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STUDIES ON THE BIOSYNTHESIS OF GANGLIOSIDES

by
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STUDIES ON THE BIOSYNTHESIS OF GANGLIOSIDES

Subhash Chandra Basu

Abstract

Recently, Kuhn and Wiegandt produced conclusive evidence that disialoganglioside has the following structure:

Galactosyl-(β,1→3)-N-acetylgalactosaminyl-
(2→3)
NAN

(β,1→4)-galactosyl-(β,1→4)-glucosyl-ceramide
(2→3)
NAN

This thesis provides evidence that several of the enzymes involved in the biosynthesis of monosialo- and disialogangliosides are present in a particulate fraction of embryonic chicken brain.

The following components were sialic acid acceptors in this system: lactose > ceramide-tetrasaccharide > monosialoganglioside > lactosyl-ceramide > ceramide-trisaccharide. The following compounds were inactive: galactosyl-ceramide, galactosyl-sphingosine. At least two transferase activities were detected in the particulate fraction, one of which transferred sialic acid to ceramide-disaccharide, while the other transferred the sugar to monosialoganglioside, ceramide-tetrasaccharide and lactose.

The particulate enzyme preparation from nine-day old chicken embryo brains catalyzed the synthesis of

sialyl→lactosyl→ceramide from CMP-NAN and lactosyl-ceramide. The maximum activity was observed with brains from 9-11 day chicken embryos. The radioactive product was isolated and purified by Florisil, Silicic acid and thin-layer chromatography. The molar ratios of the constituents in the product were in good agreement with that expected for hematoside.

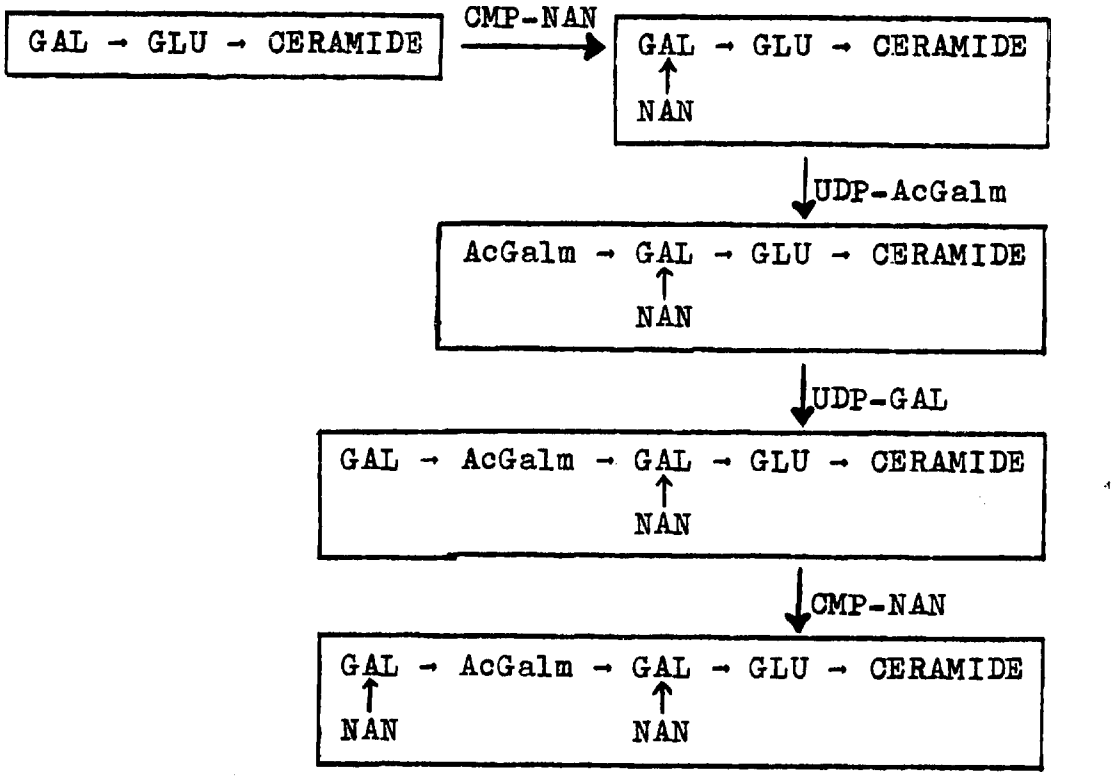
The particulate preparation from embryonic chicken brain also contained a galactosyltransferase that catalyzed the following reaction:

Tay-Sachs ganglioside + UDP-galactose → monosialoganglioside (+ UDP?)

Galactosyltransferase activity was detected in homogenates of brain from embryonic chicken (7 to 20 days), pig fetus (82 mm), and rat (9 days); maximal specific activity was observed with brains from 13 to 18 day-old embryonic chickens. EDTA completely inhibited the enzymatic activity, while Mn^{++} was required; Co^{++} was about 20% as effective as Mn^{++} when tested at the same concentration. Tay-Sachs ganglioside was the most active acceptor. N-acetylglucosamine and orosomucoid (treated with sialidase and galactosidase) were also active. The activities toward N-acetylglucosamine and glycoprotein were markedly heat-labile compared with the activity towards the glycolipid. Mixed substrate experiments also suggested that the transferase acting on Tay-Sachs ganglioside differed from the system active with N-acetylglucosamine and glycoprotein. The radioactive enzymic product was isolated, purified and

analyzed. Molar ratios of the components were in good agreement with those expected for monosialoganglioside. Preliminary experiments with the particulate system also suggested the presence of a N-acetylgalactosaminyl-transferase. The most active lipid acceptor for this transferase system was hematoside.

While the structures of all of the enzymatic products are not yet determined, the present studies suggest that the biosynthesis of gangliosides proceeds in the following manner:



To my parents

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ABBREVIATIONS

AcGalm	N-acetylgalactosamine
ATP	adenosine-5'-triphosphate
BSM	bovine submaxillary mucin
C-M	chloroform-methanol in mixtures (v/v)
Cer	ceramide or N-acylated sphingosine (the acyl group is fatty acid)
Cer-mono	glucosyl-ceramide
Cer-di or, Cytolipin-H	galactosyl-(β,1→4)-glucosyl→ceramide
Cer-tri	N-acetylgalactosaminyl-(β,1→4)-galactosyl-(β,1→4)-glucosyl→ceramide
Cer-tetra	galactosyl-(β,1→3)-N-acetylgalactosaminyl-(β,1→4)-galactosyl-(β,1→4)-glucosyl→ceramide
CDSTase	CMP-N-acetylneuraminic:ceramide-disaccharide sialyltransferase
CTSTase	CMP-N-acetylneuraminic:ceramide-tetrasaccharide sialyltransferase
CMP	cytidine-5'-monophosphate
CoA	coenzyme-A
CTP	cytidine-5'-triphosphate
DEAE-cellulose	diethylaminoethylcellulose
DNAase	deoxyribonuclease
DS-DGal-OM	desialized, degalactosized orosomucoid (i.e., orosomucoid pre-treated with sialidase and β-galactosidase)
EDTA	ethylenediamine tetraacetic acid
Ether	diethyl ether
F.A	fatty acid
GLC	gas liquid chromatography
GM_1 or G_1	monosialoganglioside
Hm	hematoside (or sialoceramide-disaccharide)

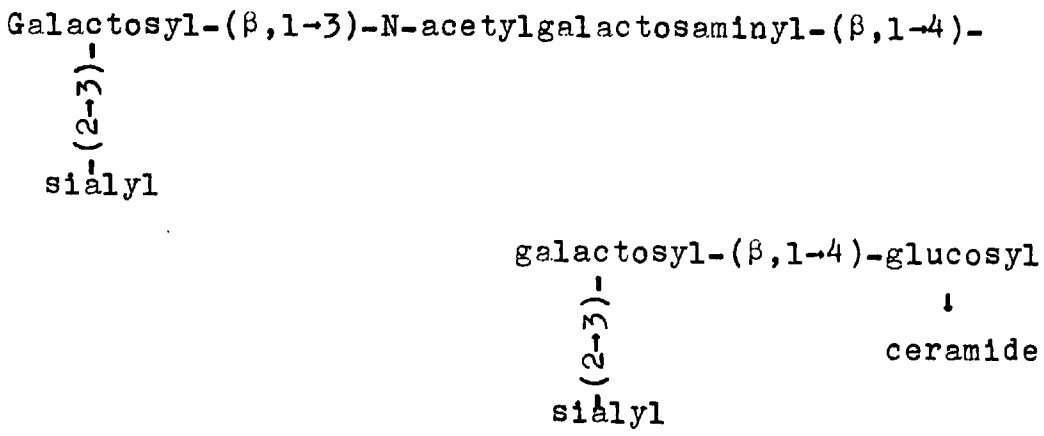
ABBREVIATIONS (cont'd.)

MeOH	methanol
ME	2-mercaptoethanol
Mn^{++}	manganese divalent cation
N-AcGm	N-acetylglucosamine
NAN	N-acetylneuraminic acid
NGN	N-glycolylneuraminic acid
OM	orosomucoid (α_1-acid globulin, isolated from human serum)
OSM	ovine submaxillary mucin
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
PPO	2,5-diphenyloxazole
PSM	porcine submaxillary mucin
r.b.	round bottom
RNAase	ribonuclease
TLC	thin-layer chromatography
Tris	tris (hydroxymethyl) aminomethane
TSG	Tay-Sachs ganglioside
UDP	uridine-5'-diphosphate
UMP	uridine-5'-monophosphate

GENERAL INTRODUCTION

INTRODUCTION

The term ganglioside was first suggested by Klenk (2,3) to denote a glycosphingolipid containing N-acetylneuraminic acid (abbreviated NAN; one of the sialic acids) which he first isolated from mammalian brain. The word ganglioside now refers to a family of compounds in which there are variations in the number and type of sugars in the oligosaccharide chain, in the types of sphingosine bases, and fatty acids. The structure of gangliosides is derived from studies from many laboratories, particularly those of Klenk (27,33,93), Kuhn (5,6), and Svennerholm (4). Structural studies, particularly by Kuhn and Wiegandt (6) suggested that disialoganglioside[1] is



[1] The nomenclature used above is as follows. Ceramide = N-acylsphingosine where the acyl group is primarily stearic acid; sphingosine = the C_{18} and C_{20} class of sphingosines and the corresponding dihydrosphingosines; all sugars are of the D configuration, and presumably pyranose ring structures; the location of glycosidic bonds is indicated by parentheses as shown in the abbreviated structure for disialoganglioside, where β refers to anomeric configuration of the sugar to the left of the parenthesis, while the arrow indicates where the anomeric carbon of the sugar at the left (C-1 except for sialic acid) is attached to the sugar at the right of the parenthesis; sialic acid = N-acetylneuraminic acid. By removal of 1 or more sugar residues from disialoganglioside, a series of ceramide oligosaccharides is obtained.

The major ganglioside found in Tay-Sachs brain, and also occurring in small amounts in normal brain, lacks the terminal sialyl-galactosyl disaccharide unit in the structure shown above (7,8).

In contrast to extensive studies on the chemistry of gangliosides, a few reports have appeared concerning their biosynthesis, most of these being *in vivo* tracer experiments (10,98). Burton *et al.* (10) showed *in vivo* incorporation of radioactive glucose, galactose and serine into brain gangliosides of young rats. Recently Kanfer *et al.* (9) reported the transfer of NAN from CMP-NAN (cytidine-5' monophosphate-N-acetylneuraminic acid) to ceramide-trisaccharide when these compounds were incubated with rat kidney homogenates.

The present study is concerned with the *in vitro* biosynthesis of gangliosides. Embryonic chicken brains were used for these studies because of a previous report by Garrigan, *et al.* (11), that this tissue rapidly accumulates glycosphingolipids.

Part I of this thesis is concerned with studies on the biosynthesis of hematoside (monosialo ceramide-disaccharide), monosialo ceramide-tetrasaccharide, and disialo ceramide-tetrasaccharide (12,13). A particulate preparation from embryonic chicken brain was employed that catalyzed the *in vitro* synthesis of these compounds when the system was incubated with the corresponding sialic acid free glycolipids and CMP-NAN. The following reactions were catalyzed

by the particulate sialyltransferase system of chicken embryonic brain:

1) Ceramide-disaccharide + CMP-NAN → Ceramide-disaccharide ← NAN (+ CMP?)

2) Monosialoganglioside + CMP-NAN → Disialoganglioside (+ CMP?)

3) Ceramide-tetrasaccharide + CMP-NAN → Ceramide-tetrasaccharide ← NAN (+ CMP?)

4) Ceramide-tetrasaccharide + 2 CMP-NAN → Ceramide-tetrasaccharide ← $(NAN)_2$ (+ 2 CMP?)

Part II concerns the biosynthesis of monosialoganglioside from Tay-Sachs ganglioside and UDP-galactose; the embryonic chicken brain particulate system (14,15) also contained this activity. The reaction is as follows:

Tay-Sachs ganglioside + UDP-galactose → monosialoganglioside (+ UDP?)

A. HISTORICAL REVIEW OF GANGLIOSIDES

1. Discovery

In 1935 Klenk et al. (1) isolated a substance-X from the brain tissue of a child who had died of infantile amaurotic idiocy of the Tay-Sachs type. In 1938, Blix (16) isolated a similar substance from normal brain, but in much smaller amounts. Subsequently the name "ganglioside" was given to that substance. Folch et al. (17), in 1951, described the preparation of strandin from normal brain; this substance resembled the gangliosides. The word ganglioside now has a generic connotation, and refers to a family of glycolipids that contain sphingosine and sialic acid. The sphingosine is acylated by fatty acid, mostly stearic acid, and is called ceramide. The glycose fraction contains sialic acid linked to an oligosaccharide chain containing galactose, N-acetylgalactosamine, galactose, and glucose.

The major chemical difference between the brain gangliosides of Klenk (21), strandin of Folch et al. (17,18) and the brain mucolipids of Rosenberg and Chargaff (19) was that the latter compounds liberated amino acids after hydrolysis. The isolation of the pure gangliosides is difficult because they consist of a lipophilic portion and therefore form micelles in water and organic solvents (20). Gangliosides sometimes act as colloid-protecting agent for other lipids or low molecular weight compounds such as amino acids and peptides. These properties may be the reason why gangliosides prepared by some procedures

contained peptides. Various laboratories (22,23) have reported that brain ganglioside mixtures could be separated into several components by means of silicic acid chromatography. During the last few years, reports have appeared on the structures of different gangliosides isolated from brain and purified by column chromatography and thin layer chromatography (60). Svennerholm and Raal (24) isolated mono- and disialogangliosides from human and calf brains; Kuhn, Wiegandt, and Egge (25) found one monosialoganglioside, two disialogangliosides, and one trisialoganglioside in human and calf brain; Dain et al. isolated (26) four components from ox brain which had the same chemical composition as the substance found by Kuhn et al. Klenk et al. (28) isolated the hexosamine-containing and hexosamine-free monosialogangliosides. Tettamanti et al. isolated seven pure gangliosides from pig brain (29). Four of these corresponded to the G_I, G_{II}, G_{III}, G_{IV}, gangliosides of Kuhn (5); the fifth had the same chemical composition as that of Klenk (28); the sixth was a trisialoganglioside; the seventh was reported to be new in that it contained four moles of neutral hexose, and two moles of sialic acid, for each mole of ceramide.

A monosialoganglioside lacking the galactose in the terminal position constitutes about five percent of the total ganglioside fraction of normal human brain, but it is the major ganglioside in brain tissue from patients with infantile amaurotic idiocy (Tay-Sachs disease). Johnson et al. (30) isolated and purified gangliosides from normal

human gray matter. The resolution of 8 gangliosides by thin layer chromatography has been achieved; 4 of these were the major components reported by Kuhn et al. (5). One of the 4 minor gangliosides was a monosialoganglioside containing equimolar amounts of glucose, galactose, and galactosamine, corresponding to Tay-Sachs ganglioside.

Recently Gatt et al. (34) reported two new glycosphingolipids in brain tissue in cases of Tay-Sachs disease. One of the two contained sphingosine, fatty acid, and two moles of galactose, and the other contained equimolar ratios of sphingosine, fatty acid, glucose, galactose, and galactosamine, the last being present in higher concentration (1 mg/gm of brain, wet weight). Neither of these two compounds could be detected in normal human brain of adult or infant. A ceramide-trihexoside with one mole of glucose and two moles of galactose has been isolated and tentatively characterized as a stored lipid in the kidney in cases of Fabry's syndrome by Sweeley et al. (36). Ceramide-digalactoside has also been isolated by Sweeley et al. but in much smaller quantities. A number of sphingoglycolipids have been isolated from bovine spleen by Rapport (39,40). Cytolipin-H was first isolated by Rapport et al. (37,38) from human epidermoid carcinoma ($H.E_p3$); this ceramide-lactoside was also obtained by Makita and Yamakawa from bovine, equine, and human spleen (41). A molecule with this composition and similar physical properties was first found in ox spleen by Klenk and Rennkamp (42).

In 1951, Klenk and Lauenstein (46) reported the presence of a galactosamine containing glycolipid in spleen, and the substance was named "globoside" by Yamakawa et al. (47-51). Globoside, the main mucolipid of human erythrocytes, was purified by repeated silicic acid column-chromatography, and the results led to a constitutional formula of N-acetylgalactosaminyl-(β,1→6)-galactosyl-(β,1→4)-galactosyl-(β,1→4)-glucosyl-ceramide. Rapport et al. (53) recently showed that human kidney contains cytolipin-K, which has the same chemical structure as globoside (51,52) and is immunochemically indistinguishable from it. Yamakawa et al. (54,55) and Klenk and associates (57) have isolated a ganglioside, G_{M3} (hematoside), from canine and equine erythrocyte stroma, which contains fatty acid-sphingosine-hexose-sialic acid in a molar ratio of 1:1:2:1, and has the following structure:

N-glycolylneuraminic acid-(2→3)-galactosyl-(β,1→4)-glucosyl-(1→1)-ceramide

A ganglioside with a similar structure has been found in human spleen, liver, and brain (58). Recently Kuhn (59) reported the existence of small amounts of 4 different gangliosides in human brain, as follows:

a) G gal (1 mole ceramide, 1 mole galactose, and 1 mole NAN)

b) G Lact (1 mole ceramide, 1 mole galactose, 1 mole glucose, and 1 mole NAN)

c) G′ Lact (1 mole ceramide, 1 mole glucose, 1 mole galactose, and 2 moles of NAN)

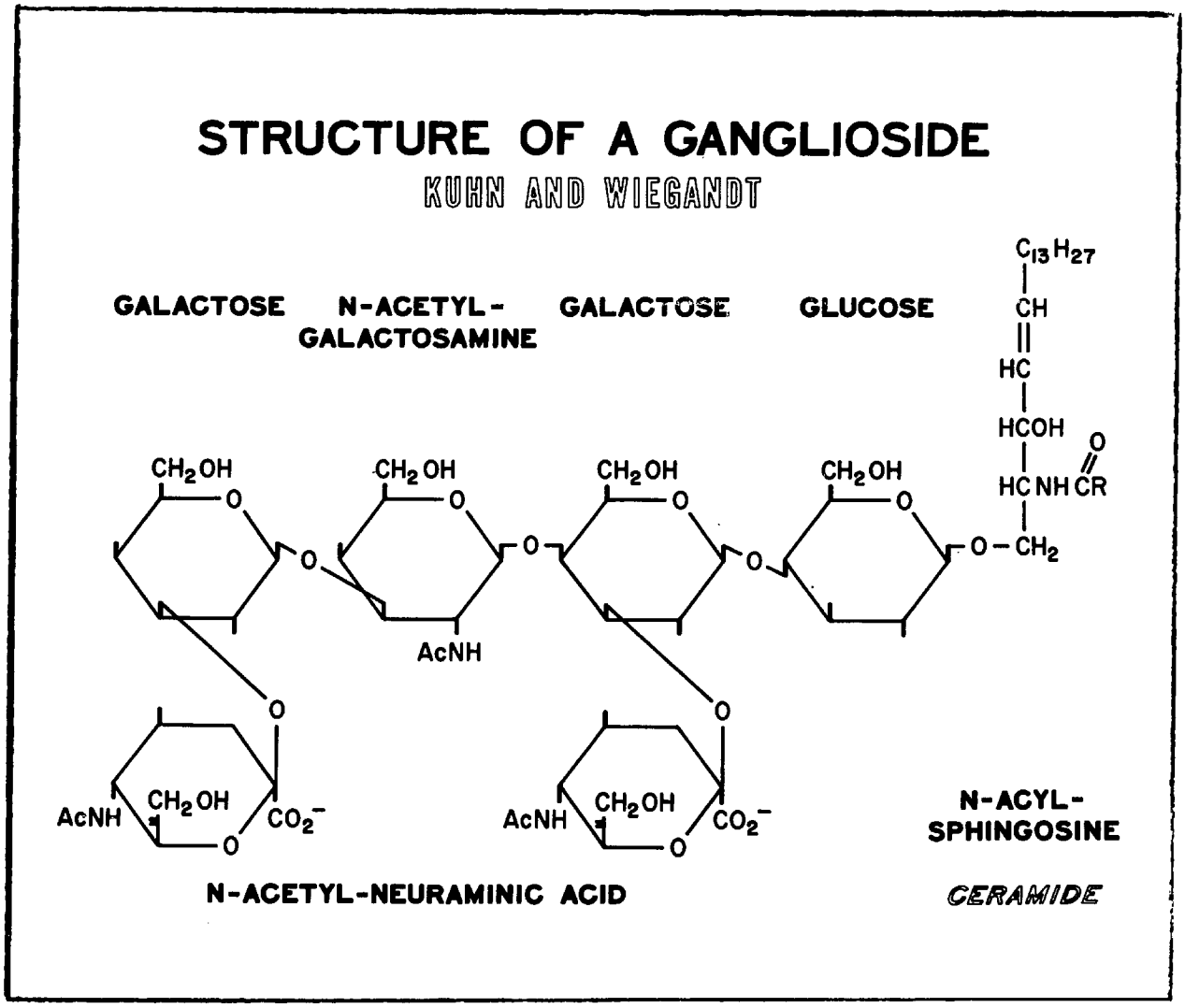
d) G' GNTr11 (1 mole ceramide with 1 mole glucose, 1 mole galactose, 1 mole galactosamine and 2 moles of NAN)

Lieb in 1924 found the accumulation of a glycolipid, cerasine, in the spleen of a patient with Gaucher's disease. Later, Klenk (35) isolated cerebroside or cerasine (containing ceramide and galactose) and a ceramide dihexoside (containing 1 mole glucose and 1 mole galactose). Cerebroside was first isolated from brain tissue by Thudichum (79) and was named according to the source of the lipid (cerebrum). The occurrence of cerebroside has also been demonstrated in different systems, such as white matter of the mammalian brain (61), gray matter of the brain (62,63), nervous tissues, and various organs in Gaucher's disease (64). In Gaucher spleen, ceramide glucoside and ceramide lactoside have been found by Suomi et al. (65), but not ceramide galactoside. Substances like cerebroside have been isolated from bacteria and plant tissues (66). Carter et al. (67) have shown the presence of "sphingolipids," containing phytosphingosine, glucose, and hydroxy stearic acid, in wheat flour.

2. Structural Studies

E. Klenk (3) identified the structural units of the ganglioside in the course of his investigations. He found six different structural units from ganglioside after hydrolysis, namely stearic acid, sphingosine, glucose, galactose, galactosamine, and sialic acid. Because of the varying composition of different ganglioside preparations, during the last four years reports have appeared from many

FIGURE 1


STRUCTURE OF A GANGLIOSIDE
KUHN AND WIEGANDT
GALACTOSE
N-ACETYL-GALACTOSAMINE
GALACTOSE
GLUCOSE
N-ACYL-SPHINGOSINE
CERAMIDE
N-ACETYL-NEURAMINIC ACID

laboratories on the complexity of brain gangliosides. As soon as it was proved that brain gangliosides could be separated chromatographically, work on the elucidation of the exact constitution of different gangliosides was started in various laboratories. Recently Kuhn and Wiegandt (6) have produced conclusive evidence that disialoganglioside, G_{M1} or G_1 (parent ganglioside of the gray matter) has the structure shown in Fig. 1.

Gangliosides are named monosialo-, disialo- and trisialogangliosides according to the number of sialic acid molecules attached to the ceramide-oligosaccharide chain. Kuhn et al. (5) found one monosialoganglioside, two disialogangliosides and one trisialoganglioside in human and calf brains. A uniform nomenclature for the gangliosides has not been adopted. In the following chart symbols chosen by Kuhn et al. (5) and Svennerholm (4) are reviewed.

The ganglioside G_{M1} (or G_1) is composed of 1 molecule each of stearic acid, sphingosine, glucose, N-acetyl-galactosamine, and 2 molecules each of galactose and N-acetylneuraminic acid. The chemistry of each component is discussed as follows:

1. Sphingosine

Klenk in 1929 (68) established the formula of sphingosine as $C_{18}H_{37}NO_2$. At that time the chemical structure was given as 1,2-dihydroxy-3-amino-4-octadecene. Carter et al. (69) have established the structure as 1,3-dihydroxy-2-amino-4-octadecene. The amino group at

Symbol* 1	Symbol* 2	Compound	Ceramide	Glucose	Galactose	N-Acetyl galactose-amine	Sialic
		Normal brain					
	G_{M3}	Ceramide-lactose-N-acetylneuraminic acid	1	1	1	--	1
G_o	G_{M2}	Ceramide-N-triose-N-acetylneuraminic acid	1	1	1	1	1
G_I	G_{M1}	Ceramide-N-tetraose-N-acetylneuraminic acid	1	1	2	1	1
G_{II}	G_{D1a}	Ceramide-N-tetraose-di-acetylneuraminic acid	1	1	2	1	2
G_{III}	G_{D1b}	Ceramide-N-tetraose-di-acetylneuraminic acid	1	1	2	1	2
G_{IV}	G_{T1}	Ceramide-N-tetraose-tri-N-acetylneuraminic acid	1	1	2	1	3
		Tay-Sachs brain					
	G_{M2}	Ceramide-N-triose-N-acetylneuraminic acid	1	1	1	1	1

* Symbols in column 1 used by Kuhn and Wiegandt (5); in column 2 by Svennerholm (4).

C-2 position was found to be in the D-configuration (70). The C-3 hydroxy group of sphingosine bears an erythro relationship to the C-2 amino group. Carter et al. (71) obtained only erythro sphingosine from cerebroside under non-acid hydrolytic conditions. The sphingosine moiety of ceramides isolated from beef brain and chicken liver has recently been characterized as having the erythro stereochemical configuration (72,73). From infrared spectroscopic studies before and after hydrogenation of sphingosine, it was shown that the double bond has trans-configuration. Recently, Shapiro and Flowers (76) have chemically synthesized isomers of cerebroside and sphingomyelin which are the same as naturally occurring compounds (i.e., the sphingosine molecule contained the erythro configuration and the trans double bond). Cleland and Kennedy (138) showed that a brain microsomal enzyme catalyzed the formation of ceramide-galactoside quite actively when the configuration of sphingosine was erythro- trans. Fujino and Zabin (77) showed that dihydrosphingosine, formed enzymatically in rat brain homogenates, had the erythro configuration. In the glycolipid fraction of equine and bovine brain, a sphingosine homologue with 20 carbon atoms was detected (78). Klenk and Gielen (80) found that in bovine brain gangliosides the C_{20} homologue of sphingosine was about 50% of the total, but they were unable to detect any such homologue in human brain gangliosides. Stanacev and Chargaff (81) found in bovine brain ganglioside 50.0% C_{18}-sphingosine, 3.45% dihydrosphingosine (C_{18}), and

46.5% C_{20}-sphingosine. Rosenberg and Stern (82) found that ganglioside contained C_{18}-sphingosine at birth, but subsequently the C_{20}-sphingosine content increased with age. This type of change they followed in both human and rat brains. In Tay-Sachs disease, they found that the ganglioside fraction contained predominantly C_{18}-sphingosine.

ii. Fatty Acids

Fatty acids of the gangliosides are present in amide linkage with the sphingosine moiety. Klenk (3) first showed that the major fatty acid of brain gangliosides was n-octadecanoic acid (or stearic acid). Subsequently, using gas liquid chromatography many laboratories (24,25, 83,84) found stearic acid to be 80-90% of the total fatty acid of brain gangliosides. Lignoceric acid constituted about 75% of the fatty acids in the glycosphingolipids of equine erythrocytes (52). Recently (86-88), a large number of isomers and homologues of unsaturated normal and hydroxy acids have been demonstrated in brain glycosphingolipids. Kishimoto *et al.* (89) analyzed the fatty acid composition of cerebroside and ganglioside (isolated from human and rat brain). They found that normal saturated fatty acids of cerebroside contained mostly stearic and lignoceric acids but also fairly large amounts of C_{22}, C_{23} and C_{25} acids were present. In gangliosides they found mostly C_{18} saturated acid. In cerebrosides the unsaturated acids of both classes contained the C_{24} acid as the major constituent, but these included

the odd- and even-numbered monoenoic acids 22-26 carbon atoms long, as well as C_{24}, C_{25}, C_{26} dienes. Small amounts of trienoic acids were also found in brain cerebrosides.

iii. Hexosamine and Hexose

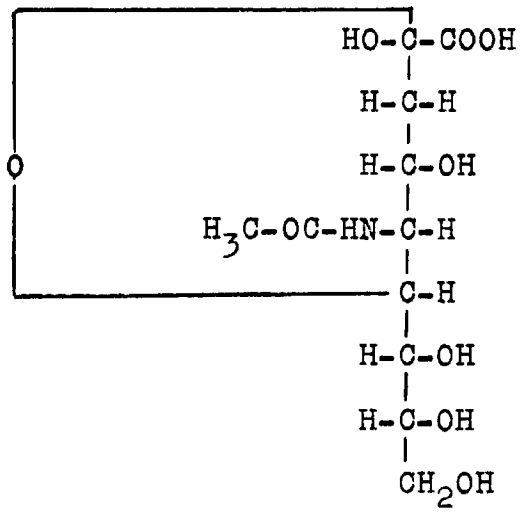
Most gangliosides contain a hexosamine. In brain ganglioside only D-galactosamine (4,91,92) has been found. Recently Hakamori and Jeanloz (101) found a fucose-containing ceramide-tetrasaccharide in tumor tissue in which N-acetylglucosamine was present.

Galactose and glucose are the only two hexoses detected in brain gangliosides and other glycosphingolipids. It is now well established that the sialic acids in disialoganglioside (G_{D1}) are linked to the two galactose molecules of the glycose chain. In trisialoganglioside, one mole of sialic acid is attached to another mole of sialic acid.

iv. Sialic Acid

The sialic acids, first reported by Blix (149), are a family of compounds derived from neuraminic acid (2-keto-5-amino-3,5 dideoxynononic acid). The structure of neuraminic acid is a modification of that proposed by Gottschalk (150) and is based on the observation by Comb and Roseman (151,152) that an aldolase isolated from Cl. perfringens reversibly cleaves N-acetylneuraminic and N-glycoylneuraminic acids (NAN and NGN) to pyruvate and the corresponding N-acyl-D-mannosamines. At that time the steric configuration at C_4 of sialic acid was not established, but Kuhn et al. (245) have now shown that the

hydroxyl group at C_4 is situated on the right in the Fischer projectional formula as given below.



β-D(-)-N-acetylneuraminic acid

No O-substituted sialic acid has been found in gangliosides, the presence of only N-acetylneuraminic acid has been indicated (22,74,75), but in hematoside of equine erythrocyte stroma exclusively the glycolyl type (56) is present.

3. Intermediary Metabolism

1. Biosynthesis of Gangliosides and Cerebrosides

The ganglioside pattern is similar in the fetal, child, and adult brain although the amounts of disialoganglioside and trisialogangliosides are small in the infant brain. The ganglioside concentration increases from the fetal stage up to about five years of age in the human; the level remains constant until 40 years of age and then drops slowly. Decreased concentrations of gangliosides are also observed in the cerebral cortex of brains at necropsy in cases of presenile and senile dementia, cerebral arteriosclerosis, leukodystrophy, and

leukencephalitis. Pritchard et al. (102) obtained purified ganglioside preparations from the gray matter of rat cerebral cortex at various ages. The NAN and hexose contents were determined at each age after hydrolysis of gangliosides. It appeared that NAN and hexose accumulated at the same rate in cerebral ganglioside during early growth.

Incorporation of hexose and sialic acid units:

Injection of labeled compounds in vivo resulted in the incorporation of D-glucosamine-1-^{14}C, D-glucose-1-^{14}C, D-galactose-1-^{14}C and serine-^{14}C into brain gangliosides. Mild acid hydrolysis, and selective degradation with neuraminidase, of the labeled ganglioside indicates that the radioactive hexose moieties arise from the injected radioactive glucose and galactose. D-Glucosamine is incorporated into N-acetylneuraminic acid and galactosamine in the ganglioside molecule. The serine radioactivity is distributed mainly in N-acetylneuraminic acid and sphingosine, but a small amount is incorporated into all the sugar moieties. Suzuki et al. (99,100) have presented evidence for the incorporation of labeled glucose into gangliosides in vitro. Kanfer et al. (9) have reported the incorporation of labeled NAN-^{14}C from CMP-NAN into ceramide-trisaccharide, the reaction was catalyzed by a cell-free preparation of rat kidney tissue. Ceramide-trisaccharide was a more effective acceptor for NAN than asialoganglioside (ceramide-tetrasaccharide).

