0°C, membranous layers formed at the gradient interfaces, which are named as shown on the right, above. These subfractions were removed by Pasteur pipet and were washed in PBS and recentrifuged to form pellets which were used for the assays and electron microscopy which follows.

Fig.3.37: low magnification electron micrographs of the P2 subfractions shown in fig.3.36. The fields shown are representative of the entire subfraction and are magnified X 7,100. These views are useful in estimating the enrichment of specific structures in the subfractions. A hand lens maybe useful in examining these photographs.

Fig.3.38: high magnification electron micrographs of the P2 subfractions "a" through "d". The magnification is X 78,100. These micrographs show morphological detail of the most significant structures in each subfraction.

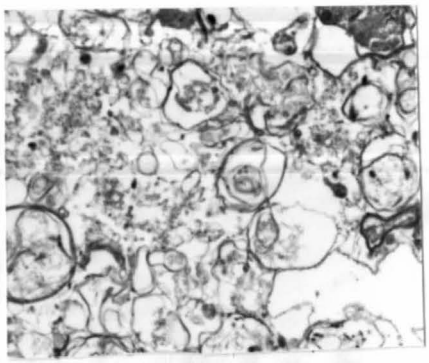
Fig.3.39: high magnification electron micrographs of the P2 subfractions "e" through "mit". The magnification is X 78,100. P2-e and P2-f are both highly enriched in typical synaptosomal structues. There is much less intra-synaptosomal detail than can be seen in fig.3.3. This is probably due to the more severe fixation procedures used (2 hours in 4% glutaraldehyde in PBS, in suspension then pelleting by centrifugation, and postfixing <u>en bloc</u> overnight in 1% OsO_4). This method was used because it is the method of choice for visualizing golgi membranes, and noting golgi contamination was one of the goals of this analysis.

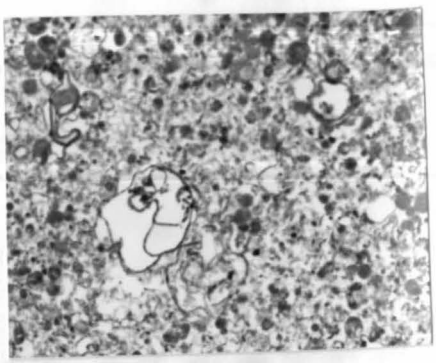
FIG.3.37–"P–2" SUBFRACTIONATION

EM MORPHOLOGY; LOW MAGNIFICATION

UNBIASED FIELDS OF EACH FRACTION

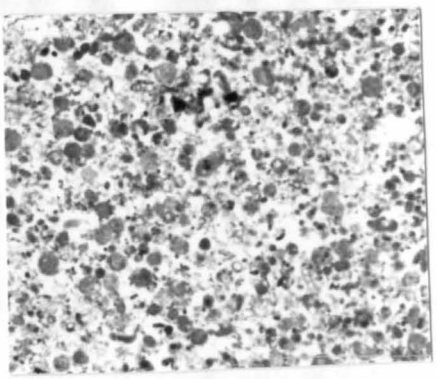
P2 -a

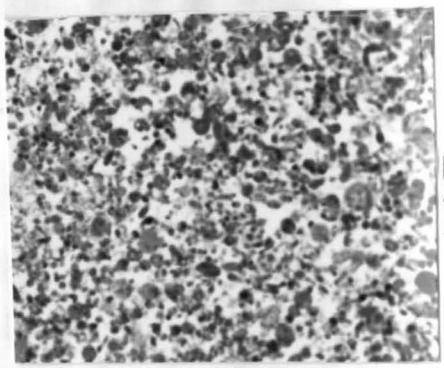




P2 -b

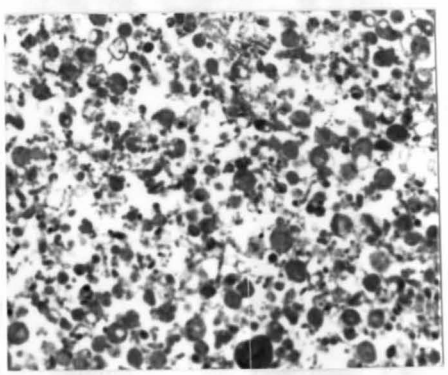
P2 -c

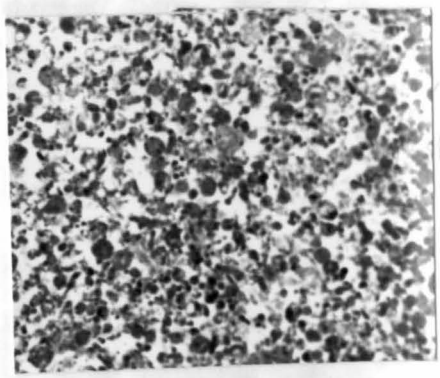




p2 -D

P2 -e





P2 -f

P2- mit.

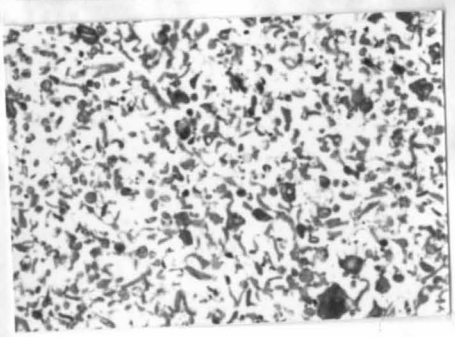
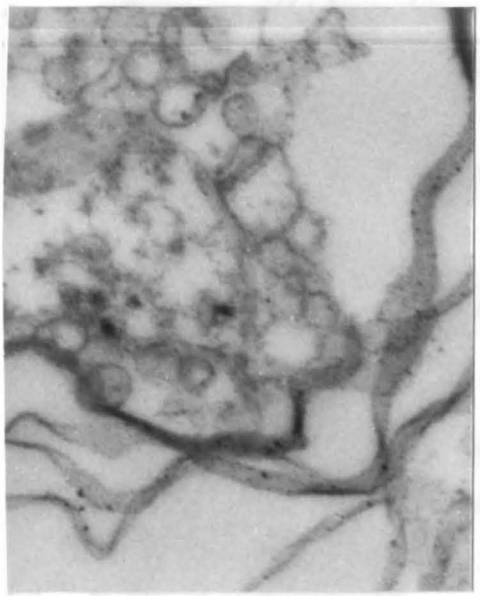


FIG.3.38–"P2" SUBFRACTIONATION

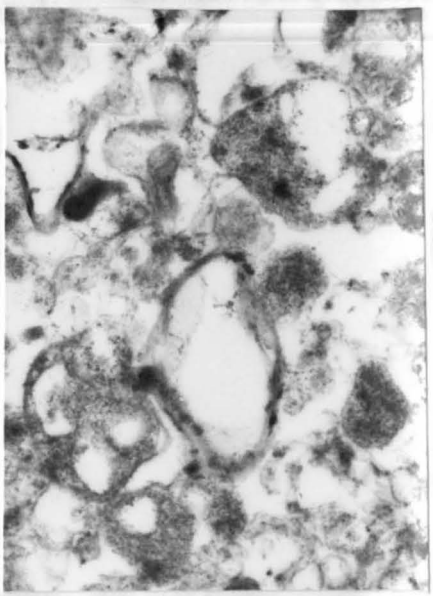
EM MORPHOLOGY–HIGH MAGNIFICATION

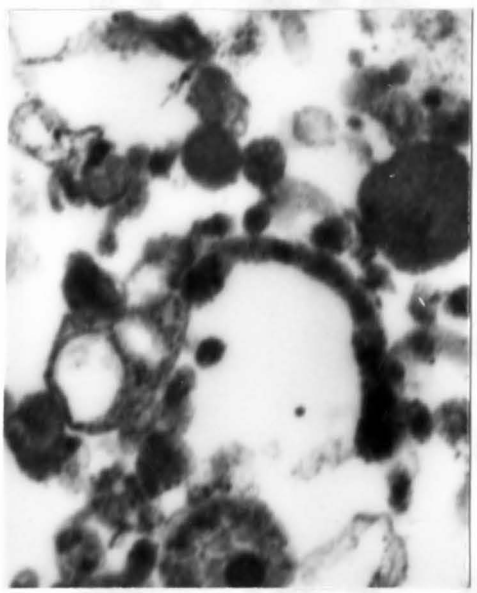
SIGNIFICANT STRUCTURAL DETAILS

P2-a

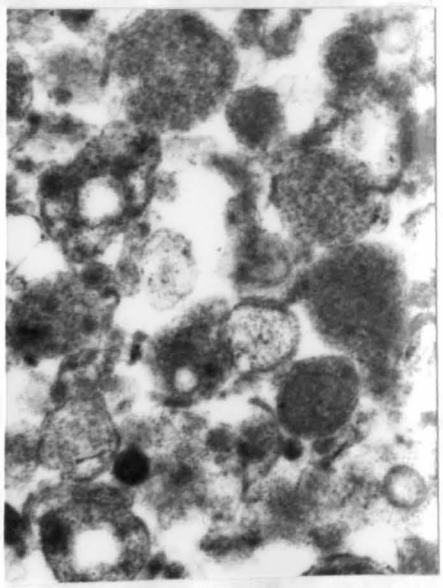


P2-b





P2-c



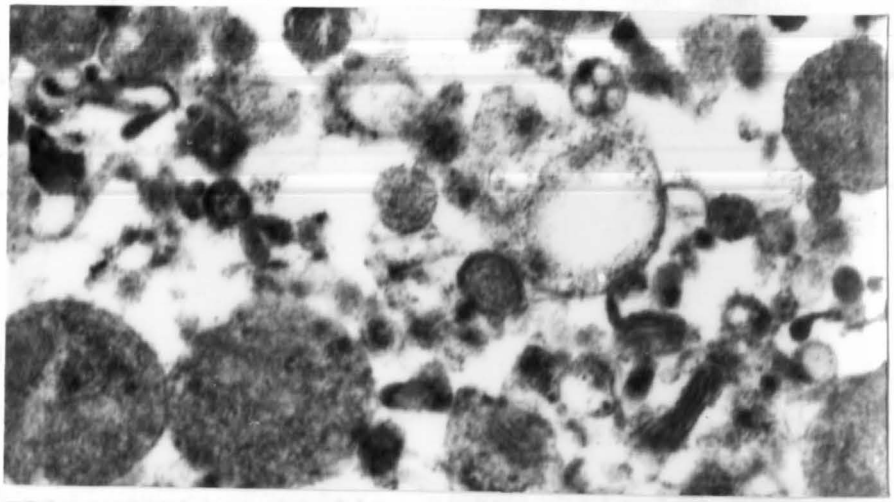
P2-d

FIG.3.39–"P2" SUBFRACTIONATION

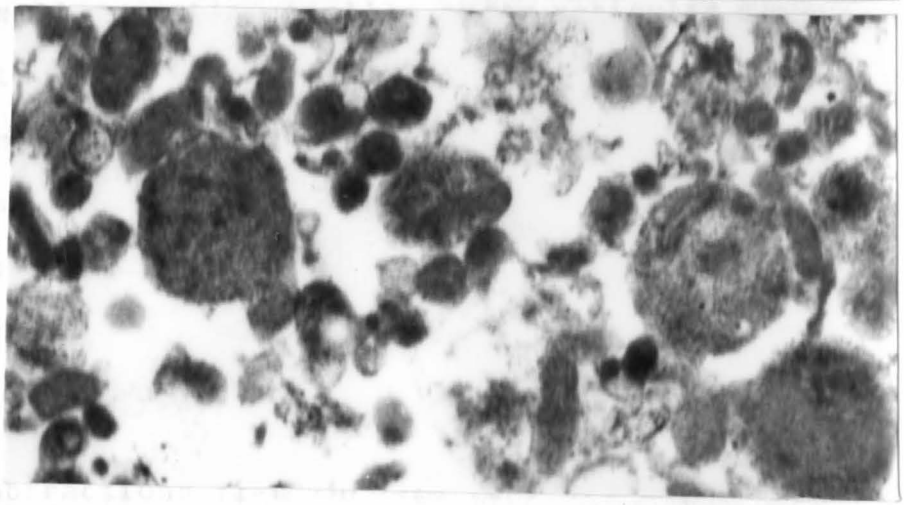
EM MORPHOLOGY–HIGH MAGNIFICATION

SIGNIFICANT STRUCTURAL DETAILS

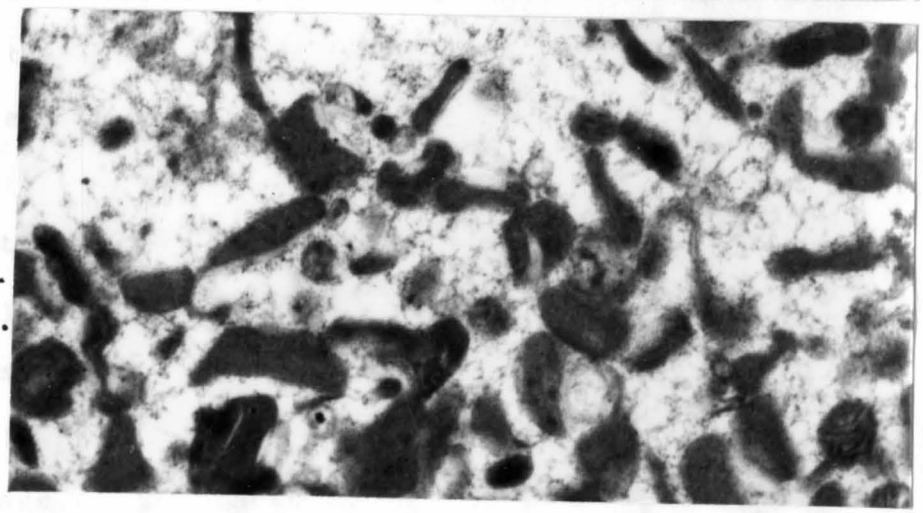
P2–e



P2–f



P2–
mit.



shown in the electron micrographs of figures 3.37 through 3.39. These micrographs are of a lower magnification than those of the microsomal subfractions, therefore, the structures are much larger here, than in the microsomal subfractions. In order to detect golgi membranes in these subfractions, they have been fixed overnight in 1% O_sO_4. Thus the synaptosomal detail is somewhat less than optimal, due to excessive protein crosslinking.

The P2-a sample contains a major proportion of dark, multilamellar myelin, smaller empty vesicular membranes, and membrane fragments. The P2-b has less myelin, and more nondescript membranes. The P2-c fraction has very little myelin. Most numerous here are relatively small, closed spherical membranes, filled with variably staining granular material. Definite synaptosomal structures appear in this fraction.

Subfractions P2-d through P2-f are characterized mainly by synaptosomal structures. These can be seen as relatively large, spherical membrane enclosed entities, containing secretory vesicles, and mitochondria. Possibly, the postsynaptic density can be seen on some of these synaptosomes (e.g. fig. 3.39, P2-f). Estimating the synaptosomal purity is best done by looking at the low power views, fig. 3.37. The P2-e sample shows a very high percentage of the large spherical synaptosomal bodies.

The P2-mit subfraction is very different from the others. The most numerous structures here are very dark staining, oblong bodies of medium size, presumably mitochondria. In some of these the intramitochondrial detail can be seen. Some extra large, and extra dense staining synaptosomes are also seen in the P2-mit fraction.

Structures resembling golgi membranes are very rare in these samples. There are possibly some golgi present in the P2-c and less in the P2-d fractions, but they are large in size. From the morphological analysis, the golgi contribution to the STase activity should be minor, but this is an objective conclusion, and could be biased in interpretation.

The marker enzyme analysis is summarized in fig. 3.40. Fraction P2-f has the highest ChAT and 5'Nucleotidase activity, followed by P2-e and P2-d. These enzymes should be enriched in synaptosomes. P2-a shows an enrichment in 5'nucleotidase activity, a marker for plasma membrane. The golgi marker (CST) is very low in the fraction showing the synaptosomal morphology: P2-f; P2-e; and P2-d. CST activity is increased in P2-a and P2-b, but most of this activity is in the microsomal, or "P3" fraction that was obtained during the preparation of these samples. P2-mit has very low specific activity in all these marker

FIG.3.40 SUBFRACTIONATION OF THE P2 PELLET

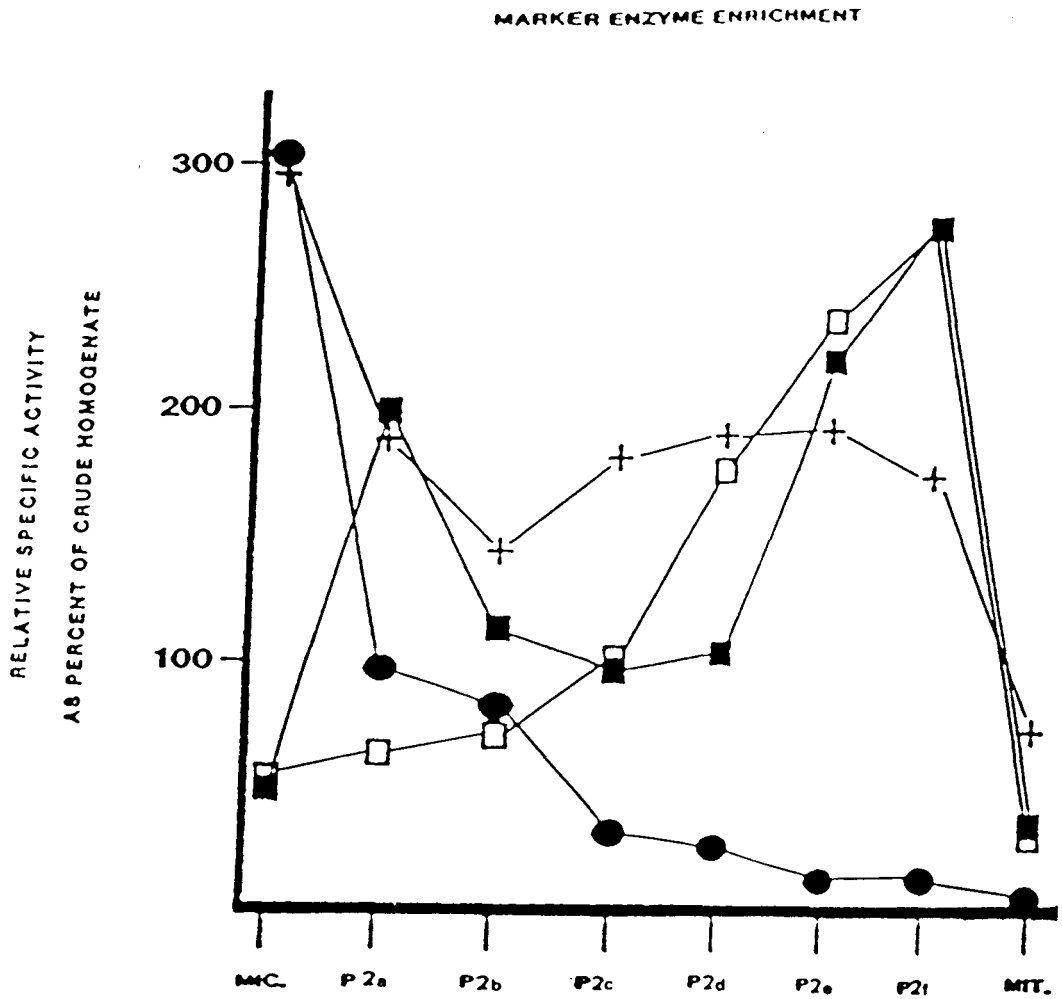


Fig.3.40: marker enzyme analysis of the subfractions obtained from the P2 fraction of rat brain. the assay procedures are described under materials and methods. The Specific activities of each enzyme were compared to the specific activity of a crude rat brain homogenate, and are expressed as a percent of the activity in crude homogenate for a given enzyme, in a given subfraction. The enzymes assayed are: ■, 5' nucleotidase; +, acetyl choline esterase; □, choline acetyl transferase; ●, cerebroside sulpho-transferase. The subfractions are listed in order of decreasing density along the bottom of the graph. The points represent the mean of six samples.

enzymes. P2-e, followed by P2-f, are the purest synaptosomal fractions judged by marker enzyme analysis and morphologically.

The total STase specific activity and the specific ganglioside concentration of the P2 subfractions are shown in figure 3.41. The P2-e subfraction has the highest values for both of these measurements. P2-d and P2-f also are enriched in STase activity. The microsomal subfraction has 3-4 fold greater STase activity than the synaptosomal enriched sample (P2-e). The P2-e STase activity is approximately the same as the activity measured from the traditional synaptosomal preparation in part 1 (above). These data are not very strong in support of a synaptosomal STase. But the contamination of the P2-e by golgi membranes as estimated by morphology and marker enzyme analysis, is not great enough to account for the STase activity measured here. The enzymatic analysis data from the subfractions of the P2 pellet are summarized in table 3.4.

