1984

Effects of Maternal Ethanol Consumption on CNS Myelin and Axolemma Development in Rat Offspring

Jean Mary Gnaedinger
Loyola University Chicago

EFFECTS OF MATERNAL ETHANOL CONSUMPTION ON CNS MYELIN AND AXOLEMMA DEVELOPMENT IN RAT OFFSPRING

by

Jean Mary Gnaedinger

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

January

1984

ACKNOWLEDGEMENTS

I would like to thank my advisor, Mary Druse-Manteuffel, for her encouragement, advice and guidance throughout my studies. I would also like to express my appreciation to Antonio Noronha for the lengthy discussions he devoted to the project and for his support during difficult times. Thanks also to my classmates and our laboratory technicians for their help and encouragement.

VITA

The author, Jean Mary Gnaedinger, is the daughter of Dr. Robert J. Gnaedinger Jr. and Agnes (Wierer) Gnaedinger. She was born August 25, 1954, in Champaign, Illinois.

Her secondary education was obtained at Highland Park High School, Highland Park, Illinois where she graduated in 1972.

In September, 1972, she entered Carleton College, Northfield, Minnesota and in January, 1975, transfered to the University of Illinois, Champaign-Urbana, Illinois. She received the degree of Bachelor of Science with a major in chemistry in January, 1977. After graduation she was employed as a research assistant in the Department of Food Science and Nutrition at the University of Illinois.

She entered the Department of Biochemistry and Biophysics at Loyola University of Chicago in July, 1978 and was awarded a Basic Science Fellowship soon thereafter.

In September, 1983, Jean will begin a junior staff fellowship under the direction of Dr. Stanley I. Rapoport at the Laboratory of Neurosciences, National Institute on Aging, National Institutes of Health.

Jean is a member of the American Chemical Society, the American Association for the Advancement of Science and an associate member of Sigma Xi, the Scientific Research Society.

PUBLICATIONS

Gnaedinger, J., Noronha, A., and Druse, M., (1983) Myelin Gangliosides in Developing Control and Ethanol Rats. (Submitted for publication).

Gnaedinger, J., and Druse-Manteuffel, M., (1982) Gangliosides in Axolemma from Developing Control and Ethanol Pups. Neurosci. Abs. 8:182.

Noronha, A., Gnaedinger, J., and Druse-Manteuffel, M., (1981) Effects of maternal alcoholism on myelin gangliosides in offspring. Neurosci. Abs. 7:910.

Noronha, A., Gnaedinger, J., and Druse, M., (1981) Synaptosomes and microsomes in offspring of 'alcoholic rats. Trans. Am. Soc. Neurochem. 12:88.

Druse, M., Noronha, A., Gnaedinger, J., and Smith, D. M., (1980) Effects of maternal alcohol consumption and maternal malnutrition on CNS membranes in offspring. Teratology 21:36.

Gnaedinger, J., and Druse, M., (1980) Myelin-associated glycoproteins in the offspring of alcoholic rats. Trans. Am. Soc. Neurochem. 11:153.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF FIGURES

CONTENTS OF APPENDIX

LIST OF ABBREVIATIONS

Abbreviation	Identity
Abs	Absorbance
ADH	Alcohol Dehydrogenase
2',3'-cAMP	2',3'-Cyclic Nucleotide (Adenosine) Monophosphate
2'-AMP	2'-Adenosine Monophosphate
ANSA	1-Amino-2-Naphthol-4-Sulfonic Acid
ATP	Adenosine Triphosphate
Na^+-K^+-ATPase	Magnesium Dependent; Sodium, Potassium Stimulated, Ouabain Sensitive, Adenosine Triphosphate Phosphohydrolase
BIS	N,N'-Methylene Bisacrylamide
CNPase	2',3'-Cyclic Nucleotide 3'-Phosphohydrolase
CNS	Central Nervous System
CPM	Counts Per Minute
CSF	Cerebral Spinal Fluid
DPM	Disintegrations Per Minute
EEG	Electroencephalogragm
EGTA	Ethyleneglycol-bis-(δ-amino ethyl ether) N,N'-Tetraacetic Acid
FAS	Fetal Alcohol Syndrome
Fuc	Fucose

LIST OF ABBREVIATIONS CONTINUED

Abbreviation	Identity
Gal	Galactose
GalNAc	N-Acetylgalactosamine
Glc	Glucose
GlcNAc	N-Acetylglucosamine
HPTLC	High Performance Thin-Layer Chromatography
HSA	Human Serum Albumin
K	Kilodalton
Kcal	Kilocalorie
Kg	Kilogram
MAG	Myelin-Associated Glycoprotein
Man	Mannose
MW	Molecular Weight
NAD	Nicotinamide Adenine Dinucleotide
NANA	N-Acetylneuraminic Acid
NL	Neutral Lipid
nm	Nanometers
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
P_i	Inorganic Phosphate
PI	Phosphatidylinositol
PL	Phospholipid

LIST OF ABBREVIATIONS CONTINUED

Abbreviation	Identitiy
POPOP	(p-bis[2-(5-Phenyloxazolyl)] -Benzene
PPO	2,5-Diphenyloxazole
PS	Phosphatidylserine
R_f	Retention Factor
rpm	Revolutions Per Minute
RSA	Relative Specific Activity
SDS	Sodium Dodecyl Sulphate
SPM	Synaptic Plasma Membrane
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
TES	N-[2-hydroxy-1,1-bis(hydroxy-methyl)] Ethyl Taurine
TLC	Thin-Layer Chromatography
UV	Ultraviolet

CHAPTER I

BACKGROUND AND LITERATURE REVIEW

INTRODUCTION

Fetal alcohol syndrome or fetal alcohol effects (FAS/FAE) is characterized by a variety of abnormalities in the offspring of chronic alcoholic women. The defects include growth retardation, abnormal facial characteristics and many central nervous system (CNS) abnormalities. Alterations in CNS structure and function are considered by many in the FAS field to be the most significant outcome associated with this disorder. Over 50% of FAS children are mentally retarded. It is likely that there are also many children of alcohol consuming women who lack the external features and diagnosis of FAS, but who are mentally retarded or who have minimal brain dysfunction (MBD) because of *in utero* exposure to ethanol.

Studies using animal models of the fetal alcohol syndrome have demonstrated numerous biochemical, behavioral, anatomical and physiological alterations in developing

and mature offspring. Previous studies have demonstrated biochemical abnormalities in myelin maturation. However, the studies of myelinogenesis have produced conflicting results about the effects of ethanol on the development and maturation of CNS myelin.

It is the objective of this investigation to study the effects of maternal ethanol consumption on the development and maturation of CNS myelin and the axolemma membrane ensheathed by myelin by examining the content and composition of these membranes and the incorporation of radioactive precursors into specific membrane constituents. The constituents chosen are myelin and axolemma glycoproteins and gangliosides as well as axolemma phospholipids. Since detailed information is lacking about axolemma and myelin gangliosides in developing rats as well as about axolemma glycoproteins and phospholipids, this dissertation will also provide new information about the normal development of these membranes.

The study of the axolemma membrane was chosen because of its close relationship with myelin. Much work has been done to demonstrate that the signal for myelination lies in the axon, presumably in its outer surface (the axolemma membrane). The recognition process and the dynamic nature of the myelinated axon make these membranes especially interesting to study in pathological or abnormal processes as well as normal development.

Membrane glycoproteins and gangliosides were chosen for study because of their prominent roles in cellular recognition processes as components of cell surface receptors, as mediators of adhesion processes and as cell surface signals.

In contrast to gangliosides, phospholipids constitute a much greater proportion of the dry weight of membranes. In a structural capacity, they act as the backbone of the membrane bilayer. In addition, the phospholipid content and composition of membranes can affect the function of membrane proteins (e.g. enzymes, receptors). Minor phospholipids such as phosphotidylinositol and phosphatidylserine have been shown to interact with certain receptors and/or ion channels. Thus it is of great interest to look at these components in the offspring of chronically alcohol-fed females to see whether CNS abnormalities may be reflected in alterations in phospholipid content and composition.

As this dissertation will assess the content and synthesis of phospholipids, gangliosides and glycoproteins, a review of their structure and metabolism will be presented. Following this, I will discuss CNS development with emphasis on studies of the functional roles of complex carbohydrates and phospholipids in developing myelin and axolemma. Next I will describe the structure and components of myelin and axolemma-enriched fractions with parti

cular emphasis on complex carbohydrates and phospholipids. I will continue with a brief description of the isolation procedure used for myelin and axolemma. This was included primarily to describe the axolemma isolation procedure which was just recently reported by DeVries (1981). Lastly, I will review the literature on FAS giving special attention to animal studies of myelination.

CNS GANGLIOSIDES: STRUCTURE AND METABOLISM

Gangliosides are glycosphingolipids containing one or more sialic acid residues. Sialic acid is a general name for a neuraminic acid that contains either an N-acetyl or N-glycolyl side group. The sialic acids of mammalian brains are N-acetylneuraminic acids. In addition, hydroxyl side groups can be methylated to produce other forms of sialic acids. The sphingolipid portion of the molecule consists of a sphingosine and a fatty acid that can vary in chain length. Ceramide is formed by the condensation of sphingosine with free fatty acid. Common to all the major gangliosides is a neutral carbohydrate chain that is four glycose units long and is attached to a ceramide moiety: galactosyl-β-(1→3)-N-acetylgalactosaminyl-β-(1→4)-galactosyl-β-(1→4)-glucosyl-ceramide. The glucosyl moiety is attached to the ceramide by the primary hydroxyl of the sphingosine terminal. This is illustrated in the figure of G_{M1} ganglioside (Figure 1). The neuraminic acid in G_{M1} is attached to the inner galactosyl residue. Sialic acids are

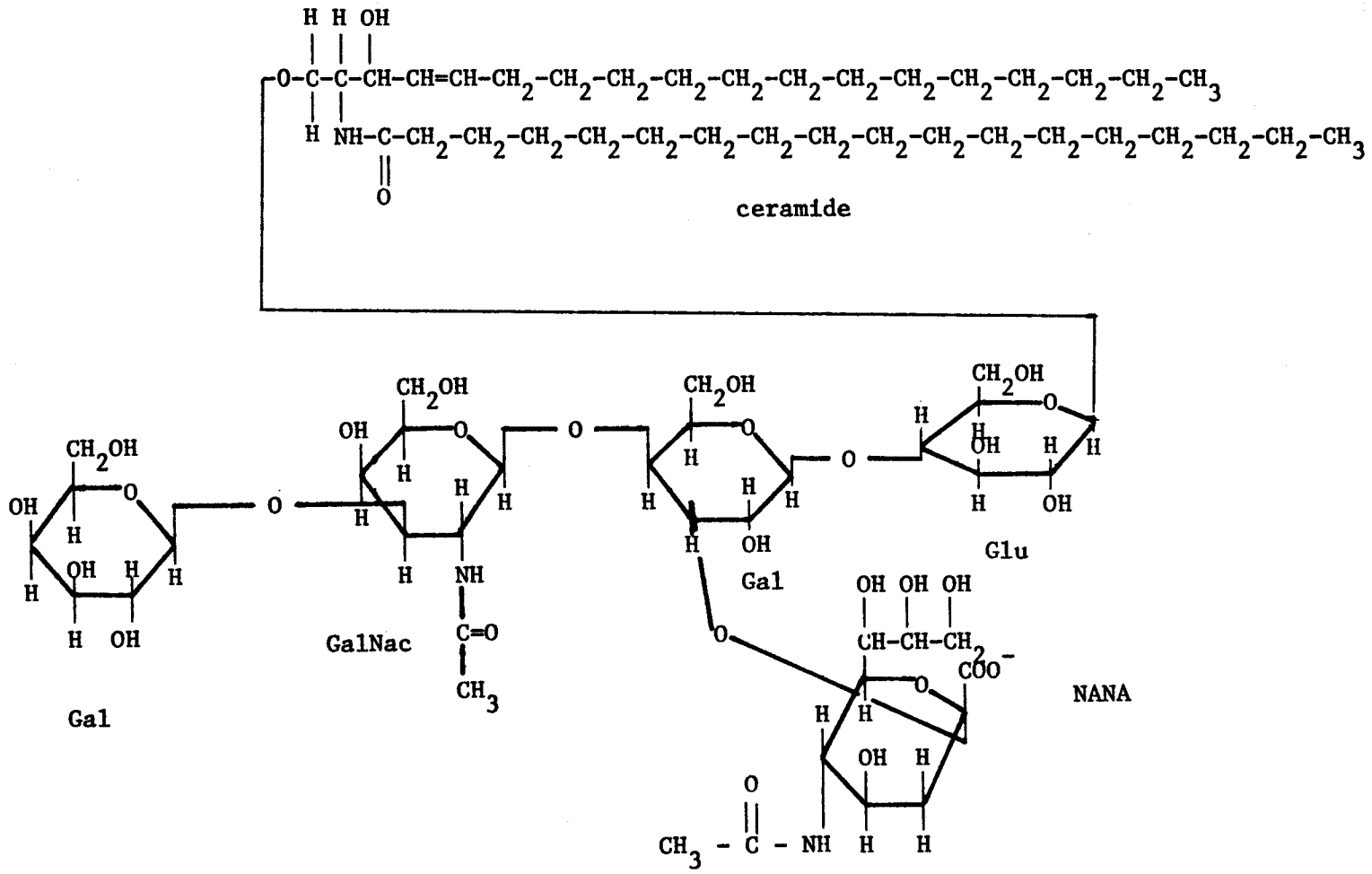


Figure 1. G_{M1} Ganglioside

attached to only the inner and outer galactosyl sugar residues of the backbone and they are always found in terminal positions except in cases where two or three sialic acid residues are bound together.

Sialic acid is formed in a series of reactions beginning with the conversion of UDP-N-acetyl-glucosamine to N-acetyl-D-mannosamine. After phosphorylation, N-acetyl-D-mannosamine-6-phosphate undergoes a condensation reaction with phosphoenolpyruvate. The product, N-acetyl-neuraminic acid-9-phosphate, is subsequently dephosphorylated to give the end product N-acetylneuraminic acid.