Biosynthesis of sphingosine:

From the in vivo experiments it was established that carbon atoms 1 and 2 and nitrogen atom of sphingosine were derived from L-serine (107,108), whereas carbon atoms 3 to 18 originated from acetate (116). Brady et al. (104) described the preparation from rat brain tissue of an enzyme system which catalyzed the formation of sphingosine from palmitoyl CoA and L-serine in the presence of TPNH and Mn^{++}. Later they showed that palmitaldehyde in the absence of TPNH could replace palmitoyl CoA as the substrate. These authors also showed that dihydrosphingosine is dehydrogenated with a flavin enzyme to form sphingosine (103).

In explanation of the mechanism of this reaction between palmitaldehyde and L-serine, it was postulated that the α-carbon atom of the amino acid is activated by formation of a Schiff's base-metal complex of serine, pyridoxal phosphate, and Mn^{++} (105). The Schiff's base-metal complex undergoes decarboxylation instead of proton loss from the α-carbon of serine. This hypothesis was suggested by Weiss (106) from his experiment in vivo, where D-L-serine-2,3-H^3 was utilized for the biosynthesis of sphingosine. Fujino and Zabin (77) have reported that the sphingosine synthesized in vitro is the naturally occurring sphingosine, D-erythro-1,3-dihydroxy-2-amino-4-octadecene.

Biosynthesis of fatty acids:

Brady and Gurin (109) showed that in the soluble supernatant fraction of a pigeon liver homogenate (presumably free of all mitochondria and microsomes), radioactive acetate was incorporated into fatty acids at a very significant rate in the presence of mitochondrial lysate, citrate, ATP, CoA, bicarbonate, Mg^{++} and pyridine nucleotides. Brady et al. (110) later showed that mitochondrial lysate was not necessary for the synthesis of fatty acids from acetate and this result was confirmed by Popjak et al. (111) and Wakil et al. (112). This system can also be prepared from rat mammary gland (111), chicken liver (113), rat liver, and rat kidney (119). It was concluded from these experiments that the enzymes of fatty acid synthesis are localized in the cytoplasmic portion of the cell. Wakil et al. (114) split the crude enzyme into two fractions, and the synthesis of fatty acids from acetyl CoA depended on the presence of both the fractions. The first fraction (acetyl CoA carboxylase) was found to catalyze the following reaction (113).

$$CH_3COSCoA + CO_2 + ATP = CH_2\begin{matrix}\diagup COOH \\ \diagdown COSCoA\end{matrix} + ADP + Pi$$

The second enzyme fraction was shown to convert malonyl CoA to palmitoyl CoA in the presence of acetyl CoA and TPNH (115). The overall reaction can be presented by the following equation:

$$AcCoA + 7\ \text{Malonyl CoA} + 14\ TPNH + 14\ H^+ \rightarrow CH_3(CH_2)_{14}\ CO\text{-}SCoA + 14\ TPN^+ + 7\ CO_2 + 6\ H_2O + 7\ CoA\text{-}SH.$$

Wakil et al. (118) have recently reported the elongation of fatty acids in a mitochondrial system by the successive addition of acetyl CoA to intermediate chain length fatty acyl CoA's (C_{10}-C_{16}). Stumpf and Barber (120) have reported the chain elongation process of fatty acid in avocado mesocarp mitochondria. Recently Hulsmann (121,122) has reported the de novo synthesis in the rat.

Biosynthesis of Cerebroside:

Sribney and Kennedy (137) suggested that galactocerebroside might be formed from UDP-galactose and ceramide by analogy to the formation of sphingomyelin. However, Cleland and Kennedy (138) found a microsomal enzyme in the brain of guinea pigs and young rats that catalyzed the formation of psychosine from sphingosine and UDP-galactose. The enzyme is highly specific for UDP-galactose. The most active acceptors are the naturally occurring erythro-trans and erythro-dihydrosphingosines. These authors suggested the formation of cerebroside from psychosine by acylation. Later, Brady (139) isolated a microsomal enzyme from brain tissue which catalyzed the condensation of stearoyl-CoA with galactopsychosine to form galactocerebroside. The pathway for the galactocerebroside synthesis therefore appears to be:

1) Palmitoyl CoA + TPNH → Palmitaldehyde,

2) Palmitaldehyde + Serine → Dihydrosphingosine,

3) Dihydrosphingosine → Sphingosine,

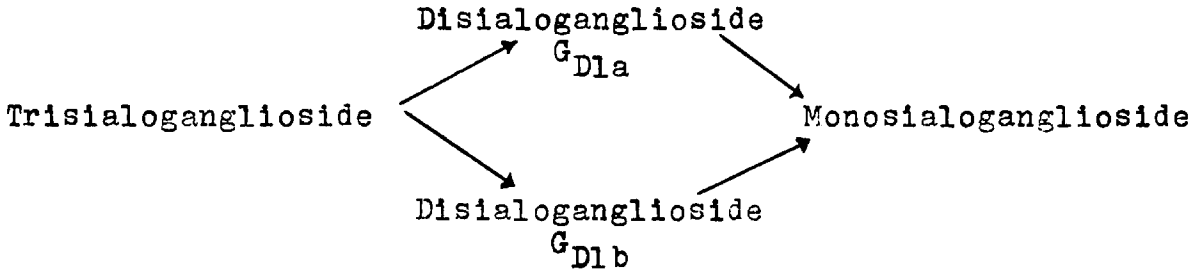
4) Sphingosine + UDP-gal → Psychosine + UDP,

5) Psychosine + Stearoyl CoA → Galacto-cerebroside + CoA-SH.

ii. Degradation of Gangliosides and Cerebrosides - Cleavage of Sialic Acid

Di- and trisialogangliosides are soluble both in chloroform-methanol and water. Part of the sialic acid from di- and trisialogangliosides are labile to enzymatic hydrolysis and dilute acid hydrolysis. Burton (123) showed that gangliosides from brain tissue were partially hydrolyzed by a Clostridium perfringens exoenzyme, neuraminidase (or sialidase), to yield N-acetylneuraminic acid (NAN) and a glycolipid residue. The glycolipid residue contained NAN which was resistant to the enzyme but was acid labile. The Cl. perfringens exoenzyme neuraminidase had a sharp pH optimum near 5.1 and $K_m = 4 \times 10^{-4}$ M. Recently Cassidy et al. (124) showed that a purified preparation of sialidase from Cl. perfringens hydrolyzed 44% of the total sialic acid of calf brain gangliosides.

Korey et al. (125,126) demonstrated a multienzyme gangliosidase system in rat and human brain capable of degrading gangliosides derived from bovine brain and from human brain, both normal and affected by Tay-Sachs disease. A number of neuraminidases (128) were unable to remove the internal N-acetylneuraminic acid (NAN) from monosialo-ganglioside, but the brain gangliosidase could degrade the individual species of gangliosides, including monosialo-ganglioside. By the action of sialidase (neuraminidase) the degradation occurs according to the following scheme: (129).



Cleavage of hexoses:

An enzyme preparation was obtained from mammalian kidney by Sandoff et al. (134), which hydrolyzed ceramide-tetrasaccharide and ceramide-trisaccharide to ceramide via ceramide-disaccharide and glucocerebroside. Hajra and Radin (131) showed the presence of a hydrolytic enzyme in pig brain which hydrolyzed galactocerebroside to galactose and ceramide. Brady et al. (132) purified (82-fold) a glucocerebroside-cleaving enzyme from rat and human spleen tissue. The products of the reaction were identified as glucose and N-stearoyl-sphingosine. The highly purified enzyme fraction did not catalyze the hydrolysis of galactocerebroside. Recently Brady et al. have isolated the gluco- and galactocerebrosidase from rat intestine (133). Gatt (136) has isolated from beef brain a glucocerebrosidase and a galactosidase which cleaves ceramide-disaccharide.

Cleavage of ceramide:

A soluble enzyme preparation was obtained by Gatt (135,136) from rat brain which catalyzed both the hydrolysis of ceramide to sphingosine and fatty acid, and its synthesis from these components. A different system, reported by

Zabin (116) and Sribney (126), catalyzed the enzymatic synthesis of ceramide from fatty acyl CoA and sphingosine.

4. Immunochemical Studies

W. E. van Heynigen (143) found that crystalline gangliosides specifically fixed tetanus toxin. Tetanus toxin is a highly potent poison to animals; 10^{-7} mg is the lethal dose for a mouse. It had long been suspected that such a toxin must have a highly specific point of attack in order to account for its great activity. Kuhn and Westphal (127) showed that injection of both mono- and disialogangliosides in mice increases their resistance to coli bacteria (110-200 times the lethal dose). Since that time there has been intensive research in many laboratories on the immunochemical properties of gangliosides and related sphingoglycolipids. Interest in this field has been renewed by the recent findings of Rapport et al. (144) that the injection of cytolipin-H (isolated from human epidermoid carcinoma) caused the production of a specific antibody. Recently Yokoyama et al. (141) showed that the injection of purified gangliosides caused the production of specific antibodies in rabbits when administered with a carrier substance such as rabbit or sheep erythrocytes, whole human serum, or bovine serum albumin. A different antibody was produced when erythrocyte was coated with a sialoganglioside before injection. Antibodies to gangliosides were demonstrated by hemagglutination, complement fixation, and passive tests of cutaneous anaphylaxis. In hemagglutination inhibition tests, gangliosides exhibited blood-group-M

and Rh_0 (D) activity, and some weaker activity of blood-group substance A and N. Asialogangliosides and crystalline N-acetylneuraminic acid were inactive in this respect. The hapten inhibition study has been used recently (142) to obtain information about the chemical structure of ganglioside. Studies with a variety of sugars and sugar derivatives indicated that inhibition is encountered only with a glycosidically bound β-D-galactopyranose or 2-acetamide-2-deoxy-β-D-galactopyranose moiety.

5. Chemical Synthesis of Glycolipids

Galactocerebroside and the glucocerebroside found in Gaucher spleen have been synthesized by Shapiro and Flowers (148). Since the natural and synthetic compounds were identical, the β-configuration has been assigned to the glycosidic bond of the natural cerebrosides.

Cytolipin-H was isolated from extracts of human epidermoid carcinoma by Rapport et al. (37). Shapiro and Rachaman (145) have reported the synthesis of N-lignoceroyl-1-D-sphingosyl-β-lactoside by an unambiguous procedure, the same procedure used for the synthesis of the cerebrosides. Schram et al. (147) also reported the synthesis of cytolipin-H which consisted of the condensation of ceramide with acetobromolactose in the presence of silver oxide.

Shapiro et al. (146) have also synthesized galactopsychosine, glucopsychosine, and dihydropsychosine. The general procedure was condensation of N-dichloroacetyl-3-O-benzoylsphingosine with acetobromoglucose or acetobromogalactose. The resulting product was deacylated by sodium

methoxide to the N-dichloroacetylcerebroside and hydrolyzed by warming with 0.3 N barium hydroxide at 70-80° for 90 min, giving the desired psychosines.

6. Sialyltransferases

Sialic acids are generally found as constituents of polymers including plasma glycoproteins (154), submaxillary mucins (155), glycolipids such as the gangliosides (4), and oligosaccharides of milk and colostrum (156,157,158). Colominic acid, the simplest known polymer of sialic acid, has also been isolated by Barry and Goebel (159,160). The partial structure of colominic acid has been determined by McGuire and Binkley (161). In all of these polymers, the sialic acids are glycosidically bound to galactose, N-acetylgalactosamine, or sialic acid residues. The position of the linkages may vary with species, e.g., bovine colostrum contains sialyl-(2→3)-lactose, while human colostrum contains sialyl-(2→6)-lactose.

Almost all of the natural sugars occur in polymers, and have been isolated as nucleotide derivatives; these serve as glycose donors in polymerization reactions. For example, a number of enzymes called sialyltransferases have been reported that catalyzed a general reaction , the transfer of sialic acid from its cytidine-5'-monophosphate derivative (CMP-NAN) to suitable acceptors containing galactose, N-acetylgalactosamine, or sialic acid as shown below:

$$\text{CMP-NAN} + \text{R} \rightarrow \text{R-NAN} + \text{CMP}$$

The different transferases show the following specificities toward the acceptor:

(a) A particulate preparation obtained from E. coli K-235 catalyzes the synthesis of colominic acid in the presence of suitable endogenous or exogenous colominic acid acceptors (162).

(b) The rat mammary gland sialyltransferase is a particulate enzyme system (163) which catalyzes the transfer of NAN from CMP-NAN to lactose (and also many other β-galactopyranosides) with the formation of sialyl-(2→3)-lactose.

(c) Soluble transferases have been demonstrated in colostrum from human, cow and goat (164). The enzyme from goat colostrum has been purified about 200-fold. With lactose as a substrate, this enzyme yields primarily the 2→6 isomer of sialyllactose. Rat mammary-gland sialyltransferase showed little or no reactivity with desialized glycoproteins, whereas with the enzyme from colostrum, sialidase pretreated orosomucoid and other glycoproteins acted as the most efficient acceptors. The result suggested that NAN was incorporated as the final step in glycoprotein synthesis, and that the incorporation of NAN was dependent on the position to which galactose was attached to the penultimate sugar residue.

(d) The sheep submaxillary gland sialyltransferases reported by Carlson et al. (165) is also a soluble enzyme system. It requires N-acetylgalactosamine as the terminal sugar

residue in the glycoprotein chain (such as ovine submaxillary mucin).

The new sialyltransferase system obtained from chicken embryonic brain shows, in contrast to the other transferases, marked preferences towards certain glycolipids. The differences are discussed in detail in Part I of this thesis.

7. Galactosyltransferases

Galactose is one of the important sugar residues in various glycoproteins, glycolipids, and oligosaccharides. A disaccharide 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine, obtained from fetuin (166), has been isolated from a variety of glycoproteins, including gastric mucin (167,168), meconium (169), blood group substances (170) and the α_1-acid-glycoprotein (171), suggesting that this may also represent a common structural pattern. Svennerholm (31) found two galactosamine-containing disaccharides after mild acid hydrolysis of monosialoganglioside. They were both composed of galactose and N-acetylgalactosamine; in one of the sugars the galactosamine moiety had a free-reducing group, while in the other this was contained in the galactose. The former disaccharide was also isolated by Klenk *et al.* (172) and later by Kuhn *et al.* (6). It was shown to be O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose and identical with a disaccharide of human blood-group substance isolated by Painter *et al.* (173). Kuhn and Wiegandt also isolated a disaccharide from monosialoganglioside which was identical with lactose

(galactose (β,1→4) glucose). Recently Kaufman *et al.* (174) have isolated a glycolipid from *Diplococcus pneumoniae* type XIV, and have established its structure as follows:

α-D-galactopyranosyl-(1→2)-α-D-glucopyranosyl-(1→3)-diglycerides.

The cell wall of most gram-negative organisms contains a complex lipopolysaccharide. Lipid-free polysaccharide can be obtained by mild acid hydrolysis of the cell wall lipopolysaccharide of *Salmonella typhimurium* (wild type). This polysaccharide contains 2-keto-3-deoxyoctonate (175), L-glycero-D-mannoheptose (176), D-glucose, D-galactose, and small amounts of glucosamine, in addition to mannose, rhamnose, and abequose. The configuration of the galactosyl linkage was established by treating the labeled polysaccharide with α- and β-galactosidases; it appears that galactose is linked to the 3-position of polysaccharide-linked glucose (177).

In view of the large variety of galactosyl-glycosides obtained from different sources, a number of enzymes may be expected to be involved in their synthesis. Several enzymes for the synthesis of these compounds have been isolated and are described below:

(a) A galactosyltransferase in milk and in mammary gland tissue has been reported by Hassid *et al.* (178) which catalyzed the formation of lactose when glucose was the acceptor. Some mammary gland preparations also utilized N-acetylglucosamine, yielding N-acetyllactosamine (partially characterized by Watkins and Hassid (179).

(b) Recently, several laboratories (180,181) have reported the existence of enzyme systems which catalyze the incorporation of D-galactose into the cell wall lipopolysaccharide of gram-negative enteric bacteria. The cell membrane fraction of mutant of Salmonella typhimurium that lacks UDP-gal-4-epimerase contains a UDP-galactose: lipopolysaccharide galactosyltransferase system which catalyzes the transfer of galactose from UDP-gal into the endogenous, galactose-deficient cell wall lipopolysaccharide (182,183). The galactose is transferred to the 3 position of glucose residue of the galactose-deficient polysaccharide.

(c) Kaufman et al. (174) have reported the presence of a specific galactosyltransferase system in Diplococcus pneumoniae Type XIV which transferred the galactosyl residue from UDP-gal to α-D-glucopyranosyl-(1-3)-diglyceride.

(d) Orosomucoid, fetuin, and other glycoproteins contain oligosaccharide side chains terminating in a trisaccharide:

Sialic acid → galactose → N-acetylglucosamine → glycoprotein.

Recently, McGuire et al. (186) isolated a soluble galactosyltransferase from goat colostrum which catalyzed the transfer of galactose from UDP-gal to the terminal N-acetylglucosamine units of pretreated orosomucoid. Orosomucoid, the α_1-acid glycoprotein (184), was pretreated with purified sialidase and β-galactosidase (185) and the resulting glycoprotein was used as substrate. This enzyme

system was also active with UDP-N-acetylgalactosamine and UDP-glucose when orosomucoid (pretreated with sialidase and β-galactosidase) and N-acetylglucosamine were employed as acceptors, and showed an absolute requirement for Mn^{++} ion.

The galactosyl-glycolipid transferase system isolated from chicken embryonic brain described in this thesis also showed an absolute requirement for Mn^{++} ion. It is important to note that the goat colostrum galactosyltransferase system isolated by McGuire *et al.* (186) was not active with glycolipids as acceptor. Studies of the activities, specificity, and kinetic aspects of the chicken embryonic brain galactosyltransferases are described in detail in Part II of this thesis.

B. MATERIALS

CMP, CTP and other nucleotides were purchased from California Biochemical Corp. Absolute methanol (ACS grade), ACS toluene, and USP chloroform were used during the course of work. The silicic acid used for chromatography was "Unisil"[1]. The Florisil[2] used was 60-100 mesh. The silica gel-G[3] contained binder.

The galactostat enzyme kit was purchased from Worthington Biochemical Corp. Glucose-6-phosphate hydrogenase and hexokinase were obtained from C. F. Boehringer and Sons, Mannheim, Germany. We are most grateful to the following individuals for their gifts of valuable compounds: Dr. T. Yamakawa for hematoside and globoside, Dr. Lars Svennerholm for monosialoganglioside and disialoganglioside, Dr. Abraham Rosenberg for Tay-Sachs ganglioside, and Dr. Edward J. McGuire for the glycoproteins used in this study. Other chemicals were obtained from commercial sources or were prepared as indicated.

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[1] "Unisil": activated silicic acid from chromatography, Clarkson Chemical Company, Williamsport, Pennsylvania.

[2] Floridin Company, Tallahassee, Florida.

[3] Silica gel-G, E. Merck, Darmstadt, Germany.

C. METHODS

1. Isolation, Extraction and Concentration of Gangliosides

Brain gangliosides were isolated by Klenk in 1942 by means of selective extraction with organic solvents. Folch (17) extracted crude lipid from brain tissues with chloroform-methanol (2:1, v/v), and ganglioside was isolated from the crude lipid by dialysis of the chloroform-methanol-water mixture against water. This partition technique was an excellent method for isolation of ganglioside, because ganglioside stays in the upper phase while most of the contaminating lipids were found in the organic layer. For the purification of gangliosides, cellulose-column chromatography was used by Svennerholm (190). Recently, ganglioside fractions were obtained in pure form (188,189) by silicic acid column chromatography. Trams and Lauter (188) extracted ganglioside with tetrahydrofuran from a phosphate buffer homogenate of brain tissue; the addition of ethyl ether induced two phases, with the ganglioside located in the water phase. Ionic nonlipid material was removed by passing the substance through a mixed bed resin, and the ganglioside was precipitated as the barium salt. Methods for extraction, purification, and separation of gangliosides on silicic acid are described in detail in a review article by Svennerholm (189). The method followed for isolation of purified mixture of ganglioside from calf brain is described in section C.6.

2. Chromatography

i. Silicic Acid Chromatography

The term silicic acid, or silica gel, refers to powdered solids of the general formula SiO_2, xH_2O, consisting of porous, three-dimensional siloxane (tetrahedral Si.O.Si.) structures with surface silanol (SiOH) groups. The fundamental adsorbent property of silicic acid is attributed to the silanol groups. The modern theory of adsorption and application of silica gel in lipid chemistry has been reviewed in detail by Warren (192). Weiss et al. (191) first used silicic acid column chromatography for separation of various sphingolipids. In this method, cerebrosides (194) and gangliosides (195) are eluted with phospholipids; cytolipin-H is eluted before phosphatidylethanolamines (193). Recently, however, it was found that if a Florisil column is used before the silicic acid column to remove phospholipids, the glycolipids can then be fractionated readily on the silicic acid column with increasing concentrations of methanol in chloroform (40). In our study we used "Unisil", a special type of activated silicic acid, 200-325 mesh, acid washed (purchased from Clarkson Chemical Company).

ii. Florisil Chromatography

Commercial Florisil consists chiefly of activated magnesium silicate, with a small amount of sodium sulphate (1-0.5%). Radin et al. (196) first described its use for the separation of cerebroside from other polar lipids such as phospholipid. They also used florisil for the

preparative isolation of cerebrosides (197). Florisil has been used for purification of glycolipids (198), steroids (200), fatty acid esters (199), and phospholipids (201). A chloroform-methanol mixture was used as the eluting solvent for glycolipids; increasing concentrations of methanol resulted in the elution of cerebroside, ceramide-disaccharide, ceramide-trisaccharide, and ceramide-tetrasaccharide. With diethyl ether, cholesterol can be eluted out from the florisil column leaving cerebroside on the column (199). The florisil column was used during the course of our work for purification of gangliosides from a crude mixture of calf brain gangliosides. The column was washed with 10 volumes of anhydrous methanol and finally with chloroform. The exact procedure is discussed below, in section C.7. It was also observed that the adsorption characteristics of florisil changed upon storage in the laboratory.

iii. Thin-Layer Chromatography

Thin-layer chromatography has been used for micro-analysis of lipids since 1961 (203-209). The apparatus developed by Stahl (202) for producing thin adsorbent layers has been generally used.

Procedure: Five glass plates were spread with a thin layer of mixture containing 30 g of silica gel G in 65 ml of H_2O; the gel and water were first shaken for 2 min in a 250 ml round-bottom flask. The plates were allowed to air dry 10-15 min for maximum gel formation and then dried at

105-125° for 1 hr. The plates were stored in a cabinet over a dehydrating agent.

To ensure the separation of closely related glycolipids without trailing or spreading, the compounds were spotted over the plated gel in narrow bands. The plates were then developed in different solvent mixture (ascending chromatography) according to the nature of compounds, in a closed glass jar. When the solvent reached almost to the upper edge of the plates, they were removed from the jars, dried at room temperature and sprayed with 0.04% bromothymol blue reagent in 0.01 N NaOH (210), to locate the lipids. The wet lipid spots were greenish-yellow on a light blue background, and when dried, the color changed to bluish-gray on a bluish-white background. Exposure to ammonia intensified the lipid color, and the plate was photographed with a Polaroid camera on regular high-contrast film. For preparative isolation of radioactive products, a series of bands were spotted on each thin layer silica gel plate (5-6 bands per plate). Each band was 1.5 cm wide and contained 200-300 µg of semipurified lipid.

After developing and spraying, the yellowish lipid bands were removed from the plate, transferred to small column, and the column eluted with acetone to remove the dye. Finally the lipids were extracted from the gel with C-M-H_2O mixture (2:1:0.2) until no detectable radioactivity (or, anthrone color) remained. For location of radioactive compounds on the plates, silica gel (2 cm^2 area) was scraped off the plates from the origin to the solvent front,

transferred directly to counting vials, 15 ml of toluene scintillation solvent added to each vial, and after thorough shaking, the samples were counted in a Packard Liquid Scintillation Spectrometer.

The following solvents were used during the purification of glycosphingolipids and for the isolation of radioactive products:

SOLVENTS FOR THIN-LAYER CHROMATOGRAPHY OF GLYCOSPHINGOLIPIDS

No.	Solvent	Type of lipid
T-I	C-M-Water (65:25:4)	Cytolipin-H, hematoside, ceramide
T-II	C-M-Water (20:10:2)	Ceramide-oligosaccharides
T-III	1-propanol-Water (7:3)	Gangliosides
T-IV	1-propanol-0.1 M KCl (7:3)	Gangliosides
T-V	1-propanol-1% sodium tetraborate (8:2)	Tay-Sachs gangliosides and normal gangliosides
T-VI	1-propanol-Water-15N-ammonium hydroxide (7:2:1)	Cytolipin-H and ceramide

iv. Gas-Liquid Chromatography

The term "gas chromatography" refers to all chromatographic methods in which the moving phase is a gas. This important technique has numerous applications in many branches of analytical chemistry (211-219). A gas chromatographic apparatus consists of four elements: (1) sample injecter; (2) column; (3) detector; (4) collector.

For this investigation a gas chromatography apparatus manufactured by the F and M Instrument Co. (Model 609) was

used. The column was 6 mm in diameter, and 6 feet long, and was packed with 10% DEGS (diethylene glycol succinate) on "gas chrom-G." Methyl esters of unknown fatty acid with internal standards in hexane solution (2-5 µl) were injected in the column. Dr. Y. Kishimoto kindly performed the fatty acid analyses.

v. Paper Chromatography

Partition chromatography (128, 263) on paper, which may be silica-impregnated (220), is another method for determining the purity of gangliosides. The following systems were used:

1. Solvent systems for untreated Whatman No. 1 papers:
   (a) Tetrahydrofuran-diisobutyl ketone-10% acetic acid (50:5:9), (221).
   (b) Diisobutylketone-acetic acid-water (40:30:7), (222).
   (c) n-Butanol-pyridine-water (3:1:1), (223).
2. Solvent for silica-gel impregnated paper:
   Diisobutyl ketone-acetic acid-water (40:25:5), (224).

The gangliosides were detected with Rhodamine-B or, with Cresyl violet (31).

3. Determination of Radioactivity

^{14}C-Labeled compounds were analyzed in the Packard Tri-carb Liquid Scintillation Spectrometer in a toluene system (225,226) containing 300 mg POPOP and 12.00 g of PPO in 3 liters of toluene (reagent grade). Radioactive lipids were spotted on filter paper (or Whatman No. 3 MM) and the paper strips were counted in the toluene system. Substances insoluble in the toluene or in dioxane, such as silica gel,

(obtained from radioactive lipid areas in thin-layer chromatography) were also counted in the toluene system.

4. Hydrolysis of Glycosphingolipids

The cleavage of esters and amides by ethanolysis or methanolysis has been practiced for many years (227,228). HCl in dry methanol, 5% (W/V), was used for methanolysis of glycosphingolipids in specially designed hydrolysis tubes (229) in order to avoid loss of HCl during heating. The glycosphingolipid, 1-5 μmoles samples, were mixed with 1 ml of 5% HCl in dry MeOH, and the tube closed with the screw clamp. The tubes were kept for 16-17 hours at 75°. After hydrolysis, the sample was quantitatively transferred to a 3 ml volumetric flask with anhydrous MeOH. Sphingosine was determined on an aliquot of this solution. Another aliquot of the MeOH-HCl solution was dried under nitrogen and rehydrolyzed with 1 ml of 1 N HCl for 3 hr in a boiling water bath. The hydrolyzed product was neutralized by passage through mixtures of Dowex-1-$CO_3^=$ and Dowex-50-H^+ resins, concentrated, quantitatively transferred to a 5 ml volumetric flask, and used for determining glucose and galactose.

Hydrolysis for hexosamine:

Direct hydrolysis of the lipid (0.1-0.5 μmole) was performed with 1 ml of 4 N HCl for 5 hr at 100° in teflon-coated screw capped tubes.

Hydrolysis for sialic acid:

Lipid samples (0.1-0.5 μmole) were hydrolyzed with 1.0 ml of 0.1 N H_2SO_4 for 1-2 hr at 80°. After cooling, 2 ml

of C-M (2:1) was added to the sample, and the upper water layer was taken for sialic acid analysis by Warren's thiobarbituric acid method (235) or by the resorcinol method (231). The lower, organic layer was concentrated and subjected to thin-layer chromatography for characterization of other hydrolysis products.

5. Analytical Methods for Quantitative Estimation

Protein was determined by a modification of the method of Lowry et al. (232), total hexose by a modified anthrone method (233), sialic acid by the Warren's thiobarbituric acid (235) or the resorcinol (231) method, hexosamine by a modified Elson-Morgan method (236), sphingosine by a colorimetric method (237), total nitrogen by the micro method of Nessler (238). Esters were measured colorimetrically by the neutral hydroxamate procedure (239) and by gas-liquid chromatography (240) after the methanolysis reaction. The composition of fatty acids was also determined by GLC and the fractions were collected according to the procedure of Hajra et al. (241). Glucose was determined by a coupled enzyme reaction with hexokinase, and glucose-6-P dehydrogenase (242). Galactose was determined by the galactose oxidase method (243), and also with galactose dehydrogenase (244).

6. Isolation of Cytolipin-H from Ox Spleen

The method used for isolation of cytolipin-H (ceramide-lactoside) was based primarily on the method published by Rapport et al. (40).

Ox spleen (40 kg) that had been stored at -18° was thawed and chopped in an electric meat grinder, and dehydrated by suspension in 80 liters of acetone. After standing overnight at room temperature, the mixture was filtered through a double-layer of gauze; the residue was re-extracted with acetone. The second residue was air-dried, and the resulting brown powder was extracted with 60 liters of chloroform-methanol(2:1). The mixture was stirred mechanically for 6 hr after the addition of 3 liters of H_2O. After filtration, the brown filtrate was shaken with 13 liters of H_2O and placed in the cold room (4-5°) overnight to break the emulsion. This step was not successful, and separation of phases was achieved by addition of KCl (15 ml of saturated KCl solution per 20 liters of emulsion). The lower, chloroform, layer was concentrated to a thick mass in a flash evaporator. Finally the lipid was dried in a Swissco rotary evaporator, to completely remove the solvent. From 40 kg of tissue, 728 g of crude lipid were obtained.

The crude lipid (720 g) was dissolved in 4.5 liters of diethyl ether containing 105 ml of water. The solution was divided equally among 3 glass-stoppered graduated cylinders (2 liter capacity), and 465 ml of acetone added to each. After these components were allowed to stand in a cold room (4-5°) for 5 days, the precipitated lipid was carefully collected by cold centrifugation, dissolved in C-M (2:1), and evaporated to dryness, after which 300 g of yellowish-brown lipid remained. The 300 g of ether-acetone

insoluble lipid were then suspended in 1,400 ml of glacial acetic acid (at 50°) and 1,800 ml of acetone were added. The undissolved residue was removed by centrifugation at room temperature and the precipitate was washed three times with 1 liter portions of ether acetone (4:1) at 5°. The residue was dissolved in 2 liters of C-M (2:1), filtered through fritted glass, and dried in a rotary evaporator. Thus, 51 g of acetic acid-acetone insoluble lipid were obtained. The lipid was dissolved in 400 ml of C-M (2:1) and passed through a short florisil column (30 g of florisil in a 4.5 x 85 cm column). The column was washed with 400 ml of C-M (2:1) and the combined eluent was evaporated to dryness, yielding 36.5 g of lipid.

Florisil column chromatography:

Florisil, 250 g, suspended in 600 ml of C-M (4:1), was placed in a column (4.3 x 85 cm); the column was washed with 800 ml of the same solvent, and a solution of 12 g of the above lipid (dissolved in 20 ml of C-M, 2:1) was applied to it. The composition and volume of fractions eluted from column are given below.

Fractions	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
C-M	4:1	4:1	4:1	4:1	2:1	2:1	2:1	2:1	1:1	1:3	MeOH
Volume (Liters)	1	0.25	0.25	0.25	3	1.5	1.0	0.5	3	1	1

Fractions V thru VIII showed the maximum anthrone reaction, and were combined. Material from 3 fractionations were combined and 645 mg of lipid were obtained.

Silicic acid column chromatography:

Unisil, 400 g, was washed with 1 liter each of methanol, acetone, and chloroform in the indicated order. The column (3.5 x 85 cm) was packed from a suspension in 1 liter $CHCl_3$. A solution of 645 mg of florisil-purified lipid in 25 ml of chloroform-methanol (95:5) was placed on the column, and fractions were collected by discontinuous gradient elution as given below:

Fractions	I	IIA	IIB	IIIA	IIIB	IIIC	IVA	IVB
C-M	$CHCl_3$	95:5	92:5	92:8	92:8	92:8	80:20	80:20
Volume	2.5	2	3	3	3	3	3	2

Fractions IIIC and IVA were combined and evaporated (250 mg) to dryness, giving a yellowish viscous lipid; 20 ml of dry ether was added to the syrup, and white flakes of ceramide-disaccharide precipitated. The lipid was filtered through a fritted glass funnel, and was characterized by TLC and by infrared spectroscopy. The material was analyzed by the indicated methods, and the following molar ratios were obtained: sphingosine, 1.00 (methyl orange); glucose, 1.04 (hexokinase, glucose-6-P dehydrogenase); galactose, 1.01 (galactose oxidase).