The gangliosides extracted from the P2 subfractions after STase assays, as separated by TLC, are shown in fig. 3.42. The lanes were analyzed by densitometric integration. Again the P2-d, P2-f, and especially P2-e fractions showed the percent ganglioside distribution patterns that are similar to the pattern found for traditional synapto-

FIG.3.41

SUBFRACTIONATION OF THE P-2 PELLET TOTAL SIALOSYLTRANSFERASE ACTIVITY AND SPECIFIC GANGLIOSIDE CONCENTRATION

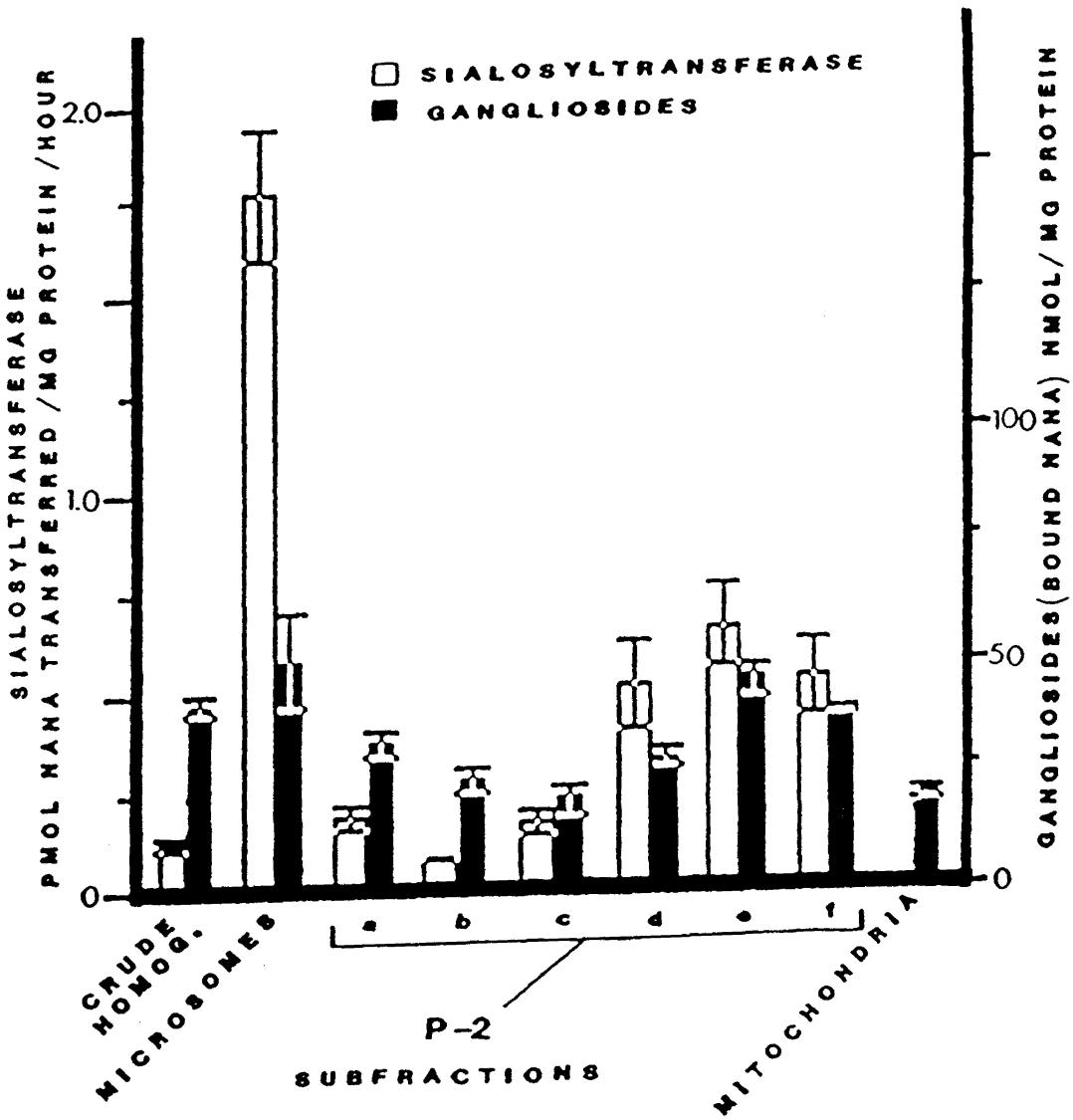


Fig.3.41: total specific STase activity and specific ganglioside concentration of the subfractions of the P2 pellet of rat brain. STase units are pmoles NANA incorporated into the total endogenous gangliosides of each subfraction, per mg protein, per hour. Ganglioside concentration is expressed as the nmoles of glycolipid bound NANA per mg protein in each fraction. "Microsomes" is the total microsome fraction (P3). The subfractions of P2 are listed in order of decreasing density.

TABLE 3.4

SUMMARY OF DATA FROM THE P2 SUBFRACTIONS

The values given are the means of 6 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each assay are: 5'-nucleotidase (5'-NT), micromoles of AMP hydrolyzed per mg protein per hour; choline acetyl transferase (ChAT), micromoles choline transferred per mg protein per minute; cerebroside sulpho transferase (CST), picomoles sulfate transfered per mg protein per hour; total sialosyltransferase (STase), picomoles NANA transferred per mg protein per hour; ganglioside specific concentration (Sp. Gang. Comc.), nanomoles lipid bound NANA per mg protein per hour.

	SUBFRACTIONS						
	P2-a	P2-b	P2-c	P2-d	P2-e	P2-f	P2-mit
5'-NTase	1.42 (0.02)	0.77 (0.04)	0.66 (0.05)	0.72 (0.04)	1.54 (0.05)	2.06 (0.29)	0.25 (0.01)
ChAT	0.13 (0.03)	0.14 (0.03)	0.21 (0.01)	0.37 (0.04)	0.49 (0.04)	0.58 (0.19)	0.06 (0.02)
AChE	3.25 (0.15)	2.45 (0.63)	3.11 (0.08)	3.29 (0.08)	3.34 (0.45)	2.99 (0.13)	1.24 (0.56)
CST	1.45 (0.01)	1.26 (0.04)	0.49 (0.02)	0.39 (0.03)	0.17 (0.01)	0.16 (0.01)	0.09 (0.01)
STase	0.19 (0.04)	0.09 (0.01)	0.17 (0.04)	0.53 (0.13)	0.66 (0.12)	0.53 (0.12)	0.04 (0.01)
Sp.Gang. Conc.	34.34 (1.42	238.06 (3.44)	22.65 (3.32)	33.54 (1.69)	52.51 (3.90)	44.03 (0.70)	23.50 (1.52)

some (part 1). There were consistant differences noted in the lighter subfractions. P2-a had many minor resorcinol positive bands running between GM1, GM2, GM3 and above. These were present to a lesser degree in P2-b and P2-c. P2-c seemed to consistantly show an increased percent content of GD3. It would be interesting to investigate what structures these ganglioside differences are associated with, but this would be a long and tedious task. Qualitatively, the "extra" bands are similar to those seen in the microsomal subfractions.

The lanes of the TLC plates were scraped and counted as usual. The mean percent distribution of radioactivity among the ganglioside species is shown in fig. 3.43 (A = P2-a, B = P2-b,...). The microsomal fraction showed a NANA incorporation pattern consistant with the preceeding microsomal subfractionation experiment, especially in the area of the UN peak. The UN band has incorporated label in the P2-a fractions, and decreases thereafter.

The pattern of NANA transfer can be seen to change from fraction to fraction, and at the P2-e and P2-f fractions, the pattern can be recognized as identical to the pattern previously established for synaptosomes in part 1. Note that the percent incorporation of radioactivity into GD3 surpasses that of GD1a in both P2-e and P2-f. These two subfractions are the purest in synaptosomes. The incorp-

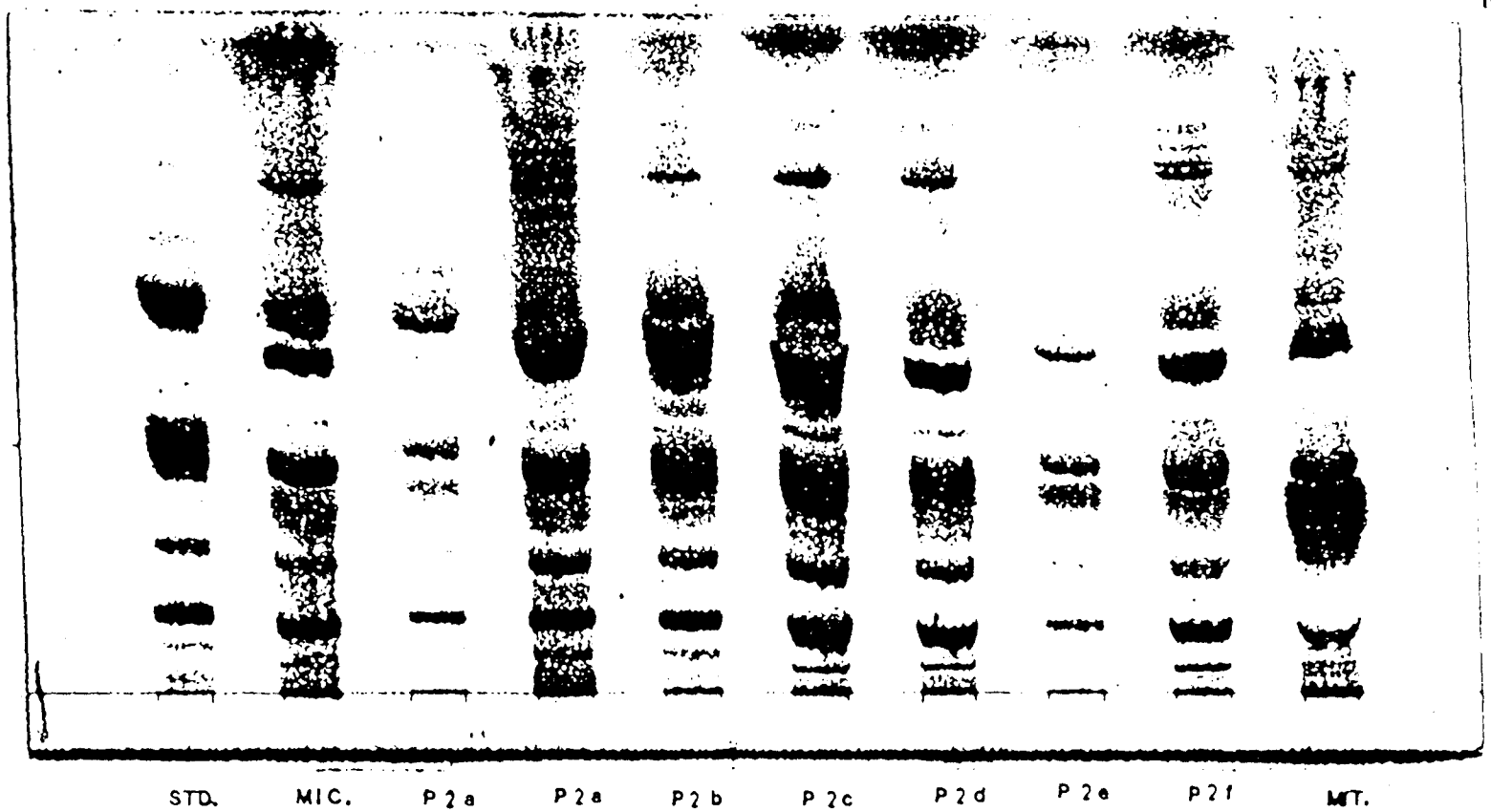
Fig.3.42: ganglioside patterns from the subfractions obtained from the P2 pellet of rat brain. The subfractions were assayed for STase, the gangliosides were extracted and purified, and then chromatographed as usual. The developed and resorcinol stained TLC plate was placed on a photocopy machine and the resulting copy is shown in this figure. Along with the lanes for the P2 subfractions are a lane for bovine brain ganglioside standards, and the gangliosides from the total microsome fraction (P3).

Fig.3.43: ganglioside labeling patterns of the STase from the subfractions obtained from the P2 pellet of rat brain. The details of the procedures used are described under materials and methods. The TLC plates of the ganglioside from the subfractions after STase incubation, as shown in figure 3.42, were scraped, counted, and the DPM incorporated into each ganglioside species were obtained. A separate bar graph for each subfraction is shown. The radioactivity of each ganglioside species is expressed as a percent of the total radioactivity incorporated into all gangliosides of a given sample. Subfractions "e" and "f" are the richest in true synaptosomes, and a clear difference in the pattern of labeling of ganglioside species can be seen when compared to the other subfractions and the crude microsomal fraction, "MIC" (P3). The length of each bar represents the mean percent of 7 samples, with the S.E.M. shown.

SUBFRACTIONATION OF THE P2 PELLET

FIG. 3.42

TLC OF THE LABELED GANGLIOSIDES EXTRACTED FROM EACH FRACTION



SUBFRACTIONATION OF THE P-2 PELLET

Product Specificity of Sialosyltransferase

Bars Represent Percent of Total DPM

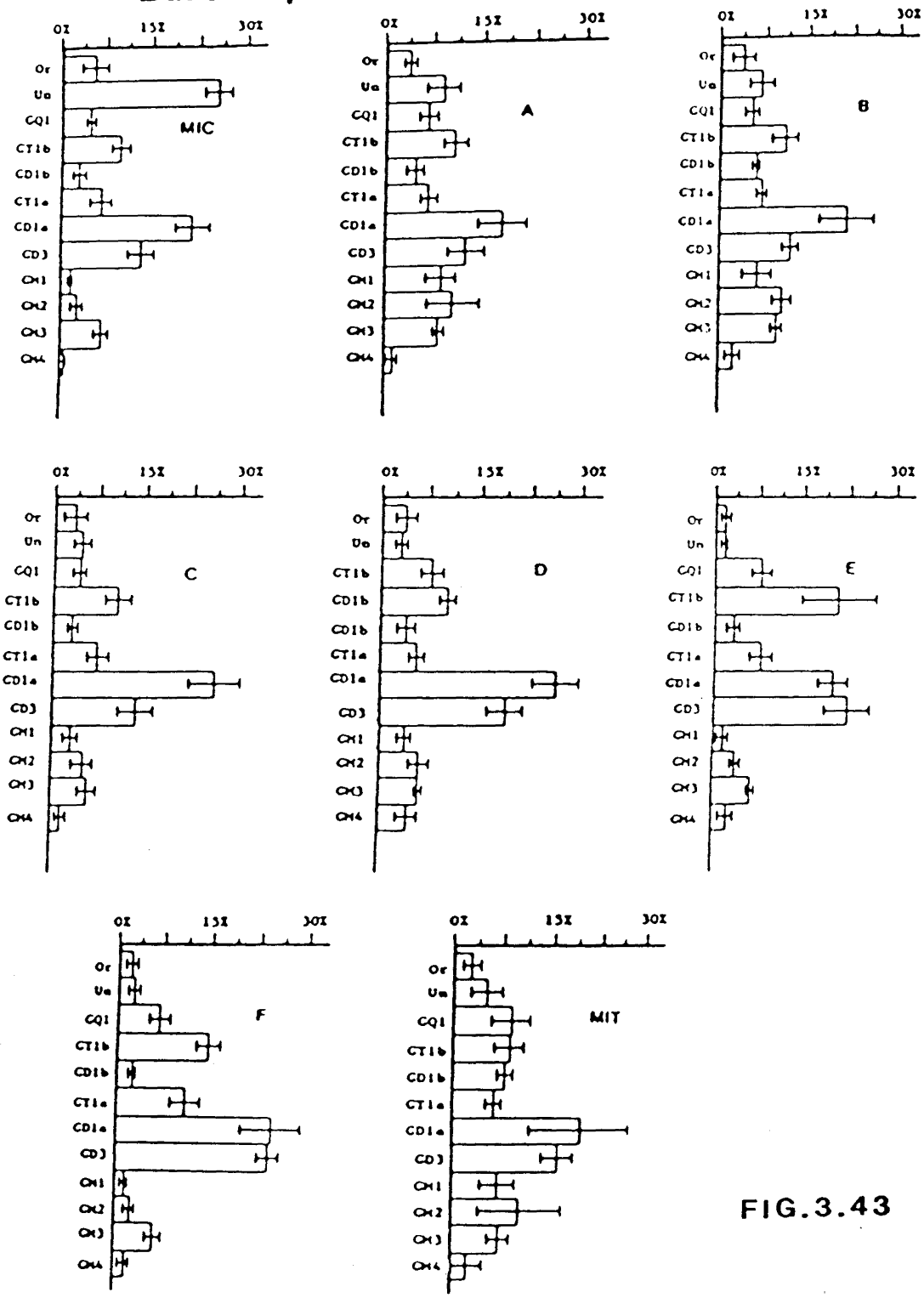


FIG.3.43

oration of label into the UN area is low in these fractions.

The STase data for the individual ganglioside species are tabulated in table 3.5. The RSRA values show the great product specificity for GD3 in the subfractions enriched in synaptosomes (P2-e, P2-f). These values are 3 to 4 fold greater than the RSRA of other gangliosides. GQ, GT1a and b, GM2, GM3, and GM4 all display specificity because their RSRA values are above 1.0.

In every sample studied, microsomes (and golgi enriched fractions) transfer NANA into the UN band most specificly, followed by the polysialosyl gangliosides. On the other hand, all the synaptosomal enriched fractions show a high specific STase incorporation into GD3, with low incorporation into the UN area. These, thus far, are consistant qualitative differences.

To summarize briefly, discontinuous ficoll density gradient centrifugation of the basic P2 fraction produced certain subfractions (P2-e and P2-f) that are highly enriched in synaptosomal morphology and marker enzymes, and virtually uncontaminated by Golgi morphology or marker enzyme. These synaptosomal subfractions contained a ganglioside STase activity, which was lower than Golgi enriched preparations, but probably is significant. The

TABLE 3.5

SUMMARY OF GANGLIOSIDE DATA
FROM THE P2 SUBFRACTIONS

The values given are the means of 6 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each ganglioside analysis value are: percent ganglioside distribution (% DIST.), the percent ot the total resorcinol positive area for a given ganglioside species as measured by densitometric integration of TLC lanes; percent distribution of radiolabel by STase (% DPM), expressed as a percent of the total radiolabel present in the total ganglioside sample; and the relative specific radioactivity of each ganglioside (RSRA), which is %DIST / %DPM. (ND = not determined)

		SUBFRACTIONS						
		P2-a	P2-b	P2-c	P2-d	P2-e	P2-f	P2-m
UNK	%DIS	ND	ND	ND	ND	ND	ND	ND
	%DPM	8.80	6.61	5.01	2.99	1.54	2.56	4.92
		(2.4)	(1.9)	(1.6)	(0.9)	(0.6)	(1.0)	(2.3)
	RSRA	ND	ND	ND	ND	ND	ND	ND
GQ1	%DIST	3.27	2.95	2.33	4.55	7.02	6.45	2.38
		(0.7)	(0.6)	(0.5)	(0.3)	(0.8)	(0.9)	(0.8)
	%DPM	7.02	5.38	4.38	7.52	8.60	7.28	8.17
		(1.7)	(1.3)	(1.4)	(2.0)	(1.5)	(1.9)	(1.0)
	RSRA	2.97	1.76	1.93	2.05	1.38	1.40	11.36
		(1.3)	(0.5)	(0.6)	(0.7)	(0.4)	(0.5)	(6.8)
GT1b	%DIST	6.27	7.02	7.25	8.97	13.30	12.32	8.30
		(1.1)	(0.7)	(1.5)	(1.6)	(2.3)	(1.8)	(1.2)
	%DPM	9.70	9.95	11.17	9.35	20.67	13.97	8.23
		(1.3)	(2.4)	(2.8)	(1.2)	(6.0)	(2.1)	(1.4)
	RSRA	1.94	1.51	2.11	1.39	2.04	1.19	1.11
		(0.8)	(0.4)	(0.7)	(0.4)	(0.8)	(0.2)	(0.3)
GD1b	%DIST	8.47	9.40	6.77	9.35	11.98	12.63	10.40
		(0.9)	(1.3)	(2.4)	(0.7)	(1.5)	(0.8)	(1.6)
	%DPM	4.25	5.70	8.18	2.78	3.18	2.03	7.67
		(1.3)	(0.74)	(2.3)	(0.6)	(1.0)	(0.3)	(0.7)
	RSRA	0.59	0.66	2.00	0.31	0.25	0.16	0.82
		(0.3)	(0.1)	(0.9)	(0.1)	(0.1)	(0.02)	(0.1)