Biosynthesis of gangliosides has been shown to occur by the stepwise addition of sugars. Sugars, including sialic acid, are activated before addition to the ceramide backbone. Most sugars are activated by linkage to uridine diphosphate (UDP) while fucose (found predominantly in glycoproteins) and mannose are activated by attachment to guanosine diphosphate (GDP). Sialic acid is activated by linkage to cytosine monophosphate (CMP). Activated sugars are then added via specific glycosyl or sialosyl transferases to the ceramide moiety. I will discuss only the first few glycosylations and sialylations to provide an overview of the complexity of the biosynthetic processes involved. It is generally believed that there is a membrane bound multiglycosyltransferase system that mediates these reaction sequences. Appearance of gangliosides of a

particular structure provide partial evidence for the existence of a particular glycosyl transferase or sialosyl transferase.

For all gangliosides other than G_{M4} (see below) the first step is mediated by a glucosyl transferase to give glucosyl ceramide (glucocerebroside). This glucosyl ceramide can be acted upon by a galactosyl transferase followed by a sialosyl transferase to give G_{M3} (NANA-Gal-Glu-ceramide). The next transfer reaction could be the enzymatic addition of N-acetylgalactosamine to the galactose to give G_{M2} (GalNAc-{NANA}-Gal-Glu-ceramide). Alternatively, the next transfer to G_{M3} could be mediated by a sialosyl transferase to give G_{D3}. This could be considered the divergent point in ganglioside biosynthesis. Further glycosylation of G_{M3} will give gangliosides with only one sialic acid on the inner galactose while sialosylation of G_{M3} to give G_{D3} will give the starting point for gangliosides with two or more sialic acids on the inner galactose. In the biosynthesis of G_{M4} (sialosylgalactosylceramide), which is an unusual ganglioside because it lacks the glucose linked to ceramide, the first addition is mediated by a galactosyl transferase. Figure 2 is a general scheme of biosynthesis that is accepted though not fully proven (Schachter and Roseman, 1980).

The <u>in vivo</u> turnover rates of brain gangliosides are estimated to be ~21 days in the rat (Suzuki, 1967).

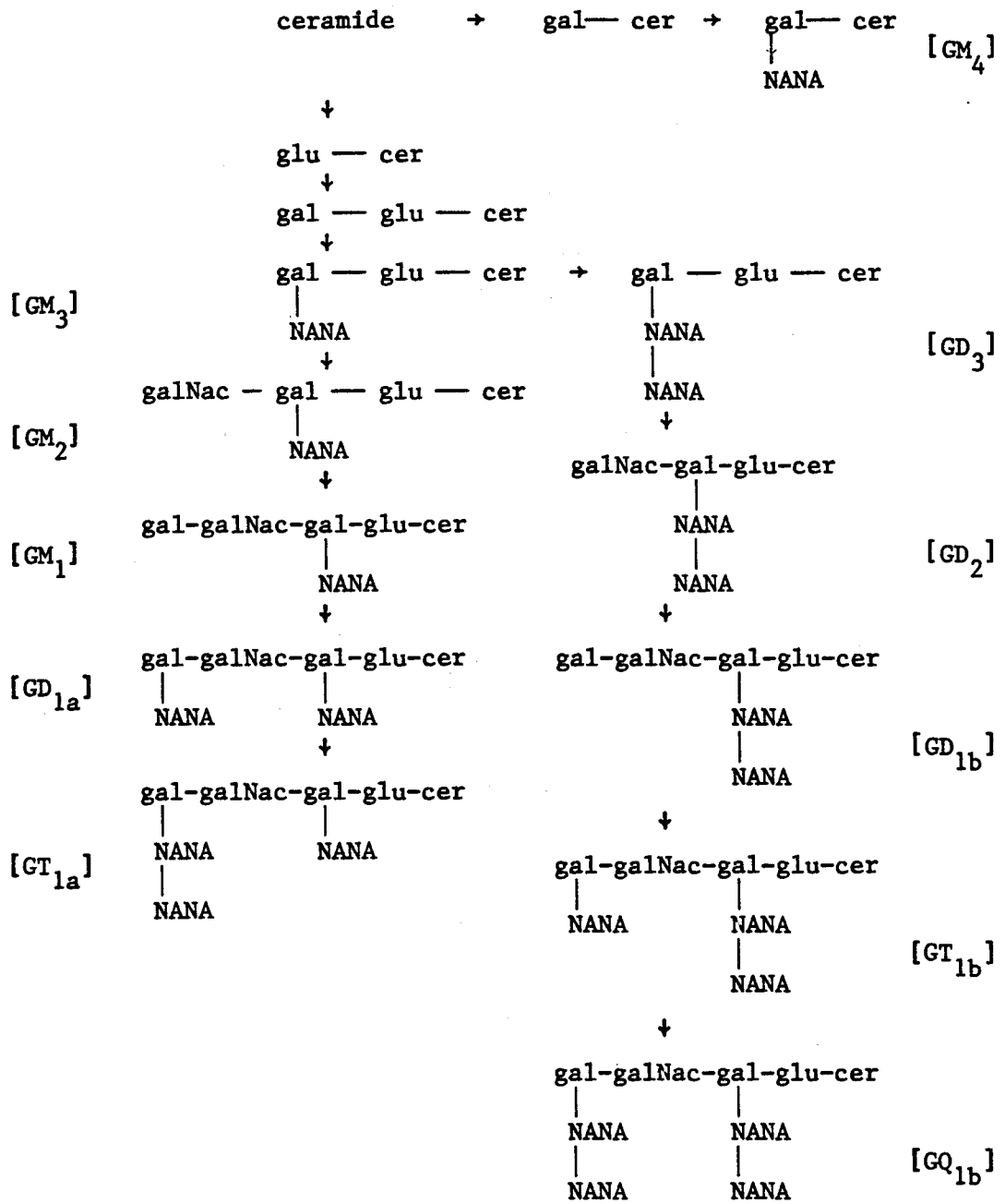


Figure 2. Ganglioside Biosynthesis

The individual sugars in a ganglioside have similar half-lives (Holm and Svennerholm, 1972), suggesting that ganglioside molecules as a whole may turn over.

Lysosomal glycohydrolases are thought to remove the glycose units of gangliosides in a sequential manner. Several of these enzymes have been extracted and partially purified from brain. These include β-glucosidase, β-galactosidase, β-N-acetylhexosaminidase and neuraminidase. These enzymes are all enriched in lysosomal particles and have acidic pH optima. Working in concert, they have the potential of degrading gangliosides to their ceramide backbone (Gatt, 1980). Neuraminidase removes external sialic acid residues only, not internal ones, as found in G_{M1}.

CNS GLYCOPROTEINS: STRUCTURE AND METABOLISM

There are two major classes of glycoproteins based on the linkage between the carbohydrate and protein. One class is known as the N-linked glycoproteins in which the amide nitrogen of asparagine is linked to an N-acetylglucosamine at the reducing end of the oligosaccharide. The other class is characterized by a linkage between the hydroxyl group of a serine or threonine residue and N-acetylglucosamine and are called O-linked glycoproteins. Approximately 85-90% of the carbohydrates in brain glycoproteins are N-linked. The sugars in rat brain glycoproteins are N-acetyl-D-glucosamine, N-acetyl-D-galactosamine,

D-mannose, D-galactose, L-fucose and sialic acid. The presence of glucose has not been definitively shown since the possibility of contamination by sucrose, used in membrane isolation procedures, is great (Margolis and Margolis, 1979a).

Five types of O-linked oligosaccharide cores have been determined in rat brain. The most common one contains two N-acetylneuraminic acids attached to a galactose which is in turn attached to the N-acetylgalactosamine on the hydroxyl of the serine or threonine (N-acetylneuraminyl-(2→3)- β-galactosyl-(1→3)-[N-acetylneuraminyl-(2→6)]-N-acetylgalactosamine-protein). The others are variations of this one, based on the anomeric positions of the sugars and on the number of sialic acids present. These are α-galactosyl-(1→3)-N-acetylgalactosamine, β-galactosyl-(1→3)-N-acetylgalactosamine, β-galactosyl-(1→3)-[N-acetylneuraminyl-(2→6)]-N-acetylgalactosamine, and N-acetylneuraminyl-(2→3)- β-galactosyl-(1→3)-N-acetylgalactosamine (Margolis and Margolis, 1979a).

All N-glycosidic linkages contain a common pentasaccharide at the point of attachment to the protein. This is a trimannosyl-di-N-acetylglucosamine. The microheterogeneity of these N-linked oligosaccharides arises from the variety of sugars that are attached to this inner core. The oligosaccharide moieties of N-linked glycopeptides (partial acid hydrolysates of glycoproteins) have been

separated into three groups based on their retention on affinity chromatography columns using Concanavalin A (a lectin specific for α-mannose and α-glucose residues). One fraction (the neutral fraction) contains only mannose and N-acetylglucosamine in the oligosaccharide. This fraction contains the simple, mannose-rich glycoproteins, while the other two acidic fractions contain various numbers of sialosylgalactosyl-N-acetylgalactosamine branches (complex glycoproteins).

The peptide backbone of glycoproteins are synthesized like all proteins under the direct control of the genetic code. For O-linked oligosaccharides the carbohydrate addition to the peptide backbone is a co-translational event in which serine or threonine residues in the newly synthesized chain are acted upon by glycosyl transferases in a sequential manner so that the product of each addition is a substrate for the next enzyme until the chain is complete (Waechter and Scher, 1979). Therefore, the genetic control of glycosylation is an indirect process, via the production of the various glycosyl transferases which are the primary gene products. This kind of synthetic process is less accurate than the synthesis of the peptide backbone and results in a heterogeneity in the carbohydrate moiety attached to a single type of protein backbone. As in ganglioside biosynthesis, the added sugars are activated by the addition of nucleotide bases.

The first step in the synthesis of O-linked oligosaccharides in brain is the attachment of N-acetylgalactosamine to the serine or threonine residue of the protein. This step is catalyzed by UDP-N-acetylgalactosamine: polypeptide α-N-acetylgalactosaminyl transferase and is illustrated in Figure 3a. This first step is critical since the following glycosylations depend on the product as a substrate for the various glycosyltransferases. The individual mucin glycosyl transferase enzymes have been studied in greatest depth and include CMP-sialic acid: GalNAc-mucin α2,6-sialyltransferase, UDP-Gal: GalNAc-mucinβ1,3 galactosyltransferase, GDP-Fuc: β-D-galactoside α1,2-fucosyltransferase, UDP-N-acetylgalactosamine: mucin α1,3-acetylgalactosaminyltransferase and CMP-sialic acid: β-D-galactoside α2,3-sialyltransferase. Each enzyme transfers the nucleotide activated sugar to its appropriate sugar-peptide substrate. Figures 3b,c and d illustrate some of the reactions mediated by these enzymes. It is important to note that the addition of sialic acid precludes any further glycose additions to that branch and therefore sialic acid is always found in a terminal position.

The glycosylation of the N-linked glycoproteins is, in contrast to the O-linked glycoprotein synthesis, a unique process that begins with the transfer of a presynthesized oligosaccharide from a dolichol-pyrophosphoryl donor to the asparagine residue of the protein. This

Figure 3

Reactions of Glycosyltransferases Involved in Synthesis of O-Linked Glycoproteins

a). UDP-αGalNAc + Ser(Thr)-peptide $\xrightarrow{E1}$ UDP + GalNAc-α-Ser(Thr)-peptide

b). CMP-Sialic Acid + GalNAc-α-Ser(Thr)-peptide $\xrightarrow{E2}$ Sialic Acid-α2,6-GalNAc-α-Ser(Thr)-peptide + CMP

c). UDP-Gal + GalNAc-α-Ser(Thr)-peptide $\xrightarrow{E3}$ Gal-β1,3-GalNAc-α-Ser(Thr)-peptide + UDP

d). GDP-Fuc + Gal-β1,3-GalNAc-α-Ser(Thr)-peptide $\xrightarrow{E4}$ Fuc α1,2-gal-β1,3-GalNAc-α-Ser(Thr)-peptide + GDP

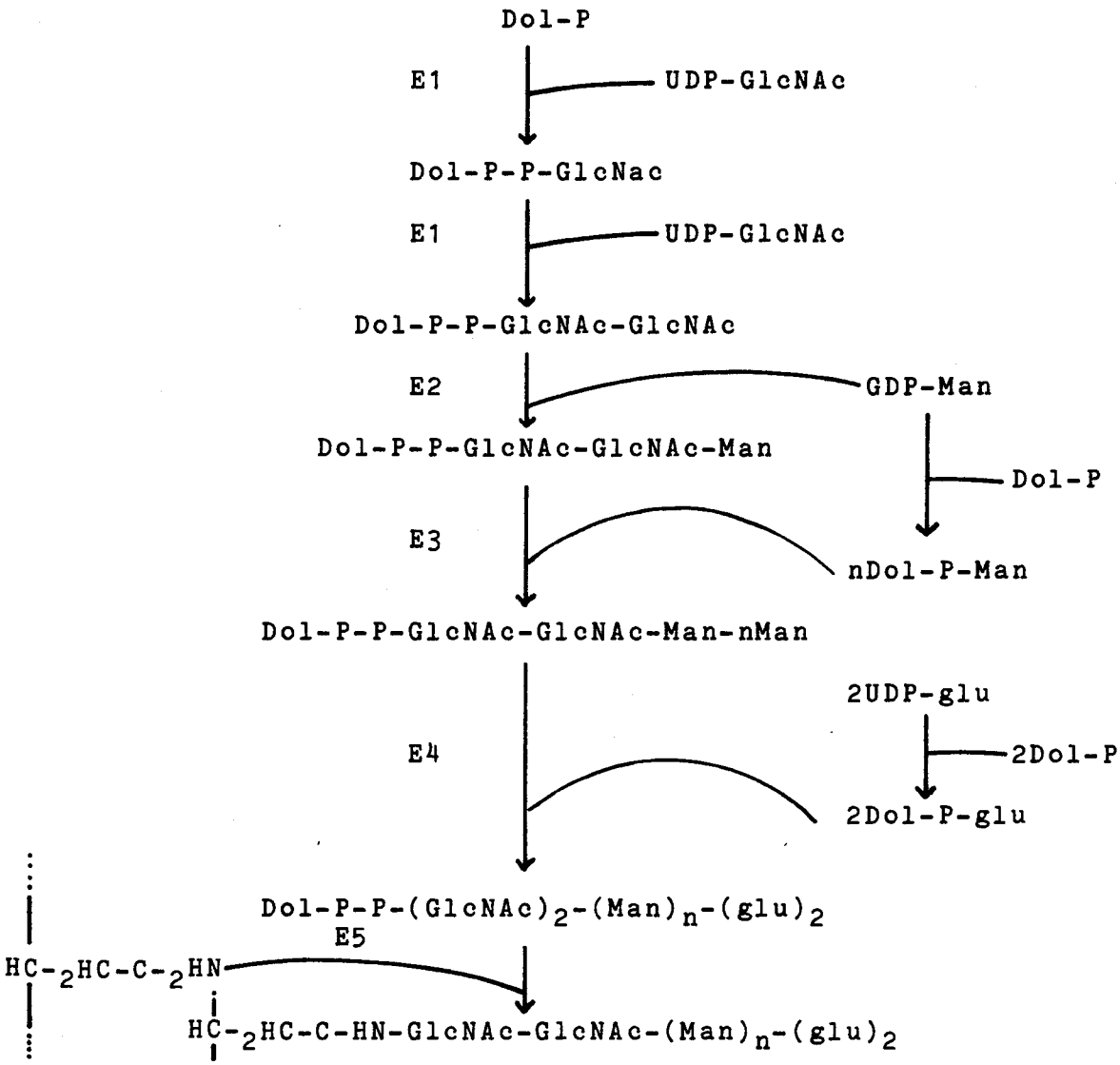
==========

Abbreviations: GalNAc= N-acetylgalactosamine, Ser= serine, Thr= threonine, UDP= uridine diphosphate, CMP= cytidine monophosphate, Gal= galactose, Fuc= fucose, GDP= guanosine diphosphate, E1= UDP-GalNAc: polypeptide αN-acetylgalactosaminyl transferase, E2= CMP-sialic acid: GalNAc-mucin α2,6-sialyltransferase, E3= UDP-Gal: GalNAc-mucin β1,3galactosyl transferase, E4= GDP-Fuc: β-D-galactoside α1,2-fucosyltransferase.