7. Isolation of Gangliosides from Calf Brain

The crude ganglioside was isolated from calf brain by a modification of Rosenberg and Chargaff's procedure (19). Using a Waring Blendor, 5.9 kg of frozen calf brain were treated 5-10 min with a 10-fold volume (v/w) of C-M (2:1, v/v) previously chilled to 4° C. The resulting mixture was filtered by suction, and 0.2 vol of water (12 liters) was

added to the filtrate and vigorously stirred. A cloudy upper layer separated and was recovered. The lower chloroform layer was washed twice with 0.5 vol C-M-H_2O mixture (5:48:47). The upper layers were combined, adjusted to pH 9.0 with NH_4OH after concentration to a final volume of 2.5 liters. The concentrated upper layer contained crude ganglioside and was dialyzed against distilled water for 7 days in the cold room (4-5°). After dialysis, the contents of the bags were evaporated to dryness, dissolved in 200 ml of C-M (2:1), and kept at -20° for 2-3 months. Before purification of the crude gangliosides, the chloroform-methanol mixture was filtered through fritted glass and subjected to florisil column chromatography, yielding 14 g of crude ganglioside from 5.9 kg of calf brain (wet weight).

Purification of ganglioside by florisil column chromatography:

Florisil, 200 g was washed in a 2 liter beaker with a total of 4 liters of MeOH and then with 3 liters of C-M (2:1), until the supernatant fluid was clear. The column (4.5 x 30 cm) was packed from a suspension of florisil in C-M (2:1). A solution of 12.5 g of crude ganglioside in 100 ml of C-M (2:1) was placed on the column, and fractions were collected using a discontinuous gradient elution procedure as outlined below:

Fractions	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
C-M	2:1	--	--	1:1	--	--	--	--	1:2	--	--	MeOH	--
Volume (liters)	2	1.5	0.5	2	2	2	2	2	2	2	0.5	3	1
Lipid (g)	--	1.5	--	2.5	2.7	--	2.4	--	--	1.4	--	1.5	--

The total recovery of lipid was 95%. Fractions IV, V and VI thru VIII were characterized as ganglioside mixture (mono and disialo) free of cerebroside or other glycolipids.

8. Preparation of Ceramide-Oligohexosides from Calf Brain Gangliosides

The major lipid fraction (7.6 g), obtained with C-M (1:1) was concentrated to dryness, and hydrolyzed in 0.15 N H_2SO_4 at 100° for 2 hr. The hydrolysate was neutralized with Dowex-1 HCO_3-form resin, filtered, and the lipids extracted by the procedure of Folch et al. (17). The mixture of ceramide-monosaccharide and ceramide-oligosaccharide (4 g) was separated by silicic acid (Unisil) column chromatography (3.7 x 70 cm column). The column was eluted successively with 2 liters of chloroform, 6 liters of C-M (92:8), 5 liters of C-M (80:20), and 5 liters of C-M (1:1). The ceramide glycosides were obtained in the following fractions:

(a) Ceramide-monosaccharide (46 mg) in the first 3 liters of C-M (92:8)

(b) Ceramide-disaccharide (340 mg) in the first 3 liters of C-M (80:20)

(c) Mixture of ceramide-disaccharide and ceramide-trisaccharide (70 mg) in the remaining 2 liters of C-M (80:20)

(d) Mixture of ceramide-trisaccharide and ceramide-tetrasaccharide (740 mg) in the initial 3 liters of C-M (1:1)

FIGURE 2

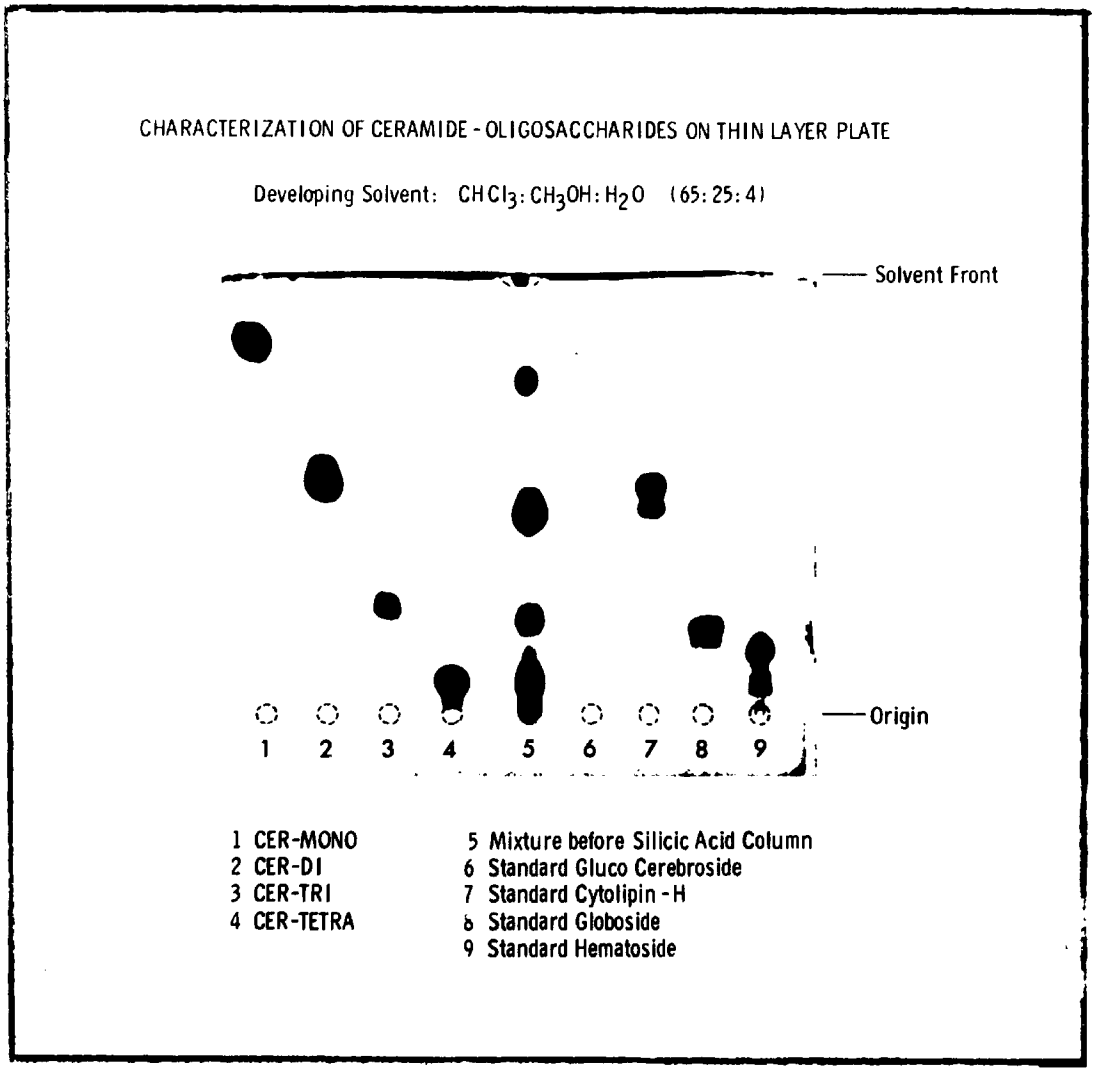

CHARACTERIZATION OF CERAMIDE - OLIGOSACCHARIDES ON THIN LAYER PLATE
Developing Solvent: $CHCl_3 : CH_3OH : H_2O$ (65:25:4)
Solvent Front
Origin
1 2 3 4 5 6 7 8 9
1 CER-MONO
2 CER-DI
3 CER-TRI
4 CER-TETRA
5 Mixture before Silicic Acid Column
6 Standard Gluco Cerebroside
7 Standard Cytolipin - H
8 Standard Globoside
9 Standard Hematoside

TABLE 1

Analyses of Ceramide Oligosaccharides

(Isolated from Acid Hydrolyzed Gangliosides)

	Sphingo-sine	Fatty Acid	Glucose	Galac-tose	Hexos-amine
Ceramide-monosaccharide	1.00	0.99	0.90	0.00	0.00
Ceramide-disaccharide	1.00	0.99	0.82	0.89	0.00
Ceramide-trisaccharide	1.00	1.07	0.91	1.30	0.94
Ceramide-tetrasaccharide	1.00	0.99	0.74	2.12	0.98

Molar ratios relative to sphingosine.

(e) Ceramide-tetrasaccharide (370 mg) in the final 2 liters of C-M (1:1).

The components of fractions (c) and (d) were separated by thin-layer chromatography on silica gel-G with solvent T-I, and eluted with C-M (1:2). The final products were chromatographically homogeneous, as shown in Figure 2. They also showed the expected analytical values for each of the constituents as shown in Table 1.

9. Purification of Tay-Sachs Ganglioside

The Tay-Sachs ganglioside (TSG) obtained from Dr. Abraham Rosenberg contained some impurities and was purified by thin-layer chromatography, using 3 different solvent systems in the following order: (1) solvent T-IV, (2) solvent T-V, and (3) solvent T-II. After developing the plates with each solvent the heavy TSG areas were monitored and detected with the bromothymol blue spray (.04% in 0.01 N NaOH), dried, removed from the plate, put in a small column (1 x 20 cm), extracted with acetone (to remove dye) and then with C-M-H_2O (20:10:2) to elute the purified lipid. The eluent was concentrated and rechromatographed until pure Tay-Sachs ganglioside was obtained. About 65% of purified Tay-Sachs ganglioside was obtained from the crude material. This preparation was used as a substrate for the galactosyltransferase system.

10. Preparation of Labeled CMP-NAN

Preparation of CMP-NAN synthetase:

Frozen slices of hog submaxillary gland, 400 g were placed in 800 ml of 0.1 M K-phosphate buffer, pH 7.6, in a

2 liter flask. The tissue suspension was shaken for 5-6 hr, then filtered through cheesecloth. The filtrate was centrifuged (39,000 x g) for 30 min. The supernatant was filtered through glass wool (to remove mucin) and dialyzed for 6-8 hr against 0.01 M Tris-HCl buffer, pH 7.6. The dialyzed, crude enzyme extract was recentrifuged for 15 min at 39,000 x g, and stored in a freezer overnight at -18°.

DEAE-Cellulose column chromatography:

Commercially available DEAE-cellulose was treated with KOH (1 N) and washed with water until pH of eluent was 7.5. After removing fines by washing with water in a beaker, a column (6 x 15 cm) was prepared, washed with 1 liter of 1 M KCl, 1.5 liter of water (until Cl^- free), and finally with 2 liters of 0.01 M Tris, pH 7.6. The crude enzyme, 650 ml, was put on the column (the rate of flow of liquid was 1 ml per 2 min). Hemoglobin was eluted with 1,500 ml of 0.01 M Tris (pH 7.6) containing 1% ME, and finally the enzyme was eluted with 4.5 liters of 0.01 M Tris containing 0.075 M KCl. To the 4.5 liters of eluent, 2.7 kg of solid ammonium sulphate was added gradually over about 1 hr, with gentle but constant stirring (0-90% saturation). The solution was stirred for an additional hour. The mixture was filtered through a 1 cm celite pad (previously washed with 90% ammonium sulphate mixture containing Tris, ME, KCl), without exposing the pad to air at any time. The celite pad was extracted with 150 ml of 0.05 M Tris (containing 0.01 M KCl, 1% ME solution), centrifuged and the residue was reextracted with 200 ml of

the above buffer mixture. Both the supernatants were combined (350 ml) and dialyzed against 6 liters of 0.05 M Tris (containing KCl and pH 7.6 and 1% ME) overnight (6-8 hr) in the cold room (4-5°).

The enzyme was assayed before the start of a large batch incubation by a method described by Kean and Roseman (234); 8.8 μmoles of CMP-NAN were formed per ml of enzyme per hr.

Large scale incubation:

The initial incubation mixture contained the following components: 100 ml of enzyme, 270 μmoles of ^{14}C-N-acetyl labeled NAN (4.4×10^6 cpm/μmole), 232 mg of unlabeled NAN, 6 ml of CTP (1.3 g of di K^+ salt of CTP were dissolved in 10 ml Tris buffer pH 9.0), 10 ml Tris (1 M $MgCl_2$:1 M Tris, 1:9, pH 9.0).

The mixture was incubated for 1 hr at 37°; then 3 ml of CTP solution and 20 ml of enzyme were added and incubated for another hour at 37°. After the second hour 1 ml of CTP and 10 ml of enzyme were again added, and the mixture incubated for one hour. After a total incubation of 3 hr, a 0.5 ml aliquot was removed for assay of CMP-NAN, and the remainder of the incubation mixture was frozen.

Separation of CMP-NAN and NAN:

A Dowex 1-HCO_3-column was prepared (450 ml of packed resin in a 2.5 x 50 cm column), washed with 3-4 volumes of 1 M $KHCO_3$, and finally with water until the pH of the eluent was 6.5. A 150 ml portion of the incubation mixture was placed on the column. A linear gradient of 1.5 liters of

water *vs.* 1.5 liters of fresh 0.60 *M* triethyl ammonium bicarbonate (TEA-HCO_3^-), pH 7.4, was used for elution, and 20 ml fractions were collected. The fractions were assayed for radioactivity, the second ^{14}C-peak contained CMP-NAN-^{14}C. The fractions containing CMP-sialic acid were pooled in a 2 liter flask and evaporated 3 times with 1% TEA to about 50-75 ml. The pH was continually checked and maintained between 7.5-8.0 by adding 1% triethylamine in water. The yellowish syrup was transferred to a small flask, evaporated 4 or 5 times with 2% NH_4OH in water, and then 4 or 5 times with 2% NH_4OH in absolute alcohol, until a white precipitate appeared. The white CMP-NAN residue was separated by centrifugation, washed once with 2% NH_3 in absolute alcohol, and redissolved in 10 ml of 1.0% NH_4OH in a 12 ml clinical centrifuge tube. Since the solution was slightly turbid, it was recentrifuged and the supernatant then contained purified CMP-NAN. Aliquots (200 µl) of this solution were placed in vials, evaporated, and stored at -20° for use in studying the sialyltransferase reaction.

11. Chemical Synthesis of Labeled UDP-Galactose

^{14}C-UDP-galactose was prepared chemically by modifying the method of Roseman *et al.* (246). A lithium salt solution of ^{14}C-gal-1-PO_4 (186 µmole, 4 µc/µmole) was passed rapidly through a 5 ml column of Dowex-50 H^+ form resin (200-400 mesh), and the eluent was collected directly in a 100 ml round-bottom flask containing 1 ml of pyridine. The solution was dried and the following compounds were then added consecutively: 0.2 ml H_2O, 1.0 ml dry pyridine, and 0.24 ml

of trioctylamine. The mixture was dried in a Swissco evaporator, 25 ml of pyridine was added, and the mixture again dried. Solid UMP morpholidate 94 μmole (94 mg) and 25 ml of anhydrous pyridine was added and shaken until the solution became clear. Water was removed in a specially designed apparatus by the repeated addition of anhydrous pyridine (dried over CaH_2) followed by evaporation. Finally, 2-5 ml of pyridine was added to dissolve the trioctylamine salt of gal-1-PO_4 and the UMP-morpholidate. The flask was closed with a glass stopper, covered with tinfoil, and placed in a dessicator over KOH and $CaCl_2$. The reaction mixture was maintained for 5 days at room temperature.

The mixture was dried at 30° C to completely remove the pyridine. Water (20 ml) and 0.6 m$\underline{M}$ of sodium acetate were added to the dried mixture, and then 20 ml of ether was added. The lower layer contained all the radioactivity. Gal-1-PO_4 (^{14}C) and UDP-galactose (^{14}C) were separated on a 2 x 10 cm column of Dowex-1 (Cl^-) resin (200-400 mesh). Compounds were eluted using a linear gradient: the mixing vessel initially contained one liter of 0.04 $\underline{M}$ LiCl in 0.003 N HCl, and the storage vessel contained one liter of 0.08 $\underline{M}$ LiCl in 0.003 N HCl. Fractions of 20 ml were collected. Gal-1-PO_4 appeared in fractions 9-23 and UDP-galactose in fractions 53-90. Fractions 61-90 were combined and the initial pH of 2-3 was adjusted to pH 7.0 (glass electrode) with solid LiOH as rapidly as possible. The neutralized fraction was evaporated to syrup, 10 ml of MeOH

and 100 ml of acetone were added, and the mixture evaporated to dryness. The syrup was dissolved in 5 ml of anhydrous MeOH, acetone was added (90-100 ml) gradually until a flocculent precipitate appeared. The cloudy solution was allowed to stand overnight at room temperature. The precipitate was then centrifuged in a 40 ml clinical centrifuge tube. The supernatant was decanted and the tube was covered with filter paper and placed in a desiccator over KOH and $CaCl_2$. After drying overnight, the residue was suspended in 3 ml of MeOH, stirred and centrifuged; the supernatant contained UDP-gal. The undissolved residue was washed twice with MeOH (total of 3 ml). The MeOH extracts were combined (6 ml) and 5-8 vol of acetone were added gradually until a flocculent precipitate appeared. The precipitate was collected by centrifugation, washed with acetone, dried in a desiccator and characterized by borate high voltage paper electrophoresis.

12. Incubation of Chicken Eggs and Isolation of Embryonic Brains

Fertilized hen's eggs of a Leghorn strain* were incubated in a humidified atmosphere at 37°. The stage of development of the embryos was determined by the time of incubation. The embryos were removed at the indicated times from the egg, and freed from embryonic membranes and external fluid. The brains were dissected from embryos by two different processes:

* Irish Hills Pullet Farm, Clinton, Michigan.

(1) For embryos 9-13 days old, brains were removed by pressing the neck gently toward the forehead.

(2) For embryos above 13 days, a sharp scalpel was used to open the head, and the brain was removed with the help of a "V" shaped stainless steel spatula.

Quantities of 200-400 specimens were obtained at one time and kept frozen at -20° until used in the study.

13. In Vivo Incorporation of Labeled Sugars in Embryonic Chicken Brain Ganglioside

The incorporation in vivo of D-glucose-^{14}C, D-glucosamine-1-^{14}C and sialic-^{14}C, into ganglioside fractions of chicken embryonic brain tissue has been studied. Solutions of the labeled sugars were injected into the yolk sac of 11 day old chicken embryo, incubated for 4 days and brains were extracted with chloroform-methanol (2:1). The chloroform extract was washed according to the procedure described by Folch et al. (17), dialyzed overnight and the radioactivity of both the upper water layer and the lower chloroform layer was determined. Attempts were made to characterize radioactive lipids on thin-layer plates with different solvent systems. Incorporation of different radioactive sugars into embryonic chicken brain glycolipids is given in the following table.

	Glucose-U-^{14}C		Glucosamine-^{14}C		Sialic-^{14}C	
No. of eggs injected	2		2		2	
Cpm per egg	2.15×10^6		2.25×10^6		5.6×10^4	
Volume of C-M (2:1) extract	30 ml		30 ml		20 ml	
Total cpm in lower chloroform layer after dialysis	1.28×10^4		7×10^2		6×10^3	
Total cpm in upper water layer after dialysis	1.66×10^4		7.7×10^3		1.53×10^4	
Total cpm in water layer evaporated and re-extracted with C-M (2:1)	6.8×10^3		3.4×10^3		6.1×10^3	
Mobility on thin-layer same as given lipids	C-M layer	water layer	C-M layer	water layer	C-M layer	water layer
	hemato-side	—	—	ganglio-side	hemato-side	ganglio-side

Specific activities: glucosamine, 4.4×10^6 cpm per μmole and sialic acid, 1.14×10^6 cpm per μmole.

Mild acid hydrolysis of labeled glycolipid isolated from sialic-^{14}C injected embryonic brains indicated that the radioactivity was incorporated in sialic acid. The radioactive sialic acid was identified by high voltage borate paper electrophoresis.

PART I

STUDIES ON THE SIALYLTRANSFERASES OF EMBRYONIC CHICKEN BRAIN

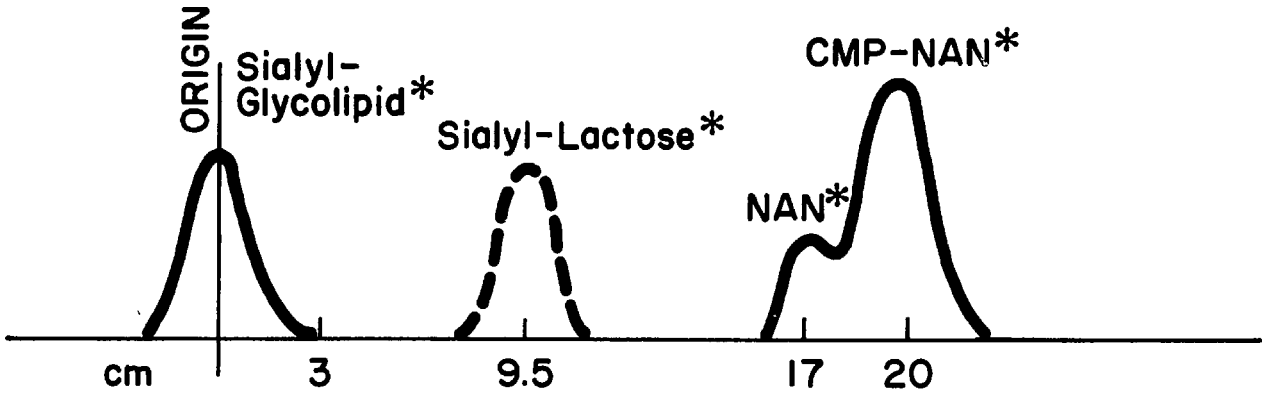
A. PREPARATION OF ENZYME SYSTEM

All steps in preparing the enzyme were performed at 0° to 4°. Fresh or fresh-frozen brains were obtained from 9 to 11 day chicken embryos and were homogenized with a glass Potter-Elvehjem homogenizer in a solution containing 0.25 M sucrose, 0.001 M EDTA (pH 7.0) and 0.014 M 2-mercaptoethanol. Four specimens (600-800 mg) were used per ml of sucrose solution. Nuclei, debris, and whole cells were removed by centrifugation at 750 x g for 10 min. The enzyme fraction sedimented in a light particulate layer (called the buffy coat) overlaying a packed heavy layer, when the supernatant from low-speed centrifugation was centrifuged at 18,000 x g for 30 min. The buffy coat was washed with 5 volumes of the sucrose solution described above, resuspended in 2 volumes of fresh solution, ruptured in a French pressure cell (6,000 lb/sq in), and centrifuged at 39,000 x g for 1 hour. The enzyme activity was located in the supernatant fluid, but could be sedimented at 100,000 x g for 1 hr. Incorporation of radioactivity into endogenous acceptors in the enzyme fraction was lowest when 9 to 11 day old chick embryos were used for preparation of the enzyme.

FIGURE 3

ASSAY FOR SIALYLTRANSFERASES IN EMBRYONIC CHICKEN BRAIN

(PARTICULATE FRACTION)



Paper Electrophoresis – 0.05 M Borate, pH 9.0, 50 min, 3500 volts.

Incubation Conditions – (μmoles/0.20 ml)
CMP-NAN*, 0.1; Cer-Di, 0.1;
Cacodylate-HCl Buffer pH 6.4, 20;
$MgCl_2$, 2.0; Triton CF-54, (400 μg);
Tween-80, (200 μg); Protein, 0.55 mg.
Incubated at 37°C for 2 hours.

B. ASSAY OF ENZYMIC ACTIVITY

The following reagents were required for the determination of enzyme activity: 1.0 M cacodylate-HCl buffer, pH 6.0; 0.2 M $MgCl_2$; 0.01 M glycosylsphingolipid (ceramide-disaccharide or ceramide-tetrasaccharide) in C-M (2:1); mixture of 0.4% Tween-80 and 0.8% Triton CF-54 in C-M (2:1); 0.005 M CMP-NAN-^{14}C (acetyl or carboxyl labeled; specific activity, 1.0 x 10^6 cpm per μmole), adjusted to pH 7.5 with $KHCO_3$ and stored at -18°; enzyme (10 mg protein per ml).

Procedure: The incubation mixture contained the following components in final volumes of 0.10 ml: cacodylate buffer, 10 μl; $MgCl_2$, 10 μl; CMP-NAN-^{14}C, 10 μl; Tween-80 + Triton CF54 solution, 25 μl; ceramide-tetrasaccharide (or ceramide-disaccharide), 10 μl; and 50 μl of enzyme. The lipid and detergents in C-M (2:1) were dried in the incubation tubes prior to adding the other reagents. After incubating for 2 hours at 37°, 10 μl of C-M (2:1) was added and the contents of each tube were quantitatively transferred to dry Whatman 3 MM paper with more C-M (2:1). The paper was carefully saturated with 0.05 M borate, pH 9.0 and the solution was allowed to diffuse from both sides toward the origin. After electrophoresis for 50 min, at 67 volts per cm as described above, the radioactive gangliosides remained at the origin, whereas other charged substances, such as CMP-NAN and NAN migrated 17 to 20 cm as shown in Fig. 3. Control tubes lacked either glycolipid acceptor, or contained heat-denatured enzyme.

Storage and stability of enzyme preparation: The partially purified enzyme preparation contained 10 mg protein per ml, and was stable to storage at -20° for at least 3 months when ceramide-disaccharide or lactose was used as the acceptor (Table 2).

In addition to the abbreviations given at the beginning of this thesis, the following are used in the next section: ^{14}C-acetyl (carboxyl)-labeled N-acetylneuraminic acid = NAN* or C^{14}-NAN; detergent mixture = a 2:1 (volume/volume) mixture of Triton CF-54 and Tween-80.

TABLE 2

Fractionation of Sialyltransferases Activity and

Effect of Freezing and Thawing

A. Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.12 ml: ceramide-disaccharide, 0.05; Tween-80, 100 μg; Triton CF-54, 200 μg; cacodylate-HCl buffer, pH 7.0, 12.5; lactose, 2.0; $MgCl_2$, 1.0; glycine, 2.5; CMP-NAN, 0.05 (1.08×10^6 cpm per μmole); enzyme 0.4-0.6 mg of protein.

B. All the components were the same as above, except for the enzyme fractions which were frozen and thawed once (after 12 hours) prior to assay. All mixtures were incubated at 37° for 4 hours.

Exp. No.	Enzyme preparation	NAN* incorporated (cpm/mg protein/4 hr)	
		Cer-di	Lactose
A	Homogenate	370	3,430
	"Buffy Coat"		
	1. Unwashed	930	6,100
	2. Washed	2,020	12,710
	3. Ruptured in French Press	2,380	4,450
B	Homogenate	460	4,320
	Ruptured in French Press	2,070	4,600

C. EXPERIMENTAL AND RESULTS

1. Preliminary Kinetic Studies with the Enzyme System

Incubation mixtures were assayed by high voltage paper electrophoresis as described above unless otherwise indicated.

i. Requirements for CMP-NAN:Glycolipid Sialyltransferase(s)

The conditions required for the transfer of ^{14}C-NAN to ceramide-disaccharide and ceramide-tetrasaccharide from labeled CMP-NAN are given in Table 3. The enzyme system contained endogenous acceptors; these have not yet been characterized. However, the synthesis of ^{14}C-sialoglycolipid was stimulated four-fold to ten-fold by adding ceramide-disaccharide or ceramide-tetrasaccharide. The experiment indicated that detergent was required by the system, but it is not clear whether Mg^{++} is or is not required. $MgCl_2$ did not stimulate the incorporation into ceramide-disaccharide, while it appeared to stimulate incorporation into ceramide-tetrasaccharide. The effects of metal ions are discussed below in greater detail.

ii. Effects of Incubation Time and Protein Concentration

The incorporation of ^{14}C-NAN was linear with time (Fig. 4), and with protein concentration (Fig. 5) within the given range (0.2-0.8 mg) in the standard incubation mixture. In these experiments ceramide-disaccharide and ceramide-tetrasaccharide were used as the NAN acceptors.

iii. Effect of pH

The incorporation of ^{14}C-NAN as a function of pH is shown in Figure 6. Endogenous values (incubations lacked

TABLE 3

Requirements for CMP-NAN:Ceramide-Disaccharide and CMP-NAN:Ceramide-Tetrasaccharide Sialyltransferases

A. The complete incubation mixture, contained the following components (μmoles) in final volumes of 0.2 ml; ceramide-disaccharide, 0.2; detergent mixture containing Tween-80 and Triton CF-54 (1:2 v/v), 600 μg; cacodylate-HCl buffer, pH 6.3, 20; $MgCl_2$, 2.0; CMP-NAN-^{14}C, 0.1 (1.08×10^6 cpm/μmole); enzyme 0.86 mg protein.

B. The components were the same as above, except that ceramide-disaccharide was omitted and half the quantities of the other substances in final volumes of 0.1 ml were used; the amount of ceramide-tetrasaccharide was 0.05 μmoles, and the pH was 6.0.

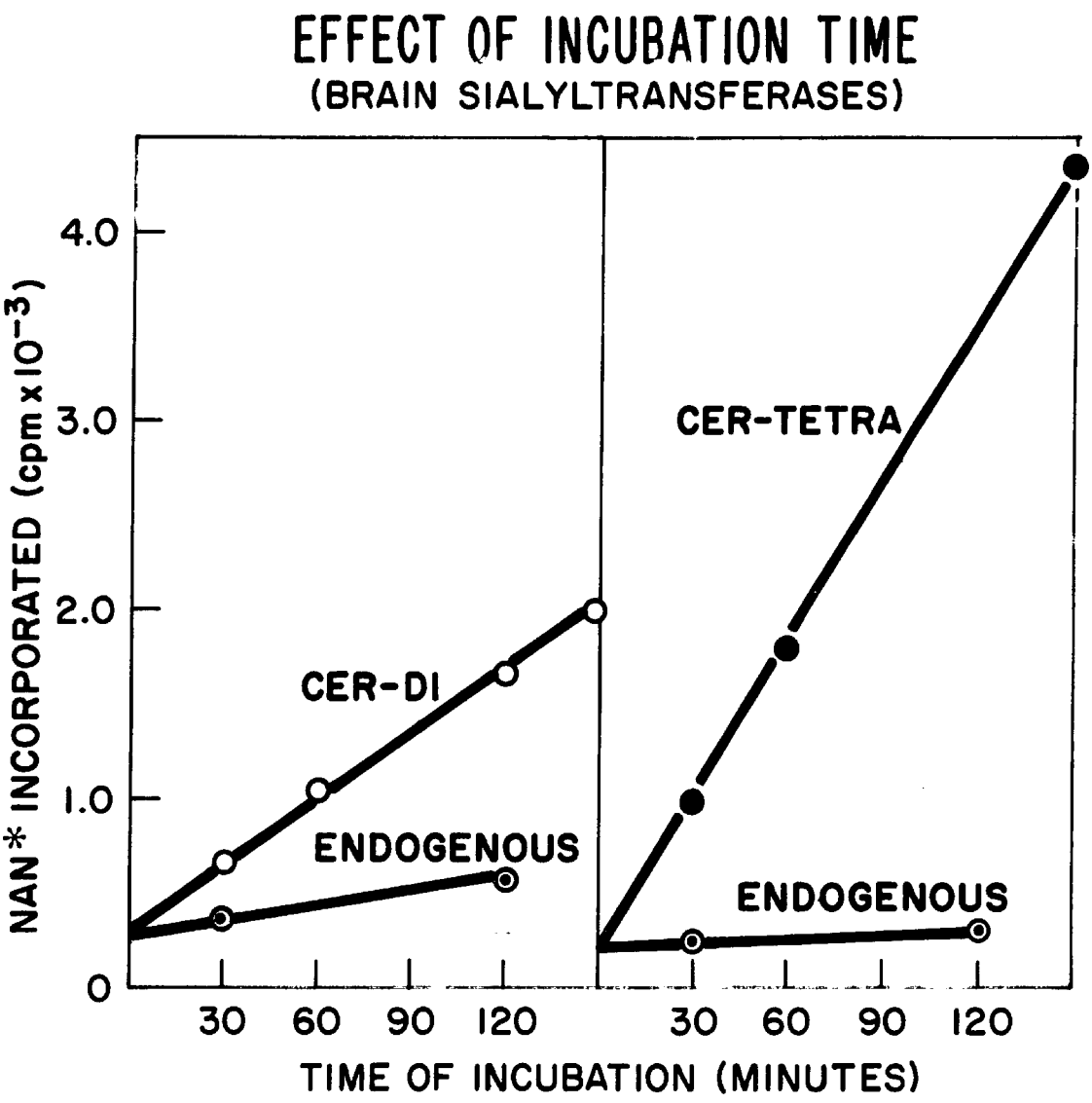
The samples were assayed, after two hours incubation at 37°, by paper electrophoresis in 1% sodium tetraborate, pH 9.0, at 55 volts per cm for 50 minutes, followed by ^{14}C determination by liquid scintillation methods.

Exp. No.	Incubation mixture	NAN* incorporated (cpm)
A	Ceramide-disaccharide	
	Complete systems†	2,763
	Minus ceramide-disaccharide	542
	Minus detergent mixture	579
	Minus $MgCl_2$	2,694
B	Ceramide-tetrasaccharide	
	Complete system‡	4,786
	Minus ceramide-tetrasaccharide	296
	Minus detergent mixture	316
	Minus $MgCl_2$	2,694

† Product formation was proportional to time of incubation for 2 1/2 hours and to protein concentration over the range of 0.2-0.8 mg per incubation.