TABLE 3.5 CONTINUED

		SUBFRACTIONS						
		P2-a	P2-b	P2-c	P2-d	P2-e	P2-f	P2-m
GT1a	%DIST	5.18	4.83	6.77	6.32	4.20	5.43	3.80
		(0.9)	(1.1)	(2.4)	(2.1)	(1.0)	(1.4)	(1.0)
	%DPM	7.38	6.60	8.18	5.77	7.61	9.77	5.92
		(1.2)	(1.0)	(2.3)	(1.3)	(1.8)	(2.3)	(0.8)
	RSRA	1.92	1.08	2.00	0.99	1.21	3.30	2.00
		(0.7)	(0.9)	(0.9)	(0.3)	(0.4)	(1.4)	(0.5)
GD1a	%DIST	20.83	21.78	27.90	28.30	32.32	33.70	21.66
		(2.5)	(2.1)	(3.4)	(5.3)	(2.8)	(2.5)	(4.0)
	%DPM	14.28	20.33	29.85	27.40	19.90	21.52	19.18
		(3.8)	(5.2)	(5.3)	(3.8)	(2.4)	(4.3)	(5.0)
	RSRA	0.72	0.99	1.60	1.30	0.64	0.62	1.05
		(0.2)	(0.2)	(0.5)	(0.4)	(0.1)	(0.1)	(0.1)
GD3	%DIST	7.75	7.63	9.35	5.22	1.78	1.30	3.82
		(1.2)	(1.3)	(1.5)	(0.9)	(0.6)	(0.5)	(1.1)
	%DPM	10.42	11.27	14.67	19.08	22.15	25.02	15.56
		(3.9)	(1.6)	(3.6)	(3.1)	(3.7)	(2.0)	(1.4)
	RSRA	1.66	1.70	1.51	4.7	18.79	16.27	10.48
		(0.7)	(0.3)	(0.4)	(1.4)	(5.0)	(1.2)	(3.3)
GM1	%DIST	24.72	28.10	27.02	15.75	15.05	15.65	20.22
		(4.4)	(2.3)	(2.8)	(2.7)	(3.1)	(2.3)	(3.8)
	%DPM	9.92	6.20	3.40	4.17	1.72	1.79	6.7
		(2.8)	(2.7)	(1.7)	(1.2)	(0.9)	(0.7)	(1.7)
	RSRA	0.40	0.20	0.19	0.27	0.17	0.12	0.37
		(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.04)	(0.1)
GM2	%DIST	9.97	8.99	7.62	5.38	2.42	1.92	6.67
		(0.5)	(1.9)	(1.6)	(1.3)	(0.6)	(0.3)	(1.3)
	%DPM	12.03	10.83	6.35	6.18	3.13	1.79	9.70
		(4.9)	(1.8)	(2.2)	(1.5)	(0.7)	(0.6)	(3.9)
	RSRA	1.25	1.46	1.33	1.41	5.28	1.06	1.35
		(0.5)	(0.3)	(0.6)	(0.5)	(3.9)	(0.3)	(0.5)
GM3	%DIST	8.31	12.78	9.62	8.22	4.18	4.22	9.17
		(2.2)	(2.0)	(0.9)	(1.4)	(0.6)	(0.8)	(1.1)
	%DPM	9.00	8.98	7.07	6.32	6.47	6.4	7.0
		(0.8)	(1.0)	(2.0)	(0.6)	(0.6)	(1.5)	(1.1)
	RSRA	2.21	0.80	0.69	0.86	1.65	1.75	0.80
		(0.9)	(0.2)	(0.1)	(0.1)	(0.2)	(0.4)	(0.1)

TABLE 3.5 CONTINUED

SUBFRACTIONS

		P2-a	P2-b	P2-c	P2-d	P2-e	P2-f	P2-m
GM4	%DIST	2.92	4.12	3.40	2.43	0.63	1.5	5.07
		(0.7)	(1.2)	(1.1)	(0.7)	(0.3)	(0.4)	(1.1)
	%DPM	1.68	2.72	2.23	4.15	1.93	2.12	2.48
		(1.0)	(1.3)	(1.0)	(1.6)	(0.8)	(0.9)	(1.5)
	RSRA	0.88	1.51	0.88	3.01	3.12	1.91	0.79
		(0.5)	(1.0)	(0.3)	(1.7)	(0.1)	(0.3)	(0.5)

ganglioside labeling pattern of synaptosomal STase is unique. The major ganglioside for NANA incorporation is GD3, which is a minor ganglioside in quantity. There is very little incorporation of NANA into the UN area of the ganglioside fraction by synaptosomal STase.

SYNAPTIC PLASMA MEMBRANES - After preparing traditional synaptosomes (P2-B) by discontinuous ficoll density gradient centrifugation, the synaptosomes were washed and then hypo-osmotically lysed as described in the methods chapter. The resulting suspension was centrifuged at 17,000 x g to sediment any unlysed structures. The supernatant was centrifuged at 100,000 x g to pellet the lysed membranes. This 17,000 x g sedimentation decreased the yield of the lysed membrane sample tremendously, but hopefully the purity of the resulting lysed membrane sample is increased in proportion. The lysed membranes were subfractionated on a discontinuous sucrose density gradient.

A drawing of the resulting gradient is shown in fig. 3.44 (the bands were so faint a photograph was not taken). The solutions and subfractions collected are shown. Note that no fractions were found at the top two gradient interfaces.

The electron microscopy of the SPM subfractions is shown in figures 3.45 through 3.47. The magnifications

FIG.3.44 SYNAPTIC PLASMA MEMBRANES

PREPARATION BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

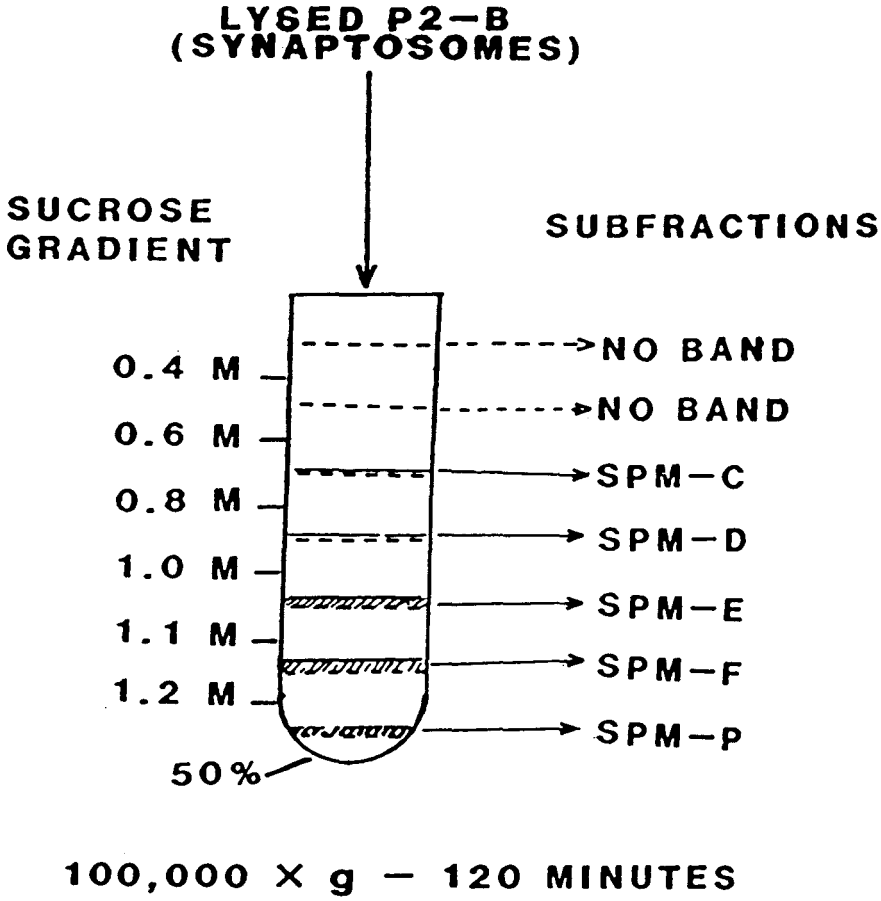


Fig 3.44: preparation of subfractions of synaptic plasma membranes by discontinuous sucrose density gradient ulta-centrifugation. Hypo osmotically lysed purified synaptosomes (P2-B) were layered on top of the gradient made of the sucrose solutions shown on the left. After centrifugation at 100,000 X g for two hours, the membranous bands shown at the right formed at the gradient interfaces. The bands were very faint, so a photograph of the tubes was not taken. The sketch above depicts the results. No material banded at the first two interfaces. Therefore the first subfraction harvested was called SPM-C, followed by -D, -E, -F, and -P was the pellet on the bottom of the tube.

Fig.3.45: electron micrographs of the subfractions obtained by discontinuous sucrose density gradient centrifugation of hypo osmotically lysed purified synaptosomes. This is a classical procedure for the preparation of synaptic plasma membranes (SPM). The gradient conditions and lysis procedures are described in materials and methods. The gradient tubes with samples were centrifuged at 100,000 X g for two hours. Membranous layers formed at the lower gradient interfaces, and were removed and washed. The resulting subfractions were termed: SPM-C; SPM-D; SPM-E; SPM-F; and SPM-P (pellet). These micrographs show the general appearance of these subfractions magnified X 16,500.

Fig.3.46: high magnification electron micrographs (X 110,000) of subfractions SPM-C and SPM-D. SPM-C was a fraction very low in yeild and was highly enriched in membranes typical of myelin. SPM-D was somewhat low yeilding, and also contained myelin morphology but more membranes of other types than SPM-C.

Fig.3.47: high magnification electron micrographs (X 110.000) of SPM subfractions E,F, and P. SPM-E yielded a substantial membranous layer from the gradient, and morphologically looks like open membranes, often with small vesicular and granular material adhered to it. In many places membranes could be seen with a dense, junction like complex on it, possibly with another membrane. These complexes were numerous and are consistent with the morphology of synaptic plasma membranes with post synaptic densities. SPM-F was even more enriched in these complexes and otherwise "cleaner" from smudges and granular material. The SPM-F field shown in this figure shows a very typical synaptic plasma membrane with the synapse in the lower left, showing intra synaptic material and dumbbell shaped post synaptic membrane adhering to it. SPM-P appears to have some synaptic plasma membranes, but is more enriched in large, closed vesicular structures, probably unlysed synaptosomes and mitochondria.

FIG.3.45– SPM SUBFRACTIONATION

EM MORPHOLOGY; LOW MAGNIFICATION

UNBIASED FIELDS OF EACH FRACTION

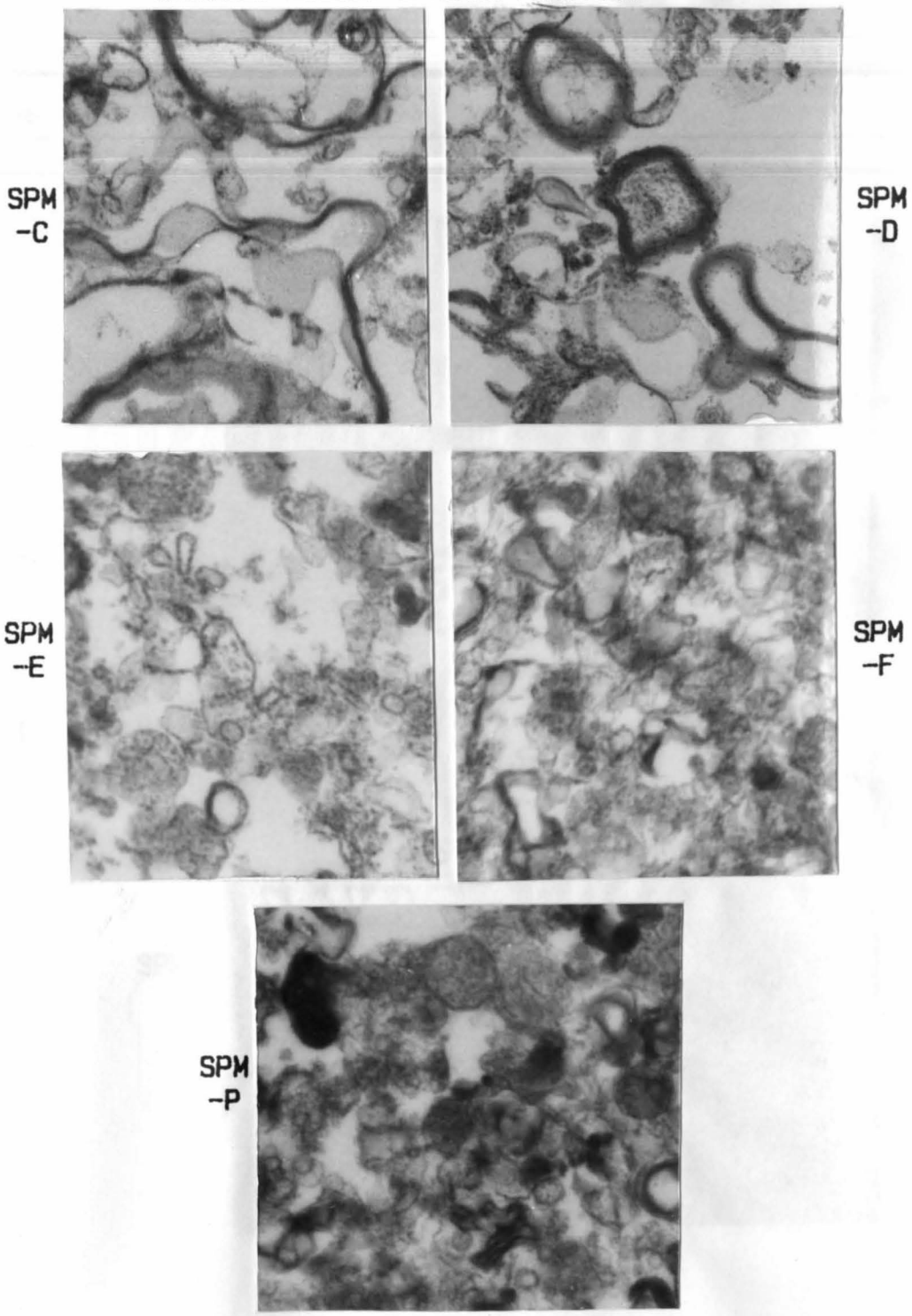
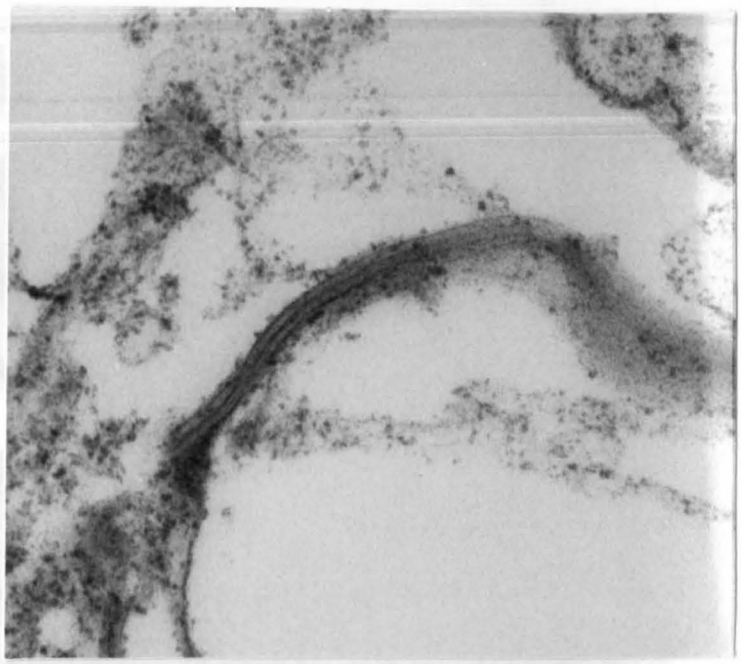


FIG.3.46–SPM SUBFRACTIONATION

EM MORPHOLOGY–HIGH MAGNIFICATION

SIGNIFICANT STRUCTURAL DETAILS

SPM–C



SPM–D

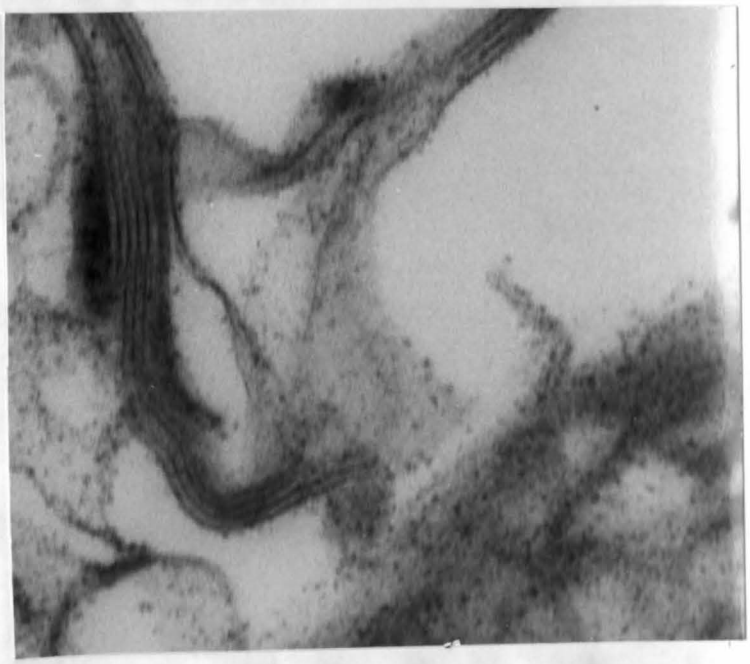
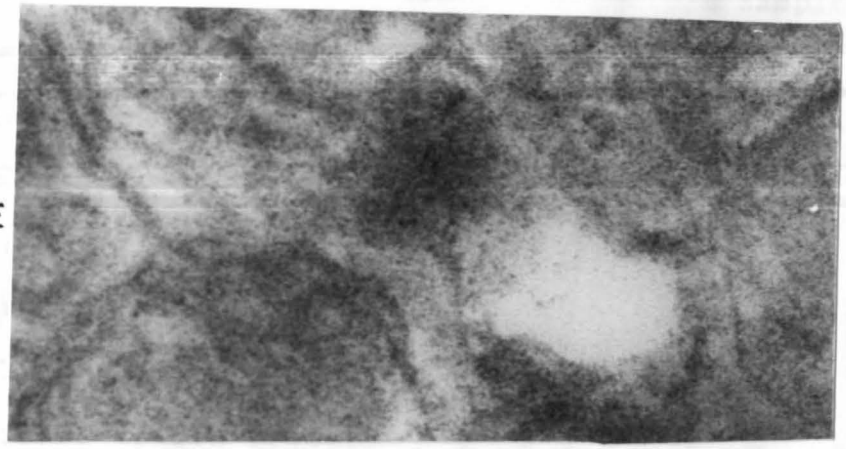


FIG.3.47—SPM SUBFRACTIONATION

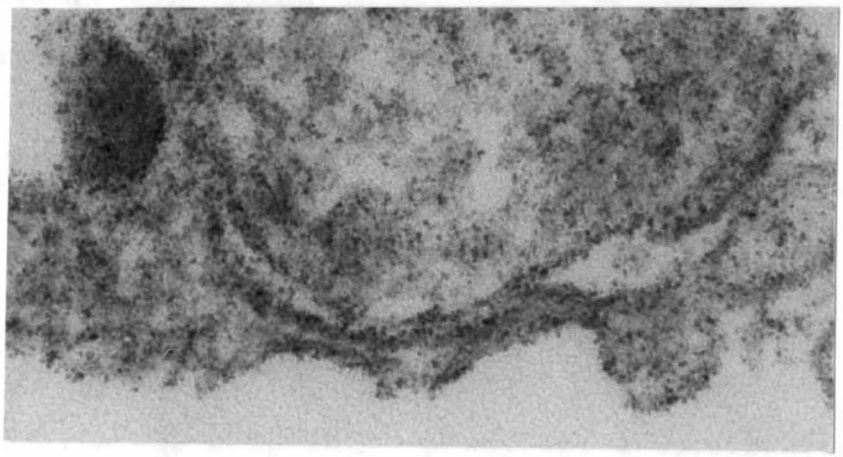
EM MORPHOLOGY-HIGH MAGNIFICATION

SIGNIFICANT STRUCTURAL DETAILS

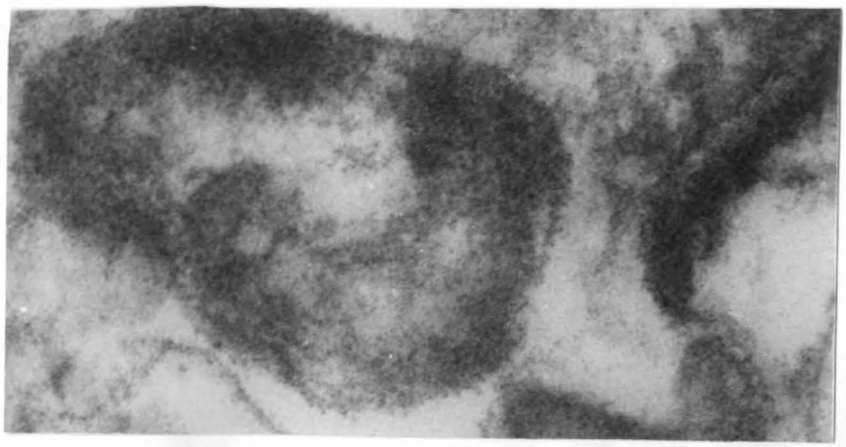
SPM-E



SPM-F



SPM-P



are greater than for the P2 subfractions, but less than for the microsomal subfractions. The SPM-C sample, which was very minute in quantity, is mainly myelin fragments and simple unidentified membrane fragments. SPM-D was slightly greater in yield, and was characterized by fewer but denser myelin, and more complex membrane fragments. There were also small vesicles present.