lipid-linked oligosaccharide has been referred to as the G-oligosaccharide and its synthesis and transfer to the protein is outlined in Figure 4. The linkage of oligosaccharide to protein is a co-translational event most likely occurring within the endoplasmic reticulum. Between 3-30 minutes after linkage, the oligosaccharide is trimmed by glucosidases and mannosidases to a heptasaccharide containing 5 mannose and 2 N-acetylglucosamine (GlcNAc) residues. This oligosaccharide core is processed further via one of two paths. It may become the substrate for a series of mannosyltransferases to form the simple mannose type of glycoproteins, or it may be acted upon by many different glycosyl transferases to form the complex glycoproteins. The trimming and elongation that characterizes the formation of complex glycoproteins is controlled by a specific N-acetylglucosaminyltransferase. After GlcNAc addition, the mannosidase, Gal-transferase, Sialic Acid-transferase, Fuc-transferase or GlcNAc-transferase II enzymes may act. Fucosyl and sialosyl transferases are enzymes which add terminal residues only (as described for the O-linked glycoproteins). Fucose addition may take place early or late in the elongation process and it appears to be added only to N-acetyl lactosamine (Gal-GlcNAc) structures. The most common attachment is to the innermost GlcNAc residues though there have been reports of attachment to outer GlcNAc and Gal residues as well (Schachter and Roseman,

Figure 4

Lipid Mediated Synthesis of the Carbohydrate Core and Subsequent Transfer to the Asparagine of N-linked Glycoproteins



Dol-P=dolicholphosphate and Dol-P-P= dolicholpyrophosphate
E1=GalNAc transferase, E2=GDP-mannosyl transferase, E3=Dol-P-mannosyltransferase, E4=Dol-P-glucosyl transferase, E5=oligosaccharide transferase

1980). These transferase enzymes have been found in the Golgi apparatus. After the final glycose additions, the membrane-destined proteins are carried in vesicles to the surface of the cell. At the cell surface they are integrated and oriented into the membrane so that the carbohydrate is exposed to the extracellular space with the protein embedded in the lipid matrix.

The catabolism of glycoproteins is not fully understood since the oligosaccharide sequences of most brain glycoproteins are not known. However, studies using artificial substrates have shown that sialidase, β-galactosidase, β-N-acetylhexosaminidase, α-L-fucosidase and α-mannosidase are found in lysosomes. Several investigators have found that some of these enzymes remove carbohydrate residues from both gangliosides and glycoproteins (Townsend et al., 1979).

Studies of the turnover half-lives of brain glycoproteins (Margolis et al., 1975) indicate a rapidly metabolized pool (t 1/2=1 to 7 days) and a slowly metabolized pool (t 1/2=2 to 6 weeks).

CNS PHOSPHOLIPIDS: STRUCTURE AND METABOLISM

CNS phospholipids are composed of sphingomyelin and several classes of phosphoglycerides. I shall discuss the phosphoglycerides only since the label used in this study ([^{3}H]-glycerol) would not be expected to appear in sphingomyelin. The parent compound for all phosphoglycerides is

phosphatidic acid which contains a glycerol backbone with fatty acids esterified to the hydroxyl groups at carbons 1 and 2 and a phosphoric acid esterified to the hydroxyl of carbon 3. The structure of phosphatidic acid is shown in Figure 5. Phosphatidic acid is an important intermediate in the biosynthesis of phosphoglycerides (see below). The different classes of phosphoglycerides are determined by the alcohol (e.g. ethanolamine, choline, serine, inositol, glycerol, phosphatidylglycerol or glycerol phosphate), that is linked to the phosphoric acid. Within a given class of phosphoglycerides (e.g. PC) there are differences in terms of the length and degree of unsaturation of the fatty acid. Phosphoglycerides may also be found in the plasmalogen form which contains an unsaturated aliphatic chain in ether linkage rather than the normal fatty acid ester linkage at the hydroxyl of C-1. PE plasmalogens are commonly found in brain, particularly in axolemma and synaptic plasma membrane. PC plasmalogens are not usually found in brain.

The biosynthesis of phospholipids occurs by one of two pathways. The first is a <u>de novo</u> synthetic pathway in which the phosphoglyceride is formed from glycerol-3-phosphate. The second type involves the modification of pre-existing phospholipids (salvage pathways). The first step in the complete synthetic pathway is the formation of L-glycerol-3-phosphate (formed from a glycolytic intermediate) either by the reduction of dihydroxy acetone phos-

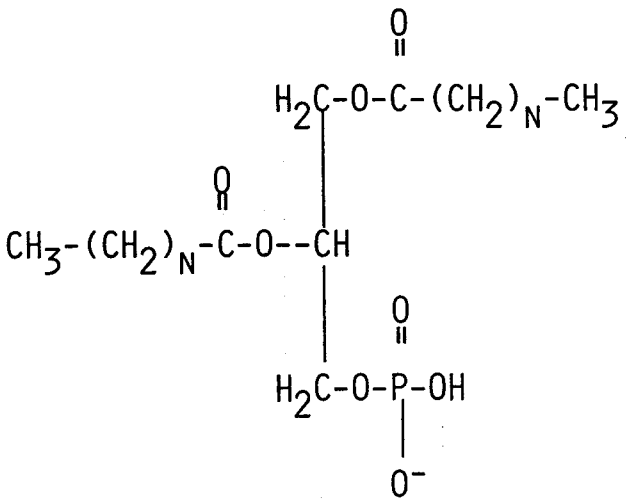


Figure 5. L-Phosphatidic Acid

phate by NADH (all tissues) or by the direct phosphorylation of glycerol by ATP (glycerol kinase, found in liver, kidney and brain). The latter pathway is of importance in the present study since we will examine the incorporation of radioactively labeled glycerol into brain phosphoglycerides (Rossiter and Strickland, 1970). L-Glycerol-3-phosphate is then acted upon by two acyl transferases to give phosphatidic acid. Subsequently, the phosphate may be removed by a phosphatase to form diacylglycerol or a reaction with CTP may occur to form CDP-diacylglycerol.

Phosphatidylethanolamine and Phosphatidylcholine

Phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) may be formed by the reaction of diacylglycerol (see above) with a sugar activated alcohol (CDP-ethanolamine or CDP-choline) in a reaction mediated by the enzymes ethanolamine-P cytidyltransferase and choline-P cytidyltransferase. Alternatively, there are also transferase enzymes (choline and ethanolamine transferases) that interconvert these phospholipids (also known as base exchange reactions). In addition, there is evidence of N-methylating enzymes that convert PE to PC by stepwise methylations (Mozzi et al., 1982). This represents a quantitatively minor pathway in the brain. Recent evidence indicates that either the ethanolamine and choline kinases or cytidyltransferases (to produce CDP-ethanolamine and -choline) are the rate limiting steps (Freysz et al., 1982)

for the pathway in which these CTP-activated alcohols are substrates.

Phosphatidylserine

Phosphatidylserine (PS) is formed in the reversible exchange of free serine with the ethanolamine base on PE; therefore, the synthesis of PS is completely dependent on PE and the activity of the serine base exchange enzyme. The reaction is also calcium dependent. This base exchange pathway appears to be the only biosynthetic pathway for PS.

Phosphatidylinositol

Phosphatidylinositol (PI) is formed in a reaction between free inositol and CDP-diacylglycerol (CTP-activated phosphatidic acid) mediated by the enzyme CDP-diglyceride: inositoltransferase. PI may be further modified by the addition of phosphates to give PI-monophosphate and PI-diphosphate.

Catabolism of phospholipids are mediated by phospholipases (Van den Bosch, 1974). Phospholipases A1 and A2 catalyze the hydrolysis of the fatty acid ester bonds at carbons 1 and 2 of the phosphoglyceride and plasmalogenase cleaves the vinyl ether bond of plasmalogens to give the lyso derivative (missing a fatty residue). Lysophospholipases act on the products of lipases A1, A2 and plasmalogenase to give fatty acids and phosphoglyerol backbone.

Phospholipid turnover measurements have demonstated

that there is a faster turnover of glycerophospholipid in neurons than in glial cells because neurons have higher activities of phospholipases A_1 and A_2. Benjamins and McKhann (1973) demonstrated that [2-^{3}H]-glycerol is an excellent precursor for studying phosphoglyceride turnover since the label is not reutilized to any appreciable extent. [1,3-^{14}C]-Glycerol was also studied and, although its distribution in phospholipids was similar to [2-^{3}H]-glycerol, the [1,3-^{14}]C-glycerol label was reutilized to a much greater extent.

MYELINATION

In rats, myelination begins 2 days after birth in the ventral and lateral tracts of the spinal cord. At 10 days the first myelin tracts are formed in the brain at the internal capsule and posterior commisure. Association areas of the cerebral cortex and the corpus callosum are myelinated at 15-21 days of age. The period of myelination occurs during a time of development referred to as the brain growth spurt or the critical period. Myelination occurs during the latter part of this period when cellular proliferation and migration have nearly been completed. It continues until at least 425 days (old age) although the rate of myelin increase is slow after 16 weeks (Jacobson, 1978).

Myelination in central and peripheral nervous systems differs due to the fact that myelin originates from

the Schwann cells in the PNS and from the oligodendrocytes in the CNS. The physical process itself appears to be similar in the two systems. When the signal has been communicated between myelin-forming cell and axon, the first step is the enfolding of the axon by the oligodendroglial cell process or by the Schwann cell. A tongue of membrane which contains cytoplasm is then extended around the axon in a spiral fashion for many turns. Myelin is ultimately compacted by the squeezing of cytoplasm from the looping membranes. After compaction, loops of cytoplasm remain in the inner and outer mesaxon, in the perinodal areas that abut the nodal regions and within the Schmidt-Lantermann clefts in the PNS (Raine, 1977).

Several studies have attempted to determine the origin of the myelination signal by using cross-anastomosed nerves, i.e. a severed nerve is inserted between the stumps of a different type of nerve. One such study, using PNS nerves, assessed the regeneration of normal myelinated and unmyelinated nerve fibers that were cross-anastomosed, as well as Schwann cell proliferation and migration (Aquayo et al., 1976). Results of these studies demonstrated that previously myelinated regenerating PNS nerves would induce Schwann cells to myelinate while those that were previously unmyelinated would not induce myelination. The evidence strongly suggests that the signal for myelination lies on the axon and any Schwann cell can be induced to myelinate

by that signal (Weinberg and Spencer, 1976).

The border between CNS and PNS is formed at the root entry zones of the spinal cord and brain stem. Schwann cells of PNS are separated from oligodendrocytes of CNS by a domed layer of CNS astrocytes. This is where CNS and PNS are divided. Following similar protocols to those described previously, the objective of the next study was to see whether a PNS nerve could stimulate an oligodendrocyte to myelinate. This was assessed by allowing the interaction of an oligodendrocyte with a transected peripheral nerve that contained a grafted segment of optic nerve (through which the peripheral nerve could regenerate). Results of these grafting experiments showed that CNS myelinating cells (oligodendrocytes) could be induced to myelinate PNS axons (Weinberg and Spencer, 1979). Other laboratories showed the reverse was true as well; i.e., that CNS axons could stimulate Schwann cells to myelinate (Blakemore, 1975; Kao et al., 1977).

The evidence to date for initiation of myelination implicates the involvement of a biochemical signal on the axonal surface. Although the nature of the myelinating signal is presently unknown, it might be in the form of a complex carbohydrate (glycoprotein or ganglioside) since these molecules appear to mediate a number of recognition phenomena and cell-cell interactions.

Some of the many processes in which gangliosides

and/or glycoproteins are postulated to play an important role shall be briefly described. This listing is not meant to be all-inclusive. Gangliosides and/or glycoproteins are thought to play a role in maintaining ionic fluxes, particularly at the synapse (McIlwain, 1961), as binding sites for neurotransmitters (Brunngraber, 1979; Salvetera and Matthews, 1980; Michaelis et al., 1981; Agnati et al., 1983), as receptor components for cholera toxin (Holmgren et al., 1975; Van Heyningen, 1974), as binding sites for tetanus toxin (Brunngraber, 1979), as mediators of cell-cell interactions (i.e., cellular adhesion, intercellular aggregation and agglutination) (Hoffman et al., 1982; Ocklind and Obrink, 1982; Ocklind et al., 1983; Nielsen et al., 1981, Yamada et al., 1983) and as mediators in behavior and performance (Morgan and Winick, 1980a; 1980b; Rick et al., 1980). Immunologically, gangliosides have been postulated to play a role in the etiology of Multiple Sclerosis based on evidence of elevated levels of antibodies to gangliosides in CSF and on blood lymphocyte hypersensitivity to gangliosides (Kasai et al., 1983; Ilyas and Davison, 1983). In addition, gangliosides may also be involved in malignant transformation and in viral infection and as receptors for interferon (Yamakawa and Nagai, 1978).