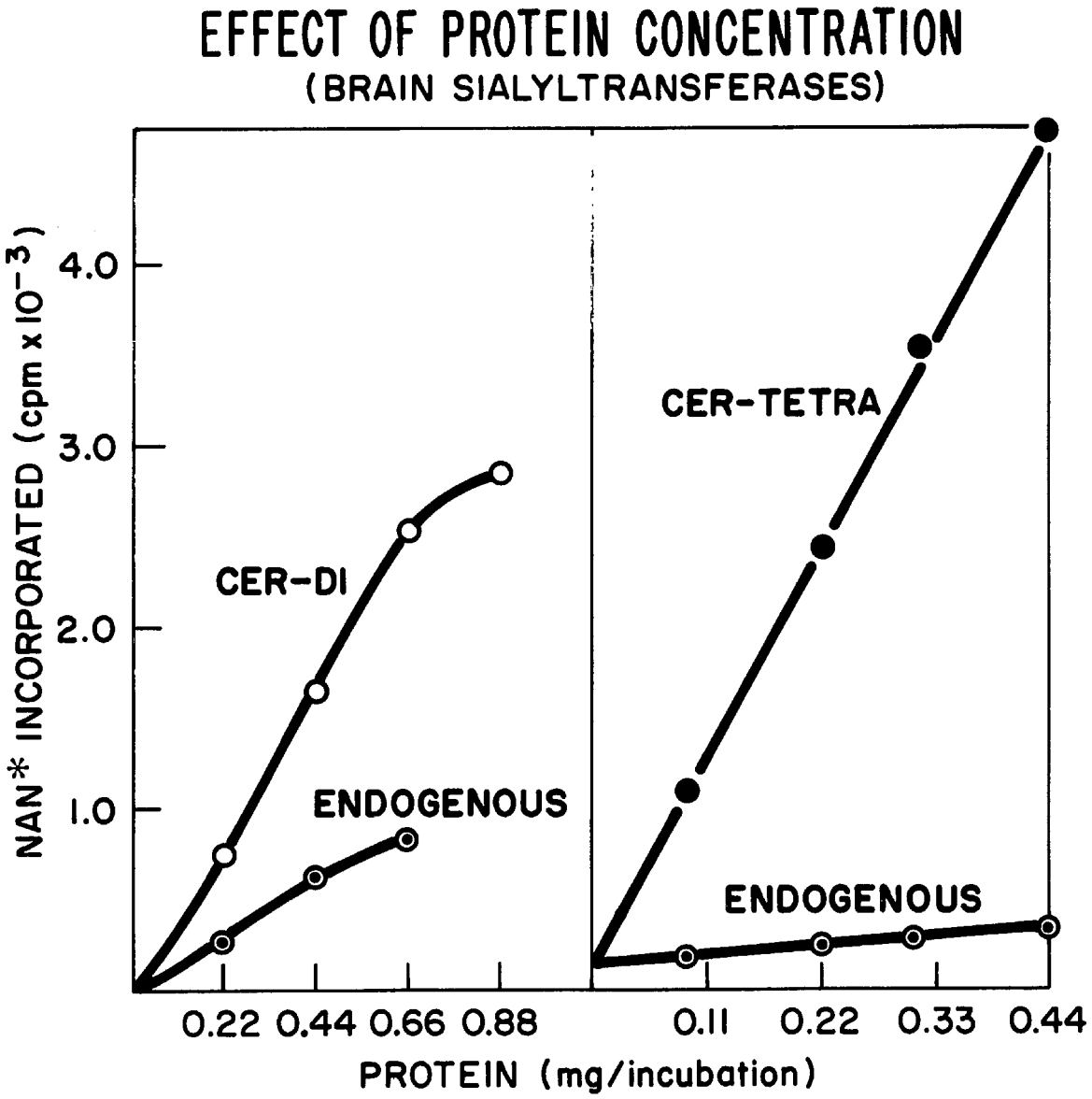
‡ Product formation was proportional to time of incubation for 4 hours and to protein concentration over the range of 0.1-0.4 mg per incubation.

FIGURE 4



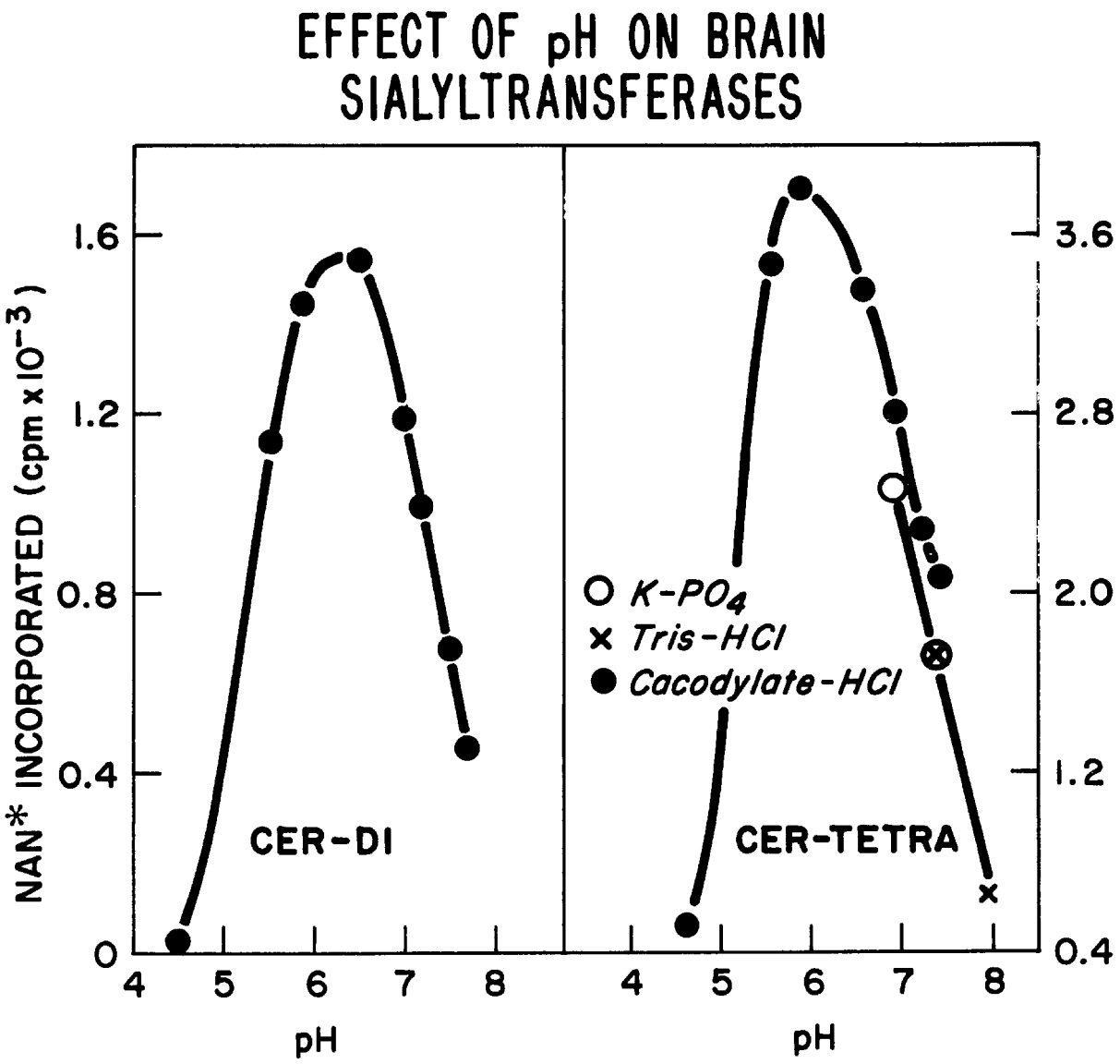
Each incubation mixture contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-tetrasaccharide, 0.05; Triton CF-54 and Tween-80 (2:1), 300 μg; cacodylate-HCl buffer, pH 6.3, 10; $MgCl_2$, 2; CMP-NAN, 0.05 (1.08×10^6 cpm per μmole); enzyme, 0.4 mg of protein. In the case of ceramide-disaccharide, all the components were doubled. Incubations were conducted at 37°; the reaction was stopped by adding 10 μl of C-M (2:1) and assayed as described in the text.

FIGURE 5



Complete incubation mixtures were prepared and assayed as described in Figure 4 except for the varying protein concentrations. Incubations were conducted at 37° for 2 hours.

FIGURE 6



Incubation mixtures were prepared as described in Figure 4, except that 0.28 mg of protein was used for the ceramide-tetrasaccharide and 0.56 mg of protein in twice the volume for the ceramide-disaccharide incubations. Incubations were conducted at 37° for 2 hours. Buffers were as indicated at 10 µmoles per incubation mixture.

glycolipids) were subtracted from values obtained with added glycolipids. The optimum pH was 5.9 for ceramide-tetrasaccharide, 6.3 for ceramide-disaccharide, and 7.0 for lactose. The nature of the buffer markedly influenced the reaction. Optimum activity was observed when cacodylate-HCl was used, while Tris-HCl or potassium-phosphate buffers inhibited the incorporation of labeled sialic acid as shown in Fig. 6.

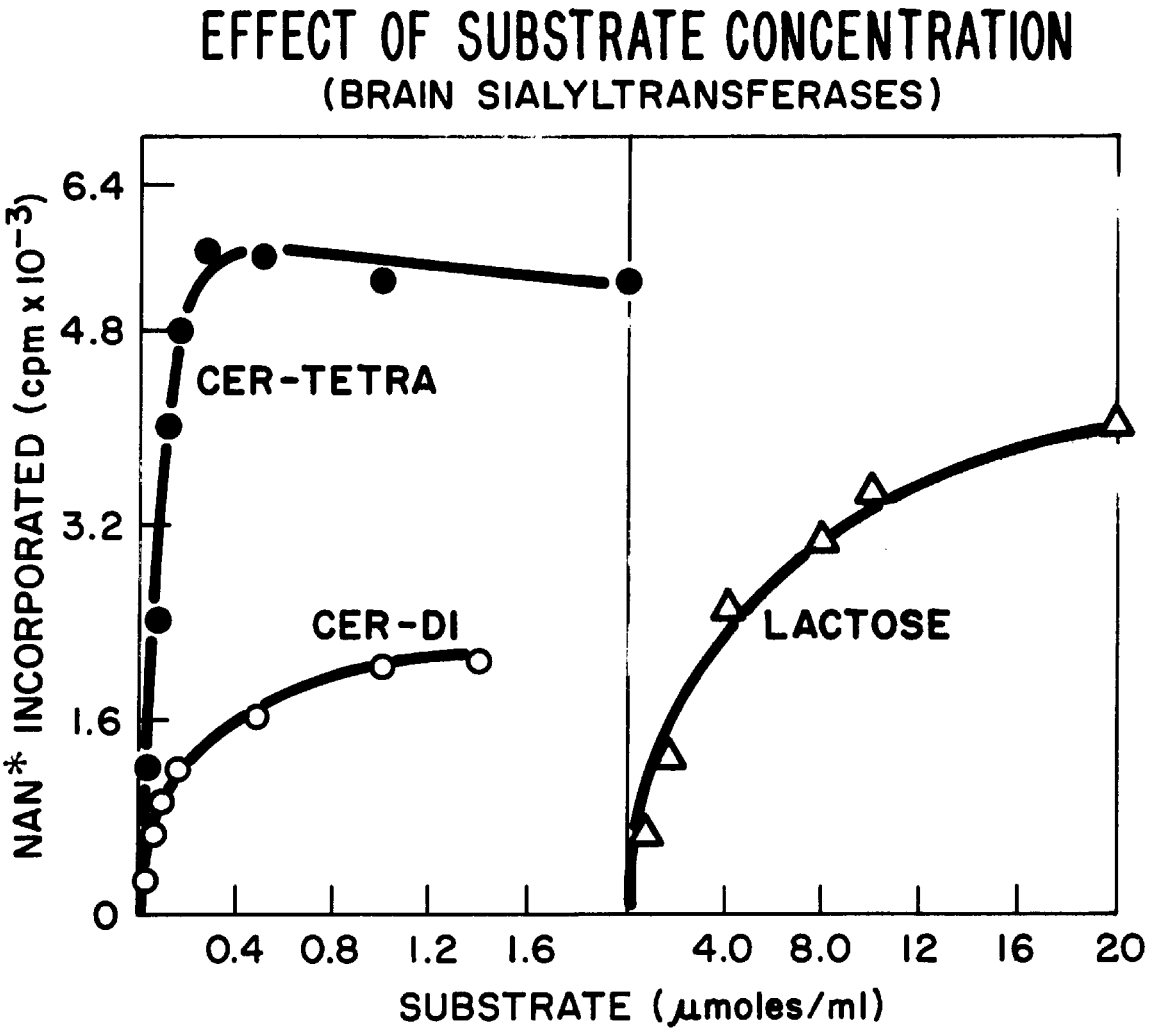
iv. Effect of Substrate Concentration

The effects of increasing concentrations of ceramide-tetrasaccharide, ceramide-disaccharide and lactose on the sialyltransferase reactions are given in Figure 7a. From the $\frac{[S]}{V}$ vs S plots (Figures 7b-7d) the following K_m values were obtained; ceramide-disaccharide, 3.0×10^{-4} M; ceramide-tetrasaccharide, 2.5×10^{-5} M; monosialoganglioside, (GM_1) 2.0×10^{-4} M; lactose, 4.0×10^{-3} M; N-acetyllactosamine, 2.6×10^{-3} M. The following relative V_{max} values were observed: ceramide-disaccharide, 1.0; ceramide-tetrasaccharide, 4.5; monosialoganglioside, 1.1; lactose, 4.6; N-acetyllactosamine, 5.8.

v. Effect of Detergents on Enzymic Activity

The effect of various detergents was tested as shown in Table 4. Maximum incorporation was obtained with Triton CF-54 (25°). A mixture of Triton CF-54 and Tween-80 (2:1) was used where the final concentration of the detergent in the incubation mixture was 3 mg/ml. The

FIGURE 7a



Complete incubation mixtures were prepared and assayed as described in Figure 4; 0.44 mg of protein was used for the ceramide-tetrasaccharide and lactose, and 0.88 mg of protein for the ceramide-disaccharide in twice the incubation volumes. Incubations were conducted at 37° for 2 hours.

FIGURE 7b

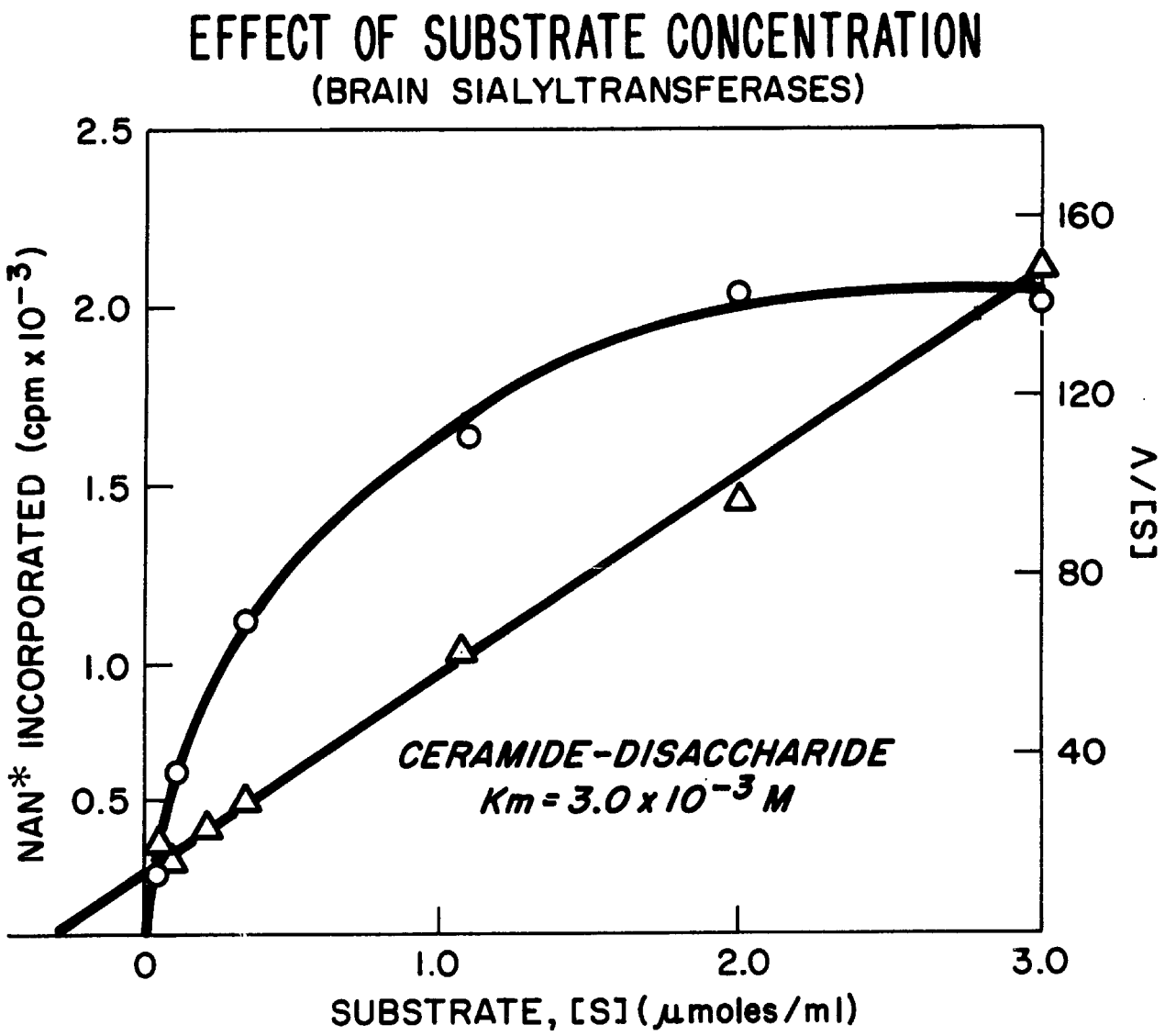

EFFECT OF SUBSTRATE CONCENTRATION
(BRAIN SIALYLTRANSFERASES)
NAN* INCORPORATED (cpm x 10⁻³)
V/[S]
SUBSTRATE, [S] (μmoles/ml)
CERAMIDE-DISACCHARIDE
Km = 3.0 x 10⁻³ M
2.5
2.0
1.5
1.0
0.5
0
1.0
2.0
3.0
160
120
80
40

FIGURE 7c

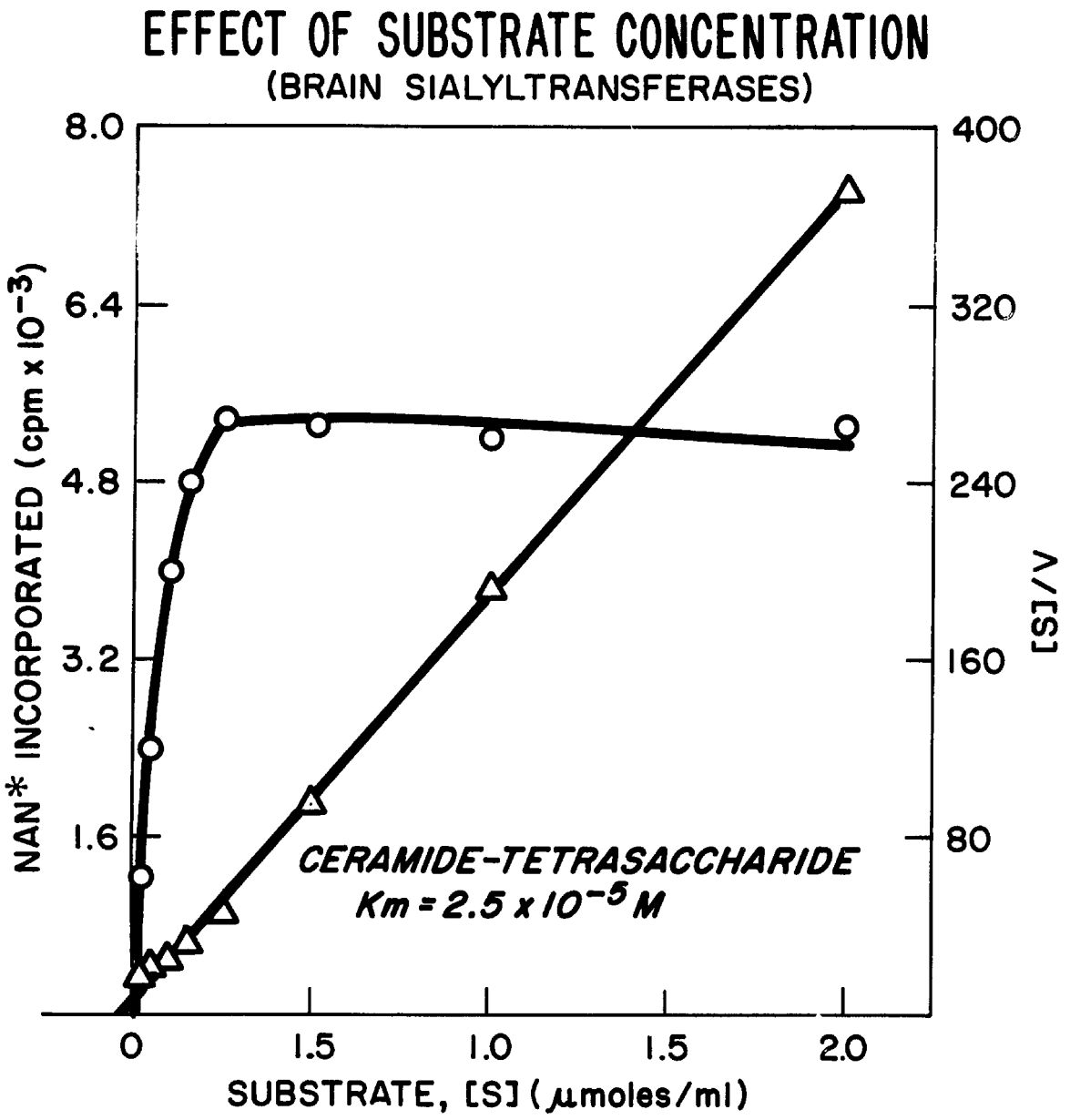
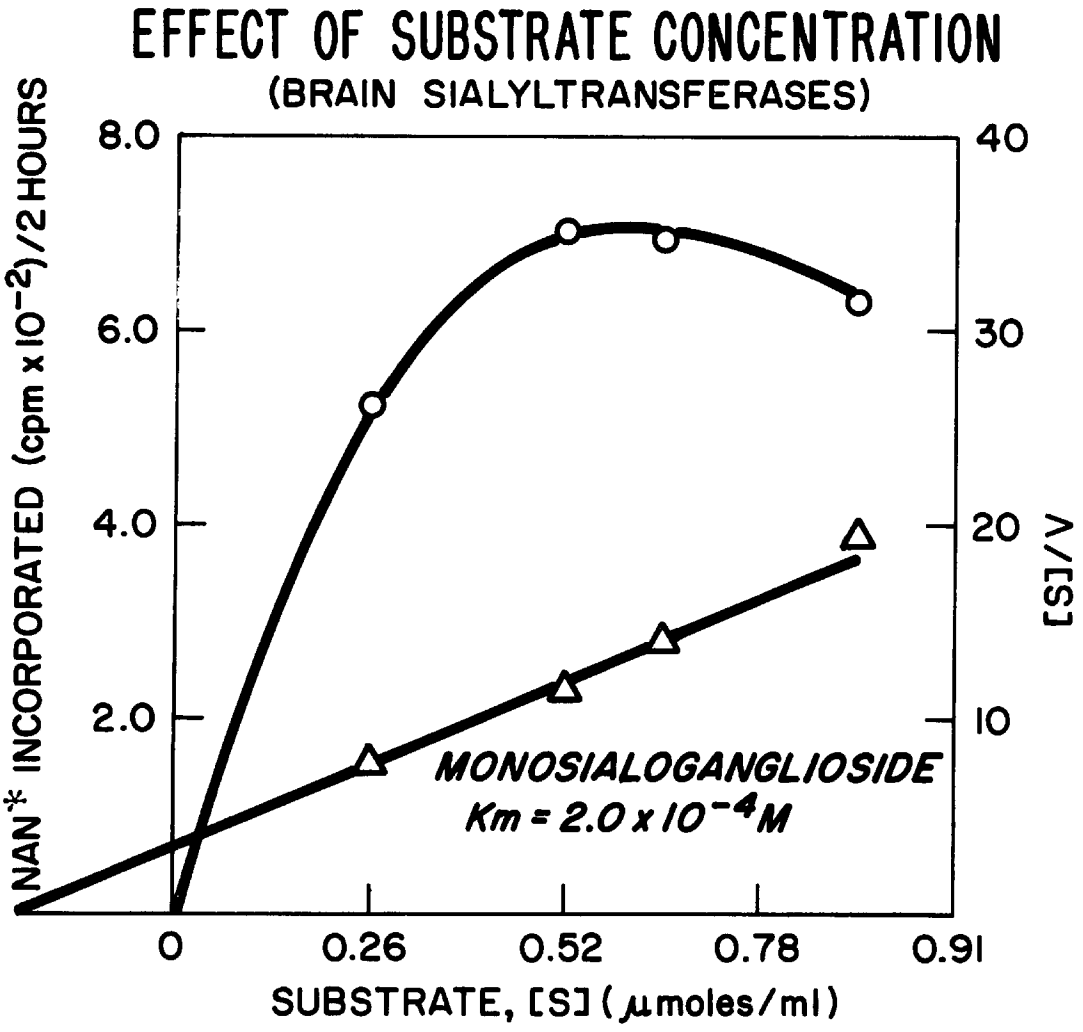

EFFECT OF SUBSTRATE CONCENTRATION
(BRAIN SIALYLTRANSFERASES)
8.0
6.4
4.8
3.2
1.6
400
320
240
160
80
NAN* INCORPORATED (cpm x 10⁻³)
[S]/V
CERAMIDE-TETRASACCHARIDE
Km = 2.5 x 10⁻⁵ M
0
1.5
1.0
1.5
2.0
SUBSTRATE, [S] (μmoles/ml)

FIGURE 7d


EFFECT OF SUBSTRATE CONCENTRATION
(BRAIN SIALYLTRANSFERASES)
NAN* INCORPORATED (cpm x 10⁻²)/2 HOURS
8.0
6.0
4.0
2.0
40
30
20
10
V/[S]
MONOSIALOGANGLIOSIDE
Km = 2.0 x 10⁻⁴M
0
0.26
0.52
0.78
0.91
SUBSTRATE, [S] (μmoles/ml)

effect of the concentration of detergent (Tween-80:Triton CF-54) mixture on the rate of reaction is shown in Figure 8.

vi. Heat Inactivation of Brain Sialyltransferases

The particulate enzyme preparation was heated at temperatures ranging between 50° and 60°, for varying periods of time, and the residual transferase activity tested with ceramide-disaccharide, monosialoganglioside, ceramide-tetrasaccharide, and lactose. The activity towards ceramide-disaccharide was far more stable than that for the other substrates. The rate of inactivation of enzyme activity at 55° is shown in Figure 9. Table 5 shows the inactivation of sialyltransferases at different temperatures. The results of partial heat inactivation experiments (Table 6) therefore indicate that the activity towards ceramide-disaccharide is distinguishable from that towards monosialoganglioside, ceramide-tetrasaccharide, and lactose. Whether one or more enzymes acts on the latter group of compounds is not known.

vii. Effect of Mg^{++} and Other Divalent Metal Ions

To determine whether the sialyltransferase system required divalent metal ion, experiments were conducted as summarized in Fig. 10, and Tables 7 and 8. The results were not easily interpretable and were variable. For example, in one experiment, the incorporation into ceramide-tetrasaccharide and lactose was stimulated 2- to 3-fold by $MgCl_2$ at relatively high concentrations, while a similar effect was noted with ceramide-disaccharide at low concentrations, followed by a decrease in activity at

TABLE 4

Effect of Detergents on Embryonic Chicken Brain Sialyltransferases

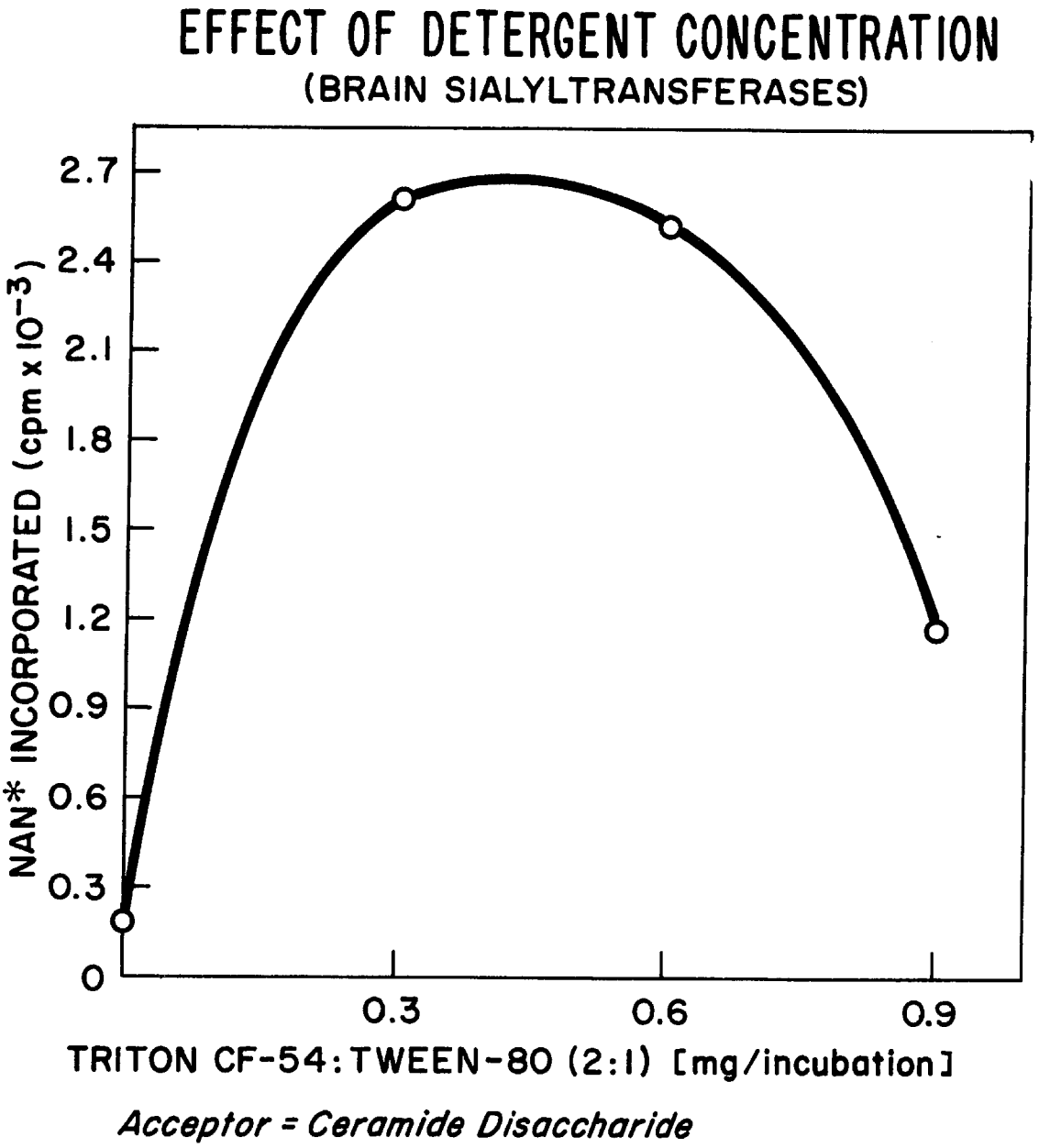
Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.025; $MgCl_2$, 1.0; K-PO_4 buffer, pH 7.1, 5; CMP-NAN, 0.18 (0.51 x 10^6 cpm per μmole); enzyme 0.4 mg of protein; detergents (as indicated in the Table). The mixtures were incubated at 37° for 12 hrs. Endogenous values were subtracted from values obtained with ceramide-disaccharide.

Group	Detergents	Concentration	NAN* incorporated (cpm)
I	Tween-20	25 μg	620
	Cutscum	" "	940
	Lysolecithin†	" "	1,010
	Bile salt	" "	140
	Surfactol	" "	740
II	Tween-80 †	125 μg	1,120
	Triton CF-54	" "	2,020
	Triton X100	" "	320
	Cholesterol + lecithin (1:1)	" "	890
III	Tetrahydrofuran	10 μl	110
	$CHCl_3$	" "	0
	$CHCl_3$ + Methanol (2:1)	" "	0

* Detergents of Group I inhibit enzyme activity at higher concentration.