The SPM-E fraction was higher in yield. There was very little myelin present. There are numerous open, dense membranes. Occasionally two membranes form dense junctions (see fig. 3.47). There are small vesicles and granular material adhering to these membranes. This description is compatable with synaptic plasma membranes. Fraction SPM-F is also greatly enriched in these SPM structures. Figure 3.47 (plate F) shows an excellent synaptic junctional complex: showing a large presynaptic terminal with the associated presynaptic apparatus and densities; a synapse with inter-synaptic material; and an attached post synaptic membrane fragment. The SPM-E and SPM-F subfractions were loaded with such structures.

The SPM-P subfraction was the largest in yield. Much of this fraction was closed spherical or oblong structures of variable size and staining density. These appear to be mitochondria, lysosomes, and unlysed synaptosomes. Many of the synaptosome-like structures are irregular in shape. These might be hypertonically crenated

synaptosomes which are said to be refractory to hypo-osmotic lysis.

The marker enzyme analysis summary of the SPM subfractions is shown in fig. 3.48. The SPM-E and SPM-F fractions, which were enriched in true SPM morphology, were enriched in the SPM marker enzymes ChAT, and 5'nucleotidase. These fractions were very low in the golgi marker, CST.

The SPM subfractions were assayed for total ganglioside STase activity as usual. These results and the specific ganglioside content of each subfraction is shown graphically in fig. 3.49. The STase specific activity is enriched in the subfractions that contain SPM morphology and marker enzyme enrichment. This STase activity is higher than that observed for intact synaptosomes by over two fold. This suggests that the STase activity in synaptosomes is located in the synaptic membrane.

The enzymatic analysis data of the SPM subfractions are summarized in table 3.6.

The gangliosides of the SPM subfractions, after STase incubation, were separated by TLC and processed in the same way as all other samples. A chromatogram is shown in fig. 3.50 in the form of a photocopy of the plate. Due to the very small yield of material from the subfractionation procedure, the entire sample was spotted

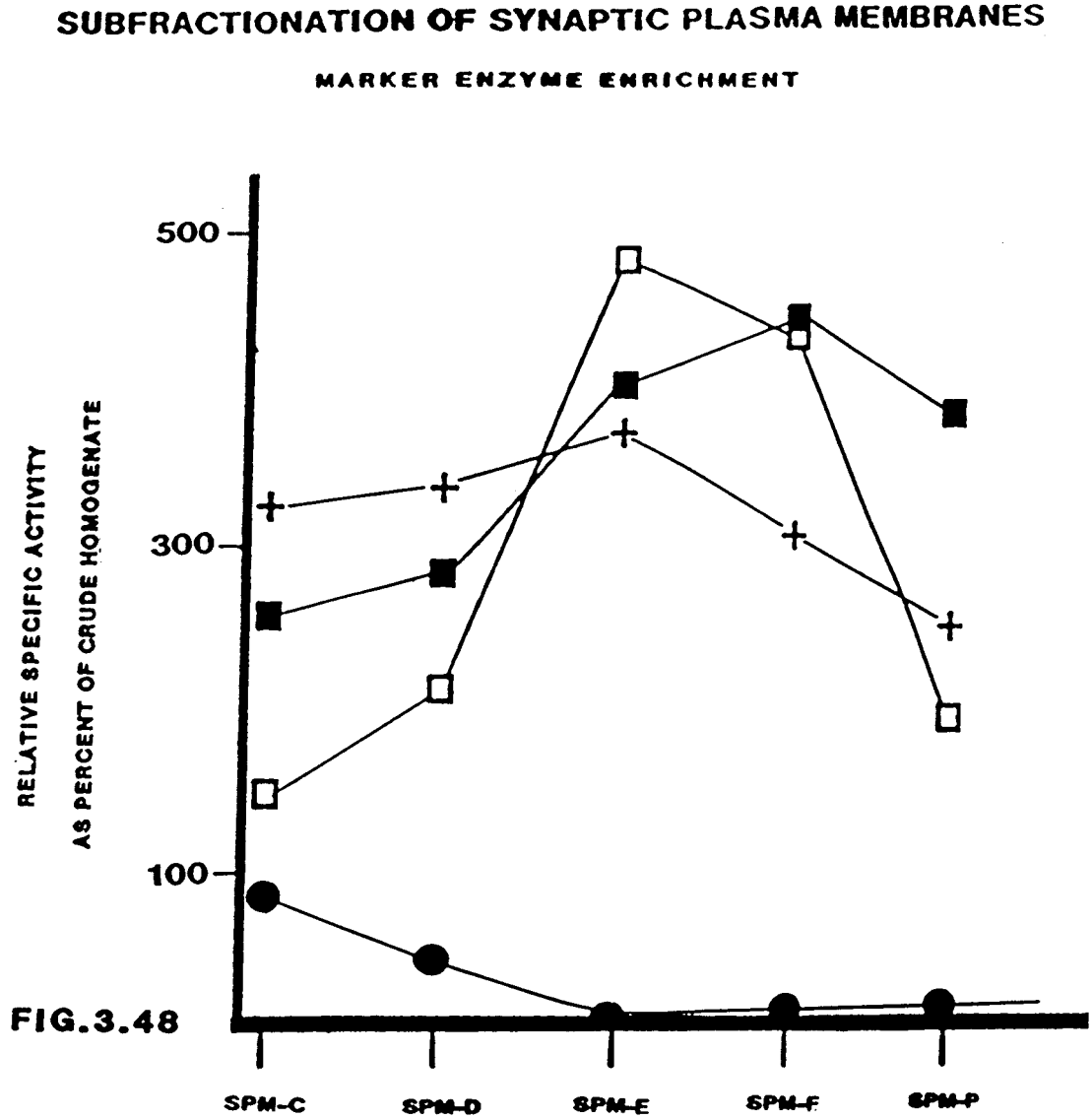


FIG.3.48

Fig.3.48: marker enzyme analysis of the SPM subfractions. The procedures for the individual enzyme assays are described under materials and methods. Each enzyme activity for a given subfraction is expressed as the percent of the specific activity of the crude brain homogenate. The enzymes assayed were: ■,5'nucleotidase; +, acetyl choline esterase; □, choline acetyl transferase; and ●, cerebroside sulpho-transferase.

FIG.3.49

SYNAPTIC PLASMA MEMBRANE SUBFRACTIONS

TOTAL SIALOSYLTRANSFERASE ACTIVITY AND SPECIFIC GANGLIOSIDE CONCENTRATION

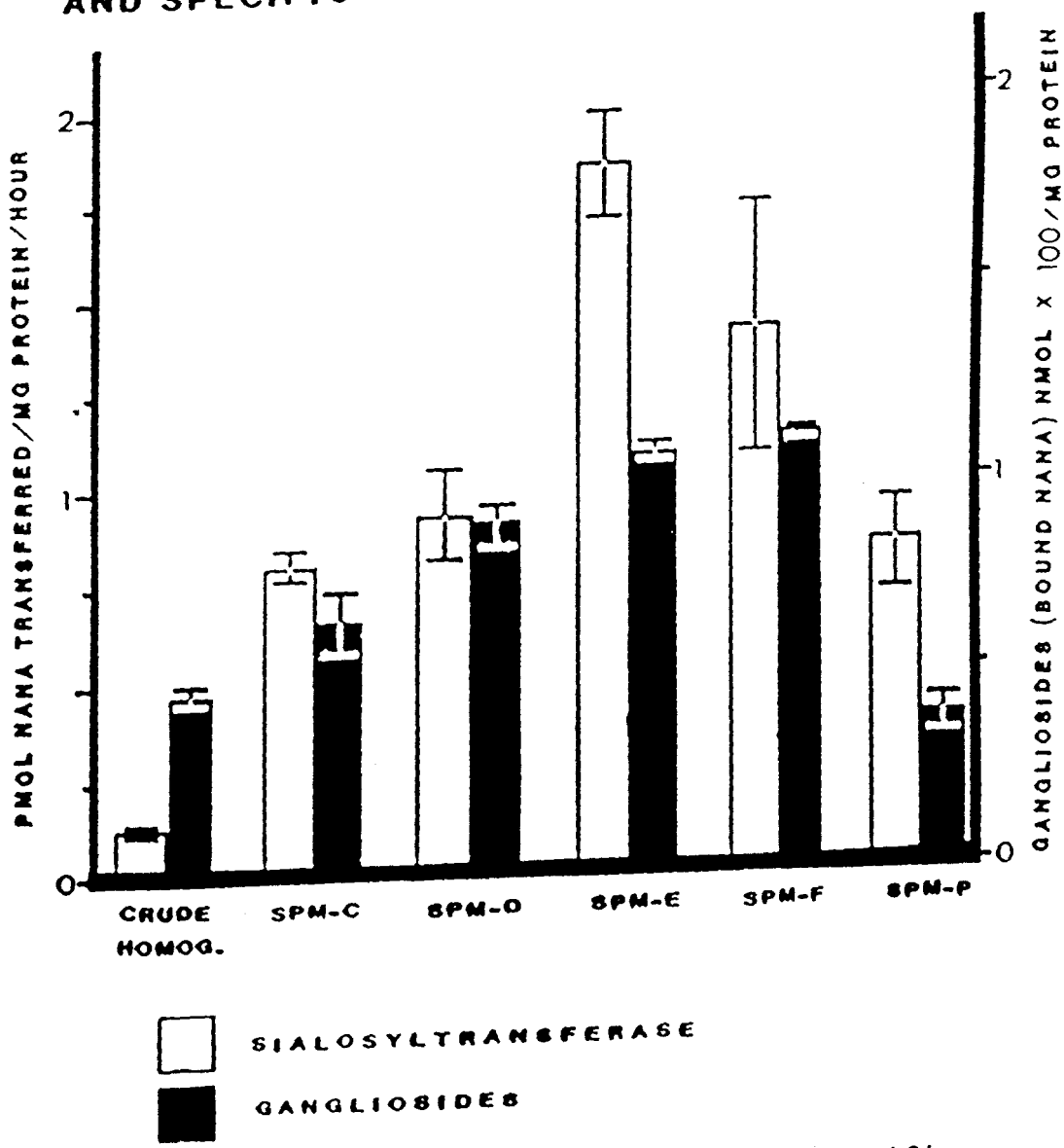


Fig.3.49: total specific STase activity and specific ganglioside concentration of the SPM subfractions. The STase activity is the pmoles of NANA incorporated in to the endogenous total gangliosides of each subfraction, per mg protein, per hour. The ganglioside concentration of each subfraction is expressed as the nmoles of NANA bound to endogenous glycolipid, per mg protein for each subfraction.

TABLE 3.6

SUMMARY OF DATA FROM SYNAPTIC PLASMA MEMBRANES

The values given are the means of 4 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each assay are: 5'-nucleotidase (5'-NT), micromoles of AMP hydrolyzed per mg protein per hour; choline acetyl transferase (ChAT), micromoles choline transferred per mg protein per minute; cerebroside sulpho transferase (CST), picomoles sulfate transfered per mg protein per hour; total sialosyltransferase (STase), picomoles NANA transferred per mg protein per hour; ganglioside specific concentration (Sp. Gang. Comc.), nanomoles lipid bound NANA per mg protein per hour.

	SUBFRACTIONS				
	SPM-C	SPM-D	SPM-E	SPM-F	SPM-P
5'-NTase	1.83 (0.04)	2.17 (0.24)	2.90 (0.23)	3.46 (0.19)	3.28 (0.490
ChAT	0.30 (0.07)	0.44 (0.12)	1.03 (0.13)	0.92 (0.17)	0.41 (0.01)
AChE	5.78 (0.52)	5.93 (0.35)	6.54 (0.25)	5.43 (0.02)	4.38 (0.14)
CST	1.30 (0.05)	0.58 (0.02)	0.02 (0.0005)	0.08 (0.004)	0.07 (0.02)
STase	0.80 (0.04)	0.93 (0.11)	1.85 (0.14)	1.41 (0.32)	0.84 (0.11)
Sp.Gang. Conc.	65.48 (7.87)	90.03 (5.19)	107.56 (2.13)	112.01 (1.55)	39.40 (4.79)

on the lanes. This intensifies the contaminants in the ganglioside extracts, which can be seen as the large band running at the level of GD1a in the bovine brain standard. This band is actually yellow. The ganglioside bands are very faint in SPM-C; SPM-D; and SPM-E due to the very low yield of material.

The percent distribution of radioactivity among the SPM subfraction ganglioside species is shown in fig. 3.53, and in table 3.7. In general, all of the subfractions showed a NANA incorporation pattern similar to the established synaptosomal pattern. Subfractions SPM-C and SPM-D had increased NANA transfer into gangliosides GM3 and GM2. These subfractions had substantial myelin morphology. Also these fractions were enriched in 5'nucleotidase activity, without ChAT enrichment. This can be interpreted as enrichment of plasma membranes, but not SPM.

There may be a STase activity in these membranes that are specific for NANA transfer to form GM3 and GM2 from the asialo counterparts of these gangliosides. GM3 is known to be the major myelin ganglioside. It is interesting to consider that various specialized cellular membranes, which are characterized by a unique ganglioside pattern, or have specific functional gangliosides, may have surface STases that function to maintain the specific ganglioside character of that membrane.

The SPM-E and SPM-F subfractions, which are enriched

Fig.3.50: ganglioside pattern of the SPM subfractions. The subfractions were assayed for STase and the gangliosides were extracted, purified, and chromatographed as before. The resulting TLC plates were placed on a photocopy machine and a copy of a TLC is shown in this figure.

FIG.3.51: ganglioside labeling pattern of the STase of the SPM subfractions. TLC plates such as shown in fig.3.49 were scraped , counted, and the radioactivity was assigned to specific ganglioside species as usual. There is a seperate bar graph for each subfraction. The DPM for a given ganglioside is expressed as a percent of the total DPM in the total gangliosides of that sample. Each bar represents the mean percent of four samples (the error bars are +/- S.E.M.).

SYNAPTIC PLASMA MEMBRANES

TLC: STase LABELED GANGLIOSIDES

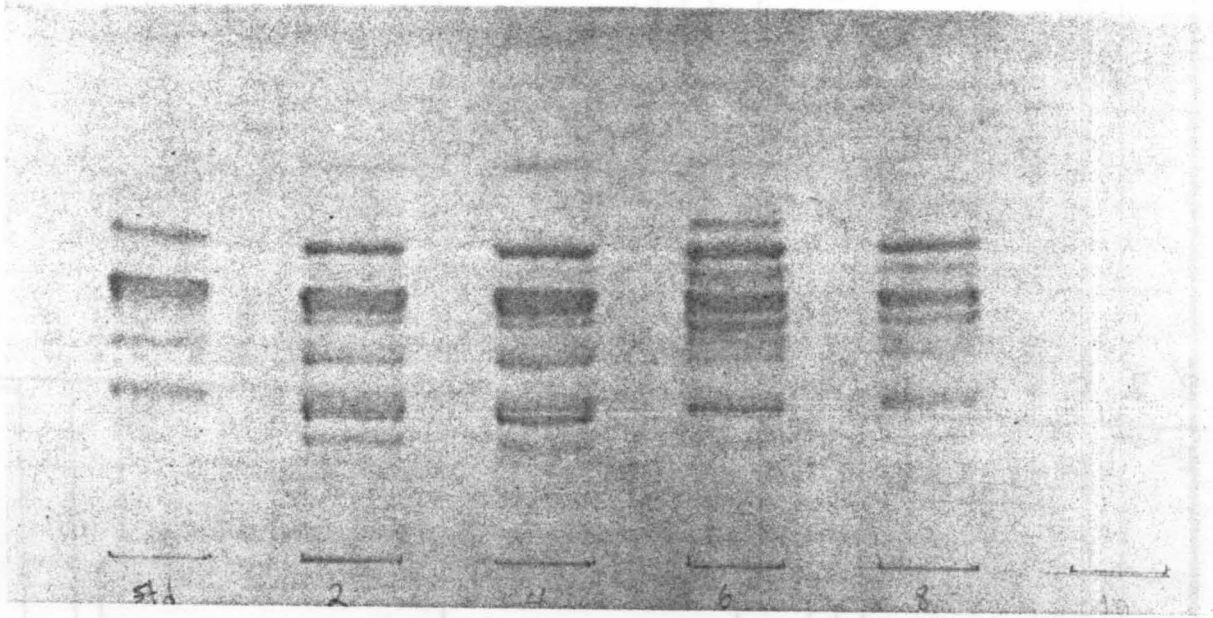


FIG. 50

SYNAPTIC PLASMA MEMBRANE SUBFRACTIONS

Product Specificity of Sialosyltransferase

Bars Represent Percent of Total DPM

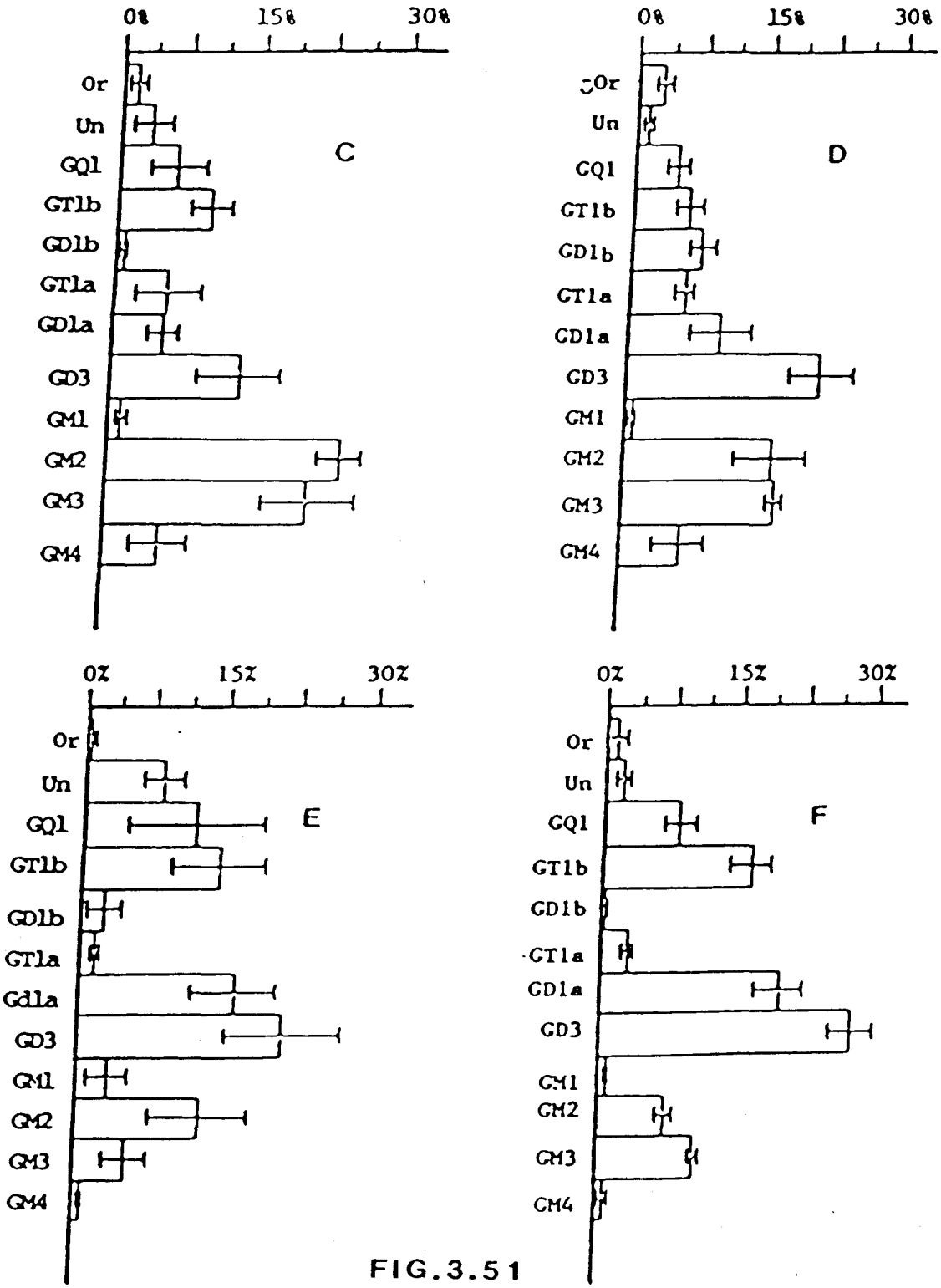


FIG.3.51

TABLE 3.7

SUMMARY OF GANGLIOSIDE DATA FROM
THE SYNAPTIC PLASMA MEMBRANE SUBFRACTIONS

The values given are the means of 4 subfractionation experiments. The standard error of the mean is shown in parenthesese. The units of each ganglioside analysis value are: percent ganglioside distribution (% DIST.), the percent ot the total resorcinol positive area for a given ganglioside species as measured by densitometric integration of TLC lanes; percent distribution or radiolabel by STase (% DPM), expressed as a percent of the total radiolabel present in the total ganglioside sample; and the relative specific radioactivity of each ganglioside (RSRA), which is %DIST / %DPM. (ND = not determined)