MYELIN MEMBRANE STRUCTURE

Myelin surrounds axons in a spiral fashion. Within the compacted layers of myelin the external proteins of

each unit membrane have fused with the external proteins of the adjacent membrane. These condensed/fused membranes are characteristically shown to have alternating protein-lipid-protein layers. Evidence for this pattern was conclusively obtained using three physical techniques: polarized light, x-ray diffraction and electron microscopy. Electron micrographs typically show alternating stained and unstained layers. The darkly staining lines, known as the major dense lines, represent the apposition of the inner membrane proteins while the less darkly staining lines, known as the intraperiod lines, represent the apposition of the outer proteins. The unstained areas represent the lipid bilayer (Figure 6). Evidence from studies using membrane permeable and non-permeable probes (Braun, 1977) suggests that the major dense line contains primarily basic protein, a major protein constituent of CNS myelin, while the Folch-Lees or proteolipid protein, another major CNS myelin protein, is primarily found in the intraperiod line. However, these findings are still controversial. Immunochemical procedures utilizing electron microscopy indicate that myelin basic protein may have antigenic sites in the intraperiod region (which corresponds to the extracellular apposition of membrane surfaces) (Braun *et al.*, 1980). Many studies using cross-linking agents show associations between basic protein molecules, proteolipid molecules and perhaps between basic and proteolipid molecules (Smith,

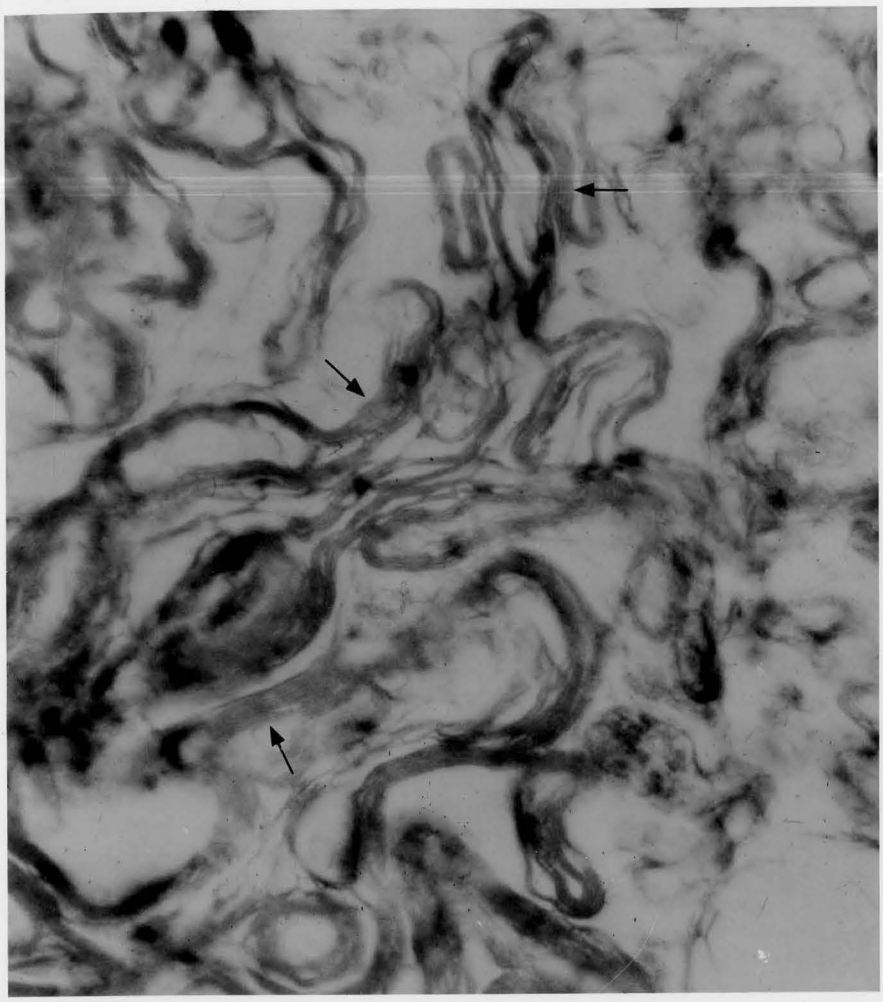


Figure 6. Electron Micrograph of Myelin

Myelin was purified by the method of Norton and Poduslo, 1973a). Arrows identify areas of compacted membrane fragments showing stained and unstained regions. (Microscopy done by Sue Simonson, pre-doctorol student, Department of Biochemistry, LUMC).

1977; 1980).

CNS myelin contains defined substructures which are distinct from the compacted layers. These substructures include the nonlamellar paranodal regions, the oligodendroglial stalks and the inner and outer mesaxons. The oligodendroglial stalks are the cytoplasmic "feet" which connect the body of the cell to the myelin on the axon. Internodes separate nodal areas which contain exposed axolemma. This nodal area is the site of impulse conduction (Rogart and Ritchie, 1977). In the paranodal regions, adjacent to the nodes, the cytoplasmic surfaces of myelin are not compacted. The glial cytoplasm is found in this area of the sheath, while it is not found in compacted lamellae. The inner mesaxon includes non-compacted myelin containing a narrow region of cytoplasm at the innermost part of the myelin sheath. The outer mesaxon is at the outer rim of the compacted myelin and contains a ridge of cytoplasm which ends in a loop of glial cytoplasm. These areas of cytoplasm are referred to as the inner and outer loops of cytoplasm (Figure 7).

MYELIN MEMBRANE COMPONENTS

GENERAL LIPIDS

There are no lipids which are unique to myelin; however, the proportion of individual myelin lipids is unique. Lipid comprises 70-85% of the dry weight of myelin. In rat, the major lipid classes are cholesterol

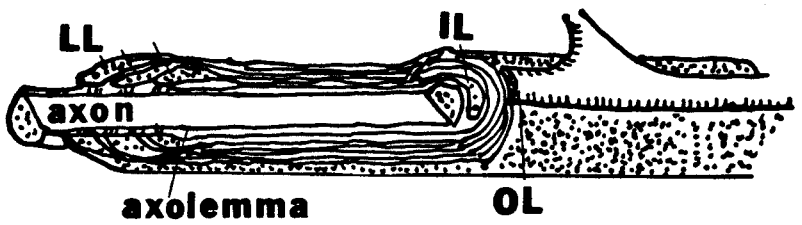


Figure 7. Stuctures of CNS myelin.

Spiral wrapping of the oligodendroglial cell process produces the structures depicted at this internode. LL, lateral loop; IL, inner loop; OL, outer loop.

(27% of total lipid), galactosphingolipid (32%) and phospholipid (44%). Of the phospholipids, the ethanolamine phosphatides, comprising 17% of total lipids, are most abundant, followed by phosphatidyl choline (11%), phosphatidyl serine (7%), phosphatidyl inositol (1%), and sphingomyelin (3%) (Norton, 1981). If lipid composition is expressed in terms of moles, cholesterol, phospholipid, and galactolipid have molar ratios of approximately 4:2:3.

GANGLIOSIDES

Gangliosides in myelin were originally thought to arise from neural tissue contaminants (Norton and Autilio, 1966; Soto et al., 1966). Since the concentration of gangliosides in rat myelin is only one tenth that of synaptic plasma membranes, it was thought that the contamination arose primarily from the axonal membrane (axolemma) which adhered tightly to isolated myelin fragments. However, work by Suzuki and coworkers (Suzuki et al., 1967,1968; Suzuki, 1970) gave tentative evidence that these constituents are intrinsic components of myelin. A recent study by Haley et al. (1981) ruled out axonal contamination as a source of the gangliosides in myelin. By purifying myelin in EGTA buffers, which dissociates the axonal membrane from myelin fragments (DeVries, 1981), it was demonstrated that axon-free myelin had approximately the same ganglioside content (concentration and distribution) as that obtained by a procedure not using EGTA (Norton and Poduslo, 1973a).

In addition, the the ganglioside compositions of axolemma and myelin are distinct (DeVries and Zmachinski, 1980).

There are several unusual features of myelin gangliosides. First the concentration of gangliosides in myelin is extremely low (<1.0 μg ganglioside NANA/mg myelin (dry weight)). Second, the distribution of gangliosides is distinct from other CNS membranes. Myelin from all mammalian species examined has a high proportion of G_{M1} ganglioside (Ledeen *et al.*, 1981). In human myelin, G_{M4} also is a major ganglioside (Ledeen *et al.*, 1973). G_{M4} is present in significant amounts in myelin from chicken and pigeon brains (Cochran *et al.*, 1981,1983). Original studies of rat myelin failed to detect G_{M4} (Suzuki *et al.*, 1967). This was probably due to loss of the less polar (monosialylated) gangliosides when solvent partitioning steps were used since a recent study of rat myelin gangliosides (isolated by non-partitioning procedures) found that G_{M4} accounts for ~4.6% of the total ganglioside sialic acid (Cochran *et al.*, 1982) in mature animals. It is interesting to note the similarity in structure of G_{M4} (sialosylgalactosylceramide) and galactocerebroside, a major component of myelin. A metabolic relationship between these two components has been described (Ledeen *et al.*, 1973; Yu and Lee, 1976), yet the exact nature of this relationship *in vivo* is unknown. Mature rat myelin also contains approximately 4% G_{Q1b}, 6% G_{T1b}, 7% G_{D1b}, 3% com-

bined G_{T1a} and G_{D2}, 6% G_{D1a}, 3% G_{D3}, 64% G_{M1}, 2% G_{M2}, 1% G_{M3} and 5% G_{M4} (Cochran et al., 1982).

PROTEINS

Rat CNS myelin proteins are composed predominantly of a small (14 K, MW) and large (18.3 K) basic protein and proteolipid protein (30 K). Other proteins include the pre-large and pre-small basic proteins, DM-20 (25 K), the Wolfgram proteins (53-55 K) (including CNP and tubulin) and several (quantitatively minor) glycoproteins. The proteolipids of myelin (approximately 40-50% of the protein content), are a heterogeneous group of hydrophobic proteins. There appears to be a single major component of 24 K. The major basic protein (large basic protein in rats) has been the subject of intensive investigation because of its antigenic properties. When myelin basic protein is injected into animals in Freund's complete adjuvant, it produces a cellular antibody response that results in an autoimmune disease of the CNS known as Experimental Allergic Encephalomyelitis (EAE) (Brostoff, 1977; Morell et al, 1981). This disease is characterized by inflammation and demyelination that is somewhat similar to that seen in Multiple Sclerosis (MS). The small basic protein differs from the large basic protein by a deletion of 40 amino acid residues within the protein. The Wolfgram proteins are found as a doublet on polyacrylamide gels with molecular weights in the region of 50 to 60 K. The major band has a molecular

weight of ~55 K and appears to be CNP (Drummond and Dean, 1980) (see enzyme section) and the minor band (MW of ~52 K) appears to be tubulin (Reig et al., 1982). Several high molecular weight, minor proteins account for 15-20% of the myelin proteins. The DM-20 doublet migrates between proteolipid and basic protein on SDS-PAGE gels. One of the DM-20 proteins has also been called the pre-large basic protein because its amino acid sequence is similar to large basic protein except for an additional polypeptide sequence.

ENZYMES

Myelin specific enzymes are cyclic nucleotide phosphohydrolase (CNPase) and pH 7.2 cholesterol ester hydrolase. Isolated myelin contains about 60% and 70-80%, respectively, of the total brain activity of these enzymes. In addition, there are several enzymes that are found in myelin as well as in non-myelin membranes. These include (Na^+-K^+)-ATPase, 5'-nucleotidase, carbonic anhydrase, UDP-galactose:ceramide galactosyltransferase, aminopeptidase, basic protein kinase, a nonspecific esterase and several enzymes involved in lipid metabolism (Norton, 1981). There is evidence that ATPase activity in myelin is not due to contaminants (Reiss et al., 1981).

GLYCOPROTEINS

Glycoproteins were first definitively demonstrated in myelin by Margolis (1967) who chemically demonstrated the presence of N-acetylgalactosamine in protein fractions.

Quarles et al. (1972) observed that the patterns of fucosylated myelin glycoproteins were distinct from those of whole particulate fractions. The prominent peak of fucose incorportion in myelin was found in the 100-110 K region. Other fucosylated myelin glycoproteins were observed also.

Although the major fucosylated myelin glycoprotein (MAG) is a quantitatively minor myelin protein (<1% of total myelin protein), it does not appear to be a contaminant. Immunocytochemical procedures (Sternberger et al., 1979) demonstrated that MAG is located exclusively in the periaxonal region and in the paranodal loops of CNS myelin. Because of MAG's location in both CNS and PNS (Sternberger et al., 1979; Figlewicz et al., 1981), a possible role for MAG in the interaction of myelin and the axon during myelination has been proposed (Quarles et al., 1983).

Analyses of MAG have shown that by weight, it is 2/3 protein and 1/3 carbohydrate. The protein portion contains a large number of acidic amino acid residues. The carbohydrate portion of MAG contains GlcNac, Man, Gal, Fuc and NANA. Labeling studies using [^{35}S]sulfate have shown that MAG is sulfated, probably on sugar residues (Matthieu et al., 1975c; Simpson et al., 1976). Experiments using trypsin indicate that MAG is an integral membrane protein (McIntyre et al., 1978a). Rodent MAG undergoes a change in molecular weight during normal myelin maturation from a

large to a small form (Quarles *et al.*, 1973b) (see Glycoproteins During Development).