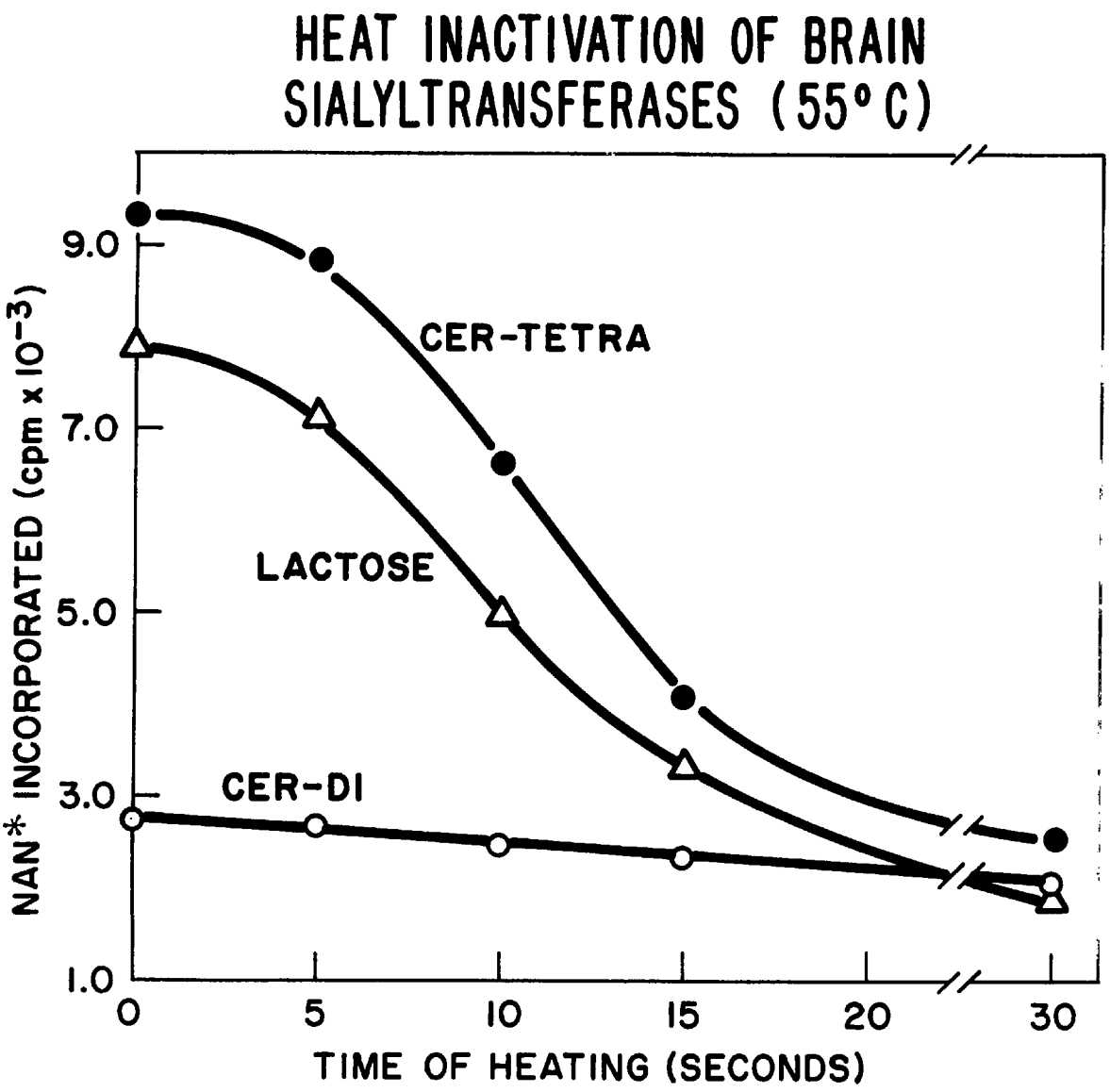
† With mixture of Triton CF-54 and Tween-80 (2:1), NAN* incorporated, 2600 (cpm); condition was maintained as given in Figure 8.

FIGURE 8



Each incubation mixture contained the following (μmoles) in final volumes of 0.095 ml: ceramide-disaccharide, 0.05; cacodylate-HCl, buffer, pH 7.0, 12.5; $MgCl_2$, 1.0; CMP-NAN, 0.09 (1.08×10^6 cpm per μmole); enzyme, 0.78 mg of protein; detergent mixture was added as indicated in the Figure. Incubated for 4 hr at 37° and assayed by borate electrophoresis.

FIGURE 9



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.2 ml: ceramide-disaccharide, 0.2 or ceramide-tetrasaccharide, 0.1, or lactose, 4.0; detergent mixture, 600 μg; cacodylate-HCl buffer (pH 6.4 for the glycolipids and 6.95 for lactose), 20; $MgCl_2$, 5.0; CMP-NAN, 0.1 (1.08×10^6 cpm per μmole); enzyme (heated at 55° as indicated and cooled to 0° C), 0.88 mg of protein. Incubations were conducted at 37° for 2 hours. The values for all points were determined from incubations in which product formation was proportional to enzyme concentration and incubation time, and independent of substrate concentration.

TABLE 5

Heat Inactivation of Brain Sialyltransferase(s)

at 50° and 60° C

Complete incubation mixtures contained the components as given in Figure 9, except for the enzyme which was first heated at 50° C and 60° C for the indicated time. All assay mixtures were incubated at 37° for 2 hours.

Time of heating (seconds)	NAN* incorporated (cpm)							
	Endogenous		Ceramide-disaccharide		Lactose		Ceramide-tetrasaccharide	
	50°	60°	50°	60°	50°	60°	50°	60°
5	421	485	3,170	3,063	7,958	6,921	10,219	10,026
10	429	447	2,890	2,473	5,534	3,122	8,276	3,956
15	380	311	3,009	2,074	5,004	1,613	7,432	1,993
30	333	317	2,815	1,674	4,422	1,368	5,777	1,415
Unheated	355		3,124		7,890		9,869	

TABLE 6

Percent Inactivation of Sialyltransferase

After Heat Treatment

A. Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.085 ml: lipid, 0.1; detergent mixture, 300 μg; $MgCl_2$, 1.0; cacodylate-HCl buffer, pH 6.5, 10; CMP-NAN, 0.05 (1.8×10^6 cpm per μmole); unheated enzyme, 0.41 mg of protein.

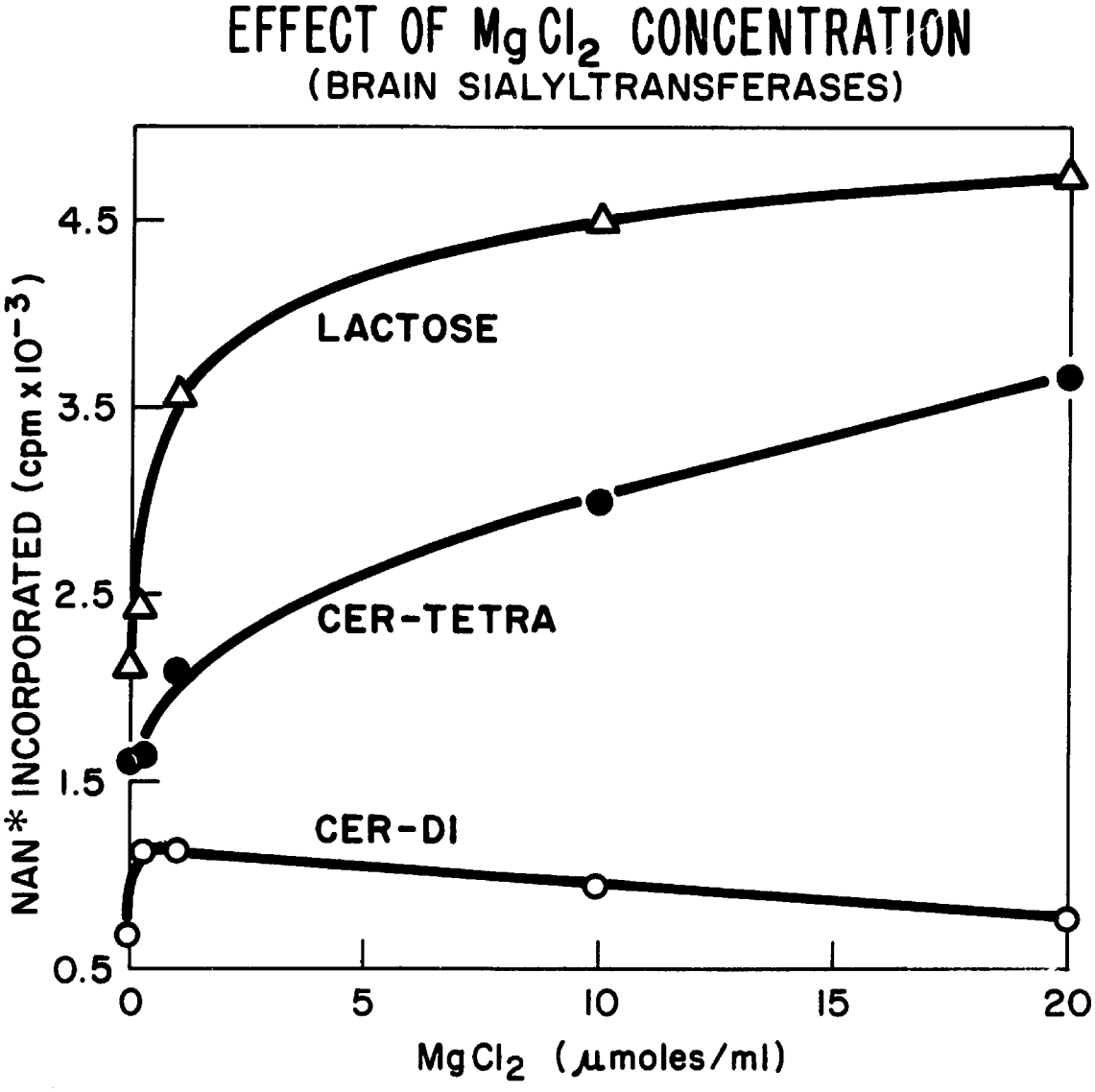
B. Complete incubation mixtures contained all the components given above except for the enzyme, which was first heated for 25 seconds at 55°, cooled to 0° and then added to the incubation mixtures.

All mixtures were incubated at 37° for 2 hours.

Substrate	NAN* incorporated (cpm)		Percent loss of activity
	(A)	(B)	
Ceramide-disaccharide	1,830	1,830	0
Ceramide-tetrasaccharide	6,630	2,880	59
Monosialoganglioside	1,990	430	80
Lactose	9,060	4,380	52
N-Acetyllactosamine	9,120	5,030	45

higher concentrations (Fig. 10). If the system required divalent cation, EDTA might be expected to inhibit the activity; these results were, in fact, obtained with ceramide-disaccharide as acceptor, but there was no significant inhibition with ceramide-tetrasaccharide and lactose (Table 7). Table 7 also indicates that with the latter substrates, $MgCl_2$ was the most effective metal ion tested. In contrast to the results shown in Fig. 10 and Table 7, when the enzyme preparation was dialyzed for 6 hr at 4° against 0.05 M EDTA, the activities towards the substrates changed as follows (Table 8): ceramide-disaccharide, increased slightly, and no effect with added Mg^{++}; ceramide-tetrasaccharide, no effect, and a slight increase with Mg^{++}; lactose, decreased about 30%, and a slight increase with Mg^{++}. The activity with monosialoganglioside was inhibited in the presence of Mg^{++}, whether or not the preparation was first dialyzed against EDTA. In the case of metal ion requirements, it is important to note that the other known sialyltransferases (e.g., with lactose, or glycoproteins), metal ions do not stimulate activity. In view of the inconsistencies in the present results, no interpretation can be made. It is possible, for example, that the effects of metal ions result from effects on lipid mi celles or on the physical properties of the particulate system, rather than an effect on the enzyme per se.

FIGURE 10



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.1 (ceramide-tetrasaccharide, 0.05 or lactose, 2.0); detergent mixture, 300 μg; cacodylate-HCl, pH 6.3, 10.0; enzyme, 0.41 mg of protein; CMP-NAN, 0.05 (1.8×10^6 cpm per μmole). Mixtures were incubated at 37° for 2 hours.

TABLE 7

Effect of Various Metal Ions on Brain Sialyltransferase(s)

A. Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-tetrasaccharide, 0.05 (ceramide-disaccharide, 0.10 or lactose, 2.0); detergent mixture, 300 μg; cacodylate-HCl, pH 6.3, 10.0; CMP-NAN, 0.05 (1.8×10^6 cpm per μmole); enzyme, 0.41 mg of protein.

B. Complete incubation mixtures contained the same components as above except for the following: ceramide-disaccharide, 0.05; CMP-NAN, 0.07 (1.08×10^6 cpm per μmole); enzyme 0.85 mg of protein.

Metal ions were used at concentrations that gave maximum activities. All mixtures were incubated at 37° for 4 hours. Values given are corrected for those obtained from control incubations (with metal ions, without substrates).

Exp. No.	Metal ions		NAN* incorporated (cpm)		
		Concentration (μmoles)	Cer-tetra	Cer-di	Lactose
A	---	---	3,570	1,120	4,920
	EDTA	5.0	4,100	630	4,110
	$MgCl_2$	1.0	4,940	930	6,480
	$MnCl_2$	1.0	4,540	700	6,440
	$HgCl_2$	0.1	3,540	1,140	4,980
B	---	---	ND	2,390	3,800
	$CuCl_2$	2.5	"	2,240	2,800
	$CaCl_2$	2.5	"	120	2,300

TABLE 8

Effect of $MgCl_2$ on Brain Sialyltransferases

A. Complete incubation mixtures contained the following components (μmoles) in final volume of 0.11 ml: lipid, 0.1 (or lactose, 2.0); detergent mixture, 300 μg; $MgCl_2$, 2.0; cacodylate-HCl, pH 6.5, 10; CMP-NAN, 0.05 (1.8×10^6 cpm per μmole); enzyme, 0.41 mg of protein (undialyzed).

B. Complete incubation mixtures contained the same components as above except for the enzyme, which was dialyzed for 6 hours against 0.25 M sucrose containing 0.05 M versene at pH 7.0.

Substrates	NAN* incorporated (cpm)				
	Undialyzed enzyme		Dialyzed enzyme		
	No metals	With $MgCl_2$	No metals	With $MgCl_2$	With KCl
-----	370	260	378	327	352
Ceramide-disaccharide	1,786	1,513	2,032	2,028	1,984
Ceramide-tetrasaccharide	4,802	5,876	5,191	7,568	4,690
Monosialoganglioside	922	693	1,016	661	936
Lactose	7,583	9,573	5,164	6,129	---

viii. Effect of Heating and Cooling of Substrates

Ceramide-disaccharide was heated at 60° C for 15 min in the presence of buffer and the other ingredients of the incubation mixture (without enzyme) and then allowed to cool slowly at room temperature. Enzyme was then added and the mixture incubated at 37°; there was no increase in activity over that of the unheated substrate (Table 9). Rothfield et al. (260) showed that the lipopolysaccharide (LPS) lipid complex prepared by heating and slow-cooling was required for binding of the UDP-galactose:LPS transferase enzymes. No such effect was observed with chicken embryonic brain sialytransferases.

2. Effect of Inhibitors on Sialyltransferase(s)

The effects of various inhibitors were studied in order to distinguish between CPM-NAN:ceramide-disaccharide sialyltransferase (CDSTase) and CMP-NAN:lactose sialyltransferase (SLTase). Iodoacetate, p-hydroxy-mercurobenzoate and mercuric chloride were used at various concentrations as indicated in Table 10. At higher concentrations iodoacetate inhibited CDSTase activity without any loss of activity of SLTase. The results suggested the presence of two different sialyltransferases in the same particulate fraction.

3. Sialyltransferase(s) Activities at Various Stages of Embryonic Development

The effect of the age of the embryo on the activity of the sialyltransferases is shown in Figure 11. Optimum enzyme activity with ceramide-disaccharide as substrate

TABLE 9

Effect of Heating and Cooling of Substrates

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.025; detergent mixture, 150 μg; cacodylate-HCl, pH 7.0, 12.5; $MgCl_2$, 2.0; glycine, 2.5; CMP-NAN, 0.063 (1.08×10^6 cpm per μmole); enzyme 0.85 mg of protein.

All mixtures were incubated at 37° for 10 hours.

Treatment	Ceramide-disaccharide	NAN* incorporated (cpm)
Unheated	-	140
	+	2,440
Heated for 15 min at 60° C then cooled slowly to room temperature	-	290
	+	2,300

TABLE 10

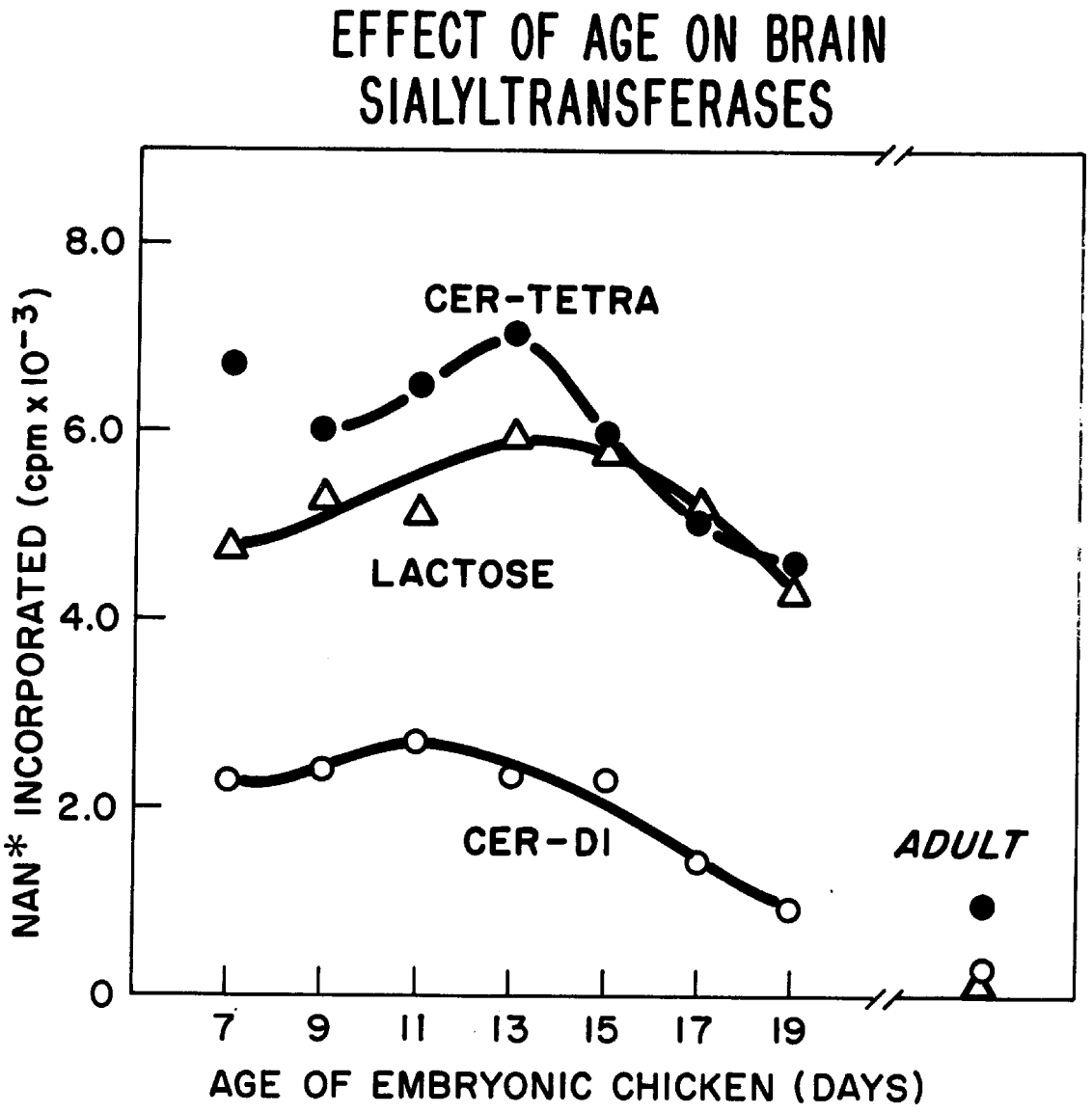
Effect of Inhibitors on Brain Sialyltransferases

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.025; detergent mixture, 150 μg; cacodylate buffer, pH 7.0, 12.5; lactose, 2.0; inhibitors as indicated in the Table; enzyme 0.85 mg of protein (enzyme was pre-incubated at 37° for 7 hours); CMP-NAN, 0.05 (1.08×10^6 cpm per μmole).

All mixtures were incubated at 37° for 12 hours.

Inhibitors	Concentration x 10^{-3} M	NAN* incorporated (cpm)	
		Cer-di	Lactose
	---	1,200	1,000
Iodoacetate	4.0	600	760
	8.0	500	920
	16.0	250	1,000
	32.0	100	1,430
p-OH mercuro-benzoate	0.85	500	630
	1.7	130	480
	3.4	130	225
	6.8	0	50
Mercuric chloride	0.031	1,170	950
	0.31	980	1,050
	3.1	590	870
	6.2	20	250

FIGURE 11



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.095 ml: ceramide-disaccharide, 0.1 (ceramide-tetrasaccharide, 0.1 or lactose, 2.0); detergent mixture, 300 μg; CMP-NAN, 0.05 (1.08×10^6 cmp per μmole); cacodylate-HCl buffer (pH 6.4 for ceramide-disaccharide and ceramide-tetrasaccharide incubations and pH 7.0 for lactose incubations), 10; $MgCl_2$, 1.0; unfractionated brain homogenates, 50 μl (0.5-1.4 mg protein). The values were plotted as cpm per mg protein per 2 hours and were calculated from the data obtained from incubations in which product formation was proportional to enzyme concentration, time of incubation, and independent of substrate concentration.

occurred when the embryo was 9-11 days old, and between 11 and 13 days with ceramide-tetrasaccharide was the substrate. The values in the Figure were corrected for those obtained from control incubations that lacked exogenous glycolipids. Table 11 gives the values obtained with endogenous acceptor.

4. Enzyme Activity in Brain Homogenates of Different Species

The activity of sialyltransferases in various brain homogenates is given in Table 12. Sialyltransferase activities were detected in homogenates prepared from brain tissues of embryonic chicken (7-21 days), pig getus (82 mm) and rat (9 days). Activity was not detected in the brain tissue from pig adult with ceramide-tetrasaccharide as substrate. Brain homogenates of adult chicken, pig and sheep showed high endogenous values; the addition of glycolipid to the incubation mixtures gave only slightly higher values. Low activity was detected in calf, sheep and guinea pig brain (last column of Table 12).

5. Study of Substrate Specificity

The transfer of NAN from CMP-NAN to various acceptors is presented in Table 13. The relative activities of the glycolipid acceptors were as follows: ceramide-tetrasaccharide > monosialoganglioside > ceramide-disaccharide > ceramide-trisaccharide > ceramide-monosaccharide. N-acetyllactosamine, lactose and fetuin (pretreated with sialidase) were also active. Sialidase treated orosomucoid, and ovine and bovine submaxillary mucin were inactive ($< 2\%$).

TABLE 11

Relationship of Brain Sialyltransferase Activity to Age of Embryo

Complete incubation mixtures contained all the components as given in Figure 11 and tested with ceramide-disaccharide as substrate.

Age (days)	NAN* incorporated (cpm/mg of protein/2 hr)	
	Endogenous	+ Ceramide-disaccharide
7	416	2,684
9	354	2,736
11	364	3,052
13	433	2,640
15	375	2,676
17	346	1,823
19	323	1,300
Adult[1]	377	661

[1] Age unknown.

TABLE 12

Sialyltransferase(s) Activity in Brain Homogenates of Various Species

A. Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.1; detergent mixture, 300 μg; cacodylate-HCl buffer, pH 6.3, 10; $MgCl_2$, 1.0; CMP-NAN, 0.05 (1.08×10^6 cpm per μmole); enzyme[1] (unfractionated homogenate) 1.0-2.0 mg of protein. All mixtures were incubated at 37° for 12 hours.

B. Complete incubation mixtures contained all the components as above except for the cacodylate-HCl buffer used, pH 6.0; CMP-NAN, 0.05 (1.8×10^6 cpm per μmole). All mixtures were incubated at 37° for 4 hours.

Source	Age	NAN* incorporated (cpm/mg of protein/4 hr)			
		(A)		(B)	
		Endogenous	Cer-di	Endogenous	Cer-tetra
Chicken embryo	9 days	204	2,041	---	---
Chicken embryo	13 days	---	---	509	10,333
Chicken	Adult	413	466	1,172	1,684
Pig embryo	(82 mm)[2]	229	2,484	461	6,248
Pig	Adult	922	840	1,274	1,281
Rat	9 days	345	757	405	1,634
Rat	Adult	513	545	503	665
Calf	?[2]	822	938	632	1,180
Sheep	Adult	541	694	1,204	1,574
Guinea pig	11 days	716	682	574	1,068

[1] Two gm of brain of each species were homogenized with 4 ml of 0.25 M sucrose containing 0.1% 2-mercapto-ethanol.

[2] Age unknown.

TABLE 13

Substrate Specificity of Embryonic Chicken Brain Sialyltransferases

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.2 ml; substrates (as indicated in the Table); detergent mixture, 600 μg; cacodylate-HCl, pH 6.3, 20; $MgCl_2$, 2.0; CMP-NAN, 0.1 (1.08×10^6 cpm per μmole); enzyme, 0.55 mg of protein. All mixtures were incubated for 2 hours at 37°.

Group	Substrates	Concentration (μmoles)	NAN* incorporated† (cpm)
Lipids	Ceramide-tetrasaccharide	0.1	4,950
	Ceramide-trisaccharide	"	140
	Ceramide-disaccharide	"	850
	Cerebroside (glucose)	"	N.D.‡
	Cerebroside (galactose)	"	N.D.
	Monosialoganglioside (G_{M1})	"	1,500
	Ganglioside mixture (calf brain)	"	480
Disaccharides	Lactose	2.0	5,544
	N-acetyllactosamine	"	6,942
Enzymatically desialyzed glycoproteins	Fetuin	0.10	970
	Orosomucoid	"	N.D.
	Ovine submaxillary mucin	0.12	N.D.
	Bovine submaxillary mucin	"	N.D.

† Corrected for endogenous value (540 cpm).

‡ N.D. = not detectable.

TABLE 14

CMP-Sialic Acid and Sialic Acid Specificities of Embryonic Chicken Brain Sialyltransferases

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: substrates (as indicated in the Table); detergent mixture, 300 μg; cacodylate-HCl, pH 6.3, 10; $MgCl_2$, 2; enzyme 0.42 mg. The samples were incubated for 2 hours at 37°. Specific activities of CMP-NAN, 1.7×10^6 cpm per μmole; CMP-NGN, 4.6×10^5 cpm per μmole; sialic-^{14}C, 1.1×10^6 cpm per μmole; sialic-9-PO_4, 4.0×10^5 cpm per μmole.

Acceptors	Concentration (μmoles/0.1 ml)	NAN* incorporated (cpm) from			
		CMP-NAN (40,000 cpm)	CMP-NGN (31,000 cpm)	Sialic-^{14}C+CMP† (180,000 cpm)	Sialic-^{14}C 9-PO_4+CMP† (18,000 cpm)
---	---	219	216	105	84
Ceramide-tetrasaccharide	0.05	4,964	2,097	142	104
Ceramide-disaccharide	0.10	639	464	158	85
Lactose	2.0	5,918	3,506	123	100
N-Acetyllactosamine	2.0	6,054	2,679	152	87

† CMP, 0.5 μmole.

Sialic acid 9-phosphate, or sialic acid, incubated in the presence of CMP, did not substitute for CMP-NAN. CMP-N-Glycolylneuraminic acid also served as a sialic acid donor; the results are shown in Table 14.

6. Substrate Competition Experiments

The partial heat inactivation and inhibitor experiments described above suggested that the particulate preparation obtained from embryonic chicken brain contained more than one sialyltransferase. This question was also approached by measuring the activity with glycolipid in the presence of lactose (Table 15). The products, sialyl-lactose and sialo-glycolipid, were easily separable by paper electrophoresis. In the mixed substrate experiments, each acceptor was used at concentrations higher than required to saturate the system. The results indicated that ceramide-disaccharide and lactose did not compete since less than 8% inhibition of activity was observed compared to the incorporation found with the total for the individual acceptors. By contrast, the combination of ceramide-tetrasaccharide and lactose resulted in 82% inhibition, and lactose with monosialoganglioside showed 24% inhibition. These data, agree with those obtained above, and indicate that the sialyltransferase active with ceramide-disaccharide can be distinguished from that (or those?) active with ceramide-tetrasaccharide, monosialoganglioside, and lactose.

TABLE 15

Effect of Combined Substrates on

Sialyltransferase Activities

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.085 ml: glycolipid, 0.1 (or lactose, 2.0); detergent mixture, 300 μg; $MgCl_2$, 1.0; cacodylate-HCl, pH 6.5, 10; CMP-NAN, 0.045 (1.73×10^6 cpm per μmole); enzyme, 0.41 mg of protein. All mixtures were incubated at 37° for 2 hours.

Substrate	NAN* incorporated (cpm)†		% Inhibition‡
	Glycolipid	Lactose	
None	460	110	
Ceramide-disaccharide	2,490	---	
Ceramide-tetrasaccharide	7,090	---	
Monosialoganglioside	2,450	---	
Lactose	---	9,450	
Ceramide-disaccharide + lactose	2,080	8,690	8%
Ceramide-tetrasaccharide + lactose	5,560	1,670	82%
Monosialoganglioside + lactose	1,700	7,190	24%

† The values were determined in incubations in which the reaction was proportional to enzyme concentration, time of incubation, and independent of substrate concentration.

‡ % Inhibition = 100 (sialyllactose + sialoglycolipid formed with the mixed substrate ÷ sialyllactose + sialoglycolipid formed with the individual substrates).

7. Attempts to Solubilize the Particulate Sialyltransferase Activities

The homogenate from embryonic chicken brain was treated with enzymes, detergents, and with ultrasonic vibration in attempts to solubilize the sialyltransferase activities.

i. Effect of RNAase, DNAase, Deoxycholate and Phospholipases

The brain homogenate was treated with RNAase (254), DNAase, and phospholipase (255) at 37° for 30 min, and centrifuged at 37,000 x g for 30 min. Enzyme activity (with ceramide-disaccharide as substrate) was primarily confined to the residue as shown in Table 16. Bile salts are sometimes useful as enzyme-solubilizing agents; in the present case, sodium deoxycholate was employed, but it inhibited the enzyme activity. Treatment of the enzyme with histone resulted in a small increase in the enzyme activity.

ii. Differential Centrifugation after Cutscum Treatment

The non-ionic detergent Cutscum (isoctyl p-benzoyl polyethoxyethanol, **Fisher Scientific Company**) was added at a concentration of 0.25% to the particulate enzyme preparation, and stirred in ice for 30 min. After centrifugation at 100,000 x g for 1 hr, it appeared that the enzyme was soluble, although 50% of the activity was inhibited. However, when the supernatant fluid was passed through small charcoal column (1 x 3 cm) to remove the detergent, and the filtrate recentrifuged at 100,000 x g, the activity was detected only in the residue. This observation indicated that in the presence of Cutscum (251), the enzyme

TABLE 16

Attempts to Solubilize Enzymatic Activity of

Sialyltransferases

(Effect of RNAase, DNAase, Phospholipase, Deoxycholate and Histone)

Twelve embryonic chicken brains (9 days old) were homogenized with 3.8 ml of 0.25 M sucrose (20 mg of protein per ml), and 1 ml aliquots of the homogenate were treated as follows:

A. 1 ml homogenate; 1 ml of 0.2 M $K\text{-}PO_4$, pH 7.2; 0.2 mg phospholipase-C; 5 mg albumin; 0.1 ml of 2% $CaCl_2$.

B. 1 ml of homogenate; 1 ml of 0.2 M $K\text{-}PO_4$ buffer, pH 6.9; 0.3 mg DNAase; 0.5 mg RNAase; 0.1 ml of 0.2 M $MgCl_2$.

C. 1 ml of homogenate; 1 ml of 0.2 M cacodylate-HCl buffer, pH 6.8; 5 mg of deoxycholate.

D. 1 ml of homogenate; 1 ml of 0.2 M cacodylate-HCl buffer, pH 6.8; 4.0 mg of histone.

Mixtures A through D were incubated for 30 minutes at 37°, and 1.5 ml aliquots were centrifuged at 37,000 x g for 30 min. Supernatants were termed as AS, BS and CS. Residues in each tube were suspended in 0.5 ml of 0.25 M sucrose and termed as AR, BR and CR enzyme fractions.

Complete incubation mixtures contained the following components (µmoles) in final volumes of 0.09 ml: lactose, 2.0; ceramide-disaccharide, 0.025; detergent mixture, 150 µg; cacodylate-HCl, pH 6.8, 12.5; $MgCl_2$, 1.0; enzyme, 0.05 ml; CMP-NAN, 0.1 (0.5×10^6 cpm per µmole).

Endogenous values were subtracted from values obtained with added substrates.

TABLE 16

Number	Enzyme treatment	Fractions	NAN* incorporated (cpm)	
			Cer-di	Lactose
Homogenate	---	Direct	1,550	5,250
A	Phospholipase	Supernatant	50	0
		Residue	1,030	3,750
B	RNase and DNase	Supernatant	570	820
		Residue	1,210	14,400
C	Deoxycholate	Supernatant	30	280
		Residue	80	20
D	Histone	Direct	1,720	6,860

particles were dispersed and were not sedimentable, but the activity was not solubilized. A gradient of enzyme activity was also observed when it was centrifuged in 0.25 M sucrose medium in the presence of 0.25% Cutscum at 100,000 x g. The effect of Cutscum concentration on enzyme activity is shown in Figure 12.