		SUBFRAFCTIONS				
		SPM-C	SPM-D	SPM-E	SPM-F	SPM-P
UNK	%DIS	ND	ND	ND	ND	ND
	%DPM	3.50 (2.11)	1.31 (0.54)	4.2 (1.12)	1.9 (0.71)	5.8 (1.93)
	RSRA	ND	ND	ND	ND	ND
GQ1	%DIST	7.75 (0.70)	7.80 (1.27)	9.73 (0.44)	5.65 (0.62)	7.88 (1.84)
	%DPM	6.38 (2.98)	14.5 (1.15)	12.00 (7.05)	8.15 (1.92)	6.38 (1.36)
	RSRA	0.79 (0.36)	0.66 (0.22)	1.16 (0.64)	1.59 (0.55)	1.07 (0.423)
GT1b	%DIST	20.35 (1.78)	24.25 (3.41)	18.95 (2.29)	10.75 (3.23)	9.78 (0.68)
	%DPM	10.30 (1.97)	5.98 (1.59)	13.95 (4.59)	19.95 (1.91)	16.95 (2.69)
	RSRA	0.50 (0.01)	0.28 (0.09)	0.78 (0.25)	2.06 (0.60)	1.72 (0.19)
GD1b	%DIST	14.40 (1.86)	12.93 (2.65)	10.73 (0.46)	10.83 (3.23)	13.00 (0.39)
	%DPM	0.90 (0.34)	7.53 (1.54)	2.53 (1.83)	1.03 (0.29)	0.75 (0.09)
	RSRA	0.07 (0.03)	0.69 (0.23)	0.25 (0.18)	0.10 (0.02)	0.06 (0.01)

TABLE 3.7 CONTINUED

		SUBFRACTIONS				
		SPM-C	SPM-D	SPM-E	SPM-F	SPM-P
GT1a	%DIST	4.80 (1.18)	8.73 (1.37)	8.68 (1.54)	5.13 (1.49)	3.78 (2.97)
	%DPM	5.95 (3.01)	5.78 (1.07)	1.63 (0.41)	4.38 (1.25)	4.38 (2.51)
	RSRA	1.24 (0.68)	0.76 (0.27)	0.23 (0.08)	1.11 (0.39)	1.77 (0.79)
GD1a	%DIST	28.78 (3.21)	27.03 (2.15)	21.18 (0.60)	27.39 (0.70)	18.50 (0.95)
	%DPM	5.68 (1.68)	11.15 (1.38)	16.38 (4.41)	20.13 (2.69)	19.35 (3.50)
	RSRA	0.22 (0.09)	0.42 (0.07)	0.79 (0.24)	0.96 (0.15)	1.07 (0.23)
GD3	%DIST	3.03 (0.73)	1.78 (1.24)	3.00 (0.72)	1.5 (0.45)	3.44 (1.53)
	%DPM	17.00 (4.42)	21.65 (3.58)	21.38 (5.87)	19.03 (0.56)	19.03 (5.84)
	RSRA	5.81 (1.57)	13.24 (2.97)	9.90 (5.15)	13.60 (2.01)	5.98 (1.74)
GM1	%DIST	8.80 (0.66)	11.78 (1.29)	12.10 (3.63)	12.60 (5.09)	16.65 (2.45)
	%DPM	1.48 (0.53)	1.05 (0.12)	3.48 (2.20)	1.70 (0.22)	1.70 (0.57)
	RSRA	0.17 (0.05)	0.09 (0.02)	0.35 (0.20)	0.08 (0.03)	0.12 (0.05)
GM2	%DIST	5.83 (0.73)	2.95 (0.67)	4.01 (0.97)	6.75 (1.14)	7.15 (2.01)
	%DPM	24.63 (2.18)	16.35 (4.09)	10.11 (2.90)	7.78 (0.95)	10.38 (0.86)
	RSRA	4.44 (0.70)	6.00 (1.11)	2.75 (1.22)	1.26 (0.40)	2.15 (0.90)
GM3	%DIST	8.10 (1.77)	8.48 (0.76)	5.15 (0.55)	11.50 (1.43)	9.03 (1.96)
	%DPM	21.35 (4.81)	16.83 (0.97)	5.83 (2.13)	10.73 (0.50)	11.58 (0.36)
	RSRA	7.83 (0.76)	1.99 (0.12)	1.20 (0.53)	0.96 (0.69)	1.49 (0.33)

TABLE 3.7 CONTINUED

SUBFRACTIONS

		SPM-C	SPM-D	SPM-E	SPM-F	SPM-P
GM4	%DIST	0.30	3.60	0.40	0.80	4.88
		(0.20)	(1.50)	(0.27)	(0.21)	(1.35)
	%DPM	6.00	6.35	0.91	1.00	3.58
		(3.05)	(2.78)	(0.24)	(0.48)	(2.08)
	RSRA	4.71	1.47	6.13	3.40	1.12
		(3.34)	(0.18)	(2.86)	(2.87)	(0.79)

in true synaptic plasma membranes, exhibit the unique ganglioside STase labeling pattern that has been established for synaptosomes above. Thus, the labeling pattern characteristic of synaptosomes is also characteristic of the synaptic plasma membrane, and the specific activity of the STase is increased approximately 2.5 fold in the SPM. This is significant evidence for a synaptic STase ecto-enzyme presence.

To summarize the experiments of part 2, extensive density gradient ultracentrifugation subfractionation of rat brain homogenates, have produced subfractions that are highly enriched in golgi membranes, synaptosomes and synaptic plasma membranes. The EM morphology of these three samples are compared in fig. 3.52. There are no similarities at all between the golgi fraction and the synaptic fractions. The SPM fraction appears to be derived from the synaptosomes found in the micrographs of the synaptosomal fractions. Marker enzyme analysis also shows that the two nerve ending derived subfractions are clearly different from the golgi membrane subfraction.

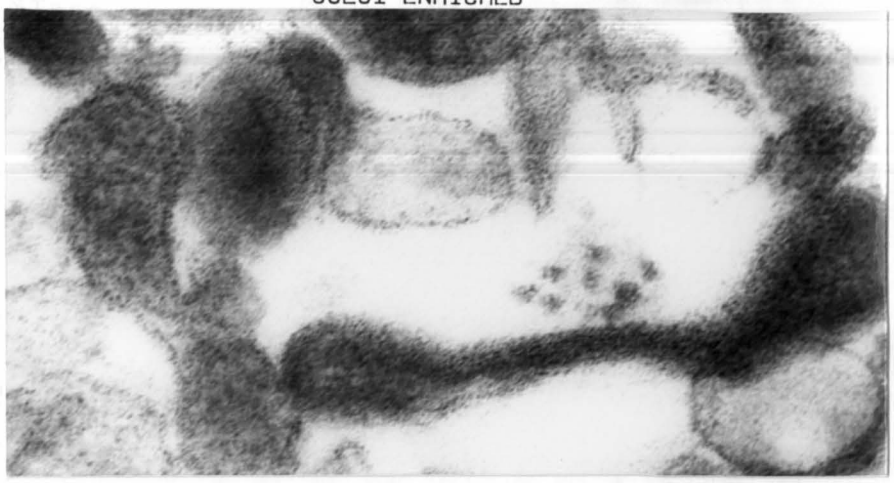
The specific activities of the total ganglioside STase of these three subfractions are compared in fig. 3.53. Golgi membranes (M-E) have the highest activity. The enzyme activity found in the intact synaptosomes (P2-e) is increased in the SPM derived from them (SPM-E). This increase is greater than the enrichment of the

Fig.3.52: morphological comparison of the golgi membrane enriched fraction (M-E), the synaptosome enriched fraction (P2-e), and the synaptic plasma membrane enriched fraction (SPM-F). The magnifications of these electron micrographs are: golgi membranes, X 310,000; synaptosomes, X 78,100; and synaptic plasma membranes, X 110,000. Clearly, the golgi preparation is very different in form than the others. Almost every structure in the preparation seems to be a densely staining dumbbell shaped crescent of small size. The synaptosome enriched preparation is mainly robust, round, closed spheres of large size containing secretory vesicles and mitochondria. The synaptic plasma membrane preparation is a homogeneous mass of open plasma membranes of large size with numerous synaptic junctional complexes visable.

FIG.3.52–MORPHOLOGIC COMPARISON
THREE MEMBRANE SUBFRACTIONS

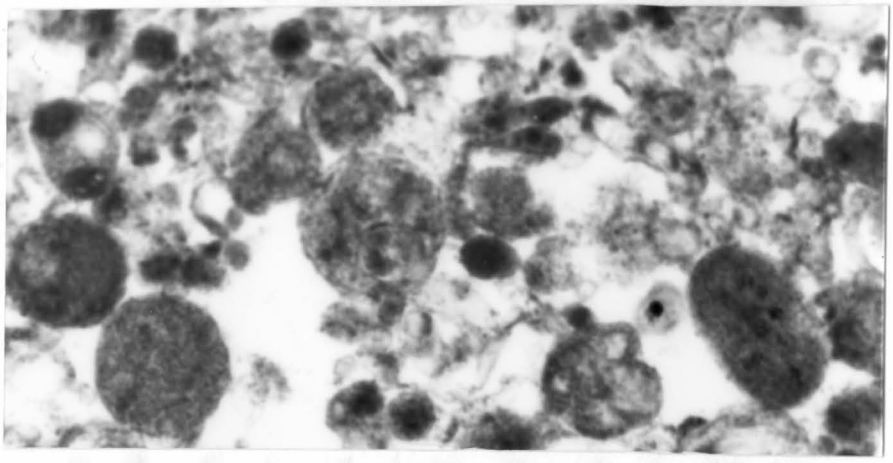
GOLGI ENRICHED

M-E



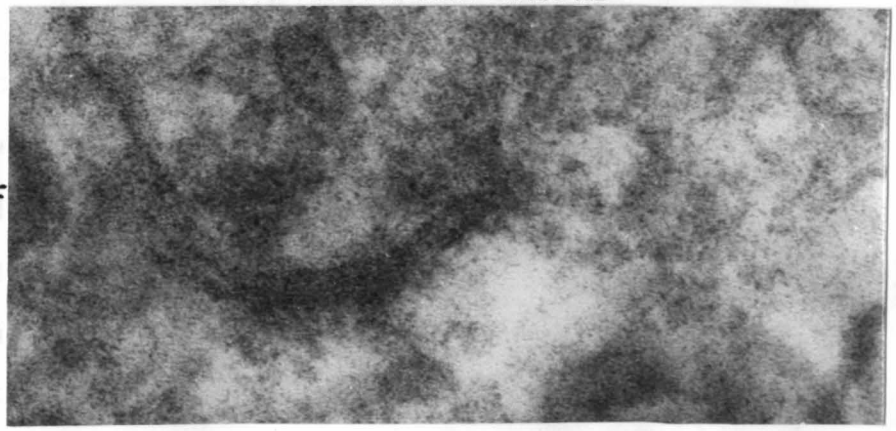
SYNAPTOSOME ENRICHED

P2-e



SYNAPTIC MEMBRANE ENRICHED

SPM-F



ganglioside concentration of SPM over the synaptosomal fraction they came from. This suggests that the location of the STase of synaptosomes is on the SPM.

The ganglioside labeling pattern of Golgi, synaptosomes, and SPM is compared in fig. 3.54. The golgi pattern is altogether different than the patterns of synaptosomes and SPM. The latter two are nearly identical to each other.

In figure 3.55 the relative specific radioactivities (RSRA) of the gangliosides labeled by STase of the three fractions are compared. Note that the Golgi STase (A.), the RSRA values are all low, and the value decreases with the number of NANA residues on each ganglioside (GQ, GT>GD>GM). Thus the Golgi STase shows little product speciicity for gangliosides. Of course there is great specificity for the "Unknown" compound, but there is no means of determining it.

On the other hand, figure 3.55 shows the high product specificity for GD3 in synaptosomal (B.) and synaptic plasma membranes (C.). The pattern is very similar in these two fractions.

These data support the concept of a cell surface ecto-sialosyltransferase enzyme activity in the nerve ending of rat brain. The substrate-product relationships of this activity are clearly different than that of the golgi. STase, involved in the <u>de novo</u> biosynthesis of .PN

FIG.3.53

COMPARISON OF GOLGI MEMBRANES, SYNAPTIC PLASMA MEMBRANES, AND SYNAPTOSOMES

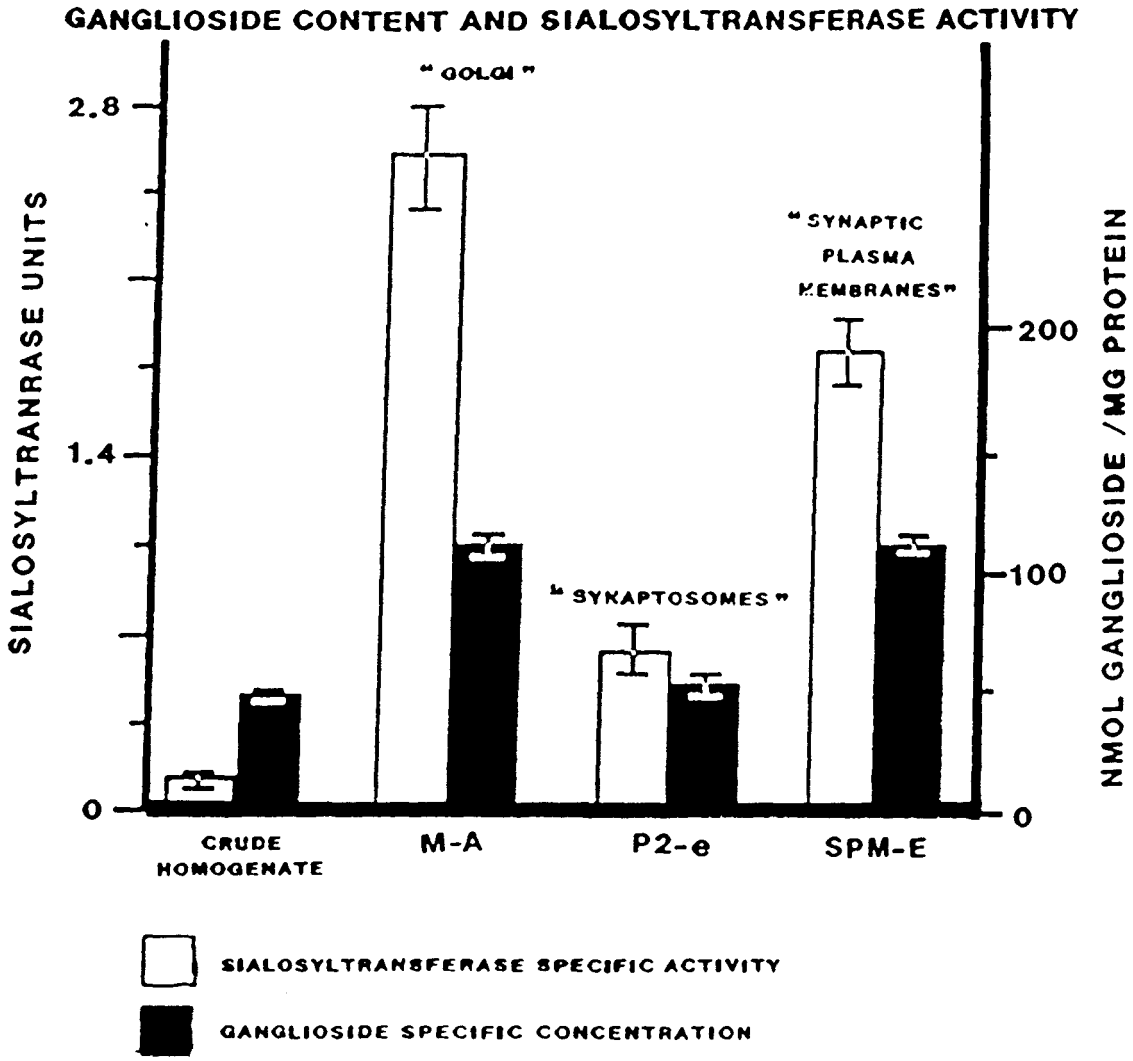


Fig.3.53: comparison of the total ganglioside specific STase activity and specific ganglioside concentration of the subfractions most enriched in golgi membranes (M-E), synaptosomes (P2-e), and synaptic plasma membranes (SPM-F). STase activity is expressed as the pmoles NANA incorporated into the endogenous total gangliosides in the given fraction per mg protein per hour. The specific ganglioside concentration of each fraction is expressed as the nmoles NANA bound to glycolipid per mg protein.

Fig.3.54: comparison of the specific ganglioside labeling patterns of the subfractions enriched in golgi membranes (M-E), synaptosomes (P2-e), and synaptic plasma membranes (SPM-F). The radioactivity incorporated into individual gangliosides by the STase of each subfraction was determined by extracting, purifying, and chromatographing the gangliosides from the samples after STase incubation (2 hr.). The TLC plates were srcaped and counted (see materials and methods for all procedures). Each bar rebresents the mean radioactivity of a given ganglioside species as a percent of the total radioactivity in the total gangliosides in the sample. A) and C) are the mean percents of four samples and B) is the mean percent of 7 samples. The error bars are the S.E.M. Synaptosomal and SPM subractions have a similar labeling pattern, but the microsomal subfraction exhibits a very different pattern.

Fig. 3.55- Comparison of the product specificity of Golgi, synaptosomal, and synaptic membrane STase by analysis of the relative specific radioactivity (RSRA) of the enriched subfractions for these membranes. The values are the means of four experiments (six for synaptosomes). RSRA is defined as % of the total DPM / % of the total gangliosides, for each ganglioside species. Values near 1.0 or below represent little or no specificity. The greater the value, the greater the enzymes specificity for a given ganglioside product.