The use of a technique which involves binding of iodinated lectins to glycoproteins that have been separated on polyacrylamide gels has revealed several glycoproteins that were not apparent by periodic acid Schiff staining or by *in vivo* fucose incorporation (Hukkanen, 1982; Poduslo *et al.*, 1980; McIntyre *et al.*, 1979). However, it should be noted that because of the greater sensitivity of the lectin studies there was a greater potential for detecting trace glycoproteins of contaminating membranes (Quarles *et al.*, 1983).

AXOLEMMA MEMBRANE COMPONENTS

The axolemma membrane is the outer plasma membrane of axons. In myelinated axons it is enveloped by myelin except at the nodal regions of the axon.

GENERAL LIPIDS

Total lipids comprise 52% (of the dry weight) of axolemma-enriched fractions isolated from 30- to 60-day-old rats. Cholesterol, total phospholipid and galactolipid comprise 21%, 67%, and 11%, respectively, of total lipids. Of the galactolipids, cerebrosides and sulfatides are found in a molar ratio of 1.3 to 1. The phospholipid fraction contains 24% ethanolamine glycerophospholipid, 28% choline glycerophospholipid, 5% serine glycerophospholipid, 3% inositol glycerophospholipid and 2% sphingomyelin (DeVries

and Zmachinski, 1980). The galactolipids are also found in myelin yet contamination by myelin seems unlikely since the estimate of myelin basic protein (contaminating axolemma) suggests that myelin could account for only 10% of the total axolemma galactolipid. Also it has been shown that the fatty acid composition of axolemma is distinct from that in myelin (Zetusky et al., 1978).

GANGLIOSIDES

Gangliosides constitute a much greater proportion of the lipids in axolemma than in myelin. Axolemma contains 11.9 µg ganglioside NANA/mg dry weight whereas myelin contains < 1.0 µg ganglioside NANA/mg dry weight. This enrichment of gangliosides in axolemma over myelin is consistent with Ledeen's proposal that gangliosides are distributed in all CNS neuronal tissue rather than being concentrated at nerve endings (Ledeen, 1978). The distribution of ganglioside types in axolemma contrasts greatly with myelin as well. A preliminary, nonquantitative study of axolemma suggests complex gangliosides, particularly G_{D1a}, G_{D1b} and G_{T1b}, are found in significant quantities. G_{M1} constitutes a much smaller proportion of total gangliosides than in myelin (DeVries and Zmachinski, 1980).

PROTEINS

Axolemma fractions have shown a heterogeneous population of proteins with molecular weights ranging from 20 Kdalton (K) to over 200 K. A prominent group of three pro-

teins is seen in a region around 50 K on the gel. These patterns of axolemma and myelin proteins are distinct. Individual axolemma proteins have not been quantitated previously.

GLYCOPROTEINS

Periodic acid-Schiff staining of axolemma proteins separated on SDS-PAGE revealed a number of glycoproteins. Axolemma was shown, by the staining procedure, to have a tenfold enrichment of glycoproteins over myelin. There appear to be six classes of glycoproteins ranging from 50 K to 200 K in molecular weight. Most of these react with the lectin Concanavalin A (Con A) (DeVries et al., 1979). The patterns of fucose incorporation into axolemma differs markedly from the patterns of myelin. Substantial incorporation occurred in the high molecular weight region of the gel particularly into one glycoprotein which migrated between MAG (~100 K) and the Wolfgram doublet (~50-60 K). There was also a peak of incorporation in a protein that migrated in the same region as the Wolfgram doublet (DeVries et al., 1978). These profiles were obtained using 15% polyacrylamide cylindrical gels, which did not appear to resolve the heavily labeled high molecular weight proteins. In this dissertation the improvement of the resolution of radiolabeled glycoproteins was attempted by the use of slab gels.

ENZYMES

Previous enzymatic studies of axolemma-enriched fractions (DeVries et al., 1978; Zetusky et al., 1979) have found a high specific activity of the plasma membrane markers, Na^+-K^+-ATPase and 5'-nucleotidase (eighteen and ten times that of homogenates). Sweadner (1979) found that the axolemma-enriched fraction contains only the alpha-(+) subunit of Na^+-K^+-ATPase. She postulated that the alpha-(+) subunit, which in axolemma is 2 K heavier than the usual alpha subunit, may play an important role at the node of Ranvier where it has been demonstrated histochemically (Wood et al., 1977). The specific activity of acetylcholinesterase, which was shown by histochemical methods to be restricted to axonal membrane (Stanely et al., 1979), is enriched in axolemma (two and one half times). The specific activity of CNPase, a myelin marker enzyme, is much lower in axolemma than in myelin. Non-axolemma membrane markers such as cytochrome oxidase and antimycin sensitive NADH-cytochrome reductase (markers of mitochondrial membranes) and NADH-cytochrome reductase (a microsomal membrane marker) are not enriched in the isolated axolemma or myelin fractions.

UDP-galactose: ceramide galactosyl transferase, an enzyme involved in the synthesis of galactosylcerebroside, has a higher specific activity in axolemma than in microsomes of young rats (15-25 days) and has a different diva-

lent cation requirement in axolemma than in microsomes (Costantino-Ceccarini et al., 1979). The activity of this enzyme in axolemma fractions declines with age while the microsomal enzyme increases with age. This axolemma enzyme appears to be distinct from the microsomal enzyme and it has been postulated that it may play a role in membrane surface phenomenon that occur during myelination.

Two enzymes involved in dolichol-linked oligosaccharide biosynthesis are also found in axolemma fractions. One enzyme, mannophosphoryl transferase, catalyzes the transfer of GDP-mannose to dolichol phosphate and the other enzyme, N-acetylglucosaminyl transferase, catalyzes the transfer of GDP-N-acetylglucosamine to dolichol phosphate. Detailed studies of the activities of these enzymes in the various membrane fractions indicate that these axolemmal activities are not due to microsomal contamination (Harford et al., 1979). The presence of these enzymes in axolemma membranes is of interest in light of their association with glycoprotein synthesis and since it has been shown that axolemma has a relatively high glycoprotein content. Lipid-mediated mannosyltransferases and sugar nucleotide pyrophosphatase activities have been described on cell surface membranes (Evans, 1974; Struck and Lennarz, 1976).

MYELIN AND AXOLEMMA GLYCOPROTEINS DURING DEVELOPMENT

Developmental studies using the isotopic precursors

[^{3}H]- and [^{14}C]-fucose and N-[^{3}H]acetylmannosamine were originally done using soluble and particulate fractions from rat brain (Quarles and Brady, 1971). It was shown that incorporation of N-acetylmannosamine into the sialic acid moieties of glycoproteins was higher than that into glycolipids at 5 days of age. In addition there was a greater amount of fucose incorporation into the high molecular weight proteins in young animals.

A developmentally related shift in molecular weight was seen in the major fucosylated myelin associated glycoprotein (MAG). At the early ages examined (12-20 days in rats), the immature, larger MW glycoprotein is present whereas at the time of most rapid myelination (20-30 days), the smaller MW form is predominant (Matthieu *et al.*, 1975a). The change in MW during myelin maturation is suggestive of a role for the immature form in glial-axon interaction, especially since the cleavage of the molecule occurs during the time of initial myelination. Myelin maturation has been assessed, using the shift in MW of MAG, in animals that have genetic, nutritional and experimental disorders that affect CNS myelin. A delay in the shift in MAG has been seen in hypothyroid (Matthieu *et al.*, 1975b), undernourished (Druse and Krett, 1979), and copper deficient rats (Zimmerman *et al.*, 1976) and in the myelin-deficient Quaking mice (Matthieu *et al.*, 1974; 1978a). It is possible that the larger form of MAG may be incompatible

with normal compaction of myelin since these mutants do not develop normal compacted myelin.

The shift was first thought to be due to a loss of glycose residues such as sialic acid. This hypothesis was disproven when it was shown that there was no difference in binding of the two forms of MAG to lectin affinity columns (Quarles et al., 1977; Quarles and Everly, 1977) and that the carbohydrate composition of the mature and immature forms is approximately the same (Quarles et al., 1979). However, when the two forms were treated with trypsin, an identical 80 K component was released from both MW forms which did not change with age (McIntyre et al., 1978a). It is presently thought that the developmental shift is due to the cleavage of a small MW polypeptide from one terminus of MAG.

Further studies of MAG demonstrated that this glycoprotein is susceptible to endogenous proteolytic cleavage, especially when isolated from human brain. The product of this cleavage, dMAG, shows the same reactivity with anti-MAG antiserum. The enzyme responsible for the cleavage is an endogeneous component of myelin (Sato et al., 1982) which also cleaves basic protein. The role of these proteases in demyelinating diseases is now being investigated.

Although DeVries et al. (1978) examined the incorporation of fucose into glycoproteins in the axolemma-

enriched fractions of young adults, no studies in developing animals have been done.

Several laboratories have, however, examined cerebellar glycoproteins during development and have found changes in the parallel axolemma fibers (Reeber et al., 1980; Zanetta et al., 1978). Con A binding proteins in cerebellum were examined from birth to 90 days (adult age) in rats. Using a crude membrane fraction, it was shown that there was a tremendous increase in accumulation of Con A binding proteins at an early age (about 13 days) which subsequently declined until ~24-40 days when a steady level was maintained. The binding sites were visualized in cerebellar tissue slices using fluorescent probes. The heavily fluorescing areas were localized primarily to the axolemma surface of the parallel fibers by light microscopy at high magnification or electron microscopy. The decrease in Con A binding glycoproteins between the 14th and 18th postnatal days appears to be due to destruction of their glycans. Although these fluorescent studies relate the accumulation of a class of glycoproteins to recognition phenomenon of axonal fibers (non-myelinated) for their destined post synaptic cells, the relevance of glycoprotein involvement in the axon-glial relationship is an important consideration.

GANGLIOSIDES DURING DEVELOPMENT

Brain gangliosides increase during postnatal

development of the rat. At day 3, the ganglioside concentration is 27% of adult levels and by day 24 it has reached 90% of adult levels. The greatest surge of synthesis occurs around 10 days, during the final phase of neuronal maturation, and just prior to myelination. G_{M1} and G_{T1} are the predominant gangliosides at birth. Their concentration drops rapidly during maturation. G_{D1a} is the major ganglioside in adult brain, although G_{M1} predominates in the cortex (Benjamins and McKhann, 1981).

During development the concentration of total myelin ganglioside sialic acid ranges from 36 to 68 µg/100mg brain tissue (Suzuki et al., 1967, no statistical analyses given). In addition, Suzuki et al. (1967) demonstrated developmentally related differences in the ganglioside profiles of myelin from rats. In Suzuki's study, the proportion of G_{M1} increased from 56 mole % of total gangliosides at 15 days to 90 mole % at 425 days of age. G_{T1}, G_{D1a} and G_{D1b} decreased from 12, 14 and 19 mole % to 2, 4 and 5 mole %, respectively, during this same time interval. According to more recent studies; however, G_{M1}, G_{D1b}, G_{D1a}, G_{T1b} and G_{Q1b} account for 45, 9, 8, 6, and 11 mole %, respectively, of adult rat myelin (Cochran et al., 1982). In addition, G_{M4} was recently reported to account for 5% total sialic acid in adult rat myelin (Cochran et al., 1983).

G_{M4} was detected in myelin from 35-day-old mice (Yu

and Yen, 1975) and in mature humans, chimpanzies and a number of avian species (Ledeen *et al.*, 1973; Cochran *et al.*, 1983). The extraction procedures used in the original studies (Suzuki *et al.*, 1967) of rat myelin probably did not adequately extract monosialogangliosides other than G_{M1}. G_{M4} has not been studied previously in immature or developing rats using non-partitioning methods which maximize its recovery.

DeVries and Zmachinski (1980) demonstrated that axolemma gangliosides are qualitatively similar to those of neuronal membrane. No quantitative, radioactive precursor, or developmental studies have been performed previously.

Functional roles for gangliosides in myelin have not been proven. There is, however, evidence to support a role in stabilization of specific protein components. There are several reports of a relationship between myelin gangliosides and myelin basic protein. Fong *et al.* (1976) found similar molar concentrations of gangliosides and P1 basic proteins in PNS myelin preparations. Mullin demonstrated an interaction between human G_{M4} and basic protein (BP) in an artificial system that utilized ganglioside liposomes (Mullin *et al.*, 1980,1981). Based on the evidence of these interactions, Mullin postulated that the interaction between G_{M4} and basic protein may be disturbed in the myelin of multiple sclerosis (MS) plaques since they contain little BP and no detectable G_{M4}. Yohe and Yu

(1982) demonstrated that in the presence of BP, G_{M4} was protected better than other gangliosides from degradation by neuraminidase. Recently Kasai et al. (1983) found significantly elevated levels of antibodies to G_{M4} and galactocerebroside in the cerebrospinal fluid (CSF) of MS patients. Interestingly, antibody levels to G_{M1} and myelin BP (in CSF) did not significantly differ from controls. There is a possibility that the functional roles of these myelin lipids are altered in MS. Poser (1978) discusses ganglioside metabolism as an important factor in the pathogenesis of dysmyelination.

Other studies examined the effect of antibody to G_{M1} on brain activity and development. Anti-G_{M1} administration was shown to induce seizure activity, inhibit passive avoidance learning, retard development and block morphine analgesia in young animals (Rapport et al., 1980). Adult animals treated with antibodies to G_{M1} during the neonatal period had significantly less ganglioside sialic acid, galactocerebroside (a myelin marker) and RNA in corticies. Golgi preparations showed a loss of thin spines on the pyramidal cell dendrites (Karsarkis et al., 1979,1981; Hess and Pope, 1972). The authors suggest that G_{M1} may act as a signal to mediate the growth of pyramidal cells or oligodendrocytes.

Immunologically mediated activities of gangliosides have been demonstrated in an experimental autoimmune di-

sease, the ganglioside syndrome. In susceptible animals, administration of G_{M1} and G_{D1a} produces paralysis and autoimmune lesions of the PNS presumably via a cell mediated response (Nagai et al., 1976). Gangliosides may also act as conformation stabilizers for proteins and glycoproteins. Nagai and Iwomori (1980) suggested that glycolipids interact with functional membrane proteins to facilitate a transmembrane signal which could then initiate a biochemical response. If the proper interaction between glycolipid and protein does not exist due to alteration in glycolipid composition/conformation, the cellular response would not occur. This mechanism is purely speculative and is difficult to prove since extraction methods tend to destroy natural conformations. However this is an intriguing potential role of ganglioside function.