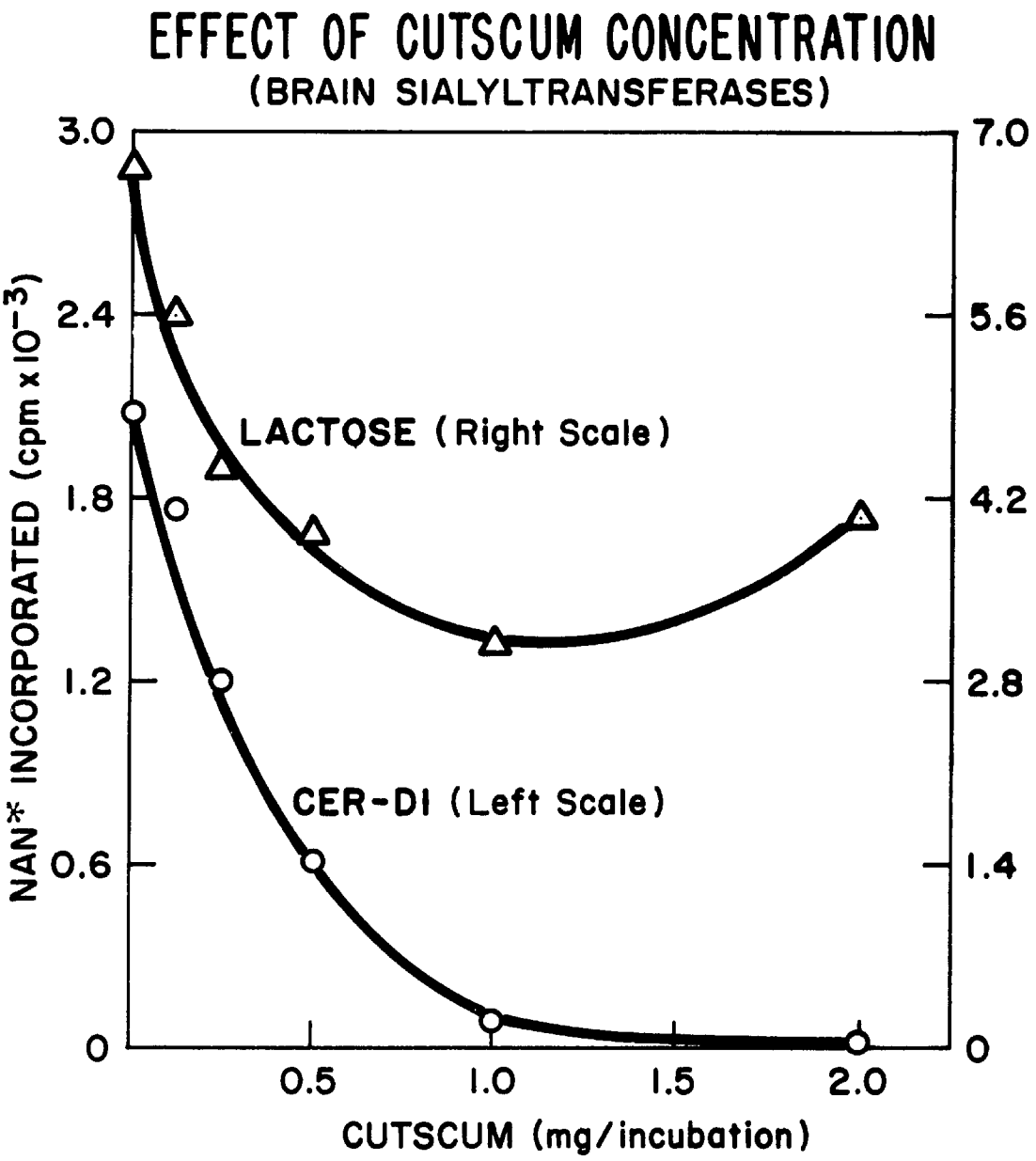
iii. Effect of Sonication

It has been shown by various workers that ultrasonic vibration helps to keep lipids or lipoprotein in solution. The particulate sialyltransferase of the chicken embryonic brain was subjected to sonication for 5-20 seconds and activities with respect to ceramide-disaccharide and lactose were determined. Incubation mixtures with or without enzyme were also sonicated but there was no increase in activity; rather it decreased with the increase of sonication time as shown in Table 17.

8. Attempt to Separate Sialyltransferases by Continuous Sucrose Gradient

The particulate enzyme preparation obtained from chicken embryonic brain was subjected to sucrose density gradient centrifugation in attempts to separate different sialyltransferases. Three ml of a 0.25 M sucrose suspension of disrupted particles (passed through the French Press), was layered on the top of discontinuous density gradients usually consisting of five successive layers (2 ml/layer) of sucrose solution; the concentration of layers increased from 0.25 M at the top to 1.5 M at the bottom of the gradient in 0.25 M increments. After centrifugation at

FIGURE 12



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.105 ml: ceramide-disaccharide, 0.05; detergent mixture, 300 μg; lactose, 2.0; cacodylate-HCl, pH 7.0, 12.5; $MgCl_2$, 1.0; glycine, 2.5; CMP-NAN, 0.05 (1.08×10^6 cpm per μmole); enzyme, 0.85 mg of protein. Cutscum was added as indicated in the Figure. All mixtures were incubated at 37° for 4 hours.

TABLE 17

Effect of Sonication on the Activity of

Particulate Sialyltransferase(s)

A. Complete incubation mixtures contained the following components (μmoles) in the final volumes of 0.08 ml: ceramide-disaccharide, 0.025; detergent mixture, 150 μg; lactose, 1.0; $MgCl_2$, 1.0; cacodylate-HCl, pH 7.0, 12.5; enzyme, 0.85 mg of protein.

B. Complete incubation mixtures contained all the components as above except that the enzyme was preincubated at 37° C for 6 hr before it was added to incubation mixtures.

All mixtures were incubated at 37° for 10 hours.

Endogenous values were subtracted from values obtained with substrates.

Exp. No.	Time of sonication (seconds)	NAN* incorporated (cpm)	
		Cer-di	Lactose
A	0	1,310	3,150
	5	1,300	2,500
	10	1,220	2,350
	20	1,020	1,990
B	0	1,100	900
	*10	1,080	990
	†10	1,070	900

* Incubation mixture was sonicated and the enzyme added.

† Whole incubation mixture was sonicated (including enzyme).

100,000 x g for 1 hr, the layers were carefully removed with a syringe, and enzyme activity in the different fractions was assayed. The layer between 0.75-1.0 M of sucrose contained the major activity. Discontinuous, sucrose density gradient did not separate the CDSTase from SLTase activity. In order to obtain better resolution of these two enzymes, continuous sucrose density gradient experiment was performed as shown in Table 18.

Experiment B suggests a slight separation of two enzymes, but the results were not conclusive.

9. Isolation of Radioactive Products

Isolation of Ceramide-Disaccharide-NAN-^{14}C

To isolate the product, an incubation mixture was prepared as follows (400-fold increased quantities over the standard incubation mixture): each of 40 tubes contained the following compounds (μmoles) in final volumes of 2.5 ml: ceramide-disaccharide, 10.0; detergent mixture, 7.5 mg; cacodylate-HCl buffer, pH 6.5, 50; $MgCl_2$, 60; CMP-NAN, 2.0 (0.3 x 10^6 cpm per μmole); enzyme, 2 ml (40,000 x g washed buffy coat; 11.0 mg of protein per ml). After 16 hr incubation at 37°, the 40 reaction mixtures were combined, divided into 6.0 ml portions and transferred to 50 ml centrifuge tubes. Ten ml of C-M (2:1) was added to each tube. After thorough mixing, 7 ml aliquots of C-M were added to each tube, mixed, and centrifuged at room temperature in the clinical centrifuge for 10 min. The supernatant fluids were collected and the residue in each tube was again washed twice with 20 ml portions of C-M (2:1).

TABLE 18

Attempt to Separate Sialyltransferases by Centrifugation in a Continuous Sucrose Gradient

The French Press preparation (1.5 ml; 16 mg of protein per ml) was layered on the top of 9.0 ml of the sucrose gradient (upper layer 0.25 M to lower layer 1.5 M) as described in the text. The mixture was centrifuged for 1 hr at 105,000 x g in a Spinco centrifuge; 0.5 ml portions of fractions were collected from the bottom, and 0.05 ml of each fraction was assayed as described below.

A. Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: ceramide-disaccharide, 0.025; detergent mixture, 150 μg; cacodylate-HCl, pH 7.0, 12.5; $MgCl_2$, 1; lactose, 1.0; CMP-NAN, 0.14 (500,000 cpm per μmole); enzyme, 0.05 ml of sucrose gradient fractions.

B. All the components were the same as above except that sucrose gradient fractions were preincubated at 37° for 3 hr and then were added to incubation mixtures. All mixtures were incubated for 12 hours at 37°.

Fraction number	NAN* incorporated (cpm)			
	(A)		(B)	
	Cer-di	Lactose	Cer-di	Lactose
5	60	520	150	600
6	600	160	660	910
7	1,120	1,730	1,300	1,490
8	1,120	2,330	1,080	1,850
9	740	1,880	1,090	2,280
10	400	1,780	920	1,150
11	250	920	620	820
12	60	600	380	460

The combined C-M extract was evaporated to dryness, dissolved in 20 ml of C-M (2:1), and mixed with 20 ml of water. The mixture was electro-dialyzed for 3 hours at 440 volts against distilled water in a specially designed apparatus (257-259). The upper aqueous layer contained 10% of the total ^{14}C, and was discarded. The lower chloroform layer was concentrated and applied to a Florisil column (2.5 x 50 cm).

The column was eluted successively with 6 liters of C-M (2:1); 6 liters of C-M (1:1), 3 liters of C-M (1:2), 2 liters of C-M (1:2) and 4 liters of C-M (1:4). The first 3 eluates were collected in 1 liter fractions and the last 6 liters of eluates were collected in 2 liter fractions; all fractions were assayed for radioactivity. The major radioactive product was eluted with C-M (1:1) and C-M (1:2); the combined fractions were concentrated to dryness. Twenty per cent of the total radioactivity was recovered from the major fractions (3 μmoles). The lipid was finally purified by TLC using solvent T-I and T-III (see page 36).

Isolation of Ceramide-Tetrasaccharide-NAN-^{14}C

To isolate the product, an incubation mixture was prepared containing 300-fold increased quantities over the standard incubation mixture. Each of two flasks contained the following substances (μmoles) in final volumes of 45 ml: ceramide-tetrasaccharide, 20; detergent mixture, 150 mg; cacodylate-HCl buffer, pH 6.4, 5 mmoles; CMP-NAN, 43 (0.4 x 10^6 cpm per μmole); enzyme, 35 ml (40,000 x g,

washed buffy coat containing 11 mg of protein per ml). The two flasks were incubated at 37° for 16 hours. The radioactive products were associated with the enzyme particles, an observation made by Dr. Kaufman, that greatly aided purification. The mixture was centrifuged at 40,000 x g for 1 hr and the particles were washed two times with 10 vol of 0.1 M KCl, twice with 5 vol of water, and the product was finally extracted with 5 vol of methanol (3 times). The methanol solution was concentrated to dryness and the residue was redissolved in 5 ml of C-M (2:1) and centrifuged. The supernatant solution was applied to silicagel-G thin-layer plates, and the plates were first developed with solvent T-I. One ^{14}C-area was detected; it also was heavily stained with bromothymol blue. The spots were eluted (see pages 102-104), concentrated and subjected to TLC with solvent T-II. Again the areas corresponding to heavy bromothymol blue stain and high radioactivity were scraped from the plate and eluted with solvent T-II after removal of the dye with acetone.

The total yield of the radioactive product was 16 μmoles (40%). Characterization of the product by thin-layer chromatography with different solvent systems, showed the presence of two compounds; these were finally separated with solvent T-V (see page 36). The products were analyzed by the procedures described in the Methods Section. Ninety per cent of the total product was monosialoganglioside and 10% was disialoganglioside.

10. Characterization of Substrates and Radioactive Products

Radioactive products were hydrolyzed with mild acid as described in the Methods Section. The original and acid-hydrolyzed products were analyzed by TLC, along with standard glycolipids; the plates were developed with solvent II for characterization of sialyl-ceramide-disaccharide (hematoside) as shown in Figure 13. The TLC plates were developed with solvent III for the identification of sialyl-ceramide-tetrasaccharide as shown in Figures 14 and 15.

Ceramide-tetrasaccharide-NAN-^{14}C, and ceramide-tetrasaccharide-(NAN-^{14}C)$_2$ were homogenous, and indistinguishable by thin-layer chromatography (as shown in the Figures 14 and 15) from authentic monosialoganglioside (G_{M1}), and disialoganglioside, respectively. (Standards obtained from Dr. T. Yamakawa and Dr. L. Svennerholm.)

11. Analyses of Substrates and Radioactive Products

The analytical procedures described in the Methods Section were employed to determine the molar ratios of the components in substrates and products. The results with the substrates are given in Table 1. Analyses for the radioactive sialyl ceramide oligosaccharides are given in Table 19. The fatty acid composition of ceramide-tetrasaccharide-NAN-^{14}C is shown in Figure 16.

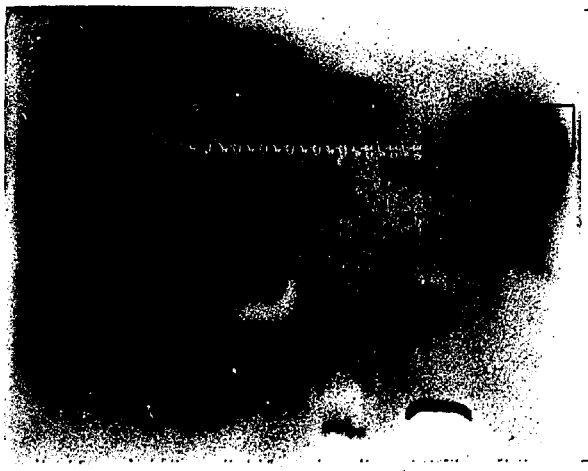
12. Treatment of Radioactive Products with Sialidase

Incubation of the ^{14}C-glycolipids with Clostridium perfringens sialidase (124) for 17 hours resulted in the release of 90, 70, and 67% of ^{14}C sialic acid from the

FIGURE 13

CHARACTERIZATION OF BRAIN SIALYLTRANSFERASE PRODUCT

(Ceramide Disaccharide←NAN*)



1. Ceramide Disaccharide
2. Ceramide Trisaccharide
3. Hematoside
4. Ceramide Disaccharide←NAN*
5. Acid Hydrolyzed - 4

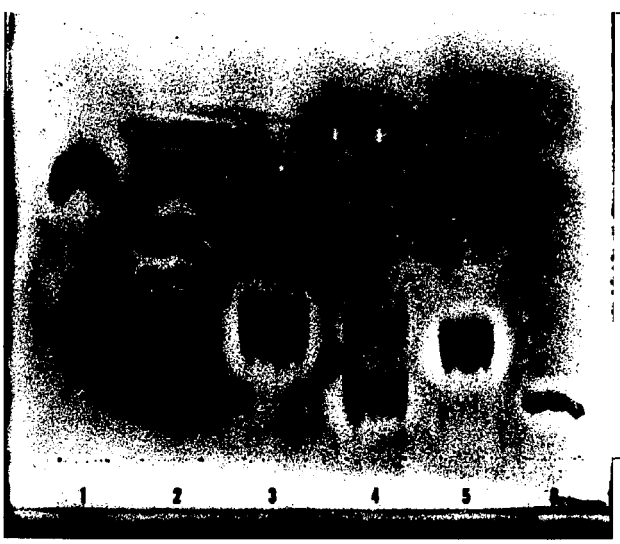
Thin layer plate of silica gel - G

Developing solvent $CHCl_3$: MeOH : H_2O (65 : 25 : 4)

FIGURE 14

CHARACTERIZATION OF BRAIN SIALYLTRANSFERASE PRODUCTS

[Cer-Tetra←NAN*; Cer-Tetra←$(NAN^*)_2$]



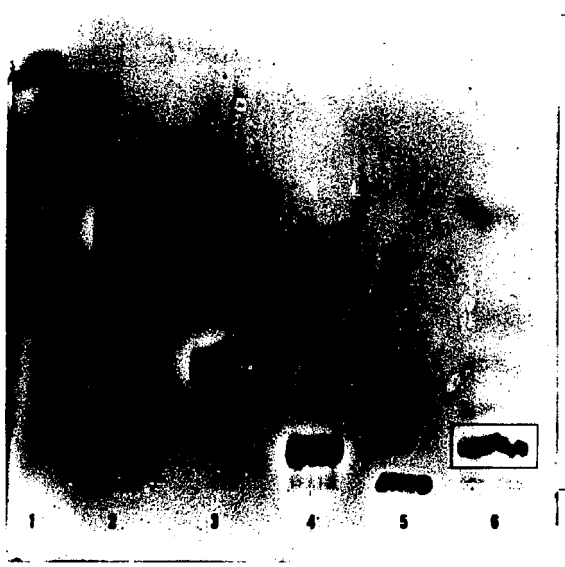
1. Hematoside
2. Tay-Sachs Ganglioside
3. Monosialo Ganglioside (Gm_1)
4. Calf Brain Ganglioside
5. Ceramide Tetrasaccharide←NAN*
6. Ceramide Tetrasaccharide←$(NAN^*)_2$

Thin layer plate of silica gel-G.
Developing solvent n-Propanol : 1% Borate (8 : 2)

FIGURE 15

CHARACTERIZATION OF BRAIN SIALYLTRANSFERASE PRODUCT

(Ceramide Tetrasaccharide←NAN*)



1. Ceramide Monosaccharide
2. Ceramide Disaccharide
3. Ceramide Trisaccharide
4. Ceramide Tetrasaccharide
5. Ceramide Tetrasaccharide←NAN*
6. Acid Hydrolyzed-5

Thin layer plate of silica gel-G

Developing solvent $CHCl_3$: MeOH : H_2O (65 : 25 : 4)

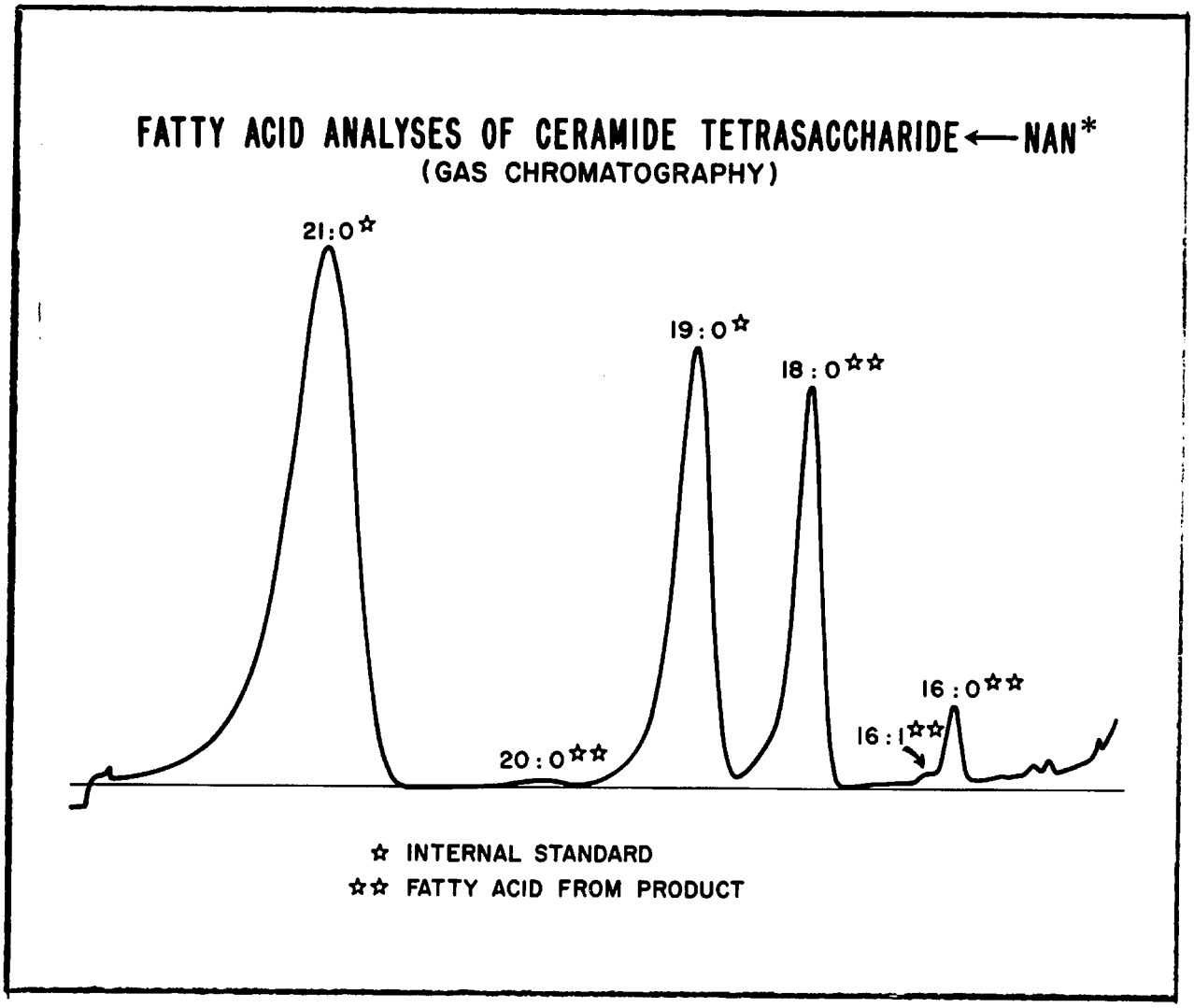
TABLE 19

Analyses of Brain Sialyltransferases Products

Component	Analytical methods	Molar ratios		
		Cer-di ←NAN	Cer-tetra ←NAN	Cer-tetra ←NAN_2
Sialic acid	Thiobarbituric acid	1.00	1.00	1.00
Sphingosine	Methyl orange	1.30	1.00	0.48
Fatty acid	Gas chromatography	0.91	1.04	0.50
Hexose	Anthrone	2.30	3.18	ND†
Glucose	Hexokinase and glucose-6-PO_4 dehydrogenase	1.02	1.14	ND
Galactose	Galactose oxidase	0.85	2.23	ND
Hexosamine	Elson-Morgan	---	0.94	ND

† ND - not determined due to insufficient material.

FIGURE 16


FATTY ACID ANALYSES OF CERAMIDE TETRASACCHARIDE ← NAN*
(GAS CHROMATOGRAPHY)
21:0☆
19:0☆
18:0☆☆
20:0☆☆
16:0☆☆
16:1☆☆
☆ INTERNAL STANDARD
☆☆ FATTY ACID FROM PRODUCT

labeled ceramide-disaccharide-NAN, ceramide-tetrasaccharide-NAN* and ceramide-tetrasaccharide-NAN_2*, respectively, as shown in Table 20.

TABLE 20

Release of Radioactive NAN from Brain

Sialyltransferase Products by Sialidase

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.035 ml: radioactive lipids, 0.04-0.1; acetate buffer, pH 5.0, 10; sialidase, 0.025 ml (0.7 units).

All mixtures were incubated at 37° for 17 hours. One unit of enzyme was defined as 1.0 μmole of NAN released per 15 minutes from sialyl-lactose (124).

Compounds	% of NAN* Released
Sialyllactose	99
Ceramide-disaccharide-NAN*	90
Ceramide-tetrasaccharide-NAN*	70
Ceramide-tetrasaccharide-NAN_2^*	67

TABLE 21

K_m Values of Different Substrates with Brain Sialyltransferase(s) and Rat Mammary Sialyltransferase(s)

Substrates	K_m Values	
	Brain sialyltransferase(s) (M)	Rat mammary sialyltransferase(s) (M)
Ceramide-disaccharide	3.0×10^{-4}	No activity
Ceramide-tetrasaccharide	2.5×10^{-5}	5.0×10^{-4}
Lactose	4.0×10^{-3}	2.0×10^{-3}
N-Acetyllactosamine	2.6×10^{-3}	1.0×10^{-3}

DISCUSSION

The results described above indicate that a particulate fraction obtained from 9 day old chicken embryonic brains catalyzed the transfer of N-acetylneuraminic acid (NAN) from CMP-NAN to three different glycosphingolipids, ceramide-disaccharide‡, ceramide-tetrasaccharide‡, and monosialo-ganglioside. In addition, the sialic acid was transferred to lactose, N-acetyl lactosamine, and one glycoprotein (fetuin). The sialyl-glycolipid transferase activity was detected in homogenates of brain from embryonic chicken (7-19 days), pig fetus (82 mm), and rat (9 day), and the adults of some species; maximum activity was observed with 9-13 day old embryonic chicken tissues as shown in Figure 11.

Detergent was required to detect enzymatic activity (Table 3). Of the several detergents tested, the mixture of Triton CF-54 and Tween 80 was most active but in inhibited activity at higher concentration as shown in Figure 8.

The incorporation of ^{14}C-NAN was proportional to time of incubation and concentration of protein. With ceramide-disaccharide as substrate, a slight lag was observed at low

‡ Acid hydrolysis of the calf brain ganglioside mixture removes the sialic acid and yields mostly ceramide-tetrasaccharide and ceramide-disaccharide and smaller quantities of ceramide mono- and trisaccharide. These glycosphingolipids were separated on silicic acid columns and found to be homogeneous by thin-layer chromatography as shown in Figure 2. The molar ratios of the constituents relative to sphingosine were determined as shown in Table 1 and were close to the theoretical values. These compounds were tested as substrates.

protein concentrations. Tszur and Shapiro (262) also noted the initial protein lag in the *in vitro* biosynthesis of neutral lipids and phospholipids with microsomes. They suggested that the lag is related to the ratio of protein, lipid, and detergent. They also proposed that microsomes synthesize lipoproteins and that lipid synthesis is limited by the amount of acceptor protein available.

The activity of the particulate fraction with different glycolipids is shown in Table 13. Ceramide-tetrasaccharide and ceramide-disaccharide were active, as were monosialo-ganglioside and the calf brain ganglioside mixture. However, there was no activity with ceramide-monosaccharide, and questionable activity with ceramide-trisaccharide. The inactivity with the ceramide-trisaccharide, in contrast to the effectiveness of the ceramide-tetra- and disaccharide, shows that this system is different from the rat kidney preparation reported by Kanfer and coworkers (9), where the opposite results were obtained.

In earlier studies (162-165), sialic acid was enzymatically transferred from CMP-N-acetylneuraminic acid or CMP-N-glycolylneuraminic acid to glycosides, disaccharides, oligosaccharides, a polysaccharide (colominic acid), glycoproteins, and mucins. These reactions are catalyzed by a class of enzymes, called sialyltransferases, that are distinguishable from each other by their specificities towards the sialic acid acceptors. In the earlier work with the different enzyme preparations, glycolipids had not been tested, or had exhibited little or no activity.

The mammalian sialyltransferase first studied, was obtained from lactating rat mammary gland, and was active with a number of β-galactopyranosides (163) including lactose and N-acetyllactosamine. This activity was demonstrated in a number of rat tissue extracts. Since it was possible that the embryonic chicken brain preparation contained one sialyltransferase of broad specificity, similar to the rat mammary gland, the two enzyme systems were compared, as shown in Table 21. The most important result of these experiments is the fact that the rat mammary gland preparation, in contrast to the brain system, showed no detectable activity with ceramide-disaccharide, but was active with ceramide-tetrasaccharide, as well as lactose and N-acetyllactosamine. These data suggest the existence of a sialyltransferase in embryonic brain that is specific for ceramide-disaccharide.

We propose, therefore, that embryonic brain contains at least two sialyltransferases, one active with ceramide-disaccharide, and the other(s) active with ceramide-tetrasaccharide, monosialoganglioside, lactose, N-acetyllactosamine, etc. This conclusion is based on the following: (a) The comparison of activities of the rat mammary gland and embryonic chicken brain preparations discussed above. (b) Partial heat inactivation (Fig. 9, Table 6) studies showed that the activity towards ceramide-disaccharide was relatively stable, under conditions where the other sialyltransferase(s) were almost inactivated. (c) Substrate competition experiments indicated that

lactose did not compete with ceramide-disaccharide. However, when lactose was mixed with ceramide-tetrasaccharide or monosialoganglioside, a mutual inhibition resulted. (d) Some of the inhibitor (Table 10) studies also indicated that the activity towards ceramide-disaccharide was distinguishable from that towards lactose.

The sum of these 4 types of results agree with the conclusion that embryonic chicken brain contains a new sialyltransferase, specific towards ceramide-disaccharide. At least one other sialyltransferase is also present in the preparation, and is active with lactose, N-acetyl-lactosamine, ceramide-tetrasaccharide, and monosialo-ganglioside. If these four compounds are substrates for one non-specific sialyltransferase, then the glycolipids appear to be much more efficient substrates, since their K_m values are 20 to 100-fold less than the values obtained with the disaccharides.

The two sialyltransferase systems catalyze the following reactions:

I. Ceramide-disaccharide + CMP-NAN → hematoside (+ CMP?)

II.

i) Lactose + CMP-NAN → sialyl-lactose (+ CMP?)

ii) N-Acetyllactosamine + CMP-NAN → sialyl-N-acetyllactosamine (+ CMP?)

iii) Ceramide-tetrasaccharide + CMP-NAN → monosialoganglioside (+ CMP?)

iv) Monosialoganglioside + CMP-NAN → disialoganglioside (+ CMP?)

Hematoside obtained from ceramide-disaccharide and monosialoganglioside obtained from ceramide-tetrasaccharide were isolated from big batch enzyme incubation and purified by florisil and thin-layer chromatography. The products ^{14}C-hematoside and ^{14}C-monosialoganglioside were homogeneous in several chromatographic systems. The purified products were analyzed and the molar ratios were in good agreement with expected values.

Upon hydrolysis of ^{14}C-hematoside and ^{14}C-monosialoganglioside in 0.1 N H_2SO_4 at 80° for one hour, the sialic acid was released with the concomitant appearance of the respective glycolipid acceptor incubated with the sialyltransferase. Incubation of ^{14}C-products with Clostridium perfringens neuraminidase (124) for 17 hours resulted in the release of 90, 70 and 67 percent of the ^{14}C-NAN from the labeled ceramide-disaccharide-NAN, ceramide-tetrasaccharide-NAN and ceramide-tetrasaccharide-NAN_2 respectively.

Neither acid hydrolysis nor neuraminidase treatment directly proved the mode of attachment of NAN to the glycose unit but suggested that at least one NAN was attached to the end of the glycose chain in each product (ceramide-disaccharide-NAN-^{14}C, ceramide-tetrasaccharide-NAN-^{14}C and ceramide-tetrasaccharide-NAN_2-^{14}C).

Sialidase treated orosomucoid, and ovine and bovine submaxillary mucins were not active ($< 2\%$), but sialidase pretreated fetuin was active (about 20% relative to cer-tetrasaccharide activity). Sialic acid or sialic acid

9-phosphate, incubated in the presence of CMP, did not substitute for CMP-NAN, whereas CMP-NGN was as effective as CMP-NAN.

PART II

STUDIES ON THE GALACTOSYLTRANSFERASES OF EMBRYONIC CHICKEN BRAIN

A. PREPARATION OF ENZYMES

Galactosyltransferase activity was detected in homogenates of brain from embryonic chicken (7-20 days). A typical preparation was obtained from frozen 13-day old embryonic chicken brain by homogenizing the thawed tissue with 4 volumes of a solution containing 0.25 M sucrose, and 0.014 M 2-mercaptoethanol. All steps in preparing the enzyme were performed at 0-4°. Nuclei, debris and whole cells were removed by centrifugation at 750 x g for 10 min. The specific activity of the preparation was increased 4-fold when a particulate fraction, sedimenting between 3,000 and 39,000 x g was collected; the activity was located in a light particulate layer overlaying a heavy residue. The light particulate layer was washed twice with 0.25 M sucrose containing 0.014 M 2-mercaptoethanol, and resuspended with a glass-Teflon Potter-Elvehjem homogenizer.

Storage and stability of enzyme preparation: After washing and resuspension of particulate enzyme, the final concentration was adjusted to 8 mg protein per ml; the activity was stable to storage for several months at -20°. Disruption of particles in a French Pressure cell (6,000 lb/sq in) resulted in loss of activity (90%).