FIG.3.54 SIALOSYLTRANSFERASE SPECIFICITY

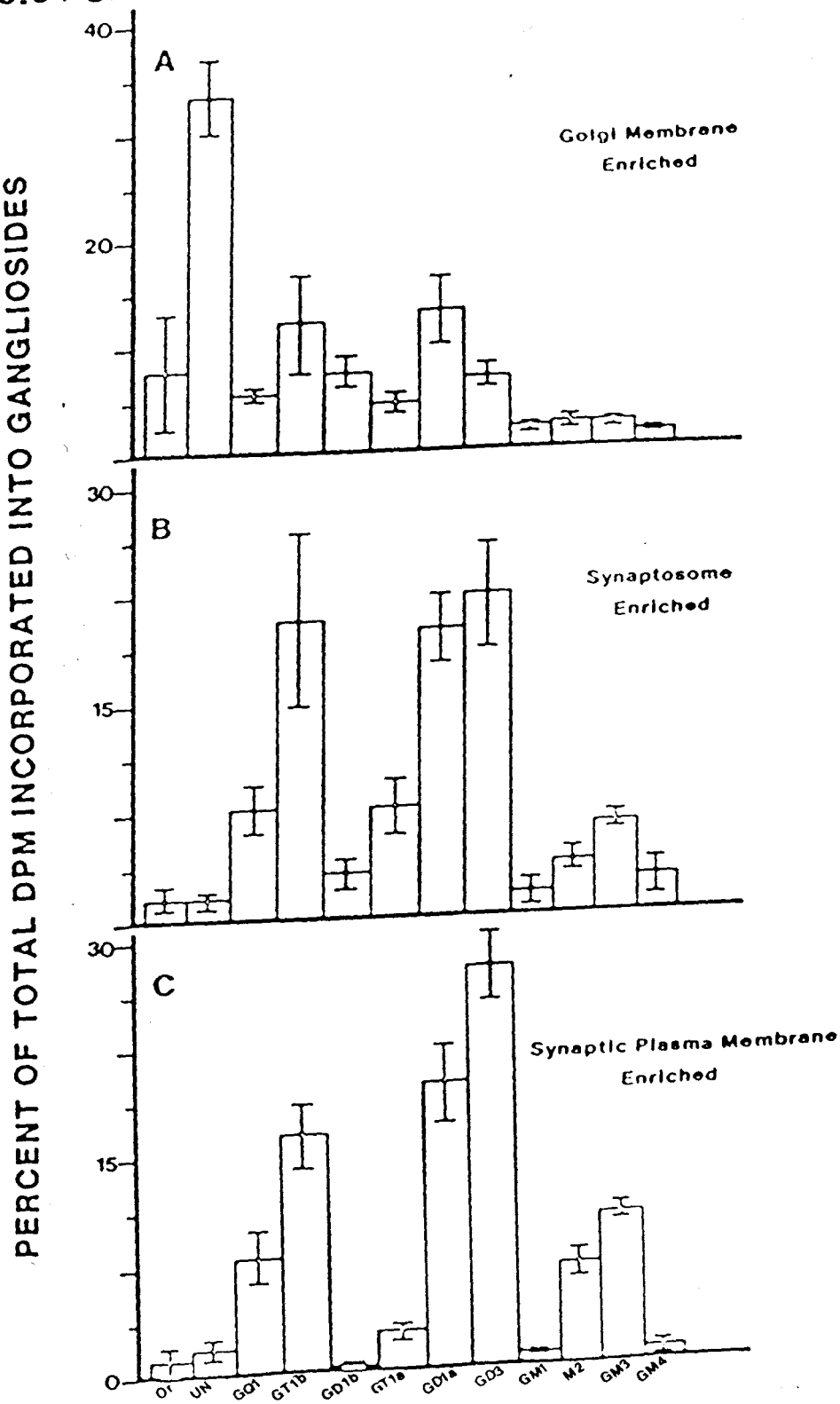
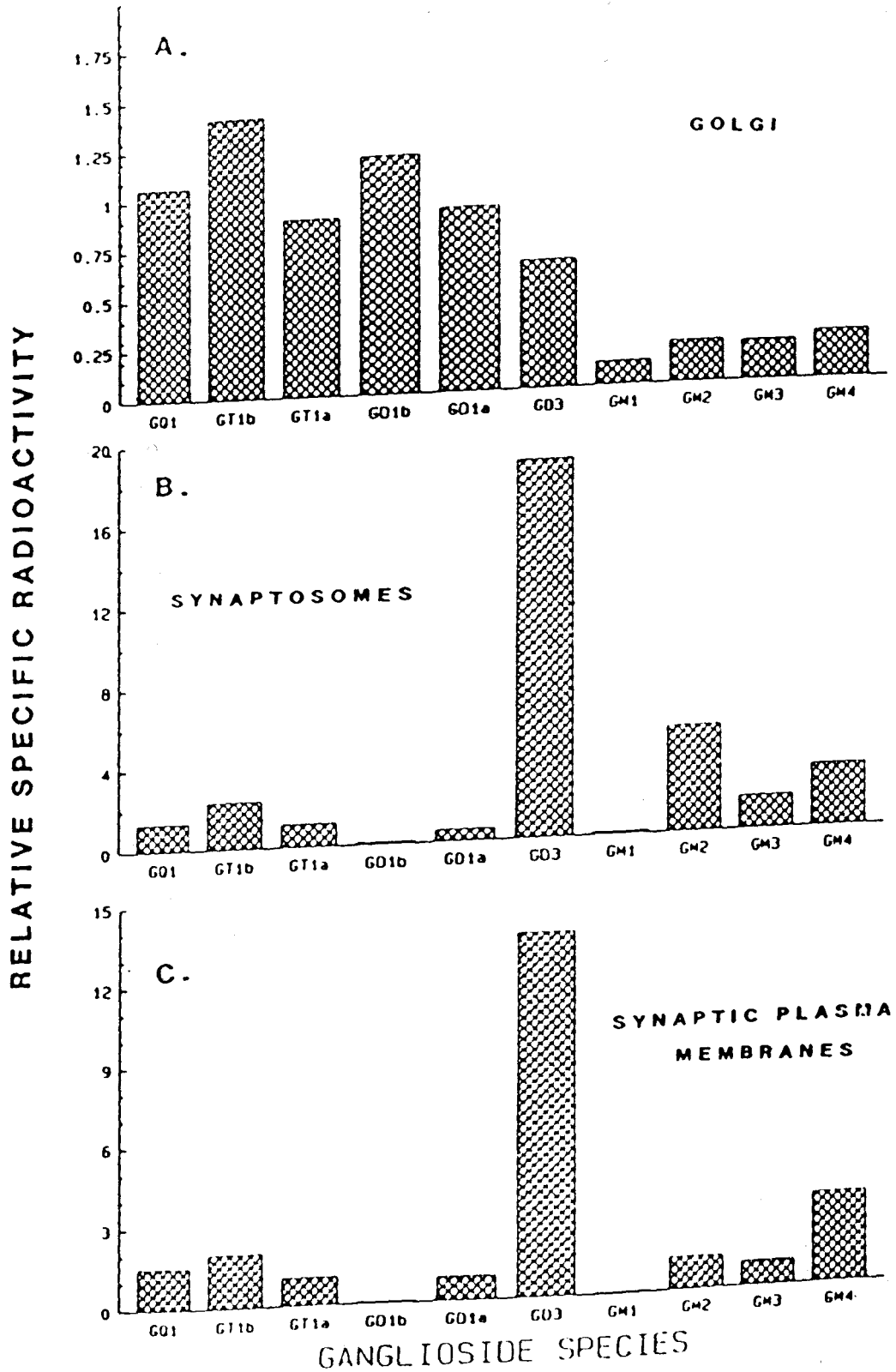


FIG.3.55 GOLGI, SYNAPTOSOME, AND SPM ST

COMPARISON OF PRODUCT SPECIFICITY



gangliosides. Possibly this represents a different function for synaptic STase. Ganglioside GD3 is highly specifically labeled by synaptic STase. This suggests a specific function for GD3 in rat brain nerve ending. But this GD3 specificity may represent favorable physical-chemical properties of this ganglioside, and/or the membrane environment of STase. The data could also support a proximal galactose disialosyl (beta 2-8) linkage specificity for the synaptic STase.

PART 3 - GANGLIOSIDE SIALOSYLTRANSFERASE IN CORTICAL BRAIN SLICES OF RAT.

The main goal of this part of my investigation is to gain additional data concerning the proposed cell surface ecto-enzyme nature of STase. The orientation of the enzyme, to be catalytically active on the outer leaflet of the plasma and/or synaptic membrane of the neuron is central to proposed functions of STase, and of CNS gangliosides.

In the cortical brain slice model, millions of neurons remain entirely intact. The nerve terminal, axon, soma, dendrites, and synaptic connections should be continuous. The metabolic pathways for CMP-NANA biosynthesis (cytoplasm and nucleus), and the centers for de novo biosynthesis of ganglioside (endoplasmic reticulum, and golgi complex) should be able to function if the neurons remain metabolically active. In addition, cell surface ganglioside metabolic pathways should be operational.

In the biosynthesis of NANA, N-acetyl-mannosamine (MANac) is locked irreversibly into the formation of NANA, and to CMP-NANA. Brain slices fed (^{14}C) MANac (incubated under the same physiologic conditions used for the membrane fractions, above) would label neuronal gangliosides. This

method has been established *in vivo* (Quarles and Brady, 1971). This process should take place through intracellular mechanisms, in the *de novo* biosynthesis of ganglioside (i.e. the golgi STase system).

Brain slices incubated with radio-labeled CMP-NANA might be more apt to label gangliosides at the cell surface. In order for this substrate to label gangliosides through the golgi STase, it would first be internalized. This probably would be by internalization of free NANA after cell surface hydrolysis of CMP-NANA, via the highly active cell surface hydrolase noted in the incubation media analysis above (part 1), and by others (Brunngraber, 1979). The internalized NANA would then be activated to CMP-NANA in the nucleus.

Incubations of cortical brain slices with these two labeled substrates were done, and the specific activities of total ganglioside STase were obtained and are shown in fig. 3.56. The specific activity of STase with the MANac substrate (graph B, "control" sample) is more than double that with CMP-NANA substrate (A, control). This may reflect the observed higher activity of Golgi STase over synaptic STase.

As an addition to the incubations, unlabeled free NANA (1.0mM) was included in the media with both

Fig.3.56: STase assay of cortical slices from rat brain. Two mm cortical slices were incubated with: A) CMP-(^{14}C)NANA ; or B) N-acetyl-(^{14}C)mannosamine, in Kreb's media at 37^{o}C in the same manner as the membrane preparations. The radioactivity incorporated into the endogenous gangliosides of the slices per mg protein was determined. Tere were no other additions to the "control" samples. The "cold NANA" samples contained 1mM unlabeled reagent grade NANA. The "monensin" samples were .2 micromolar in the ionophore monensin, which is a known inhibitor of golgi fuction. The samples marked "both" were 1mM in NANA and .2 microM in monensin. The STase units are pmoles NANA incorporated into gangliosides per mg protein per hour. The data is the mean of three samples + or - S.E.M.

Fig.3.57: comparison of the ganglioside labeling pattern of the STase of rat brain cortical slices using either CMP-(^{14}C)NANA or N-acetyl-(^{14}C)mannosamine as the substrate. After the STase incubation the gangliosides were extracted, purified, chromatographed, scraped and counted as before. The radioactivity of each ganglioside is expressed as a percent of the total radioactivity in all gangliosides. The bars represent the mean percent of 3 samples + or - the S.E.M.

Fig.3.58: STase ganglioside labeling patterns of rat brain cortical slices by the two substrates (CMP-NANA or N-acetyl-mannosamine) under the influence of two inhibitors. The radioactivity incorporated into each ganglioside is expressed as a percent of the total DPM incorporated into all gangliosides and was determined as described before. A), B), and C) are CMP-NANA incubations, and D), E), and F) are N-acetyl-mannosamine incubations. A) and D) were 1mM in unlabeled NANA. B) and E) were .2 micromolar in monensin. C) and F) contained both of these compounds in the above concentrations. Each bar is the mean percent of 3 samples + or - S.E.M.

CORTICAL BRAIN SLICE SIALOSYLTRANSFERASE
COMPARISON OF THE NANA TRANSFERRED INTO THE GANGLIOSIDE FRACTION WITH EITHER OF TWO SUBSTRATES: CMP-NANA OR N-ACETYL-MANNOSAMINE: + or − INHIBITORS

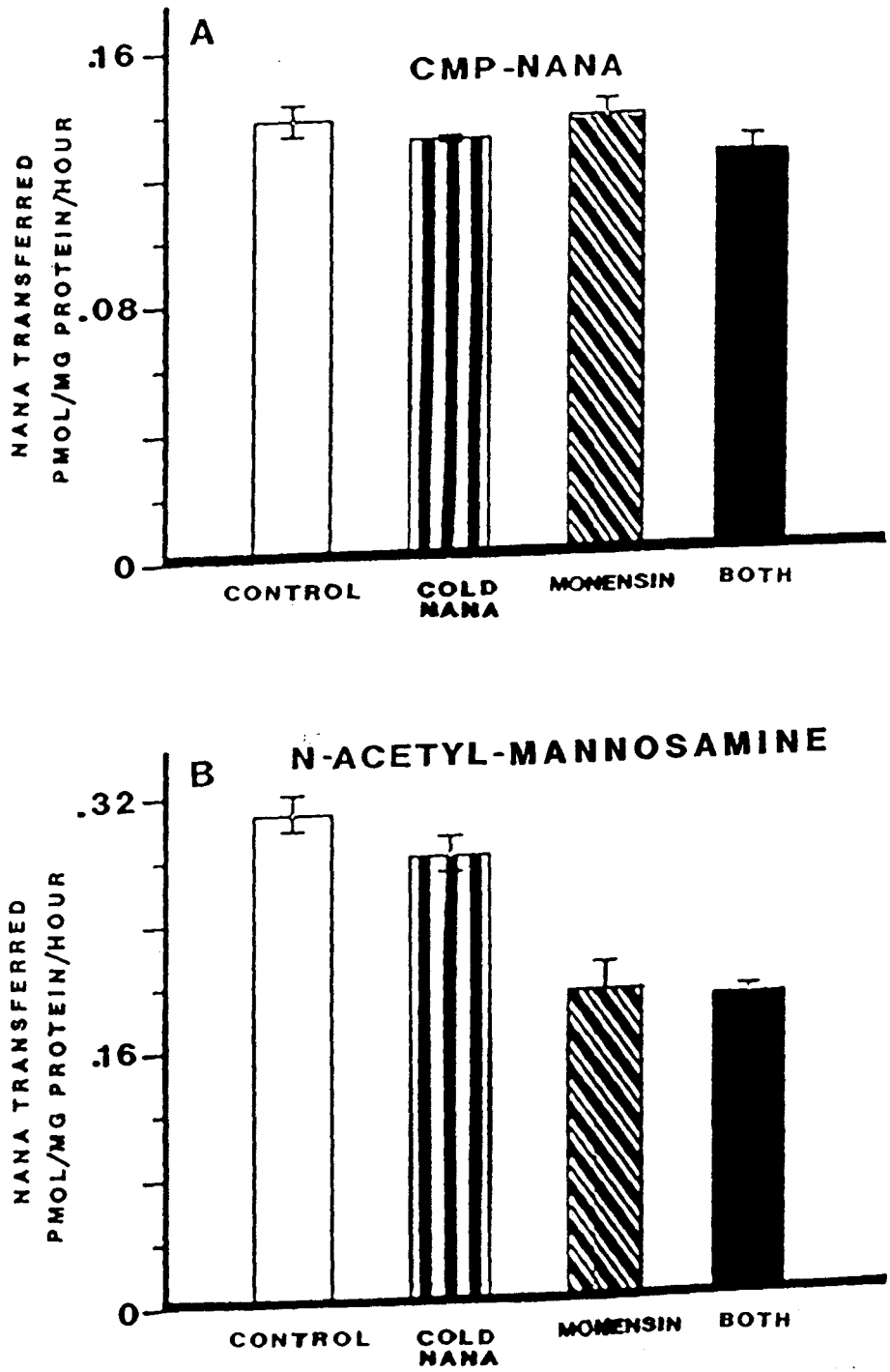


FIG.3.56

CORTICAL BRAIN SLICE SIALOSYLTRANSFERASE

COMPARISON OF THE GANGLIOSIDE LABELING PATTERNS
WHEN THE SUBSTRATE WAS CMP-NANA OR N-ACETYL-MANNOSAMINE

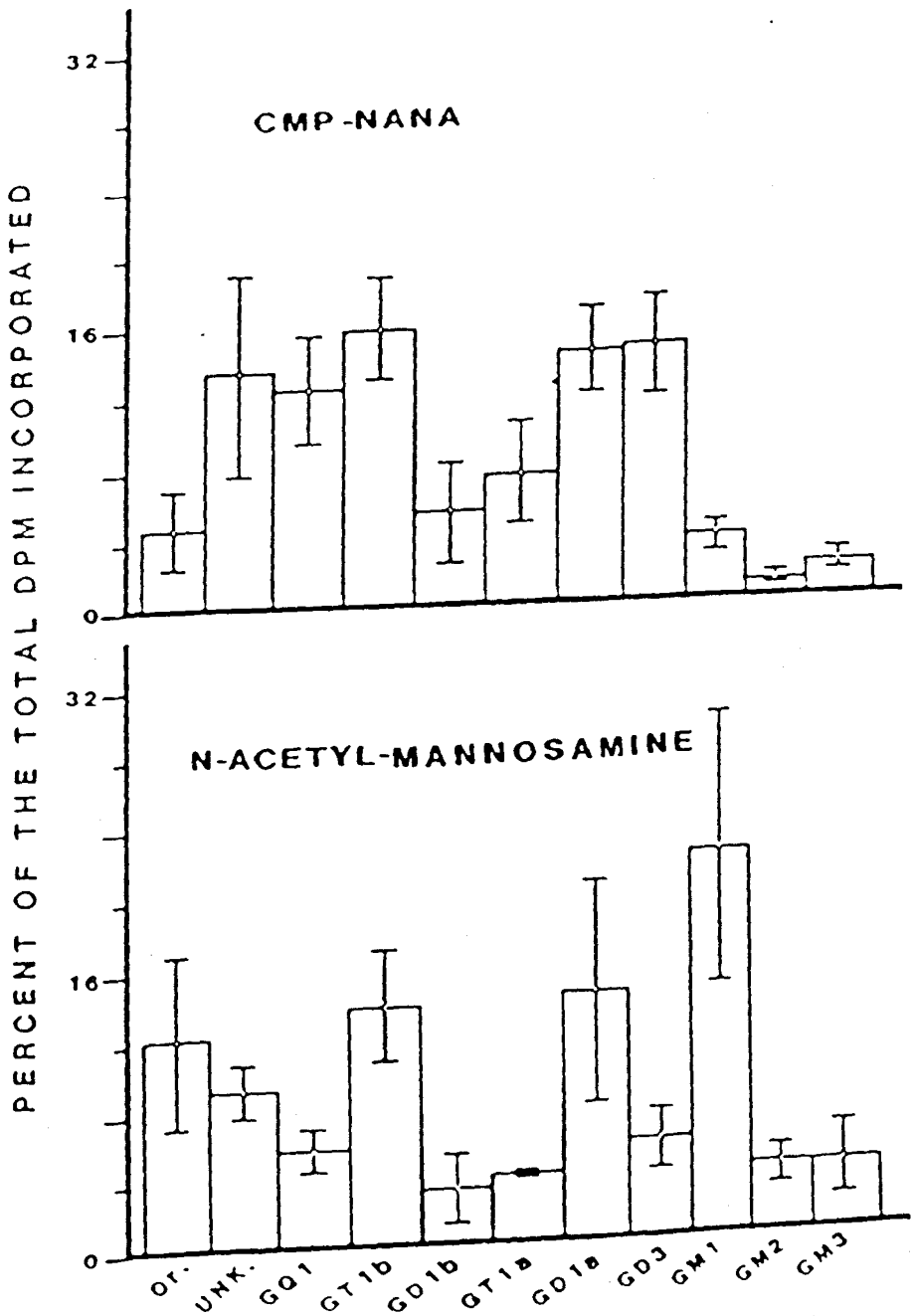


FIG.3.57

CORTICAL BRAIN SLICE SIALOSYLTRANSFERASE

EFFECT OF INHIBITORS ON THE LABELING PATTERNS OF CMP-NANA AND N-ACETYL-MANNOSAMINE

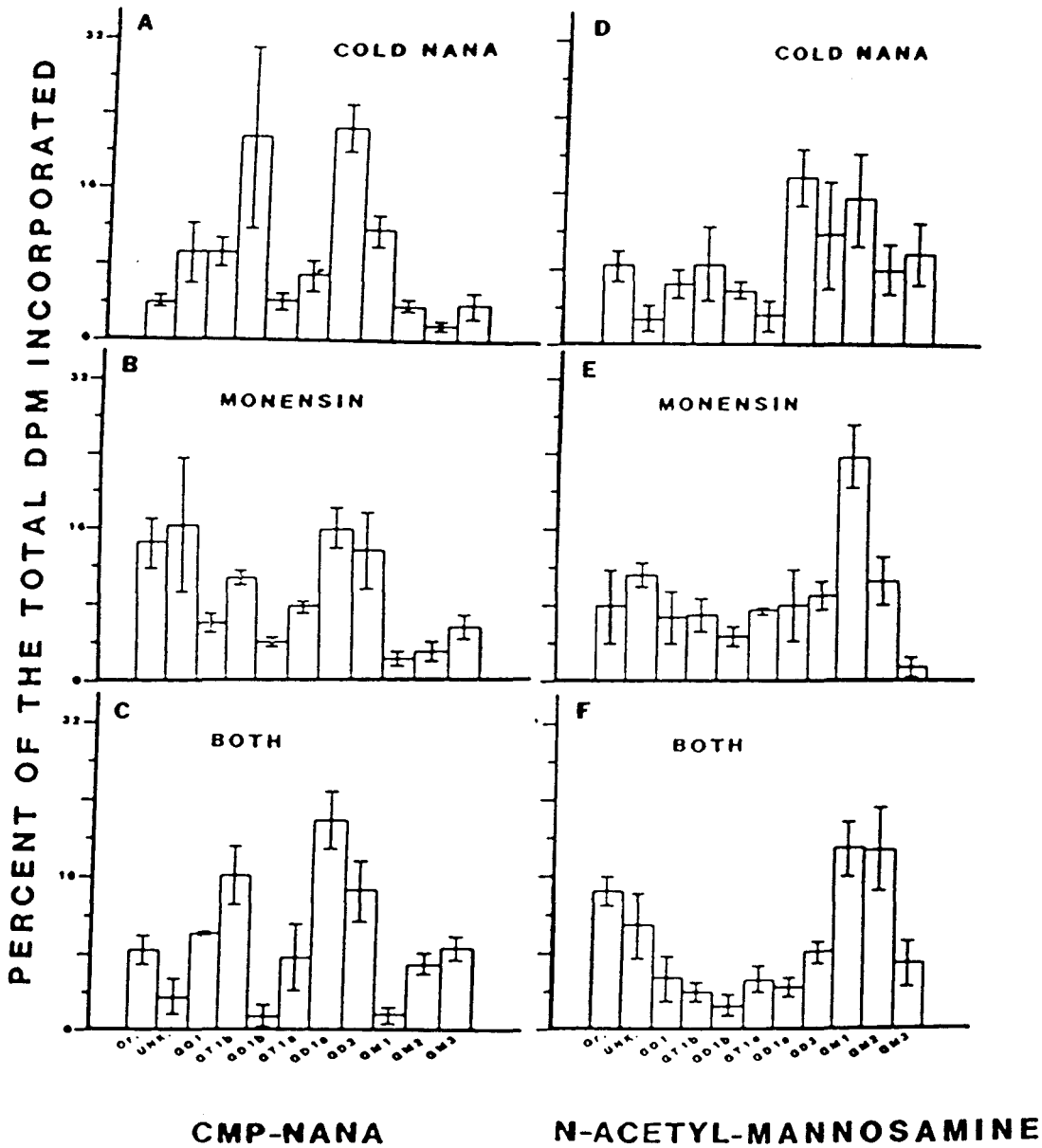


FIG.3.58

substrates. This cold NANA (in great excess) would not be a substrate for ecto-STase. To be a substrate it must be internalized and activated in the nucleus. If the CMP-NANA substrate were internalized before STase labeling, the cold NANA may reduce the labeling by dilution. This was not seen (A). With MANac there was some reduction, but is not great enough to be considered significant. The ionophore monensin has been shown to specifically inhibit the functions of the golgi complex (Tartakoff and Vassalli, 1978). It has also been shown that monensin will inhibit GSL transfer reactions of the golgi, but not affect cell surface GSL transfer reactions in experiments done on brain slices (Townsend et al., 1984). Monensin, at 0.2 microM, had little or no effect upon CMP-NANA labeling of gangliosides (A). But this level of monensin inhibited the labeling of gangliosides by MANac by approximately 30%. This is true for monensin alone, or monensin in combination with cold NANA (B). These data suggest that an ecto-STase exists on the plasma membranes of brain cells.