It is obvious that much work needs to be done to definitively elucidate the function of gangliosides in myelin; however, by virtue of their roles in other tissues, they may act as receptors or signals in myelin development. Thus it is interesting to look at myelin and axolemma gangliosides since the signal in the axon for the myelinating oligodendrocyte may be regulated or functionally altered by gangliosides.

PHOSPHOLIPIDS: FUNCTION AND DEVELOPMENT IN CNS MEMBRANE

Phospholipids are important constituents of membranessince they form the functional backbone of the lipid

bilayer. They appear to contribute significantly to the molecular structure of a membrane which is necessary for physical and chemical interactions. It has been shown that many membrane bound structures require phospholipids to properly function. Mitchell et al. (1975) has described interactions between anionic phospholipids (particularly phosphatidyl inositol) and membrane enzymes and receptors and proposed that alterations of these phospholipids may alter specific membrane functions. Many studies have demonstrated neurotransmitter-stimulated metabolism of phosphatidylinositols (see review by Hawthorne and Pickard, 1979; Michell, 1982). Some aspects of receptor mediated inositol lipid metabolism are that (1) it is always accompanied by Ca^{+2} mobilization in the cytosol, and (2) the stimulated metabolic step is inositol breakdown to diacylglycerol. There are some who conclude that the PI effect is mediated through muscarinic-cholinergic and β-adrenergic receptors and that the activation of these receptors leads to an increase in cation permeability of the cell surface (Abdel-Latif and Akhtar, 1982; Michell, 1982).

Other postulated roles of phospholipids in nervous tissue include the influence of methylating enzymes on membrane fluidity and Ca^{+2} influx and the effects of choline phospholipid metabolism on the regulation of GABAergic neurons (Crews et al., 1982; Giesing and Gerken, 1982).

Certain phospholipids are directly involved in ion transport and the biochemistry of the visual process. The analysis of the composition of purified Na^+-K^+-ATPase has demonstrated that the active enzyme complex contains 9% lipid (all are phospholipids) (Siegel et al., 1981). Photoreceptors in vertebrates contain ~40% phospholipid mainly PE and PC (Shichi, 1981). PE plays a role in regenerating visual pigments as well (by forming an intermediate in a chemical conversion).

Developmental changes in rat brain phospholipids have been described by Rappoport et al. (1971). A group of lipids consisting of ethanolamine plasmalogen, choline plasmalogen, diphosphatidyl glycerol and ethanolamine phosphoglycerol ether increase markedly in concentration from 3 to 18 days postnatally and appear to be unrelated to myelin membrane changes. Adult levels of these lipids are reached by 42 days postnatally. Another group of lipids consisting of PC, PE, and PI also dramatically increase in concentration between days 3 and 18 postnatally and reach adult levels by 24 days postnatally. These increases are directly related to the massive increase in production of membranes in the CNS.

Developmental profiles of axolemma membrane phospholipids have not been reported, though adult levels have been studied (DeVries and Zmachinski, 1980).

MYELIN AND AXOLEMMA ISOLATION PROCEDURES

Myelin was isolated in the present studies according to the method of Norton and Poduslo, (1973a). This procedure utilizes differential centrifugation to obtain a purified myelin fraction. The method takes advantage of the low density and large fragments of myelin that are produced upon homogenization of brain tissue. Reportedly, this procedure yields myelin of comparable purity at all ages of animals studied (beginning at the 15 day postnatal age). An important consideration in purifying myelin from developing animals is to maintain consistent recovery. Because a myelin subpopulation was found in young animals with a heavier density than usually seen in adults, the sucrose concentration of the lower layer was adjusted to isolate this subpopulation. In summary, the modifications Norton and Poduslo (1973a) introduced were (1) to maintain a constant concentration (5%, w/v) of brain homogenate in the initial gradient, and (2) to repeat the initial density gradient centrifugation with the crude myelin obtained from the first gradient. Developmental studies using rat brain myelin obtained by this procedure showed convincingly that the purity and yields at the younger ages correlated with those of the older ages so that accurate developmental trends could be assessed (Norton and Poduslo, 1973b).

The isolation of an axolemma-enriched fraction from

myelinated axons has recently been described by DeVries (1981). This procedure also utilizes differential centrifugation. There are several unique aspects to this isolation. The procedure began as an isolation protocol for myelinated axons. White matter was homogenized in buffered salt-sucrose solutions to yield myelinated axon fragments. When this fraction was centrifuged, a floating layer of axon fragments was obtained. Further centrifugation yielded a floating fraction that was relatively free of contaminating debris. The floating fraction was osmotically shocked so that axonal membranes were stripped from the myelin. After a final centrifugation of a sucrose gradient, the floating myelin was separated from the axonal pellet (DeVries et al., 1972). This procedure was utilized on bovine CNS, rat CNS and rat peripheral nerve starting materials. Chemical and electron microscopic analysis demonstrated that the myelin-free axon pellet was too heterogeneous to be characterized. However using the myelinated axon as a vehicle for the isolation of axons had been a success.

Further modifications were made by DeVries (summarized in DeVries, 1981) to fractionate the myelin-free axonal pellet. The shocked myelin and axon fraction was layered onto a discontinuous sucrose gradient. The axolemma-enriched fraction was obtained from the dense layer of material that formed a band at the 1.0 M/1.2 M sucrose

interface. Thus, by taking advantage of the differences in lipid content of membrane fractions both an axolemma-enriched and an intermediate axolemma fraction (0.8 M/1.0 M sucrose interface) could be obtained.

The purity of the axolemma-enriched fraction was established using electron microscopy, enzyme marker analysis, histochemistry, freeze fracture analysis and chemical analysis. It appears that the axolemma fractions are minimally contaminated with myelin and have virtually no contamination from oligodendroglial and astroglial membrane. In addition, sodium channel probes (tetrodotoxin and saxitoxin) and natural neurotoxins bind specifically to the axolemma fraction (DeVries and Lazdunski, 1982) and support the contention that this fraction is derived from axons. However, possible contamination by smooth endoplasmic reticulum and/or paranodal loop regions of myelin has not been excluded.

One important advantage of the axolemma preparation methodology is that myelin can be isolated at the same time. Myelin isolated by this protocol compares well with the Norton and Poduslo (1973a) protocol in terms of purity and yields (in terms of mg dry weight myelin/ gm wet weight tissue) (DeVries, 1981).

ANALYSIS OF GLYCOPROTEINS IN MYELIN AND AXOLEMMA

As previously mentioned, the presence of glycoproteins in myelin was not recognized until recently (for a

review, see Quarles et al., 1983). Elaborate methods were used to indisputably show that at least one of these was indeed intrinsic to myelin and not derived from a contaminating membrane (Quarles et al., 1983) Initial studies of the myelin-associated glycoprotein (MAG) were done using myelin from young rats. A sensitive in vivo labeling procedure was used. [^{3}H]- or [^{14}C]-Fucose were used as a precursor of the myelin glycoproteins since fucose is not converted to other sugars and since it is specifically incorporated into glycoproteins (Zatz and Barondes, 1971; Quarles and Brady, 1971). The radioactive glycoproteins were electrophoresed on SDS gels. Double-label procedures using this format are particularly advantageous since two animals can be injected with different isotopes ([^{3}H] and [^{14}C]) of the same precursor and the combined glycoproteins can be electrophoresed on the same gel. Since this protocol can eliminate the minor migration differences seen between gels, one has a method of detecting relatively small differences in the glycoproteins of the two samples. However, the patterns of incorporation (using single or double label methods) must be interpreted in light of the fact that they are the results of the relative rates of synthesis and turnover of the different glycoproteins.

[^{3}H]- or [^{125}I]-Labeled lectins can be used to bind bovine and human myelin glycoproteins that have been separated on polyacrylamide gels. This technique has revealed

many glycoproteins that were not detected with the fucose incorporation technique (McIntyre et al., 1979). However the localization of all of these glycoproteins in myelin has not been demonstrated unequivocally.

The most informative technique for determining the location of the glycoproteins is by immunological methods. Specific antisera to MAG have been generated to demonstrate the location of MAG within myelin. A radioimmunoassay for MAG has also been developed and provides a sensitive means of detecting MAG in tissues or fluids.

ANALYSIS OF GANGLIOSIDES IN MYELIN AND AXOLEMMA

In choosing an appropriate and sensitive analytical procedure for ganglioside extraction and analysis, we considered three major points. These included the total quantity of gangliosides in our initial membrane preparation (axolemma-enriched or myelin membrane), the types of gangliosides found in those fractions, if known, and the proper solvent system to ensure adequate separation of the gangliosides.

The standard procedure for ganglioside extraction utilizes the lipid extract procedure of Folch et al. (1957) coupled with the partitioning procedure of Suzuki (1964). However this procedure has a major shortcoming when applied to tissues such as myelin which are rich in the less polar monosialogangliosides, because these less polar gangliosides are not fully partitioned into the upper aqueous

phase. This is not a serious problem when working with tissues or fractions rich in the complex gangliosides. Preliminary analysis of axolemma-enriched membrane preparations demonstrated the presence of predominantly complex gangliosides. The only modification of the Folch and Suzuki methods that is necessary when isolating axolemma gangliosides is to desalt samples using Sep-pak C18 reverse phase columns (Williams and McCluer, 1980). This desalting procedure has the added advantage of removing CMP-sialic acid and UDP-galactose as well as the salts.

Because of the stated limitation of the Folch-Suzuki methods, we chose a different approach for isolation of myelin gangliosides which are known to have a high proportion of monosialogangliosides. The procedure utilized the techniques described by Saito and Rosenberg (1982b) which appear to maximize the recovery of G_{M2}, G_{M3} and G_{M4}.

FETAL ALCOHOL SYNDROME

HUMAN STUDIES

A group of abnormalities seen in the offspring of alcoholic women are known as the fetal alcohol syndrome or fetal alcohol effects (FAS/FAE). Since the first clinical report in the U.S. scientific literature, the experimental study of the clinically observed syndrome has grown tremendously in the last ten years.

Effects of maternal alcohol consumption on offspring were originally described in a large clinical study

by Lemoine *et al.* (1968) in France. Prominent characteristics observed were prenatal and postnatal growth deficiency, developmental delay, facial, limb and cardiac defects as well as mental retardation and microcephaly. In 1972, Ulleland (1972) described severe growth deficiency in offspring of alcoholic women. In 1973, Jones *et al.* (1973) reported on the outcome of eight pregnancies of alcoholic women and found the same type of abnormalities as described by the European studies.

Evaluation of offspring of two groups of alcoholic women indicated that the perinatal mortality rate is 17% (Jones *et al.*, 1974; Hanson *et al.*, 1976). The occurrence of the fetal alcohol syndrome is at least 1 case per 1,000 pregnancies (Hanson *et al.*, 1976). Between 30% and 50% of the offspring of alcohol consuming mothers can be expected to have serious physical or behavioral problems.

Clinical examination of FAS cases has grown phenomenally since the original 1973 study of Jones *et al.* (1973). In addition to the numerous reports on individual cases, there are now available several completed studies involving thousands of women (alcoholic and non-alcoholic) in different areas of the world as well as the United States. I will briefly comment on major findings of these studies.

In a prospective study of over 32,000 pregnant women, Harlap and Shiono (1980) documented a twofold in-

crease in spontaneous abortions during the second trimester of pregnancy in women drinking 1 or 2 drinks per day. There was a three and one half fold increase when consumption was 2 or more drinks daily. This difference was significant even after adjustments were made for age, parity, marital status, smoking and previous abortions. Another study involving 12,000 women (Sokol et al., 1980) showed similar results.

The relationship of maternal alcohol comsumption to neonatal mortality has been examined in several studies. The 17% mortality of the offspring of heavy drinkers (Jones et al. 1974) contrasts with the results of several prospective studies of women consuming less alcohol in which no significant rise in mortality was found (Kaminski et al., 1976,1981; Sokol et al., 1980).

Prenatal growth deficiency (decreased weight, length and height) are consistently seen in FAS/FAE. Decreased birth weight has been correlated with postnatal growth retardation, lower I.Q., poor sucking reflex, sleep disturbances, learning disabilities and poor school performance, hyperactivity and speech problems (Abel, 1982). When compared to a frequency of about 7% for the general population, a survey of 95 FAS offspring demonstrated a 75% incidence of low birth weight. Prospective studies by Little (1977), Ouellette et al. (1977) and Sokol et al. (1980) all reported decreased birth weight which related to

alcohol consumption during pregnancy. Ouellette et al. (1977) also reported that the greater the daily alcohol consumption of a group of women, the higher the incidence of low birth weight. This weight deficiency is apparently not overcome by postnatal enrichment (Smith, 1980).

The facial abnormalities frequently seen in FAS offspring include short palpebral fissures (perhaps due to the small eye size), small noses, a low nasal bridge, a long and smooth philtrum (area between nose and mouth), thin upper lip and underdeveloped jaw. Skeletal, cardiac and genital abnormalities are also seen (Jones et al., 1973,1974,1976; Jones and Smith, 1973; Hanson et al., 1976; Mulvihill et al., 1976; Mulvihill and Yeager, 1976; Peiffer et al., 1979).

The problems of FAS associated with CNS dysfunction are mental retardation, irritability in infancy, hyperactivity in childhood, delayed development, poor coordination, sleep disorders and abnormal EEG patterns (Abel, 1980; Clarren et al., 1978; Pierog et al., 1977; Streissguth et al., 1981; Shruygin, 1974; Peiffer et al., 1979; Root et al., 1975; Havlicek and Childaeva, 1976). Brain growth may be more severely retarded than body height or weight growth.

About half the children born to alcoholic women are mentally retarded (Jones et al., 1974; Palmer et al., 1974; Hanson et al., 1976; Mulvihill and Yeager, 1976; Mulvihill

et al., 1976; Clarren and Smith, 1978; Streissguth et al., 1978,1980). IQ's of FAS children reportedly range from 16 to 105 with an average of 65 (Streissguth et al., 1978). Studies in France (Lemoine et al., 1968) and in Germany (Majewski et al., 1976) found similar values (70-85). In Sweden, 19% of the FAS children had an IQ below 70 (Olegard et al., 1979). The degree of mental retardation has been positively correlated with the severity of the external physical features of FAS (Streissguth, 1976; Majewski et al., 1976); however, it is interesting to note that CNS dysfunction (both physical and behavioral) may occur in the absence of the other common physical abnormalities. In the studies by Clarren et al. (1978), only two of the four children showing brain malformations at autopsy had the external physical abnormalities of FAS upon which the clinical diagnosis is normally made. Behavioral abnormalities have also been seen in children that do not display the physical abnormalities. Looking at another aspect of CNS dysfunction, Shaywitz et al. (1980) studied a group of offspring of women who drank heavily during pregenancy. Despite normal intelligence (normal IQ), 15 out of 87 of these children were hyperactive and required special education.