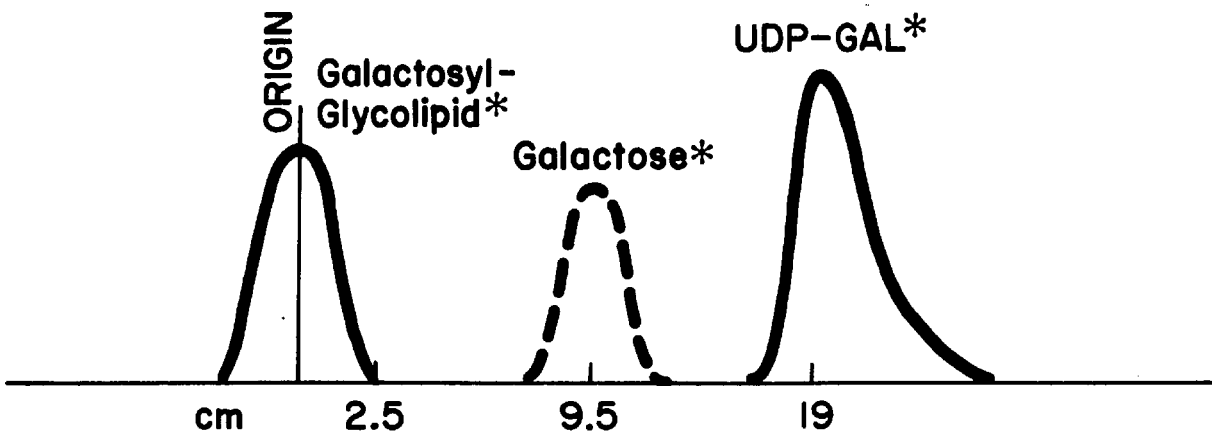
B. ASSAY OF ENZYMIC ACTIVITY

Reagents: 1.0 M cacodylate-HCl buffer, pH 7.38; 0.2 M $MnCl_2$; 0.007 M Tay-Sachs ganglioside in C-M (2:1); mixture of 0.4% Tween-80 and 0.8% Triton CF-54 in C-M (2:1); 0.006 M UDP-galactose-^{14}C (specific activity, 0.78×10^6 cpm per μmole); enzyme, 8 mg protein per ml.

Procedure: Complete incubation mixtures contained the following components in final volumes of 0.1 ml: cacodylate-HCl buffer, pH 7.3, 10 μl; $MnCl_2$, 5 μl; UDP-galactose-^{14}C, 10 μl; Tween-80:Triton CF-54 solution (1:2), 25 μl; Tay-Sachs ganglioside, 10 μl; and 50 μl of enzyme. The lipid and detergents in C-M (2:1) were dried in the incubation tubes prior to adding the other reagents. After incubating for 2 hours at 37°, 5 μl of 0.5 M EDTA was added to stop the reaction. The contents of each tube were quantitatively transferred to Whatman 3 MM paper with C-M (2:1). The paper was carefully saturated with 0.05 M borate, pH 9.0, and the solution was allowed to diffuse from both sides towards the origin. After electrophoresis for 30 min at 55-62 volts per cm, the radioactive gangliosides remained at the origin, whereas the labeled substrate and degradation products migrated as shown in Figure 17. Control tubes lacked glycolipid acceptor.

FIGURE 17

ASSAY FOR GALACTOSYLTRANSFERASES IN EMBRYONIC CHICKEN BRAIN



Paper Electrophoresis – 0.05 M Borate, pH 9.0, 30 min, 3500 volts.

Incubation Conditions – (μmoles/0.10 ml). UDP-Gal* 0.06; Tay-Sachs Ganglioside 0.07; Cacodylate-HCl Buffer pH 7.3, 10; $MnCl_2$, 1.0; Triton CF-54 (200 μg); Tween-80 (100 μg); Enzyme (0.41 mg of Protein). Incubated at 37°C for 2 hours.

C. EXPERIMENTAL AND RESULTS

1. Preliminary Kinetic Studies with the Enzyme System

The following studies were performed using the electrophoretic assay, where the electrophoretically ^{14}C-content of the immobile product was measured. Detergent mixture means Triton CF-54:Tween-80 (2:1).

i. Requirements for UDP-Galactose:Glycolipid Galactosyltransferase

The transfer of galactose-^{14}C to Tay-Sachs ganglioside is shown in Table 22. The enzyme system contained unidentified endogenous acceptors. However, the synthesis of ^{14}C-galactosylglycolipid was stimulated ten-fold by adding Tay-Sachs ganglioside to the reaction mixture. Detergent was necessary to obtain maximum activity. An absolute requirement for Mn^{++} was evident; versene inhibited the reaction completely.

ii. Effect of Incubation Time and Protein Concentration

Under the above mentioned conditions, the incorporation of galactose-^{14}C was linear with time (Figure 18) and with protein concentration (Figure 19). Product formation was proportional to time of incubation with the following substrates for the indicated times: Tay-Sachs ganglioside up to 4 hours, sialidase and galactosidase pretreated orosomucoid up to 2 hours.

iii. Effect of pH

The incorporation of galactose-^{14}C as a function of pH is shown in Figure 20. Control incubations lacked TSG and these control values were subtracted from values

TABLE 22

Requirements for UDP-Galactose:Glycolipid Galactosyltransferase(s)

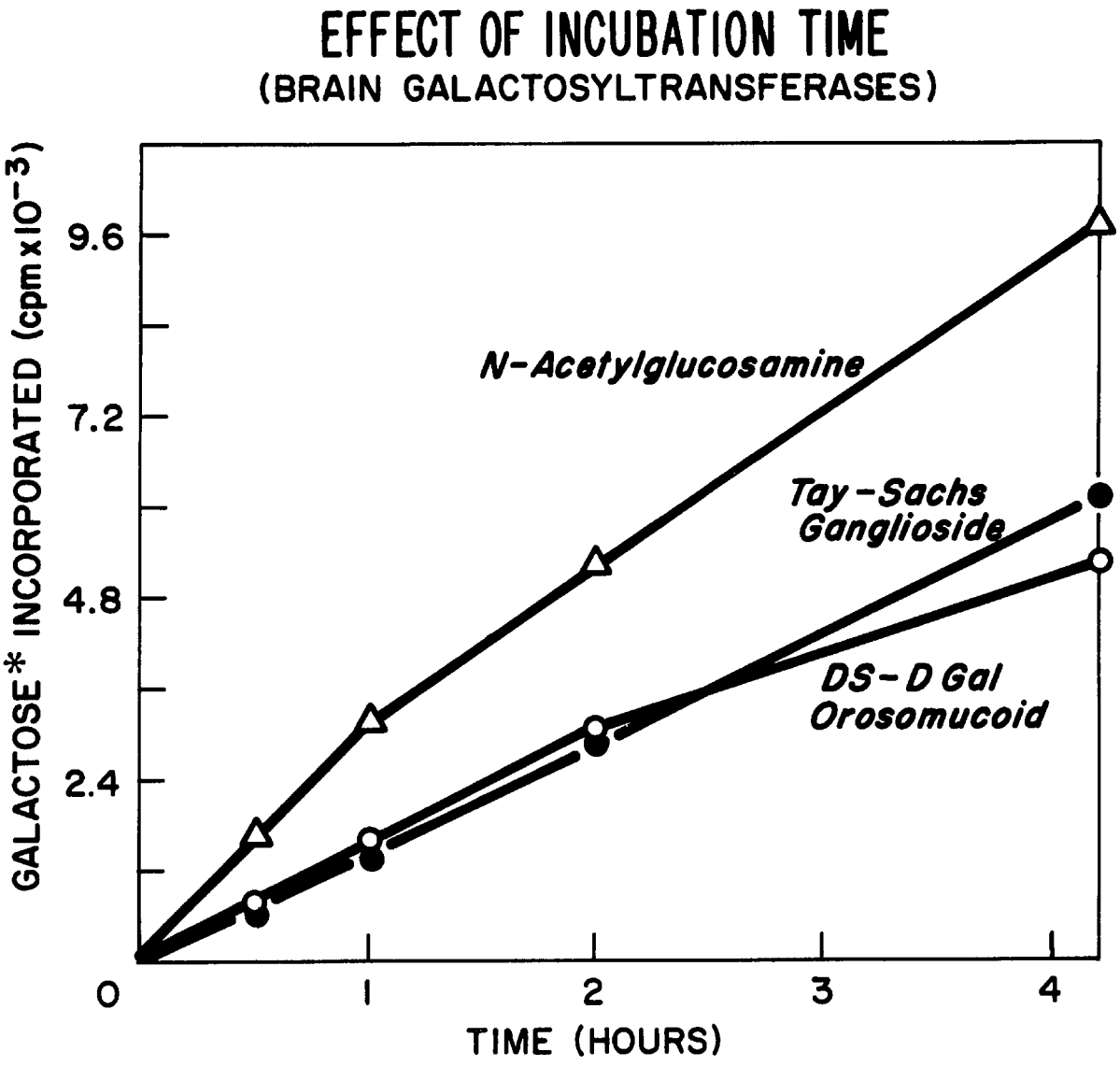
Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.10 ml: Tay-Sachs ganglioside, 0.035; detergent mixture containing Tween-80 and Triton CF-54 (1:2, v/v), 300 μg; cacodylate-HCl buffer, pH 7.3, 10; $MnCl_2$, 2.5; UDP-galactose-^{14}C, 0.07 (7.0×10^5 cpm per μmole); particulate enzyme preparation, 0.66 mg protein. After 2 hr at 37°, the mixtures were assayed by paper electrophoresis in 1% sodium tetraborate. Under these conditions, product formation was proportional to time of incubation for at least 4 hr, and was essentially proportional to protein concentration over the range 0.03 to 0.42 mg per incubation mixture. The product formed from endogenous acceptor has not been characterized, but was insoluble in chloroform-methanol (2:1). In contrast to the results obtained in the complete system, the counts incorporated into endogenous acceptor were not proportional to time of incubation; for example, 300 cpm at 30 min, and 600 cpm at 4 hr.

TABLE 22

Requirements for UDP-Galactose:Glycolipid Galactosyltransferase(s)

Incubation mixture	Galactose-^{14}C incorporated (cpm)
Complete	3,628
Minus Tay-Sachs ganglioside	416
Minus detergent mixture	950
Minus $MnCl_2$	203
Plus 5 μmoles of EDTA (pH 7.0)	52
Incubated zero time	253

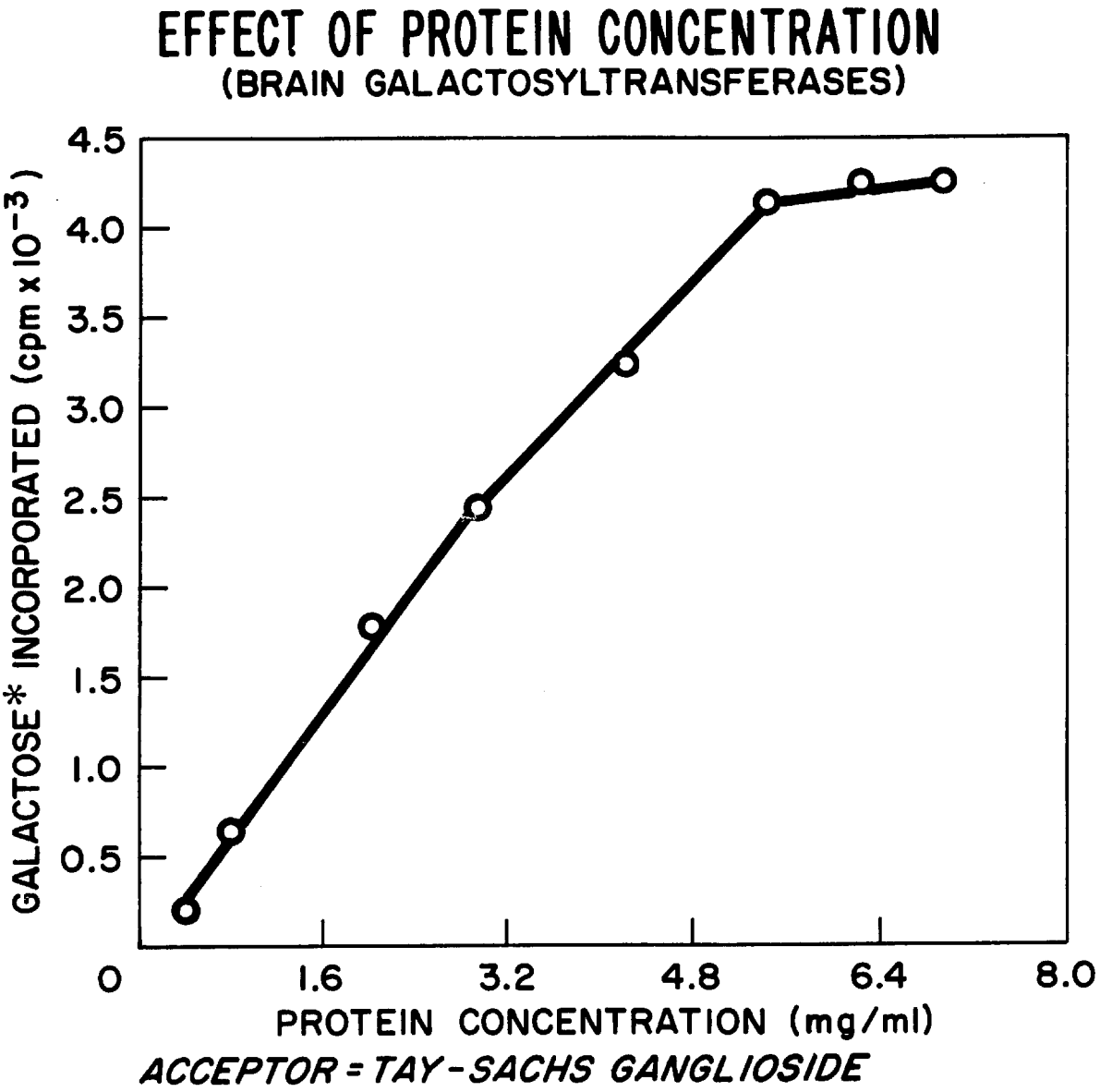
FIGURE 18



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: Tay-Sachs ganglioside, 0.07 (orosomucoid, 0.4 or N-acetylglucosamine, 2.5); detergent mixture, 300 μg; $MnCl_2$, 1.0; cacodylate-HCl, pH 7.3, 10; UDP-galactose-^{14}C, 0.07 (0.78×10^6 cpm per μmole); enzyme, 0.41 mg of protein.

All the mixtures were incubated for the indicated times at 37°, and the reactions stopped by adding 5 μmoles of EDTA (pH 7.0).

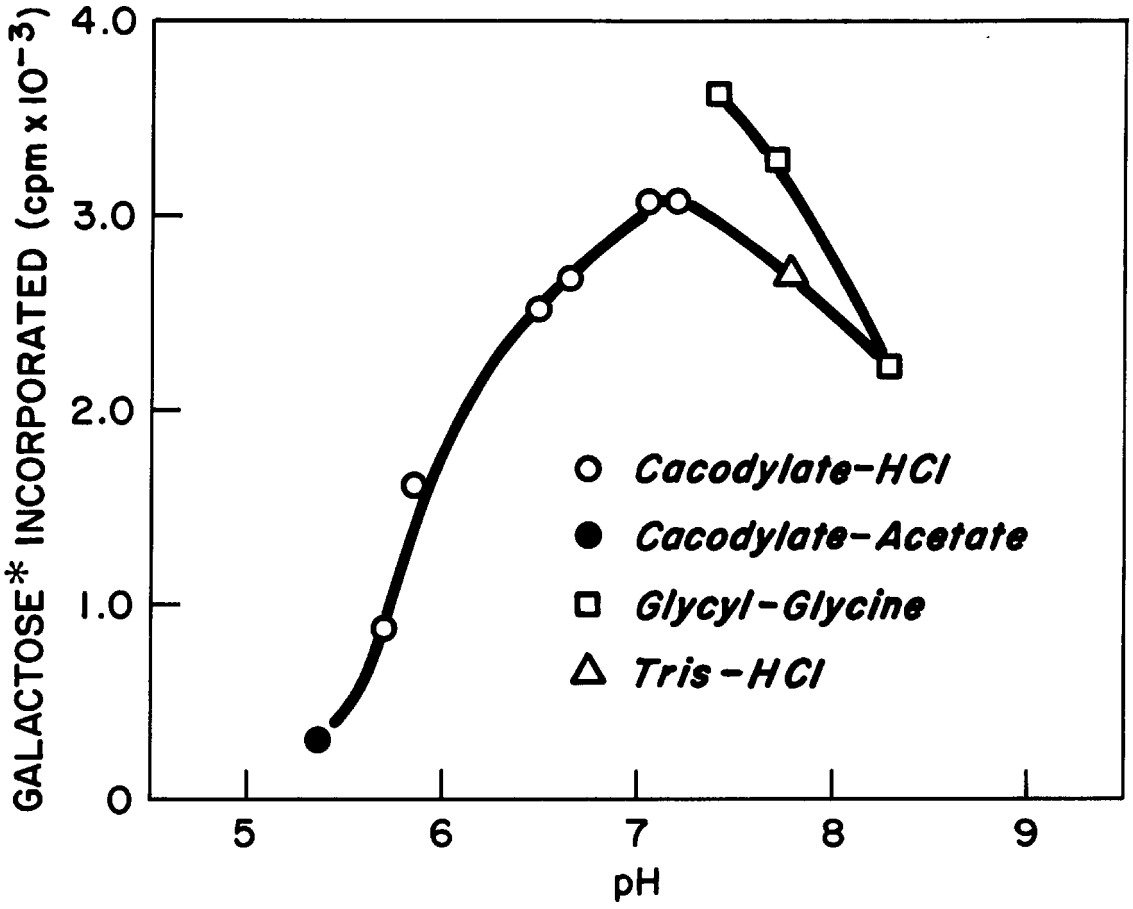
FIGURE 19



Complete incubation mixtures contained all the components as given in Figure 18 except for the varying protein concentration as shown in the Figure. All the mixtures were incubated at 37° for 2 hours.

FIGURE 20

EFFECT OF pH ON BRAIN GALACTOSYLTRANSFERASES



Complete incubation mixtures contained all the components as given in Figure 18, except for the different buffer as indicated in the Figure. The pH of the mixtures was measured after incubation at 37° for 2 hours.

obtained with complete incubation mixtures. The pH was measured after incubation, and the optimum pH with cacodylate-HCl buffer was 7.3. Enzyme activity was increased (15%) in glycyl-glycine buffer at pH 7.3.

iv. Effect of Various Metal Ions and Versene

The enzyme system required Mn^{++}. As indicated in Table 22 EDTA completely inhibited the system. Other divalent cations were tested and the results are shown in Table 23. Co^{++} was about 20% as active as Mn^{++} when tested at the same concentration (25 µmoles/ml), while the following ions were inactive: Mg^{++}, Zn^{++}, Ca^{++}, Ni^{++} and Cu^{++}.

v. Effect of Mn^{++} Concentration

The relationship of Mn^{++} concentration to enzyme activity is shown in Figure 21. Optimal enzymic activity was obtained in the range of 5-15 µmoles of Mn^{++}. Higher concentration of Mn^{++} (50 µmoles/ml) inhibited 25% of the enzyme activity.

vi. Effect of Substrate Concentration

The effect of varying substrate concentration on the "rate" of the reaction was studied as shown in Figures 22 and 23. The calculated K_m values were as follows: Tay-Sachs ganglioside, 2.0×10^{-4} M and UDP-galactose, 1.3×10^{-4} M.

vii. Effect of Heat Treatment on Galactosyltransferases

To determine whether the brain particulate system contained one or more galactosyltransferases, the enzyme preparation (0.5 ml portions) was heated for various times

TABLE 23

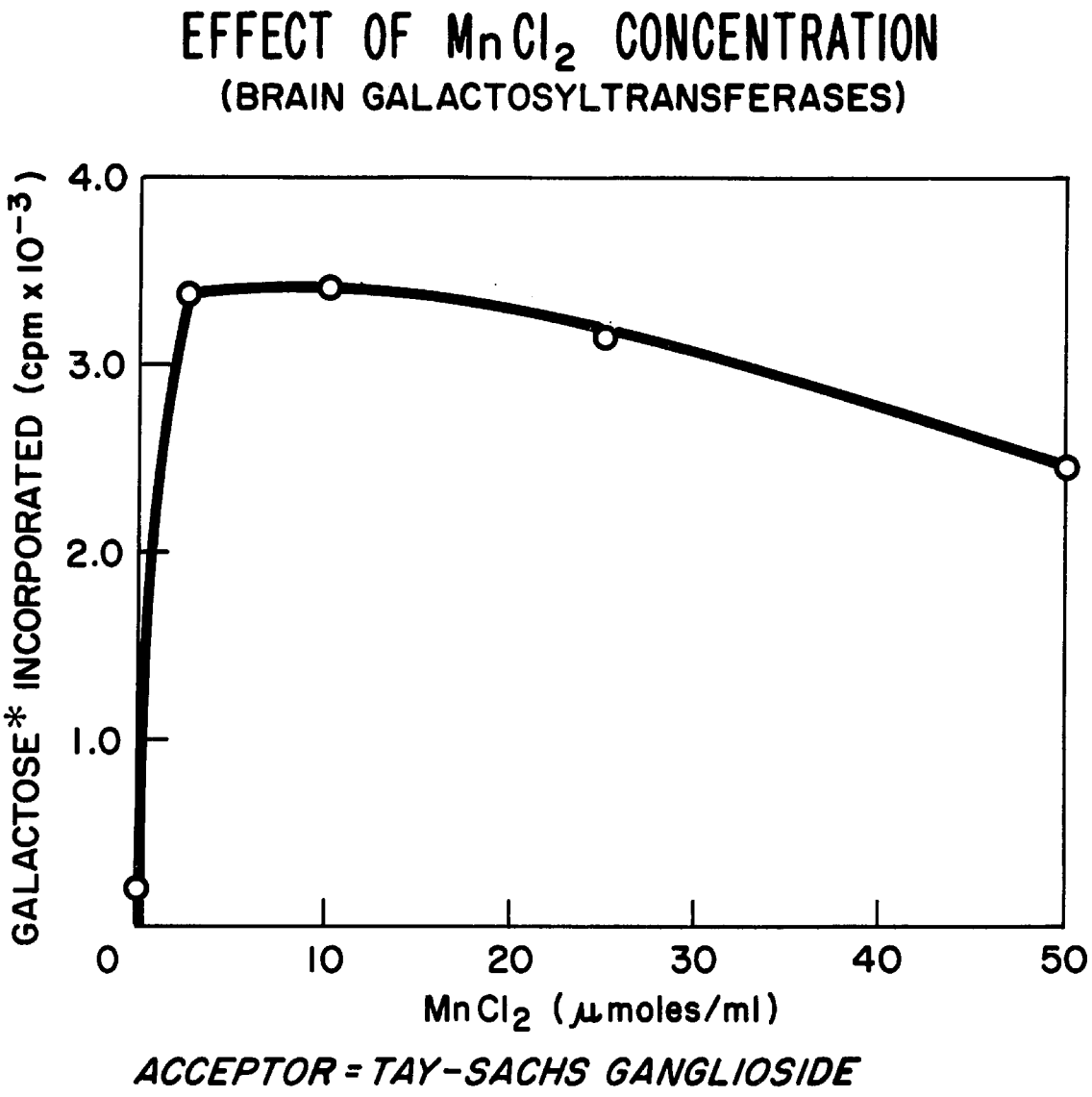
Effect of Metal Ions on Brain Galactosyltransferases

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: Tay-Sachs ganglioside, 0.05; detergent mixture, 300 μg; cacodylate-HCl buffer, pH 7.3, 10; UDP-galactose-^{14}C, 0.07 (0.78 x 10^6 cpm per μmole); metal ions, as indicated in the Table; enzyme, 0.66 mg of protein (contained EDTA, 0.05).

All the mixtures were incubated at 37° for 2 hours. Endogenous values obtained from incubation with metal and without exogenous lipid were subtracted from values obtained with Tay-Sachs ganglioside and these are given in the Table.

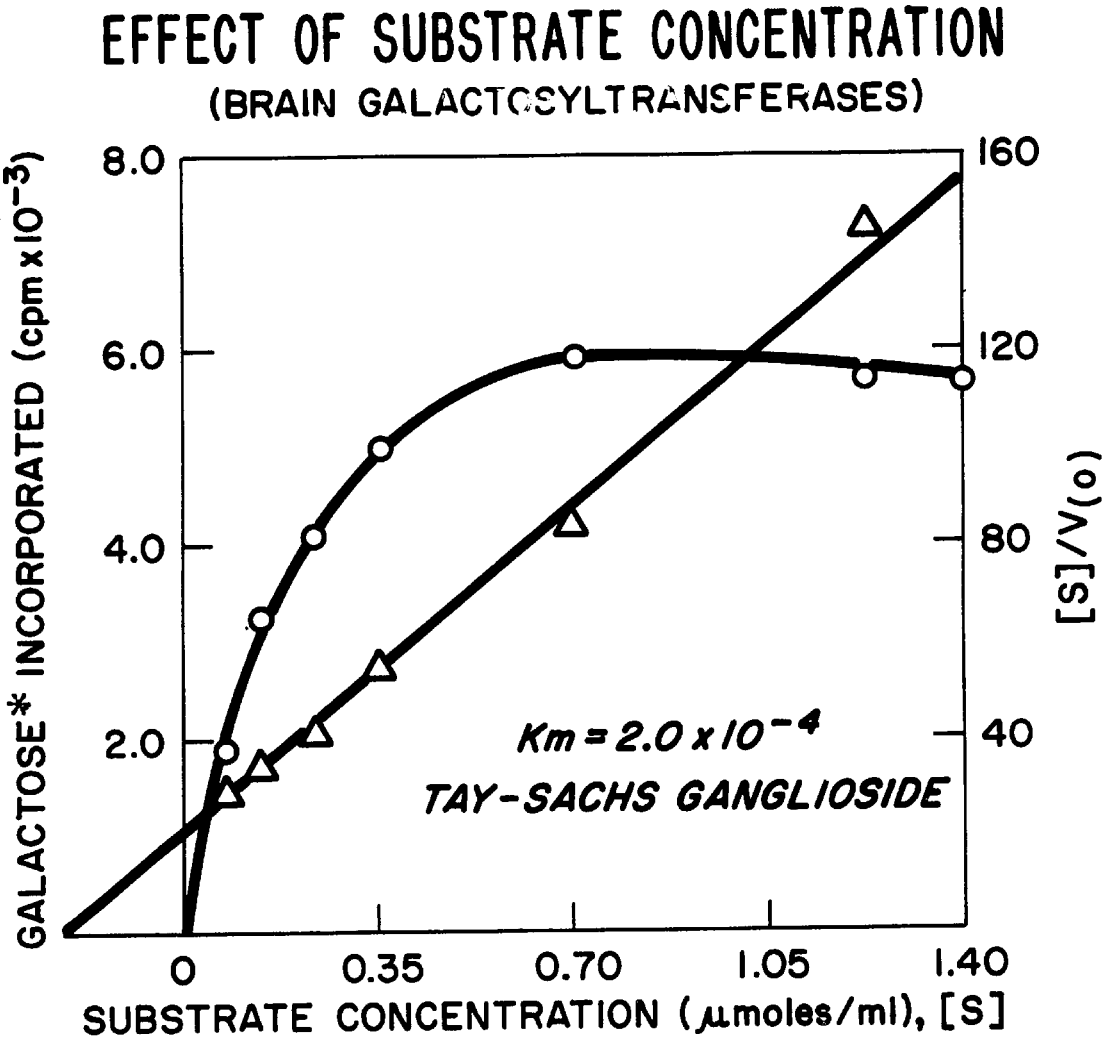
Salts	Concentration (μmoles)	Galactose-^{14}C incorporated (cpm)
---	---	340
$MnCl_2$	2.5	3,230
$CoCl_2$	"	900
$ZnCl_2$	"	100
$MgCl_2$	"	120
$NiCl_2$	"	280
$CdCl_2$	"	130
$CaCl_2$	"	210
$CuCl_2$	"	40
$MnCl_2$ + EDTA	2.5 + 5.0	0

FIGURE 21



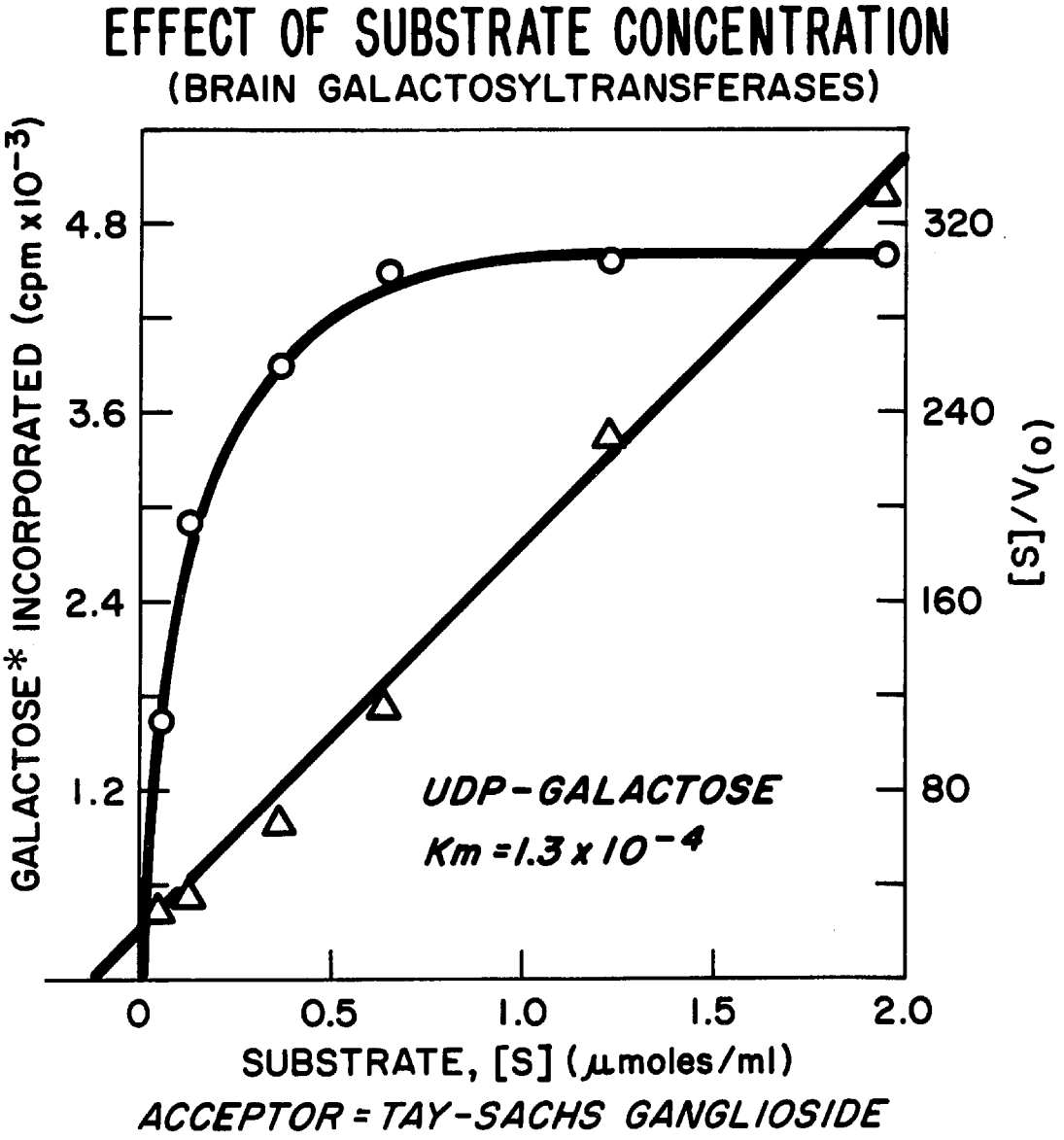
Complete incubation mixtures contained all the components given in Figure 18 except the enzyme, 0.66 mg of protein (suspension of 40,000 x g particles in 0.25 M sucrose, containing 0.001 M EDTA and 0.014 M of 2-mercaptoethanol). The mixtures were incubated at 37° for 2 hours and assayed as described in the text.

FIGURE 22



Complete incubation mixtures contained the components given in Figure 18, except the enzyme, 0.66 mg of protein, contained 0.05 μmole of EDTA. The concentration of substrate (Tay-Sachs ganglioside) was varied as indicated. The mixtures were incubated at 37° for 2 hours.