The ganglioside extracts from the above brain slice STase assays, were separated by TLC, and analyzed in the same way as all labeled gangliosides have been. The labeling patterns (mean percent distribution of radioactivity) for the CMP-NANA and MANac substrates are compared

in fig. 3.57. The pattern for CMP-NANA is recognizable as similar to the pattern obtained from synaptosomal and SPM STase assays. GD3 is a major site for DPM, yet is a very minor ganglioside, which indicates specificity. GT1b and GQ1 have incorporated increased amounts of label, over the synaptic STase labeling patterns. Also note that the UN area has incorporated significant DPM, and the level fluctuated widely from sample to sample (large S.E.M.).

The ganglioside labeling pattern is very different when the substrate was MANac. We have not seen this pattern before. GM1 is the greatest accumulator of radioactivity. GD1a and GT1b are also major sites for DPM. Since the entire cells are intact in this system, and the cells are metabolically viable, UDP-galactosamine and UDP-galactose should be available for the synthesis of GM1 from GM2 and GM3.

It is possible that the golgi is the major site for biosynthesis of GM1. As GM1 is incorporated into various neuronal membrane destinations, it could be further modified into higher gangliosides by membrane specific STases, to suit the needs of the membrane. Of course all ganglioside species are probably biosynthesized in the golgi. But the synthesis of the GM1 ganglio-tetrose backbone may be an important control point separating the ganglioseries gangliosides, which are the major brain type, from

the hemato-series gangliosides, which are the major extra-neural type. It would be interesting to include UDP-GAL and UDP-GALNac in the STase incubations of the purified golgi membranes isolated in part 2, and determine if GM1 is highly labeled, and if the labeling pattern resembles the pattern obtained here for brain slices incubated with MANac.

In figure 3.58, the labeling patterns of brain slice STace by CMP-NANA and by MANac are shown as influenced by the addition of cold NANA and/or monensin. In general, the CMP-NANA pattern remains similar to the synaptic pattern. It should be noted that certain gangliosides show marked elevations in incorporation (e.g. GT1b in graph A, and UN in graph B), but these are accompanied by large sample to sample variation (note large error bars, S.E.M., n = 3). Usually one sample was very high, resulting in a high mean value. Therefore, it is difficult to interpret these results.

The labeling patterns of the MANac substrate with the inhibitors is likewise confusing. The high GM1 labeling is consistant in all three conditions. In the incubations containing monensin (E and F) it appears that the label in GD1a, GT1b, and GQ1 are all decreased over the control labeling pattern. It is believed that monen-

sin inhibits golgi function by hindering the cis to trans intra-golgi migration of sub golgi membrane compartments. It is possible that GM1 synthesis is compartmentalized differently in the golgi than the higher species, and is thus unaffected by monensin action. But I should restrain myself from over interpreting this data.

These experiments of part 3 represent only a preliminary study of STase in the brain slice model. Thus far the data supports the existence of a cell surface ecto ganglioside STase activity. This model has exciting possibilities and many of my future research plans will utilize the brain slice.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In Part 1, rat brain synaptosomal preparations exhibited a low, but measurable ganglioside sialosyltransferase activity. The possibility exists that this activity is an artifact, due to contamination of the preparations by microsomal membranes such as Golgi complex which are known to synthesize gangliosides. The morphology of the synaptosomal preparations showed no evidence of golgi membranes. No prior reports have described the appearance of golgi membranes in synaptosomal preparations or in the nerve ending *in situ* (Tettamanti *et al.*,1980; Jones, 1975). The data of Part 1 are supportive of a synaptosomal STase activity, but difinitive existence of a synaptic plasma membrane ecto-sialosyl- transferase must be left to further proofs.

The most significant finding in part 1, is that the synaptosomal STase shows a great specificity for the ganglioside GD3. This is all the more striking since GD3 is a minor ganglioside in rat brain and that the ganglioside substrate for this transfer, GM3, is also small in quantity. A NANA transferred to GM1, to form GD1a would

be much more favorable according to the laws of mass action. But this reaction was found to be much less specific. The high GD3 specificity may be due to purely physical properties of the enzyme and substrates in the membrane, such as lateral mobility or molecular size considerations.

In part 2 rat brain homogenate was subfractionated extensively, and subfractions were produced that were highly enriched in Golgi complex membranes, in synaptosomes, and in synaptic plasma membranes. These were confirmed by marker enzyme, and EM morphological analysis. Golgi complex membranes had approximately 4 times greater STase activity than synaptosomes. Synaptic plasma membranes were about 2.5 higher in STase activity than synaptosomes. The latter suggests that the location of STase in synaptosomes is within the synaptic plasma membrane.

The ganglioside product specificities of STase activities of the three subfractions were determined. Golgi membranes showed great specificity for an unknown sialic acid containing compound (UN) in the ganglioside extract, running between the origin and GQ1 on TLC plates. Otherwise the Golgi labeling pattern showed incorporation of radiolabel roughly corresponding to the number of NANA residues per ganglioside species. This is interpreted as being nonspecific for any particular ganglioside.

The labeling patterns of purified synaptosomes and

synaptic plasma membranes were identical to each other, and were totally different from Golgi membranes. The synaptic STase had the high GD3 labeling specificity that was described for the traditionally prepared synaptosomes (above).

Brain slice experiments showed that gangliosides were labeled during incubation with N-acetyl mannosamine and had a grossly different radioactivity pattern than when CMP-NANA was the labeling substrate. The latter pattern was similar to the synaptic STase pattern. MANac must be internalized and converted to CMP-NANA before it can label gangliosides (presumably via Golgi STase). The labeling pattern difference supports the existence of a cell surface ecto-STase activity. This was enhanced by the fact that monensin (a Golgi inhibitor) reduced the MANac labeling, but not the CMP-NANA labeling.

The conclusion of these experiments is that a synaptic ganglioside sialosyltransferase ectoenzyme activity does exist in rat brain, and the activity has very high specificity for labeling GD3. It is possible that this specificity is actually for the disialo (2-8) linkage on the proximal galactose residue, but this cannot be concluded by these data alone.

The existence of this transferase, suggests a biological role for GD3 (or the proximal disialo group) in the

process of synaptic transmission, involving the metabolism of Ca^{++} (Veh and Sander, 1981; Svennerholm, 1980), or in the involvement of gangliosides in synaptic plasticity (reviewed by Ledeen, 1984; and Cotman and Nieto-Sampero, 1984).

There have been numerous reports that have suggested that ganglioside GD3 has a biometabolism that is much different than other gangliosides. GD3 belongs to the hemato- series of glycosphingolipids rather than the true ganglio- series to which most of the major brain gangliosides belong. Ando and Yu (1984) have shown in a limited study, that the hemato-series gangliosides have a different fatty acyl component in their ceramide moiety than do the other species, which may represent a branch point in ganglioside synthesis.

Developmentally, ganglioside patterns change during differentiation and maturation of the CNS. In chick brain, during the period of neuron differentiation and synaptogenisis, GD3 levels fall drastically (Dreyfus et al., 1975). The same phenomenon was noted in fetal rat and mouse brain development (Irwin et al., 1980). Many researchers have reported that gangliosides, mainly of the ganglio- series, have potent neurotrophic and neuritogenic properties, as reviewed by Ledeen, 1984. These seem to suggest that GD3 may be important to the neuron as it shifts its development from growth to functional differ-

entiation.

Eto and Shinoda (1982) have reviewed the ganglioside patterns of several transformed cell lines from the CNS, and have found a consistent elevation in GD3 levels over normal cells. A monoclonal antibody, raised against a melanoma cell line, was found to recognize O-acetylated-GD3 specifically, and does not bind to normal cells (Cheresh et al., 1983). Transformed cells by the simplest definition, are cells that have lost the ability to control their growth. Could GD3 participate in transformation?

The above observations raise questions about GD3's involvement in the control of neuronal growth (extension of axons), and the initiation of differentiation (neurite formation, synaptogenesis, and the formation of functional connections). These events correlate with a decrease in GD3 levels and increase in the complex gangliosides (Willinger, 1981; Dreyfus, 1980). In a review by Cotman and Nieto-Sampedro (1984), neurons transplanted into proper areas of the brain would surive and form connections, but axons would not regenerate long distances. On the other hand, similar transplantations of CNS neurons into peripheral nerve did show that axons would regenerate over long distances. This is interesting because a major difference in the ganglioside patterns of CNS and PNS is that

PNS has a significantly higher level of GD3 (Klein and Mandel, 1975).

During the initial contact stages of fusion of cultured myoblasts into myotubes, there is a great surge in the formation of a di-sialosyl ganglioside (Whatley et al., 1976), which was later identified as GD3 (McKay,1980). This suggests the involvement of GD3 and possibly an ecto-STase activity in the cell-cell interactions of myogenesis.

My work shows that there exists a glycolipid sialosyltransferase activity with GD3 product specificity in rat brain synaptic plasma membranes. This transferase may function to maintain and control the GD3 population of the nerve ending plasma membrane, with subsequent contribution to the cell-cell interactions of axonal extension, directional growth, and synaptogenesis.

There is new evidence which suggests another area that could involve specific function for GD3. In a most recent article by Seyfried and Yu (1985), many significant reports describing GD3 biochemistry were surveyed. The observation was made that GD3 levels were high in certain cell types. Among these are: reactive glia; glioma cells; malignant astrocytoma cells; melanoma cells; leukemic cells; adenovirus transformed cells; embryonic tissues such as endodermal and mesodermal epithelium; Meuller cells of the retina; the cell bodies of oligodendroglia

(not mature myelin); and undifferentiated neuroblasts. Mature neurons are consistently very low in GD3.

Seyfried and Yu go on to suggest that all of these cell types are characterized by having plasma membranes that are highly permeable to certain ions. These cells all absorb large quantities of metabolites from the extracellular fluid. Thus they propose a specific function for GD3 in trans membrane transport.

GD3 has a unique structure among all other gangliosides. Along with GM3, it has much greater content of C_{20} to C_{26} fatty acyl groups in its ceramide moiety. It should behave differently in a lipid bilayer than the other gangliosides due to its physical properties. Also GD3 has a divalent negative charge. It has a one to one relationship when it binds to divalent cations such as calcium ion. When such a GD3-ion complex forms the complex would be electro neutral. Since GD3 has relatively non-bulky head group, is neutral when bound with divalent cation, and has a unique lipophilic component, it is possible that these GD3-ion complexes could self aggregate into an intramembrane macromolecule that could serve as a ionophore or "ion pore".

The consideration of some cell types support this GD3-ion transport system. For instance, the oligodendrite perikaryon is responsible for the maintenance of the mye-

lin sheath. It is highly permeable to metabolites, and is rich in GD3. Myelin functions as the insulator of the neuronal axon and must closely regulate ion and metabolite access to the axon. Myelin is very low in GD3, even though it is continuous with the GD3 rich oligodendrite perikaryon.

Also consider the undifferentiated neuroblast. At this stage the cell is actively growing and extending axons. It is absorbing metabolites and ions in great quantities, and is rich in GD3. The mature, functional neuron must control its ion permeability precisely to conduct impulses. Mature neurons are very low in GD3.

There also is a possible function for the GD3 specific STase, and GD3 in synaptic transmission. The strongest evidence for this is in the *in situ* location of these components. The GD3 specific STase I have described in the synaptic membrane may be a part of a highly controlled system that regulates ion permeability of the synaptic membrane during synaptic transmission.

A remaining question that must be answered is: What is the source of the CMP-NANA substrate for this transferase? This a very important question and cannot be overlooked. The availability of CMP-NANA would be a control point for a desialosylation-resialosylation system in the synapse. There is no evidence for any source. It would not be proper to suggest any function for GD3-STase until this

source is established. Therefore the data brought forth in this dissertation open many questions, and leaves numerous avenues for further investigation.

REFERENCES

Andu S., and Yu R.K., (1984) Fatty acid and long chain base composition of gangliosides isolated from adult human brain. J. Neuroscience Res., 12:201-211.

Bassi S., Albizzati M., Sbacchi M., Frattola L., and Massarotti M.(1984) Double blind evaluation of monosialoganglioside GM1 therapy in stroke. J. Neurisci.Res. 12:493-498.

Boegmam R.J.(1976) Rat brain gangliosides following drug addiction and nutritional defieiency. Adv. Exp. Med. Biol. 71:267-273.

Booth R.F., and Clark J.B., (1978) A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. Biochem. J., 176:365-370.

Bradley W.G. (1983) Double-blind controlled trial of purified brain gangliosides in amyotrophic lateral sclerosis and experience with poriferal neuropathies. Adv. Exp. Med. Biol. 174:565-874.

Breckenridge W., Gombos G., and Morgan L. (1972) The lipid composition of adult rat brain synaptosomal plasma membranes. Biochem. Biophys. Res. Comm. 266:695-701.

Brodbeck U., Gentinetta R, and Lundin S.J., (1973) Multiple forms of a cholinesterase from body muscle and possible role of sialic acid in cholinesterase reaction specificity. Acta Chem. Scand. 27:561-572.

Brunngraber E.G., (1979) Gangliosides . In: Neurochemistry of Aminosugars. pp 3-55. Charles C. Thomas Publisher, Springfield, IL (USA).

Burton R.M. (1976) Gangliosides and Proteins of brain synaptic components. Adv. Exp. Med. Biol. 71:123-137.

Caputto R., Maccioni H.J., Arce A., and Cumar R.F.A., (1976) Biosynthesis of brain gangliosides. Adv. Exp. Med. Biol. 71:27-44.

Cheresh D.A., Varki A.P., Varki N.M., Stallcup W.B., Levine J., Keisfeld R.A., (1984) A monoclonal antibody recognizes an O-acylated sialic acid in a human melanoma-associated ganlioside. J. Biol. Chem., 259:7453-7459.

Corfield A.P., Michalski J.-C., and Schauer R., (1981) The substrate specificity of sialidase from microorganisms and mammals. Perspective in Inherited Metabolic Diseases, 4:3-70.

Corfield A.P., and Schauer R. (1982) Occurence of sialic acids. Cell Biol. Monographs, 10:5-50.

Cotman C.W., and Mathews D.A. (1971) Synaptic plasma membrane from rat brain synaptosommes; isolation and partial characterization. Biochim. Biophy. acta, 249:380-394.

Cotman C.W. (1972) Principles for the optimization of centrifugation conditions for fractionation of brain tissue. Res. Methods Neurochem. 1:45-92.

Cotman C.W., and Nieto-Sampedro M., (1984) Cell biology of synaptic plasticity. Science, 225:1287-1294.

Dawson G., and Berry-Kravis E. (1984) Gangliosides as modulators of the coupling of neurotransmitters to adenylate cyclase. Adv. Exp. Med. Biol. 174:341-354.

DeFelice S.L., and Ellenberg M. (1983) Gangliosides-clinical overview. Adv. Exp. Med. Biol. 174:625-628.

Den H.B., Kaufman B., McGuire E.J., and Roseman S. (1975) The Sialic Acids. XVIII. Subcellular distribution of seven glycosyltransferases in embryonic chick brain. J. Biol. Chem. 250:739-746.

Deppert W., and Walter G., (1978) Cell surface glycosyl-transferases-do they exist? J. Supramol. Struct. 8:19-37.

DeRobertis E., Lapetina E.G., and Fiszer de Plazas S. (1976) Subcellular distribution and possible role of gangliosides in the CNS. Adv. Exp. Med. Biol. 71:105-121.

Derry D., and Wolfe L. (1967) Gangliosides in isolated neurons and glial cells. J. Biol. Chem. 250:739-743.

Dette G.A. and Weseman W. (1978) On the significance of sialic acid in high affinity 5-hydroxy-tryptamine uptake by synaptosomes. Hopp. Saylers Z. Physiol. Chem. 359:390-406.

DeVries G.H., and Zmachinski C.J., (1980) The Lipid Composition of Rat CNS Axolemma-enriched Fractions. J. Neurochem. 34:424-430.

Dippold W.G., Lloyd K.O., Li L.T.C., Ikata H., Oettgen F., Old L.J., (1980) Cell surface antigens of a human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. Proc. Nat. Accad. Sci., 77:6114-6118.

Dimpfel W. Huang R., and Haberman E. (1977) Gangliosides in nervous tissue cultures and binding of ^{135}I labeled tetanus toxin. J. Membr. Biol. 69:85-97.

Dreyfus H., Hoflack B., Louis J.C., Harth S., and Massarelli R., (1981) Biosynthesis of glycoconjugates in pure cultures of neurons: presence of ectco-glycosyltransferases. Adv. Exp.Med. Biol. 152:325-332.

Dreyfus H., Urban P.F., Edel-Harth S., Mandel P., (1975) Developmental patterns of gangliosides and of phospholipids in chick retina and brain. J. Neurochem., 25:245-250.

Dreyfus H., Ferret B., Harth S., Gorio A., Freyzs L., Massarelli R., (1984) Effect of exogenous gangliosides on the morphology and biochemistry of cultured neurons. Adv. Exp. Med. Biol., 174:513-524.

Dreyfus H., Louis J.C., Harth S., Mandel P., (1980) Gangliosides in cultured neurons. Neuroscience., 5:1647-1955.

Eisenbarth G.S., Walsh F.S., and Nirenberg M. (1979) Monoclonal antibody to a plasma membrane antigen of neurons. P. N. A. S. 76:4913-4917.

Ellman g.l., Courtney K.D., Andres V., and Featherstone R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharm. 7:88-95.

Eto Y., Shinoda S., (1982) Gangliosides and neutral glyco- sphingolipids in human brain tumors; specificity and their significance. Adv. Exp. Med. Biol., 152:279-290.

Fishman P.H., (1982) Role of membrane gangliosides in the binding of and action of bacterial toxins. J. Membrane Biol., 64:85-97.

Folch-Pi J., Arsore S., and Meath J.A., (1951) Isolation of brain strandin, a new type of molecule tissue component. J. Biol. Chem. 191:819-831.

Fonnum F., (1975) Radiochemical Assays for choline acetyltransferase and acetylcholinesterase. Res. Method. Neurochem. 3:253-275.

Gahmberg C.G., and Hakomori S. (1973) External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. 248:4311-17.

Gasa S., Makita A., (1982) Glycolipid glycosyltransferase levels in surgical and transplanted human lung cancers. Avd. Exp. Med. Biol., 152:159-165.

Gorio A., Aporti F., DiGregorio F., Schiarinato A., Silipramdi R., and Vitadello M. (1983) Ganglioside treatment of genetic and alloxan-induced diabetic neuropathy. Adv. Exp. Med. Biol. 174:549-564.