Structural abnormalities of the brains of infants exposed to alcohol in utero have been described by Clarren et al. (1978), Peiffer et al. (1979) and Majewski and

Bierich (1979). The malformations found were similar in all the cases examined and included errors in migration of neuronal and glial elements and areas of heterotopic brain tissue usually found in the meninges.

FAS diagnoses are not easily made. There is a wide variability in the variety of effects seen in the offspring. This variability is presumably due to the extent of drinking during the pregnancy, prior drinking history, nutritional status of the mother, other drug effects (e.g. caffeine, nicotine) and genetic variability of both offspring and mothers. In support of the difference in genetic suseptibility to ethanol, Christoffel and Salafsky (1975) and Santolaya et al. (1978) reported that dizygotic twins born to alcoholic mothers had different features of FAS. In the Christoffel study, one twin had many of the FAS external features at birth while the other did not. In the other report (Santolaya et al., 1978) one twin had more severe physical abnormalities and less retarded psychomotor development than the other twin.

ANIMAL STUDIES

In recent years there have been numerous biochemical, behavioral, neuroanatomical and neurochemical studies of the adverse effect of ethanol on development in laboratory animals. These studies have used various models for the administration of alcohol that include inhalation chambers, intubations, subcutaneous implants, alcohol given in

the drinking water and alcohol given in liquid diets. The liquid diet has been the most widely used model and has now become the method of choice for animal studies of the fetal alcohol syndrome. The major advantage of the liquid diet model is that it allows one to pair-feed control and ethanol animals, provide sufficient intake of key nutrients to meet the animals' needs, to vary alcohol intake, and to measure consumption. The liquid diet model and its advantages have been recently discussed by Lieber and DeCarli (1982) and Leichter and Lee (1982). However, the original Lieber and DeCarli diet (1974) appears to lack sufficient protein to meet these extra demands of pregnancy (Weiner, 1980; Weiner *et al.*, 1981).

Animals exposed to alcohol *in utero* exhibit cardiac, kidney, skeletal and neuropathological problems (Chernoff, 1977; Randall and Taylor, 1979; Randall *et al.*, 1977). In rodents, resorptions and increased postnatal mortality have been observed (Chernoff, 1980; Henderson and Schenker, 1977; Abel, 1978; Abel and Dintcheff, 1978; Tze and Lee, 1975; Kornick, 1976; Sulik *et al.*, 1981).

Behavioral studies using animal models have demonstrated functional abnormalities as a result of *in utero* alcohol exposure. Many of these studies have primarily examined activity levels and learning and/or memory function since these are the behaviors affected in humans. There is an increase in activity levels of young animals

that were prenatally exposed to alcohol (Branchey and Friedhoff, 1976; Bond and DiGiusto, 1976; Osborne *et al*, 1980; Martin *et al*., 1978; Shaywitz *et al*., 1976) which is less severe as the animals mature. Learning and memory performance are reduced in prenatally exposed animals (Bond and DiGiusto, 1977; Riley *et al*., 1979a; Shaywitz *et al*., 1976; Abel, 1979) and in mature animals. The severity of these effects are related to the dosage of alcohol given to the mothers. Passive avoidance learning is impaired and may be indicative of the general adverse affects of prenatal ethanol exposure to response inhibition mechanisms (Riley *et al*., 1979b).

Neuroanatomical anomalies, some relating to those found in humans, have also been demonstrated in animal models of pre- and postnatal exposure to alcohol. Several studies have concentrated on histopathological changes in the hippocampal area of the brain. Barnes and Walker (1981) demonstrated a 20% reduction of pyramidal cells in the dorsal hippocampus of animals exposed to alcohol *in utero* while West *et al*. (1981) reported a pronounced alteration in the topographical organization of the mossy fibers of the ventral hippocampus in similarly treated animals. Sherwin *et al*. (1981) found a decrease in the number of dendritic spines on pyramidal cells of the hippocampus as well as abnormally short spines with fewer branches. West and Hodges-Savola (1983) have reported permanent hyper-

development of hippocampal mossy fibers after prenatal ethanol exposure.

Other studies have demonstrated alterations in other brain regions. Hammer and Scheibel (1981) found a reduction in dendritic branching and growth in the pyramidal cells in the deep layer of the cortex. The cell bodies were also smaller and less differentiated when compared to controls which indicated a delay of neuronal development. Bauer-Moffett and Altman (1975; 1977) exposed animals postnatally to alcohol and found brain weight reductions that were especially severe in the cerebellar region. They found a severe loss of Purkinje and granule cells throughout all areas of the cerebellum upon histochemical analysis. Kornguth et al. (1979) demonstrated reduced cerebellar and cerebral weight and an abnormal migration pattern of neurons in animals that were prenatally exposed to alcohol. Volk (1977) also observed abnormalities in the cerebellar maturation of the Purkinje cells of the cerebellum (Volk et al., 1981).

Neurochemical abnormalities have also been found in animals prenatally exposed to alcohol. Both Rawat (1975) and Henderson and Schenker (1977) found total RNA levels of brain to be reduced. Rawat also found decreased brain DNA synthesis rates and slightly elevated brain protein levels in these offspring. Tewari et al. (1983) found altered brain protein metabolism in offspring of mothers exposed to

alcohol during the last trimester only ([^{14}C]-leucine incorporation was increased from 6 to 34 weeks postnatally). Tewari also found an inhibition of the lysosomal enzyme N-acetyl-beta-D-glucosaminidase.

Neurotransmitter synthesis and steady-state levels of neurotransmitters have also been examined in depth in several studies. Rats exposed to alcohol *in utero* have transiently decreased levels of catecholamines in the adrenals (Lau *et al.*, 1976), a transient decrease in brain norepinephrine and a long term decrease in hypothalamic norepinephrine (Detering *et al.*, 1980). Normal brain norepinephrine in rats (Rawat, 1977) and normal brain dopamine and norepinephrine levels in mice have been reported in similar studies (Elis *et al.*, 1976,1978; Krisiak *et al.*, 1977). Such discrepancies may be explained by the method of administering alcohol, the timing of ethanol administration, and the dosage of ethanol. Other neurotransmitters affected include gamma-aminobutyric acid (GABA) (which is increased), acetylcholine (which is decreased) (Rawat, 1977) and histamine (which is increased) (Rawat, 1980).

Synaptic development in ethanol-treated offspring was examined in several studies. Thadani *et al.* (1977) looked at [^{3}H]-tyramine uptake into synaptosomes to assess the development of noradrenergic synapses. They found elevated uptake until 24 days postpartum. This suggested that the noradrenergic synapses developed earlier than

those of controls. Slotkin et al. (1980) examined the development of catecholamine (dopamine, norepinephrine) and serotonin containing synapses by assessing the uptake of these neurotransmitters. [^{3}H]-Dopamine uptake was normal in ethanol offspring while [^{3}H]-serotonin uptake was consistently decreased and [^{3}H]-norepinephrine was increased. Noronha and Druse (1982) found an abnormal distribution of radiolabeled fucose in fucosylated synaptic membrane proteins in offspring of chronically treated mothers.

Neurochemical analysis of myelin formation has been studied in offspring of animals fed ethanol chronically (1 1/2-2 months prior to gestation) and during gestation only (Hofteig and Druse, 1978; Druse and Hofteig, 1977). Normal myelination was observed in offspring of animals fed ethanol during gestation only (Hofteig and Druse, 1978). However, in offspring of the chronically fed animals there was an increase in total myelin protein early in development (18 and 25 days postpartum) which was due solely to an excess of heavy myelin, a fraction which is similar to myelin from immature brains (Matthieu et al., 1973). At 54 days of age the content of all the myelin fractions was slightly below normal. The incorporation of [^{3}H]-leucine and [^{14}C]-glucose into the myelin fractions showed the same pattern; i.e., an increase at the early ages followed by a decreased incorporation at the older periods. The results of the chronic study suggest that the onset and slowdown of

myelination occurs prematurely. Since the gestational study did not give the same shift, it is apparent that ethanol or an ethanol metabolite was not directly responsible for the abnormalities. It seems more likely that the results are the consequence of a hormonal or metabolic change due to chronic ethanol consumption or withdrawal therefrom.

Biochemical and anatomical studies done in other laboratories have reported delayed rather than premature myelination in offspring of alcohol fed females (Rosman and Malone, 1976,1979; Jacobson et al., 1979). A transient reduction in brain lipid soluble protein was seen as well as a delay in the histological staining of myelin in these studies. The difference in reports may be due to the fact that the mothers were fed alcohol during lactation in the latter studies. Since ethanol inhibits oxytocin release and therefore lactation (Fuchs and Wagner, 1963), the offspring were subjected to the combined stresses of undernutrition and alcohol exposure. In addition, Jacobson et al. (1979) did not pair-feed the animals to ensure comparable nutrition between control and experimental animals with the result that ethanol exposed offspring were growth retarded. If undernutrition were a significant factor in these studies, then delayed myelination would be expected (Figlewicz et al., 1978; Krigman and Hogan, 1976; Wiggins and Fuller, 1978).

Delayed myelination was reported by Lancaster *et al.* (1982) in offspring of animals fed alcohol during gestation only. This was based on a 30% decrease in the relative synthesis of myelin proteins at 16 days which was followed by normal synthesis and content of myelin protein at 30 days. Delayed myelination in the Lancaster study may reflect the extremely low consumption of liquid diet during gestation in their study and thus the combined stresses of alcohol and undernutrition.

RATIONALE OF THE PROPOSED STUDIES

The fetal alcohol syndrome (fetal alcohol effects) is a well documented phenomenon. A commonly occurring characteristic is CNS dysfunction as manifested by neuroanatomical, behavioral, intellectual and electoencephalogram abnormalities. CNS dysfunction may be the most sensitive marker of alcohol exposure *in utero* since it has been reported in children who do not display the common external features of the syndrome. However, the biochemical mechanisms by which these abnormalities occur have not been fully explored.

It is evident that there are biochemical and anatomical abnormalities related to myelination in animal models of FAS (Druse and Hofteig, 1977; Jacobson *et al.*, 1979; Rosman and Malone, 1976,1979; Lancaster *et al.*, 1982). However there are conflicts as to the nature of the abnormalities. It is the objective of this investigation to

closely examine the biochemical changes of myelin during myelin maturation in the offspring of animals fed chronically on an ethanol liquid diet by assessing the distribution of the glycoproteins and gangliosides in CNS myelin from these animals. Since the formation of myelin requires a signal from the axon (Aguayo et al., 1976; Weinberg and Spencer, 1979), the distribution of gangliosides and glycoproteins on the axonal surface are of interest as well. Therefore, gangliosides and glycoproteins from axolemma membrane preparations from FAS offspring will also be examined. In addition, the axolemma membranes will be examined for phospholipid content to find whether abnormalities are present.

This dissertation will also examine ganglioside, glycoprotein and phospholipid content and composition during the normal developmental process since such studies have not been previously reported, particularly for rat brain myelin gangliosides and all of the axolemma components.

All studies will be done on animals who were maintained with their biologic mother throughout the time of study or that were cross-fostered to normal chow-fed mothers during lactation.

Glycoproteins and gangliosides have been shown to play important roles in cell-cell recognition, intracellular adhesion, CNS morphogenesis and neuronal and regional

brain differentiation (Brunngraber, 1969; Barondes, 1970; Roseman, 1970; Langley, 1971; Hausman and Moscona, 1975; Gombos et al., 1977; Garber and Moscona, 1972; Margolis and Margolis, 1979b; Salvetera and Matthews, 1980). Thus, it will be of interest to see whether these components will demonstrate alterations when analyzed in myelin and axolemma sheaths.

Interest in the functional roles of phospholipids has generated studies on their involvement in neuronal activity. Phosphatidylcholine appears to play a role in regulation of GABAergic neurons. Lipid methylation itself influences membrane fluidity and calcium ion influx in neurons. Phosphatidylserine affects the regulation of cholinergic fibers. Phosphatidylinositol may play a role in synaptic receptor function, Ca^{+2} gating, prostaglandin release and as a precursor for polyphosphoinositides which have been reported to be involved in, among other physiological roles, the conduction of impulses down the axonal membrane (Hawthorne and Kai, 1970; Eichberg et al., 1982). Thus, alterations in phospholipid content or distribution may reflect altered axonal function.

CHAPTER II

MATERIALS AND METHODS

Animals

Nulliparous female Sprague-Dawley albino rats were purchased from Holtzman Co. (Madison, WI.). These rats were between 47 and 60 days of age and weighed between 160 and 200 grams at the start of each study.

Diet

All studies employed a dietary protocol calling for a prolonged period (1 1/2-2 months) of consumption of an ethanol liquid diet.

Ethanol (E) and control (C) isocaloric liquid diets were prepared as described by Lieber and DeCarli (1974). Protein, fat and carbohydrate accounted for 18%, 35% and 47% of the calories respectively. The alcohol content of the ethanol diet is 6.6% (v/v) and corresponds to 35.8% of the calories. Isocaloric control and ethanol diets differ in only one respect. The isocaloric equivalent of ethanol is replaced by maltose-dextrins in the control diet. The composition of control and ethanol Lieber-DeCarli diets is described in Table I.