FIGURE 23



Complete incubation mixtures contained all the components as given in Figure 18 except that UDP-galactose-^{14}C was used at the indicated concentrations. Mixtures were incubated at 37° for 2 hours.

at two temperatures, 55° and 60°. The activities towards N-acetylglucosamine and glycoprotein were markedly labile in contrast to the activity towards glycolipid as shown in Figure 24 and Table 24. These results suggest the existence of a galactosyltransferase specific towards the glycolipid.

viii. Effect of Mixed Substrates

When the enzyme preparation was assayed for activity in the presence of individual and mixed substrates, the results (Table 25) suggested that the glycolipid did not compete with N-acetylglucosamine or glycoprotein. These data support the concept that the particulate preparation contained a galactosyltransferase specific toward Tay-Sachs ganglioside

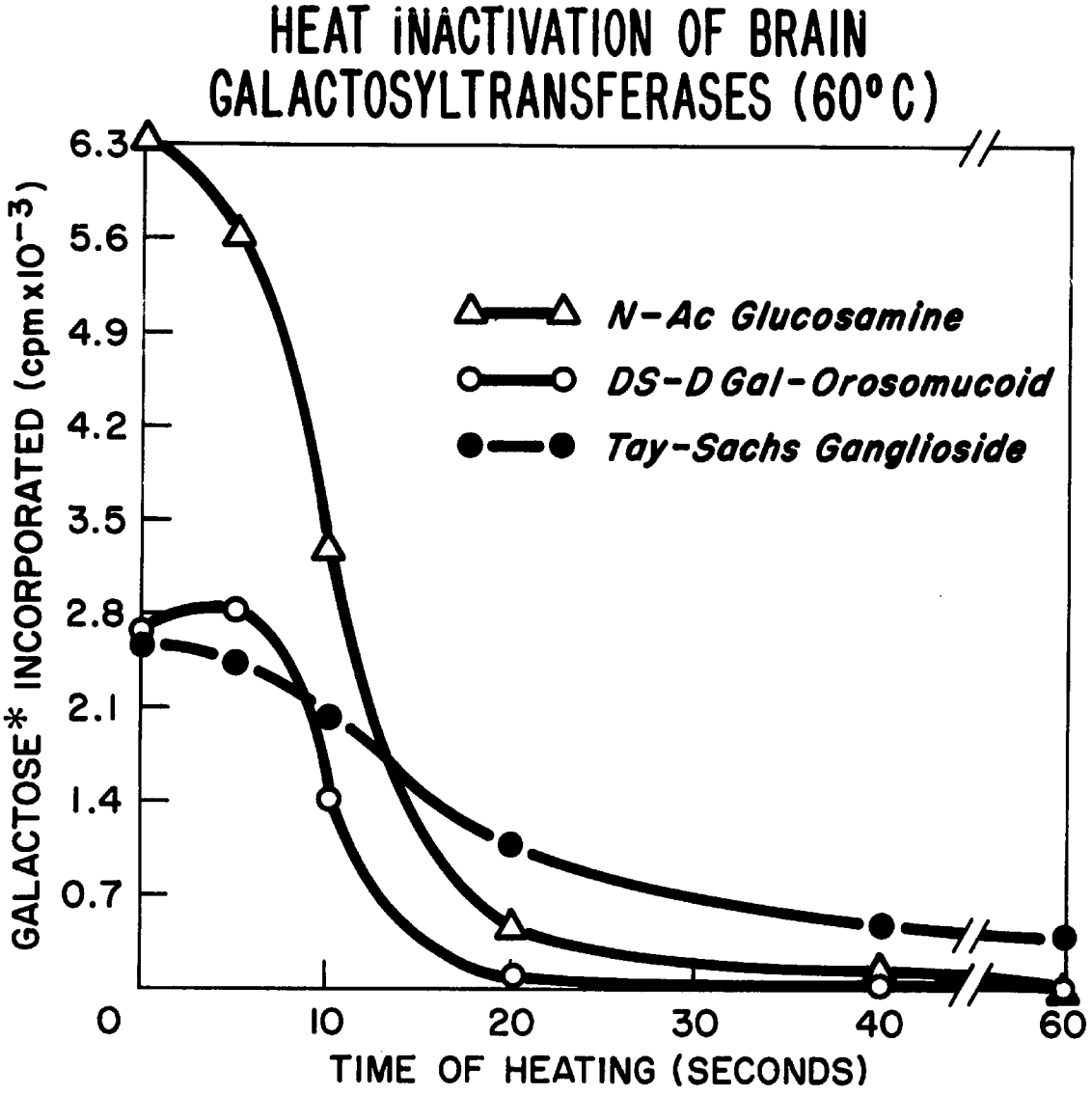
2. Relationship of Brain Galactosyltransferase Activity to Age of Embryo

The activity of the galactosyltransferase system as a function of age of the embryo is shown in Figure 25. With Tay-Sachs ganglioside as the acceptor, maximum enzyme activity was observed with brains from 11-18 day embryos. The endogenous values were subtracted from values obtained with glycolipid added to the incubation mixtures; the corrected values are plotted in the Figure. Endogenous values increased with the age of the embryos, and are shown in Table 26. These endogenous acceptor have not yet been characterized.

3. Study of Substrate Specificity

The results of substrate specificity studies with the galactosyltransferase system are shown in Table 27.

FIGURE 24



Complete incubation mixtures contained the following components (µmoles) in final volumes of 0.1 ml: Tay-Sachs ganglioside, desialized-degalactosized-orosomucoid or N-acetylglucosamine, 0.07, 0.4, and 2.5 respectively; detergent mixture, 300 µg; cacodylate-HCl buffer, pH 7.4, 10; $MnCl_2$, 1.0; UDP-galactose-^{14}C, 0.07 (0.78×10^6 cpm per µmole); enzyme (heated at 60° for the times indicated and cooled to 0°), 0.41 mg of protein. Incubations were conducted at 37° for 2 hours. The values for all points were determined from incubations in which the reaction was proportional to enzyme concentration and time of incubation, and independent of substrate concentration.

TABLE 24

Heat Inactivation of Brain Galactosyltransferases (55° C)

Complete incubation mixtures contained the components given in Figure 24, except the enzyme which was heated at 55° C for the indicated times prior to assay.

Time of heating (seconds)	Galactose-^{14}C incorporated (cpm)			
	Endogenous	+Tay-Sachs ganglioside	+Orosomucoid	+N-Acetyl-glucosamine
Unheated	357	2,184	2,257	4,869
5	352	2,091	2,367	4,363
10	348	1,799	1,349	2,650
20	336	1,107	402	668
40	244	633	253	350
60	265	522	249	258

TABLE 25

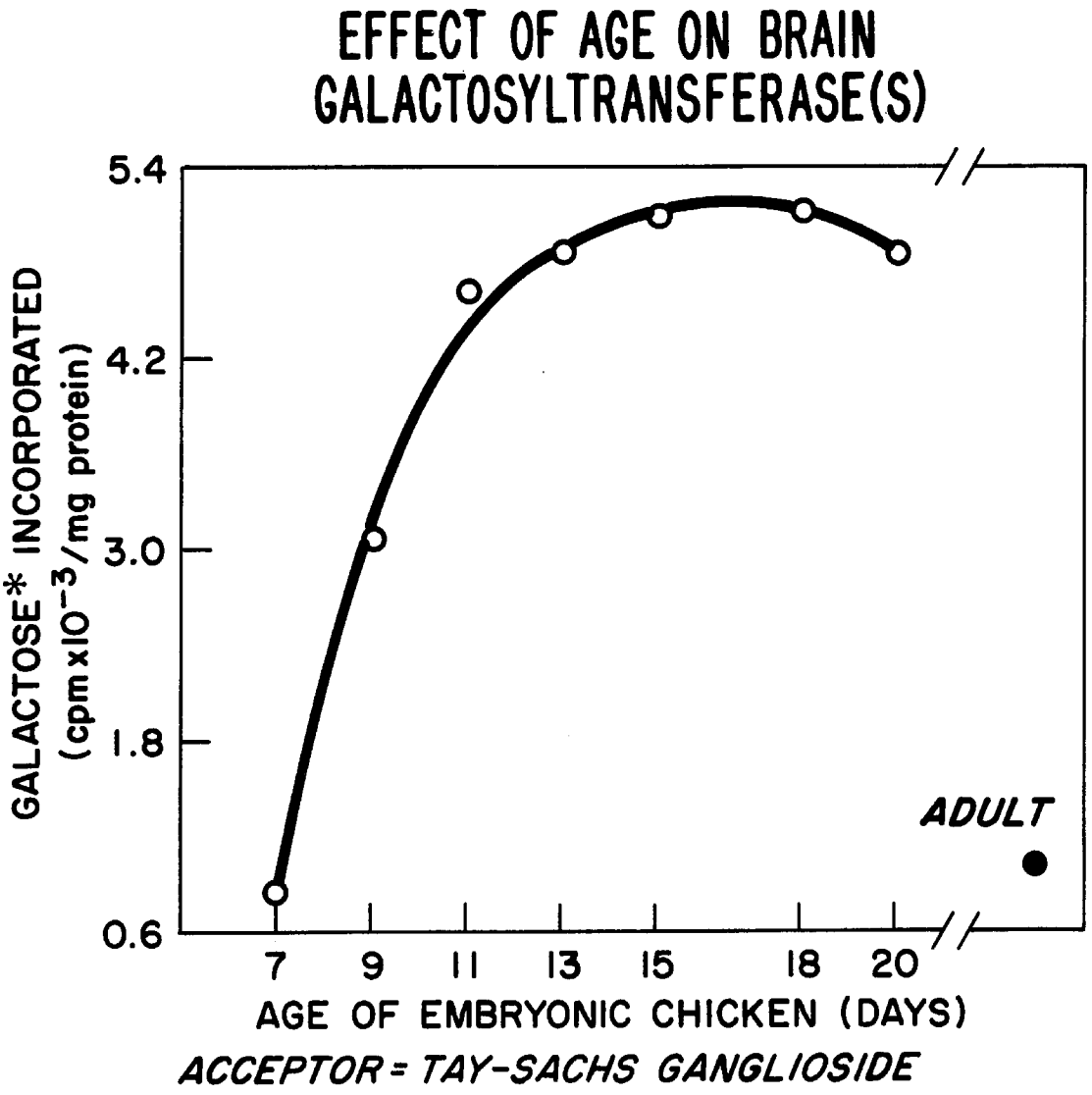
Effect of Mixed Substrates on Brain

Galactosyltransferases

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: substrate, as indicated in the Table; detergent mixture, 300 μg; $MnCl_2$, 1.0; cacodylate-HCl buffer, pH 7.3, 10; UDP-galactose-^{14}C, 0.07 (0.78 x 10^6 cpm per μmole); enzyme, 0.41 mg of protein. The substrates were present at enzyme-saturating concentrations. The mixtures were incubated at 37° for 2 hours.

Substrate	Concentration (μmole)	Galactose-^{14}C incorporated (cpm/mg of protein/2 hr)
Tay-Sachs ganglioside (TSG)	0.07	3,650
Desialyzed-degalactosized orosomucoid (OM)	0.4	3,730
N-acetylglucosamine (N-AcGm)	2.5	6,430
TSG + OM	0.07 + 0.40	7,060
TSG + N-AcGm	0.07 + 2.5	8,780

FIGURE 25



Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.075 ml: Tay-Sachs ganglioside, 0.07; detergent mixture, 300 μg; cacodylate-HCl buffer, pH 7.4, 10; $MnCl_2$, 1.0; UDP-galactose-^{14}C, 0.07 (0.8 x 10^6 cpm per μmole); unfractionated brain homogenates, 25 μl (0.4-0.86 mg of protein). The values are plotted as cpm per mg protein per two hour incubation, and were calculated from the data obtained where the reaction was proportional to enzyme concentration, and time of incubation, independent of substrate concentration, and have been corrected for endogenous values.

TABLE 26

Relationship of Brain Galactosyltransferase Activities to Age of Embryo

Complete incubation mixtures contained all the components given in Figure 25.

Age (days)	Galactose-^{14}C incorporated (cpm/mg of protein/2 hr)		
	Endogenous	+ Tay-Sachs ganglioside	Corrected
7	540	1,385	845
9	796	3,858	3,062
11	773	5,424	4,651
13	704	5,572	4,868
15	766	5,947	5,181
18	640	5,890	5,250
20	750	5,617	4,867
Adult[1]	528	1,542	1,014

[1] Age unknown.

Tay-Sachs ganglioside was the most active glycolipid acceptor; N-acetylglucosamine and several glycoproteins were also active. The following glycoproteins were active as galactose acceptors: sialidase treated ovine submaxillary mucin; sialidase and β-galactosidase treated fetuin and orosomucoid. The following compounds showed no detectableactivity: sphingosine, glucopsychosine, galactopsychosine, ceramide, glucocerebroside, galactocerebroside, ceramide-disaccharide, hematoside, sheep submaxillary mucin, pig submaxillary mucin (± sialidase treatment), fetuin, orosomucoid (± sialidase treatment), galactose and mannose.

Product formation was proportional to time of incubation and protein concentration with Tay-Sachs ganglioside, orosomucoid, and N-acetylglucosamine; at the indicated concentrations, the enzymatic activities were saturated with respect to the three substrates. The concentrations of glycoproteins are calculated on the basis of theoretical acceptor sites available (i.e., the quantity of sialic acid and/or galactose removed during pretreatment with the hydrolytic enzymes).

4. Enzyme Activity in Brain Homogenates of Different Species

The distribution of the galactosyltransferases in various brain homogenates is given in Table 28. Galactosyltransferase activity with Tay-Sachs ganglioside as acceptor was detected in homogenates prepared from the brain tissues of embryonic chicken (9-20 days), pig fetus (82 mm), and rat (9 days). Activity was not detected in the brain of

guinea pig (11 days) and calf. Brain homogenates of adult chicken and pig showed high endogenous values; the addition of Tay-Sachs ganglioside to the incubation mixtures gave only slightly higher values with chicken brain but not with the pig or rat.

5. Isolation of Radioactive Product (Monosialoganglioside)

To isolate the product, an incubation mixture was prepared as described in Table 23, but with the quantities increased 300-fold. The ^{14}C-product was sedimented with the particulate fraction; radioactive UDP-galactose and galactose were removed by washing the residue with 0.1 M KCl followed by water. Finally, the product was extracted with methanol (anhydrous), subjected to extraction by the procedure of Folch, et al. (17), and the aqueous layer, containing essentially all of the product (2.5 µmoles), was further purified by thin-layer chromatography. Tay-Sachs ganglioside and endogenous glycolipids migrated close to the ^{14}C-product in one or more solvent systems. Therefore, the radioactive product, monosialoganglioside, was purified by sequential thin-layer chromatography in four different solvent systems (a. solvent, T-III; b. solvent, T-IV; c. solvent, T-II; d. solvent, T-V). Only one discrete band containing ^{14}C-material was evident on each plate, and its position corresponded to that of standard monosialoganglioside. In order to achieve maximum purification, only the center segment of this band was eluted. Recoveries of ^{14}C-product approximated 60% at each step, giving a final yield of 0.25 µmole.

TABLE 27

Substrate Specificity of Embryonic Chicken Brain Galactosyltransferase

Conditions were the same as those described in Table 25, except for the enzyme, which contained 0.41 mg of protein, and substrates were varied as indicated below. The values given below were corrected for the endogenous value, 300 cpm. Disaccharide product was also assayed by high voltage borate electrophoresis.

Substrate			Galactose-^{14}C incorporated cpm/mg of protein/hr
Compound	Pre-treated with	Quantity per incubation (μmole)	
Tay-Sachs ganglioside		0.07	3,470
Ganglioside mixture (calf brain)		100 μg	400
Ceramide-tetrasaccharide		0.13	350
Ceramide-trisaccharide		0.10	320
Orosomucoid	Sialidase +β-galactosidase	0.40	4,630
Fetuin	Sialidase +β-galactosidase	0.25	3,610
Sheep submaxillary mucin	Sialidase	0.25	3,180
Bovine submaxillary mucin	Sialidase	0.15	1,120
N-Acetyl-glucosamine		2.5	8,120
N-Acetyl-galactosamine		2.5	260
Glucose		2.5	270

TABLE 28

Galactosyltransferase Activity in Brain Homogenates of Various Species

Each incubation mixture contained the following components (μmoles) in final volumes of 0.1 ml: Tay-Sachs ganglioside, 0.07; detergent mixture, 300 μg; cacodylate-HCl buffer, pH 7.3, 10; $MnCl_2$, 1.0; UDP-galactose-^{14}C, 0.07 (7.8×10^5 cpm/μmole); enzyme (unfractionated homogenate), 1.0-2.0 mg of protein. Incubations were conducted for 4 hours at 37°.

Source	Age	Galactose-^{14}C incorporated (cpm/mg of protein/4 hr)	
		Endogenous	+Tay-Sachs ganglioside
Chicken embryo	13 days	563	5,806
Chicken	Adult	773	1,274
Pig embryo	†	551	3,385
Pig	Adult	834	776
Rat	9 days	402	712
Rat	Adult	267	336
Calf	?	450	504
Sheep	Adult	765	952
Guinea pig	11 days	445	480

† Age unknown; length 82 mm.

6. Characterization of Nonradioactive Substrate and Radioactive Product

The radioactive product, monosialoganglioside was subjected to silica-gel TLC. Plates were developed with two different solvent systems (a. solvent T-I and b. solvent T-V) and compared with authentic standards after spraying with bromothymol blue dye. The radioactive product corresponded in rate of migration to authentic monosialoganglioside, obtained from Dr. Svennerholm, as shown in Figure 26.

7. Analyses of Radioactive Product (Monosialoganglioside)

The purified product, monosialoganglioside, was analyzed by the methods given in the Method Section, and showed the molar ratios of its constituents given in Table 29.

After methanolysis, followed by acid hydrolysis, the only ^{14}C-component detected was galactose-^{14}C, characterized by paper electrophoresis in 1% sodium tetraborate, and conversion to galactonic acid-^{14}C with galactose dehydrogenase (244).

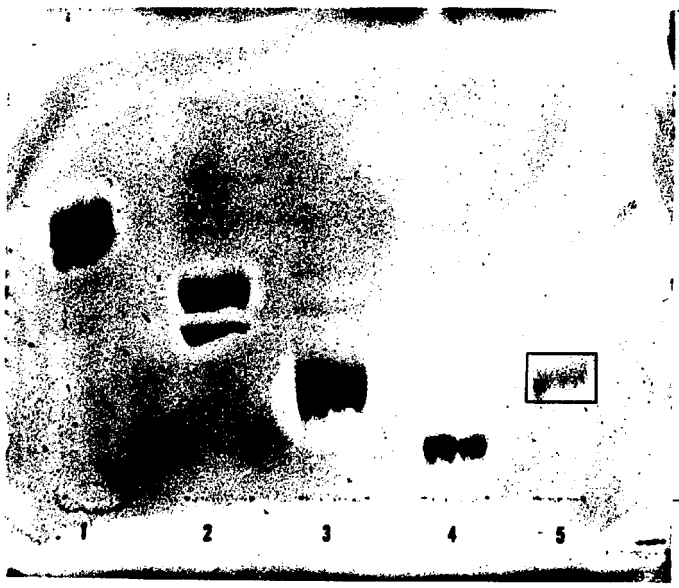
142

fig 26

FIGURE 26

CHARACTERIZATION OF BRAIN GALACTOSYLTRANSFERASE PRODUCT

(Tay-Sachs Ganglioside←Galactose*)



1. Hematoside
2. Tay-Sachs Ganglioside
3. Monosialo Ganglioside (Gm_1)
4. Calf Brain Ganglioside
5. Tay-Sachs Ganglioside←Galactose*

Thin layer plate of silica gel-G.

Developing solvent n-Propanol : 1% Borate (8 : 2)

TABLE 29

Analyses of Monosialoganglioside

(Brain Galactosyltransferase Product)

Component	Analytical methods	† Molar ratios
Sphingosine	Lauter-Trams	1.00
Galactose	Galactose dehydrogenase	1.83
Glucose	G-6-PO_4 dehydrogenase	0.87
N-Acetylneuraminic acid	Resorcinol	0.87
Fatty acid	Gas-liquid chromatography	1.04
Hexosamine	Modified Elson-Morgan	1.08

† Molar ratios relative to sphingosine.

TABLE 30

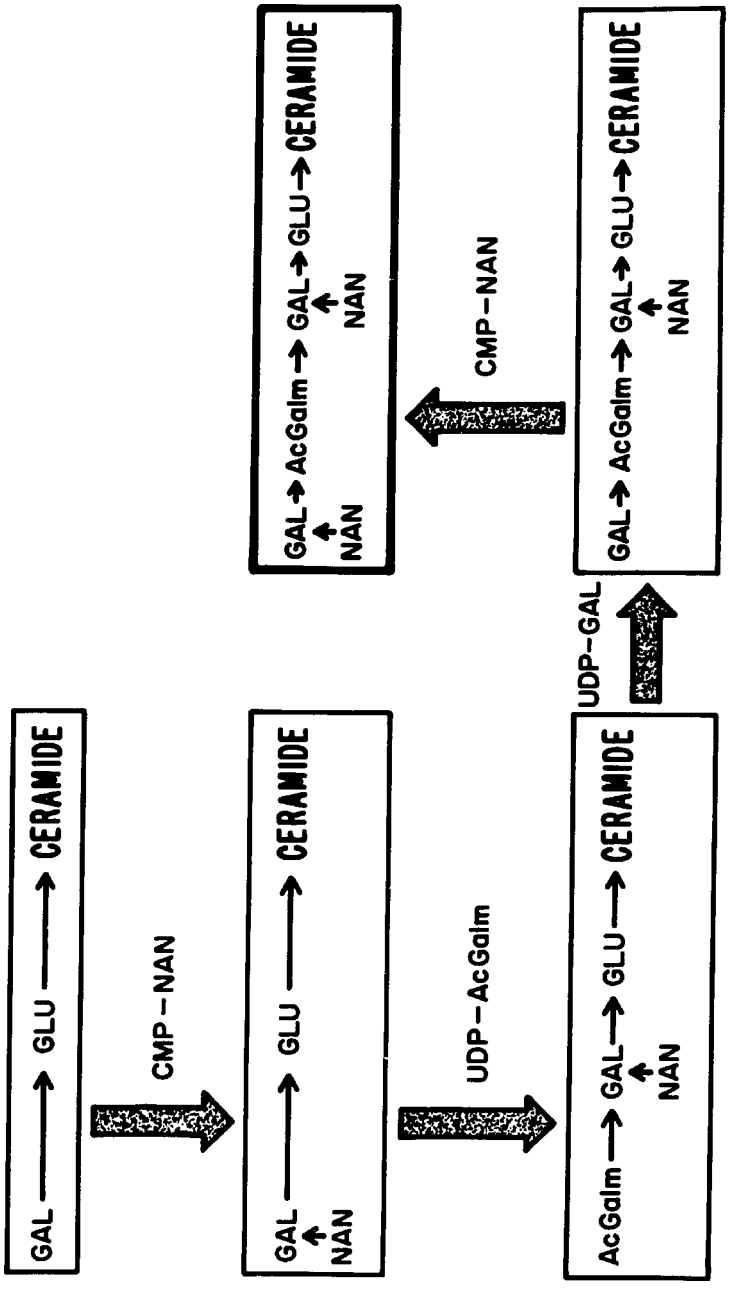
Substrate Specificity of Embryonic Chicken Brain

N-Acetylgalactosaminyltransferase(s)

Complete incubation mixtures contained the following components (μmoles) in final volumes of 0.1 ml: glycolipid, as indicated in the Table; detergent mixture, 300 μg; cacodylate-HCl buffer, pH 7.0, 10; $MnCl_2$, 2.0; UDP-N-acetylgalactosamine-^{14}C, 0.05 (4.1×10^6 cpm per μmole); enzyme, 0.6 mg of protein. After incubation at 37° for four hours, the substrate and its degradation product(s) were separated from the product by paper chromatography in 1% sodium tetraborate. The immobile ^{14}C-products were counted by liquid scintillation methods (column A), the papers placed in 15 ml of a solution of chloroform:methanol: water (2:1:0.2) at room temperature, and then again counted (column B). The difference in cpm between columns A and B represents the ^{14}C-product (glycolipid).

Substrate	Concentration (μmole)	Mn^{++}	N-Acetylgalactosamine incorporated (cpm)		
			(A)	(B)	Glycolipid
None	---	+	2,500	2,200	300
Hematoside	0.09	-	1,440	720	720
"	0.09	+	7,470	2,580	4,890
"	0.17	+	10,840	2,940	7,900
Ceramide-disaccharide	0.25	-	850	680	170
"	0.25	+	3,300	2,340	1,040
"	0.1	+	3,010	2,290	720
Glucosyl ceramide	0.1	+	2,308	2,130	178
Galactosyl ceramide	0.1	+	2,500	2,360	140
Ceramide-trisaccharide	0.1	+	2,420	2,110	310
Tay-Sachs ganglioside	0.07	+	3,946	2,620	1,320

FIGURE 27


PROPOSED PATHWAY FOR BIOSYNTHESIS OF GANGLIOSIDES
GAL → GLU → CERAMIDE
CMP-NAN
GAL → GLU → CERAMIDE
NAN
UDP-AcGalm
AcGalm → GAL → GLU → CERAMIDE
NAN
UDP-GAL
GAL → AcGalm → GAL → GLU → CERAMIDE
NAN
CMP-NAN
GAL → AcGalm → GAL → GLU → CERAMIDE
NAN

DISCUSSION

The data described above suggested that a particulate preparation obtained from embryonic chicken brain (7-20 day embryos) catalyzed the synthesis of monosialoganglioside when the system was incubated with Tay-Sachs ganglioside and UDP-galactose. The enzyme, UDP-galactose: Tay-Sachs ganglioside galactosyltransferase, was also detected in brain homogenates of pig fetus (82 mm fetus), and rat (9 day); maximum activity was observed with brains from 13 to 18 day old embryonic chickens.

The optimum conditions for determining galactosyltransferase activity are given in Figures 18-23. As indicated in Table 22, EDTA completely inhibited the system, while Mn^{++} was required. In preliminary studies with the embryonic chicken brain system, in the absence of Mn^{++} little or no activity was detected. The metal, Mn^{++}, was used when McGuire, et al. (186), observed that it was required for the UDP-galactose:glycoprotein galactosyltransferase of goat colostrum. With the chicken brain preparation, Co^{++} was about 20% as effective as Mn^{++} when tested at the same concentration, while the following ions were inactive: Mg^{++}, Zn^{++}, Ca^{++}, Ni^{++}, and Cu^{++} (Table 23). Mn^{++} at higher concentrations inhibited the reaction, as shown in Figure 21. The results of specificity studies with the galactosyltransferase system are shown in Table 27. Tay-Sachs ganglioside was the most active lipid acceptor; N-acetylglucosamine and sialidase and β-galactosidase pretreated orosomucoid were also active. The

following approximate K_m values were obtained: Tay-Sachs ganglioside, 2.0×10^{-4} M; sialidase and β-galactosidase treated orosomucoid, 2.8×10^{-4} M; UDP-galactose, 1.3×10^{-4} M. The following compounds showed no detectable activity: sphingosine, glucopsychosine (galactopsychosine), ceramide, glucocerebroside, galactocerebroside, ceramide-disaccharide, hematoside, sheep submaxillary mucin (± sialidase treatment), fetuin, orosomucoid (± sialidase treatment), galactose, and mannose.

The substrate specificity of the particulate brain galactosyltransferase system contrasted with that observed with a soluble, purified galactosyltransferase (186) isolated from goat colostrum, which was active with N-acetylglucosamine and glycoprotein, but not with Tay-Sachs ganglioside nor with other glycosphingolipids. Experiments were therfore conducted to determine whether the brain particulate system contained one or more galactosyl-transferases. Partial heat inactivation and substrate competition experiments showed that there were, in fact, two galactosyltransferase systems, and that one appeared to be specific for Tay-Sachs ganglioside. The product of the latter reaction was isolated and characterized by chromatographic methods, and by chemical analysis. After methanolysis, followed by acid hydrolysis, the only radio-active component detected was galactose-^{14}C.

Preliminary experiments resulted in the detection of yet another glycosyltransferase in the particulate fraction. The enzyme preparation catalyzed the transfer of

N-acetylgalactosamine from UDP-N-acetylgalactosamine to glycolipids, and was most active with hematoside compared with other glycosphingolipids tested (Table 30). The reaction catalyzed by this enzyme system requires further study, but appears to be:

UDP-N-acetylgalactosamine + hematoside → Tay-Sachs ganglioside (+ UDP?)

The results this far obtained with the galactosyltransferase systems found in the particulate preparation obtained from embryonic chicken brain lead to the conclusion that disialoganglioside is synthesized from ceramide-disaccharide by the pathway proposed in Figure 27.

The evidence for this porposal is as follows:

(a) The demonstration of sialyltransferase I, which appears to be specific toward ceramide-disaccharide, strongly suggests that the first sialic acid residue of disialoganglioside is introduced at this step.

(b) The most active glycolipid with each of the transferases is the product obtained from the preceding reaction in the step-wise sequence given in Figure 27.

(c) Normal brain ganglioside mixtures contain each of the compounds listed in the sequence, but to our knowledge they do not contain compounds that would indicate a different pathway, such as aminoglycolipid (ceramide-trisaccharide).

(d) If the sequence proposed above is correct, then the enzymes must be present during and possibly before, the deposition of glycolipids in embryonic chicken brain.

The pattern of enzymatic activities in embryonic brain at different stages of development correlates with the results reported by Garrigan and Chargaff **(11)**, who studied the pattern of glycolipid in this tissue at different ages, although they did not characterize the glycolipid mixtures. The specific activities of sialyltransferase I, N-acetylgalactosaminyltransferase and galactosyltransferase were maximum in brains from 9-11 day, 13-15 day, and 15-18 day chicken embryos, respectively.

One point concerning the proposed pathway is not easily interpretable. Ceramide-tetrasaccharide, a compound not included in the pathway, shows a lower K_m value with brain sialyltransferase (system II) than does monosialoganglioside. It is interesting to speculate that ceramide-tetrasaccharide is not involved in the biosynthesis of gangliosides, but has another, undefined function.

Many questions have been raised by these studies, and some of these are as follows:

(a) How is ceramide-disaccharide synthesized?
The galactosyltransferase system described above was inactive with glucocerebroside (ceramide-glucose) or glucopsychosine (sphingosine-glucose).

(b) How are the tri- and tetrasialogangliosides synthesized; i.e., is there a sialyltransferase system that requires disialoganglioside as a substrate?

(c) Are the various glycosyltransferases actually present in the same particle? If so, is there a relationship

between the biosynthesis of glycolipids, glycoproteins, and oligosaccharides? Where in brain cells, and brain cell fractions, is this particle(s) located?

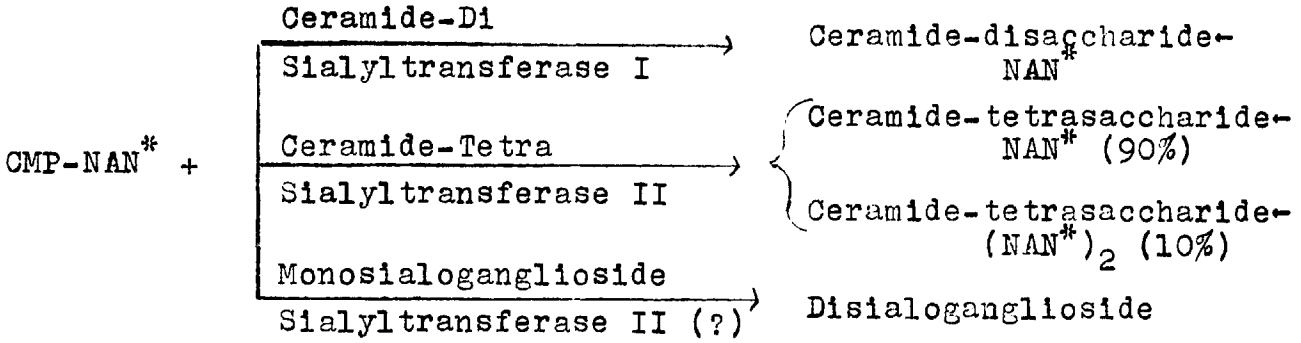
(d) Why does the specific activity of different glycosyltransferases in brain homogenates decline with age so that some of the transferases are almost non-detectable in adult animals?

(e) In Tay-Sachs disease there is an accumulation of large quantities of Tay-Sachs ganglioside. Is the defect a result of a decrease in the galactosyltransferase activity described above, or do these individuals contain an enzymatically inactive galactosyltransferase resulting from the genetic defect?

SUMMARY

In studies on the in vitro biosynthesis of ganglioside in chicken embryonic brain, a particulate fraction was isolated that catalyzed the transfer of N-acetylneuraminic acid from CMP-NAN to ceramide-disaccharide, monosialoganglioside and ceramide-tetrasaccharide. At least two transferase activities were detected in the particulate fraction, one of which transferred sialic acid to ceramide-disaccharide, while the other utilized monosialoganglioside, ceramide-tetrasaccharide and lactose.

The reaction products (shown below) formed from ceramide-disaccharide and ceramide-tetrasaccharide, respectively, were isolated from large-scale incubation mixtures, purified, and analyzed.



The data presented in Part II shows that the particulate preparation from embryonic chicken brain also contained a galactosyltransferase that catalyzed the following reaction:

Tay-Sachs ganglioside + UDP-galactose → monosialoganglioside (+ UDP)

The maximum activity was observed with brains from 13-18 day embryonic chickens. Unlike the sialyltransferase, this enzyme activity was completely inhibited by EDTA, and Mn^{++} was required.

The results also suggested that the particulate fraction contained more than one galactosyltransferase; the enzyme that catalyzed the transfer of galactose from UDP-galactose to Tay-Sachs ganglioside differed from the system active with N-acetylglucosamine and glycoprotein.

The reaction products formed from Tay-Sachs ganglioside were isolated from a large-scale incubation mixture, purified, and analyzed. The molar ratios of the constituents (relative to sphingosine) were close to the theoretical values for monosialoganglioside.

Preliminary experiments with the particulate system also suggested the presence of an N-acetylgalactosaminyl-transferase that catalyzed the transfer of N-acetyl-galactosamine from UDP-N-acetylgalactosamine to hematoside (ceramide-disaccharide-NAN). The postulated enzymatic product, Tay-Sachs ganglioside, has not yet been isolated for analyses but the product had the same mobility in thin-layer chromatography as Tay-Sachs ganglioside.

Two methods were developed for use during the course of this work:

(a) A method for the hydrolysis of ganglioside with mild acid to obtain ceramide-monosaccharide and ceramide-oligosaccharides,

(b) Improved solvent systems for the separation of Tay-Sachs ganglioside from monosialoganglioside.

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