Graus F., Cordon-Cardo C., Houghton A.N., Melamed M.R., Old L.J., (1984) Distribution of ganglioside GD3 in the human nervous system detected by R_{24} mouse monoclonal antibody. Brain Res., 324:190-194.

Gray E.G., and Whittaker V.P. (1962) Isolation of nerve endings from brain and electron microscope study of cell fragments derived by homogenation and centrifugation. J. Anat 96:79-88.

Hakomori S. (1981) Glycosphingolipids in cellular interaction, differentiation, and oncogenisis. Ann. Rev. Biochem. 50:733-764.

Hakomori S. (1984) Ganglioside receptors: a brief review and introductory remarks. Adv. Exp. Med. Biol. 174:341-354.

Hartree E.F. (1972) Determination of protein; a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48:422-425.

Hansson H.A., Holmgren J., and Svennerholm L. (1977) Ultrasrtuctural localization of cell membrane GM1 ganglioside by cholera toxin. P. N. A. S. 74:3782-3786.

Hess H.H., Leis M.B., and Derr J.E. (1978) A linear Lowry-Folin assay for both water soluble and sodium dodesyl sulfate solubilized protiens. Annalyt. Bioch. 85:295-300.

Hubel D.H. (1979) The brain. Sci. Amer.241:45-53.

Irwin C.C., and Irwin L.N., (1979) A simple rapid method for ganglioside isolation from from small amounts of tissue. Anal. Biochem., 94:335-339.

Irwin L.N., Michael D.B., and Irwin C.C., (1980) Ganglioside patterns of fetal rat and mouse brains. J. Neurochem., 34:1527-1530.

Irwin R.L., and Trams E.G. (1962) Reduction of neuromuscular blocking activity of quaternary compounds by gangliosies. J.Pharm. Exp. Therap. 137:242-248.

Janigro D., Di Gregario F., Vyskocil F., and Gorio A. (1984) Gangliosides Dual Mode of Action: A Working Hypothesis. J. Neuroscience Res. 12:2/3:499-509.

James F. and Fotherby K. (1963) Distribution of of lipid bound sialic acid and facters affecting its concentration. J. Neurochem. 10:587-592.

Jeanloz R.W., and Codington J.F. (1976) The Biological Role of Sialic Acid at the Cell Surface. In: Biological Roles of Sialic Acid (Rosenberg A. and Schengrund C.-L., eds.) pp 201-238. Plenum Press, New York.

Jones D.G. (1975) The Preparation of Synaptosomes and Derivatives. In: Synapses and Synaptosomes pp. 44-72. Chapman and Hall Ltd, London U.K.

Jonsson G., Gorrio A., Hallman H., Janigro D., Kojima H., Luthman J., and Zanoni R. (1984) Effects of GM1 ganglioside on developing and mature serotonin and noradrenalin neurons by selective neurotoxin. J. Neurosci. Res. 12:459-476.

Kanfer J.N. (1983) Sphingolipid Metabolism. In: Sphingolipid Biochemistry. Handbook of Lipid Research Vol. 3, pp 184-238. Plenum Press, New York.

Keenan T.W., Moore D.J., and Basu S., (1974) Ganglioside biosynthesis: Concentration of glycosphingolipid glycosyltransferases in golgi apparatus from rat liver. J. Biol. Chem. 249:310-315.

Kishimoto Y. (1983) Sphingolipid Formation. In: The Enzymes, Volume XVI (Boyer P.D., ed.) pp 358-401, Academic Press Inc., London, U.K.

Klein F., and Mandel P., (1975) Gangliosides of the poripheral nervous system of the rat. Life Sci., 16:751-759.

Klenk E., and Langerbeins H. (1941) Hoppe-Seyler's Z. Physio. Chem. 270:185-193.

Lacetti P., Tombaccini D., Aloj S., Grollman E.F., and Kohn L.D. (1984) Gangliosides, the thyrotropin receptor and autoimmune thyroid disease. Adv. Exp. Med. Biol. 174:355-368.

Laine R.A. (1981) Glycophosphosphingolipids; "ganglioside like" glycolipids from plants and fungi. Adv. Exp. Med. Biol. 152:115-120.

Landa C.A., Maccioni A.J., Arce A., and Caputto R. (1977) The biosynthesis of gangliosides. Separation of membranes with different ratios of ganglioside sialosylating ability.Biochem. J. 168:673-683, 325-332.

Ledeen R.W. (1978) Ganglioside structures and distribution: are they localized at the nerve ending? J. Molecular Structure 8:1-17.

Ledeen R.W., (1979) Structure and distribution of gangliosides. In: Complex Carbohydrate of the Nervous System (Margolis R.U., and Margolis R.K.,eds.). Plenum Press, New York and London.

Ledeen R. W. (1984) Biology of gangliosides: neuritogenic and neurotrophic properties. J. Neuroscience Res. 12:2/3:147-160.

Ledeen R.W., Skrivanek J.A., Nnace J., Sclafani J.R., Morton W.I.,and Farooq M., (1981) Implication of the distribution and transport of gangliosides in the nervous system. In: Gangliosides in Neurological and Neuromuscular Function, Development and Repair (Rapport M.M., and Gorio A., eds.). Raven Press, New York.

Ledeen R.W. and Yu R.K. (1976) Chemistry and Analysis of Sialic Acids. In: Biological Roles of Sialic Acids. pp 1-57, Plenum Press, New York.

Ledeen R.W. and Yu R.K. (1982) Gangliosides: structure, isolation, and analysis. Meth. Enzymol. 43:139-191.

Leon A., Benvegnu D., DalToso R., Presti D., Facci L., Giorgi O., and Toffano G. (1984) Dorsal root ganglia and nerve growth factor: a model for the understanding of GM1 effects on neuronal repair. J. Neuroscience Res. 12:2/3:277-288.

Leskawa K., and Rosenberg A. (1980) Structural modifications of gangliosides in synaptic membranes. In: Structure and Function of Gangliosides (Svennerholm L., Dreyfus H., and Urban P.-F., eds.). Plenum Press, New York and London.

Leskawa K., and Rosenberg A. (1981) The organization of gangliosides and other lipid components in synaptosomal plasma membranes and modifying effects of calcium. Cell Mol. Neurobiol. 1:373-388.

Lowry O.H., Rosenbrough N.J., Farr A.L., and Randell R.J. (1951) Protein Measurement with the Folin Phenol Reagent. J. Boil. Chem. 193:265-275.

Maggio B., Cunar F.A., and Capputo R. (1978) Surface behavior of gangliosides and other related glycosphingolipids. Biochem. J. 171:559-565.

Maggio B., Mestrallet M.G., Cunar F.A.,and Capputo R. (1977) Glucose release from liposomes cantaining gangliosides of other membrane lipids by biogenis amines and meylin basic protein. Biochem. Biophys. Res. Com. 77:267-273.

McCrea J.F., (1947) Modification of red-cell aglutinability by C. Welchie toxins. Austr. J. Exp. Biol. Med. Sci. 25:127-136.

McKay J., (1980) Gangliosides in Myogenesis. Ph.D. Thesis, Department of Biological Stucture, University of Mashington, Seattle, WA.

Morgan I.G., Tettamanti G., and Gombos G. (1976) Biochemical evidence on the role of gangliosides in nerve-endings. Adv. Exp. Med. Biol. 71:137-150.

Morgan I.G., Wolfe L.S., Mandel P., and Gombos G. (1971) Isolation of plasma membranes from rat brain. Bioch. Biophys. Acta 241:737-751.

Nagai Y. and Iwamori M. (1984) Ganglioside distribution at different levels of organization and its biological implications. Adv. Exp. Med. Biol. 174:135-146.

Ng S.-S., and Dain J.A. (1976) The natural occurence of sialic acids. In: The Biological Roles of Sialic Acids. (eds. Schendgrund C.-L., and Rosenberg A.) pp.59-102, Plenum Press, New York and London.

Ng S.-S., and Dain J.A. (1977) Sialyltransferases in rat brain: intracellular localization and some membrane properties. J. Neurochem. 29:1085-1093.

Norton W.T. and Poduslo S.E. (1970) Neuronal soma and whole neuroglia of rat brain: a new isolation technique. Science 167:1144-1145.

Ohman R. (1971a) The activity of ganglioside sialidase in different regions of human brain. J. Neurochem. 18:531-532.

Ohman R. (1971b) Subcellular fractionation of Gangliosides sialidase from human brain. J. Neurochem 18:89-95.

Ohman R., Rosenberg A. and Svennerholm L. (1970) Human Brain Sialidase. Biochemistry 9:3774-37882.

Patuszko A., Wilson O.F. and Erecinska M. (1982) Neurotransmitter metabolism in rat brain synaptosomes effect of anoxia and pH. J. Neurochem. 38:1657-1667.

Preti A., Fiorilli A., Lombardo A., Caimi L. and Tettamanti G. (1980) Occurence of sialytransferase activity in the synaptosomal membrane prepared from calf brain cortex. J. Neurochem. 35:281-296.

Purpura D.P., and Baker H.J. (1977) Meganeurites and other aberrent processes of neurons in feline GM1-gangliosidosis: a Golgi study. Brain Res. 143:13-26.

Purpura D.B., and Suzuki K. (1976) Distortion of neuronal geometry and formation of aberrent synapses in neuronal storage disease. Brain Res. 116:1-21.

Quarles R.H. and Brady R.O. (1971) Synthesis of glycoproteins and gangliosides in developing rat brain. J. Neurochem. 18:1809-1820.

Raff M.C., Fields K.L., Hakomori S., Mirsky R., Pruss R.M., and Winter J. (1979) Cell-type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Res. 174:283-295.

Rahman H. (1976) Possible functional role of gangliosides. Adv. Exp. Med. Biol. 71:151-161.

Rodgers T.B., and Snyder S.H. (1981) High affinity binding of tetanus toxin to mammalian brain membranes. J. Biol. Chem. 256:2402-2407.

Rome L.H., Garvin A.J., Allietta M.M. and Neufeld E.F. (1979) Two Spiecies of Lysosomal Organelles in Cultured Human Fibroblasts. Cell 17:143-153.

Rosenberg A. (1978) Action of rat brain sialidase on synaptic membrane components. Adv. Exp. Med. Biol. 101:439-446.

Rosengart V.I. and Taranova N.P.(1969) Vop. Med. Khim. 15:404.

Rosner H. (1981) Isolation and preliminary characterization of novel polysialogangliosides from embryonic chick brain. J. Neurochem. 37:993-997.

Saito M. and Rosenberg A. (1982) Glycolipids and their developmental patterns in chick thigh and leg muscles. J. Lip Res. 23:3-8.

Saito M. and Rosenberg A. (1982) Sialosylgalactosylceramide (GM_4) is a major ganglioside in chick embryonic liver. J. Lipid Res. 23:9-13.

Saito M. and Rosenberg A.R. (1984) Identification and characterization of N-Acetyl-2,3-didehydro-2-deoxyneuraminic acid as a metabolite in mammalian brain. Biochemistry 23:3784-3788.

Sarker C. (1976) Effect of delta-9-tetrahydrocannabinol on gangliosides and sialoproteins in subcellular fractions of rat brain. J. Neurochem. 26:721-723.

Sbaschnig-Agler M., Ledeen R.W., Grafstein B., Alpert R.M. (1984) Ganglioside change in the regenerating goldfish optic system: comparison with glycoproteins and phospholipids. J. Neuroscience Res. 12:2/3:221-232.

Schengrund C.-L. and Nelson J.T. (1975) Influence of cation concentration on the sialidase activity of neuronal synaptic membranes. Biochem. Biophys. Res. Com. 63:217-223.

Schengrund C.-L., and Repman M.A., (1982) Density-dependent changes in gangliosides and sialidase activity of murine neuroblastome cells. J. Neurochem., 39:940-947.

Schengrund C.-L. and Rosenberg A. (1970) Intracellular location and properties of bovine brain sialidase. J. Biol. Chem. 245:6196-6200.

Seifert W., and Fink H.J. (1984) In vivo and in vitro studies on gangliosides in the developing and regenerating hippocampus of the rat. Adv. Exp. Med. Biol. 174:535-545.

Seyfried T.N., and Yu R.K. (1984) Cellular location of gangliosides in the mouse cerebellum: analysis using neurological mutants. Adv. Exp. Med. Biol. 174:169-182.

Seyfried T.N. and Yu R.K. (1985) Ganglioside GD3: structure, cellular distribution, and possible function. Molec. Cell. Biochem. 68:3-10.

Siegrist H.P., Burkart T., Wiesmann U.N., Herschkowitz N.N. and Sphycher M.A. (1979) Ceramidegalactosyltransferase and cerebroside-sulphotransferase localization in Golgi membranes isolated by a continuous sucrose gradient of mouse brain microsomes. J. Neurochem. 33:497-504.

Siegrist H.P., Jutzi H., Steck A.J., Burkart T., Wiesmann U. and Herschkowitz N. (1977) Age dependent modulation of 3'-phosphoadenosine-5'-phosphosulfategalactosylceramide sulfotransferase by lipids extracted from the microsomal membranes and artificial lipid mixtures. Bioch. Biophys. Acta. 489:58-63.

Simpson L.L., and Rapport M.M. (1977) The binding of botulinum toxin to membrane lipids: sphingolipids, steroids, and fatty acids. J. Neurochem. 18:1751-1759.

Smid F. and Reinisova J. (1973) A densitometric method for the determination of gangliosides after their separation by thin-layer chromatography and detection by resorcinol reagent. J. Chromatog. 86:200-204.

Sonnino S., Ghidoni R., Fiorilli A., Venerando B. and Tettamanti G. (1984) Cytosolic gangliosides of rat brain: their fractionation into protein-bound complexes of different ganglioside compositions. J. Neuroscience Res. 12:2/3:193-204.

Steck T.L. and Dawson G. (1974) Topographical distribution of complex carbohydrates in the erythrocyte membrane. J. Biol. Chem. 249:2135-2142.

Suzuki K. (1964) A simple and accurate micromethod for quantitative determination of ganglioside patters. Life Sci. 3:1227-1233.

Suzuki K. (1965) The pattern of mammalian brain gangliosides III; regional and developmental differences. J. Neurochem. 12:969-976.

Svennerhlom L., (1957) Quantitative estimation of sialic acids; II a colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. ACTA, 24:604-611.

Svennerholm L. (1963) Chromatographic separation of human brain gangliosides. J. Neurochem. 10:613-623.

Svennerholm L. (1980) Gangliosides and synaptic transmission. Adv. Exp. Med. Biol. 125:533-544.

Svennerholm L. and Fredman P. (1980) Structure and biology of cell-membrane gangliosides. Adv. Exp. Med. Biol. 125:80-87.

Tallman J.F., and O'Brady R. (1976) Disorders in ganglioside metabolism. In: Biological Roles of the Sialic Acids. (eds. Schendgrund C.-L., and Rosenberg A.) pp. 183-200. Plenum Press, New York and London.

Tamir H., Brunner W., Casper D., and Rapport M.W., (1980) Enhancement by gangliosides of the binding of serotonin to the serotonin binding protein. J. Neurochem. 34:1719-1724.

Tartakoff A., and Vassalli P. (1978) Comparative studies of intracellular transport of secretory proteins. J. Cell Biol. 79:694-707.

Tettamanti G. (1984) An outline of ganglioside metabolism. Adv. Exp. Med. Biol. 174:197-212.

Tettamanti G., Morgan I.G., Gombos G., Vincendon G., and Mandel P. (1972) Subsypnaptosomal localization of brain particulate neuraminidase. Brain Res. 47:515-518.

Tettamanti G., Preti A., Cestaro ..B. Venerando B., Lombardo A., Ghidoni R., and Sonino S. (1979) Gangliosides, neuraminidase, and sialyltransferase at the nerve ending. Adv. Exp. Med. Biol. 125:263-281.

Tettamanti G., Venerando B., Gestaro B., and Preti A. (1976) Brain neuraminidase and gangliosides. Adv. Exp. Med. Biol. 71:65-81.

Tettimanti G., Pretti A., Cestaro B., Masserini M., and Sonnino S., (1980) Gangliosides and associated enzymes at the nerve-ending membranes. In: Cell Surface Glycolipids (Swelley C.C., ed.), American Chemical Society Symposium Series 128, USA, pp. 321-343.

Townsend L.E., Benjamins J.A. and Skoff R.P. (1983) Effects of monensin and colchicine on myelin galactolipids. J. Neurochem. 43:139-145.

Vaccari A., Vertua R., and Furlani A. (1971) Decreased calcium uptake by rat fundal strips after pretreatment with neuraminidase or LSD in vitro. Biochem. Pharmacol. 20:2603-2612.

Vna Heyning W.E. (1974) Gangliosides as membrane receptors for tetanus toxin, cholera toxin, and serotonin. Nature, 249:415-417.

Wakley S.U., Warzelman S., and Purpura D.P. (1981) Ultrastructure of neurites and meganeurites of coritical pyramidal neurons in feline gangliosidoses as revealed by the combined Golgi-EM technique. Brain Res. 211:393-398.

Veh R.W., Sander M. (1981) Differentiation Between Ganglioside and sialyllactose sialidases in human tissues. In: Sialidases and Sialidoses; Perspectives in Inherited Metabolic Diseases, Volume IV. (Tettamanti G., Durand P., and Di Donato S., eds.) pp. 71-108. Edi. Ermes s.r.l.-Via Timavo 12-Milan (Italy)

Vliegenthart J.F.C. and Kamerling J.P. (1982) Synthesis of sialic acid and sialic acid derivatives. In: Sialic Acids, Chemistry, Metabolism and Function; Cell Biology Monographs, Volume 10. pp 59-76, Springer-Verlag, Wien, F.R.G.

Warren L., and Spearing C.W. (1960) Mammalian sialidase (neuraminidase). Biochem. Biophy. Res. Comm. 3:489-492.

Whatley R., Ng S. K.-C., Roger J., McMarray W.C., and Sanwal B.D., (1976) Developmental changes in gangliosides during myogenesis of a rat myoblast cell line and its drug resistant variants. Biochem. Biophys. Res. Com., 70:180-185.

Wiegandt H. (1967) The subcellular location of gangliosides in the brain. J. Neurochem. 12:969-974.

Wiegandt H.(1972) Recent advances in the chemistry and localization of brain gangliosides and related GSL. Adv. Exp. Med. Biol. 25:127-140.

Wiegandt H. (1973) Gangliosides of extraneural organs. H.-Ss. Zschft. Physiol. Chem. 354:1049-1056.

Wiegandt H. (1976) Structure and specificity of gangliosides. In: Ganglioside Function; Biochemical and Pharmacological Implications.Adv. Exp. Med. Biol. 71:3-15..

Wiegandt H., (1982) The gangliosides. In: Avances in Neurochemistry Volume 4 (Agranoff B.W., and Aprison M.H.,eds.), Plenum Press, New York and London, pp. 149-223.

Williams M.A. and McCluer R.H. (1980) The use of Sep-Pak C_{18} cartridges during the isolation of gangliosides. J. Neurochem 35:266-269.

Willinger M., (1981) The expression of GM1 ganglioside during neuronal differentiation. In: Gangliosides in Neurological and Neuromuscular Function, Development, and Repair (Rapport M.M, and Gorio A., eds.), Raven Press, New York, pp. 17-27.

Yamakawa T., Irie R., and Iwanaga M., (1960) The chemistry of lipid of posthemolytic residue or stroma of erythrocytes, IX. Silicic acid chromatography of mamalian stroma glycolipids. J. Biochem., 48:490-495.

Yamakawa T. (1984) Wonders in Glycolipids-A Historical View. In: Ganglioside Structure, function and Biomedical Potential. Adv. Exper. Med. Biol. 174:3-11.

Yohe H., Roark D., and Rosenberg A. (1976) C_{20}-sphingosine as a determining factor in the aggregation of gangliosides. J. Biol. Chem.251:7083-7087.

Yohe H. and Rosenberg A. (1977) Action of intrinsic sialidase of rat brain synaptic membranes on membrane sialolipid and sialoprotein. J. Biol. Chem. 252:2412-2418.

Yu-Ten Li and Su-Chen Li (1982) Biosynthesis and catabolism of glycosphingolipids. Adv. Carboydrate Chem. 40:235-286.

APPROVAL SHEET

The dissertation submitted by Robert P. Durrie has been read and approved by the following committee:

Dr. Abraham Rosenberg, Director
Professor of Biochemistry and Biophysics, and
Director of the Division of Developmental and
Pathological Neurochemistry, Loyola

Dr. Michael Collins
Professor of Biochemistry and Biophysics, Loyola

Dr. Mitsuo Saito
Assistant Professor of Biochemistry and
Biophysics, and Surgery, Loyola

Dr. Nae Dun
Associate Professor of Pharmacology, Loyola

Dr. Nancy Schwartz
Professor of Pediatrics and Biochemistry,
University of Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to the content and form.

The dissertation is therefore accepted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

April 4, 1986 — Date

Abraham Rosenberg — Director's signature