Table I

Lieber DeCarli Liquid Diets

Ingredient	Grams/Liter Control	% Calories Control	Grams/Liter Ethanol	% Calories Ethanol
PROTEIN:		18%		18%
Casein:	41.4		41.4	
L-Cysteine:	0.5		0.5	
L-Methionine:	0.3		0.3	
CARBOHYDRATES (Non-Alcoholic):		47.5%		11.7%
Maltose-Dextrins:	114.0		24.4	
Dextrose Vitamin Mixture:	5.0		5.0	
CARBOHYDRATES (Alcoholic):		0.0%		35.8%
Ethanol:	0.0		50.0	
FATS:		35.6%		35.6%
Corn Oil:	8.5		8.5	
Olive Oil:	27.6		27.6	
Safflower Oil (78% Ethyl Lineolate):	3.5		3.5	
Salt Mixture:	10.0		10.0	

Daily feedings (following a pair-feeding paradigm) were given to individually housed control and ethanol animals. Pair-feeding was accomplished by feeding the control animals the same amount of diet that the ethanol animals consumed the previous day. All animals were fed between 1:00 PM and 3:00 PM. The animal rooms were maintained on a 12 hour light-12 hour dark schedule with lights on at 7:00 AM.

In this original study the pups were reared by their biologic mothers. The offspring are referred to by the abbreviations C-C and E-E which indicate the diet the biologic and lactational mothers were maintained on prior to conception and during gestation, respectively. All rat mothers were fed chow during lactation.

Revised Liquid Diet

After the studies which used the 'original' (Lieber-DeCarli diet) were completed, Weiner (1980,1981) demonstrated that the commonly used 18% protein diet did not supply adequate protein to pregnant animals. Accordingly, we modified the diet to eliminate this nutritional deficiency. The protein content of the revised diet was increased to 21% of the total calories. Chocolate flavoring was also added to the diet in the form of liquid Sego (Pet Inc., St. Louis, MO.). The composition of the revised control (C*) and ethanol (E*) liquid diets are shown in Table II. Rat offspring were maintained with their bio-

Table II

Revised Liquid Diets

Ingredient	Grams/Liter Control	% Calories Control	Grams/Liter Ethanol	% Calories Ethanol
PROTEIN:		21.1%		21.1%
Casein:	47.95		47.95	
L-Cysteine:	0.63		0.63	
L-Methionine:	0.37		0.37	
Sego:	3.75		3.75	
CARBOHYDRATES (Non-Alcoholic):		49.6%		13.8%
Maltose-Dextrins:	114.00		24.40	
Dextrose Vitamin Mixture:	5.00		5.00	
Sego:	15.00		15.00	
CARBOHYDRATES (Alcoholic):		0.0%		35.8%
Ethanol:	0.00		50.00	
FATS:		29.3%		29.3%
Corn Oil:	4.37		4.37	
Olive Oil:	26.90		26.90	
Safflower Oil (78% Ethyl Linoleate):	3.50		3.50	
Sego:	0.75		0.75	
Salt Mixture:	10.00		10.00	

logical mothers during lactation and are referred to by the abbreviations C*-C* and E*-E*. All rat mothers consumed chow during lactation.

Administration of Diets

Female rats were given the control liquid diet *ad libitum* four days after arrival at LUMC. One week later at least half the animals were fed the ethanol liquid diet. Animals were maintained on the control and ethanol diets for six to eight weeks before introducing a proven male breeder. A male was added to each cage of the individually housed females for a three day period. During this time both animals were given access to chow and liquid diet. Vaginial smears were done on one study only, to determine first day of pregnancy. The pregnant females were maintained on the C and E diets throughout gestation. In order to avoid the physiological changes which accompany abrupt withdrawal from ethanol, the female rats were given free access to laboratory chow and to half-strength ethanol (3.3%, v/v) or control diets for the first three days after parturition. Three days after parturition the animals were fed laboratory chow exclusively. Laboratory chow (Wayne Lab-Blox MRH 22/5, Wayne Laboratories, Chicago, IL.) contains protein (26%), carbohydrate (61%), fat (13%), and salt mixture and vitamin fortification (<1%). Rats had free access to water at all times. In all studies the litter size was adjusted to 10 pups at three days post-

partum.

Determination of Blood Ethanol Levels

Blood ethanol levels were determined using an enzymatic kit (Sigma, St. Louis, MO.). Control and ethanol fed mothers, maintained chronically on the diet, were bled from the tail approximately three hours after administration of fresh diet. (Animals were fasted the previous night which is the time they normally eat.) Blood samples (from tail veins) were collected in heparinized tubes that were immediately stoppered and placed on ice.

The method in the enzymatic kit involves the use of alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD) to measure the ethanol in the blood (Bonnichsen and Theorell, 1951). ADH catalyzes the conversion of ethanol to acetaldehyde. In the same reaction step, NAD is converted to NADH. The formation of acetaldehyde is favored when the reaction takes place at pH of 9.0. In addition, the acetaldehyde is trapped with semicarbazide which allows the reaction to proceed virtually to completion. The increase in absorbance at 340 nm, occuring with the conversion of NAD to NADH, provides an accurate measure of the amount of ethanol present.

Cross-Fostering Protocol

Cross foster studies were initiated only in the myelin glycoprotein study. In so doing, comparisons could be made between MAG non-cross-foster and MAG cross-foster

results, so that one could distinguish between prenatal effects of ethanol and those effects due to quality of postnatal nurturing. When cross-fostering was done, offspring of mothers fed the control or ethanol liquid diets were removed from their biologic mothers on the third postnatal day and placed with a foster mother. Foster mothers had been maintained on laboratory chow throughout the experiment and gave birth to a litter at approximately the same time (within 1-2 days) as the ethanol or control mothers. A foster mother's litter was removed just prior to the time when she received her foster litter. Since rat offspring were maintained with chow fed control mothers during lactation, they are referred to by the abbreviations C*-Ch for control cross-fostered and E*-Ch for ethanol cross-fostered. Table III is a summary of the different groups of animals used in these studies.

Isotopes

L-[1-^{14}C]-Fucose (60.6mCi/mmole), L-[1-^{3}H]-fucose (4.6Ci/mmole), N-[^{3}H]acetyl-D-mannosamine (500mCi/mmole), N-acetyl-D-[U-^{14}C]mannosamine (254mCi/mmole), [1(3)-^{14}C]-glycerol (51.3mCi/mmole) and [1(3)-^{3}H]-glycerol (2.5Ci/mmole) were purchased from Amersham Corporation (Arlington Heights, IL.). The specific activities of L-[1-^{3}H]-fucose, N-[^{3}H]acetyl-D-mannosamine and [1(3)-^{3}H]-glycerol were adjusted to 200 mCi/mmole, 110mCi/mmole and 180mCi/mmole, respectively, with either non-radioactive

Table III

Experimental Groups

Diet Consumed by the Biological (Gestational) Mother	Diet consumed by the Nursing (Rearing) Mother Prior to the 3rd Postnatal Day	Abbreviation for Animal Group
Lieber DeCarli (original) Liquid Diet		
Control (C)	Control (C)	C-C
Ethanol (E)	Ethanol (E)	E-E
Revised Liquid Diet (*)		
Control (C*)	Control (C*)	C*-C*
Ethanol (E*)	Ethanol (E*)	E*-E*
Control (C*)	Chow (Ch)	C*-Ch (Cross-fostered)
Ethanol (E*)	Chow (Ch)	E*-Ch (Cross-fostered)

Note: All rat mothers consumed chow exclusively after the 3rd postnatal day.

fucose, N-acetyl-D-mannosamine or glycerol, as appropriate, prior to usage.

Fucose was chosen as a glycoprotein precursor because of its specificity in labelling glycoproteins (Zatz and Barondes, 1971). N-Acetyl-D-mannosamine was used as a precursor of the sialic acid moiety in gangliosides (Quarles and Brady, 1971; Yohe et al., 1980) because it specifically labels sialic acid. Glycerol was used as a precursor of phospholipids (Rossiter and Strickland, 1970).

Administration of Isotope

Isotope solutions containing either 10 microcuries of the ^{14}C isotope or 30 microcuries of the ^{3}H isotope in a 10 microliter volume of sterile saline (0.9%, w/v, NaCl) were prepared. Animals were injected under light ether anesthesia. Either a single intraventricular injection was made to one side of the midline of the skull or two 5 microliter injections were made (one on each side).

Isotope Protocol

In studies where dual label format was followed, half the animals of each group (control and ethanol) received the [^{3}H] isotopic precursor and half received the [^{14}C] isotopic precursor. This was done to ensure that any differences seen in the data were not due to an isotope effect. In studies where only one isotope was utilized, the [^{3}H] isotopic precursor was used. The dual label isotope method was the technique predominantly used in the

studies described in this dissertation. It was the method of choice because of its sensitivity in detecting differences between the incorporation of isotope into the proteins and lipids of interest from control and ethanol offspring.

Those pups injected with glycoprotein (fucose) or ganglioside (N-acetyl-mannosamine) precursors were sacrificed 18 hours later (by decapitation after ether anesthesia) since the incorporation of label has stabilized by that time (Yohe et al., 1980; Quarles and Brady, 1971). Those injected with glycerol were sacrificed 14 hours later. This is a time at which one obtains maximal incorporation and a minimal amount of reutilization of glycerol (Benjamins and McKhann, 1973).

Table IV is a description of the various studies described in this dissertation. It was assembled to give a brief overview of which membrane fractions were analyzed, how they were isolated, what components were labeled, which label was used and lastly the ages that were analyzed.

At each age point examined there were usually six pairs of rats (6 control and 6 ethanol) studied. The six animals at each age came from a minimum of two different litters.

Isolation of Myelin Membrane

Each brain was homogenized in 18-18.5 milliliters (ml) of 0.32 M (w/v) sucrose (to give an approximate 5%

Table IV

Schedule of Studies

Membrane Isolated	Isolation Procedure	Molecular constituents Analyzed	Isotope Used	Ages in days postpartum of offspring when membrane was isolated (14-18 hours after injection)
Myelin	Norton and Poduslo (1973a)	Myelin-Associated Glycoprotein (MAG)	30μCi ^{3}H-fucose, 10μCi ^{14}C-fucose	14,18,23,24,26
Myelin	Cotman and Matthews (1971) with Norton and Poduslo (1973a)	Gangliosides	30μCi N-[^{3}H]-acetly-D-mannosamine, 10μCi N-acetyl-D-[U-^{14}C]mannosamine	17,24,31
Axolemma fractions	DeVries (1981)	Gangliosides	30μCi N-[^{3}H]-acetyl-D-mannosamine, 10μCi N-acetyl-D-[U-^{14}C]mannosamine	20,27,34
Axolemma fractions	DeVries (1981)	Glycoproteins	30μCi ^{3}H-fucose	21,22,31,32
Axolemma fractions	DeVries (1981)	Phospholipids	30μCi [1(3)-^{3}H]glycerol, 10μCi [1(3)-^{14}C] glycerol	20,27,34
Myelin, Axolemma and gradient fractions	DeVries (1981)	Enzymatic analyses	None	28,29

(w/v) homogenate) in a motorized tissue homogenizer. Ten up and down strokes were routinely used. One ml of homogenate was saved for analysis of protein and radioacitvity. The remaining homogenate was layered over 18.5 ml of 0.85 M (w/v) sucrose. This gradient was centrifuged in a Beckman SW 28 rotor on a Beckman model L5-65 ultracentrifuge at 75,000 x g or 24,000 revolutions per minute (rpm) for 35 minutes. Crude myelin was removed by Pasteur pipette from the 0.32 M/0.85 M sucrose interface. This fraction was osmotically shocked by dispersal in ice cold distilled and deionized water (d-water). The shocked myelin was pelleted at 24,000 rpm for 30 minutes. The pellet was dispersed in ice cold d-water and centrifuged at 10,000 rpm for 20 minutes. The loose myelin pellet obtained was dispersed in 18 ml of 0.32 M sucrose with three up and down strokes of a Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). This fraction was layered over 18 ml of 0.85 M sucrose and centrifuged for 35 minutes at 24,000 rpm. Purified myelin was removed by Pasteur pipette and dispersed in cold d-water. Myelin was washed and pelleted twice in d-water as described above. The final pellet was suspended in 1 ml d-water and stored at -20°C until further analyses were done. Samples were normally analyzed within two months of collection. Previous studies have demonstrated the stability of the membrane for long term storage (Quarles, unpublished observations). The diagram in Figure 8 describes the

Figure 8

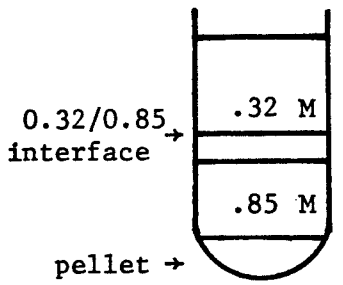
Myelin Isolation

Fresh brain

↓

Homogenize in 0.32 M sucrose (∿5%, w/v)

↓



Layer over 0.85 M sucrose (∿50% of total volume)

↓

Centrifuge at 75,000 x g, (24,000 rpm) for 35 min.

↓

Remove crude myelin at 0.32 M/ 0.85 M sucrose interface

↓

Osmotically shock in ice cold dH_2O then centrifuge at 24,000 rpm for 30 min.

↓

Resuspend pellet in ice cold dH_2O by hand homogenization then centrifuge at 13,500 x g (10,000 rpm)

↓

Suspend myelin pellet in 0.32 M sucrose by hand homogenization and layer over 0.85 M sucrose, as before

↓

Centrifuge for 35 min. at 24,000 rpm

↓

Wash myelin with dH_2O two times, as before

↓

Save pellet in 1 ml or less of dH_2O

preceding myelin isolation procedure (Norton and Poduslo, 1973a).

Myelin (a gift from Dr. Antonio Noronha) was also isolated in conjunction with the procedure used to isolate synaptic plasma membranes (Cotman and Matthews, 1971). After several initial centrifugations to remove nuclear and microsomal fractions, myelin was recovered from the 10% sucrose/7.5% ficoll interface of the first density gradient of this protocol and was further purified on the 0.32 M/0.85 M sucrose gradient as described by Norton and Poduslo (1973a). Synaptic plasma membranes (a gift from Debra Tonetti) were used for comparison of protein profiles of the different fractions used in this report.

Myelin was also isolated in conjunction with the axolemma isolation procedure of DeVries (1981). Crude myelin from the 0.75 M sucrose layer was centrifuged in the buffer (described below) and purified on the 0.32 M/0.85 M sucrose gradient as described by Norton and Poduslo (1973a). Myelin obtained from this procedure was compared to that obtained from the Norton and Poduslo procedure. It was demonstrated that although yields were not as great as in the latter procedure, the purity of the membrane was similar using both methods (DeVries, 1981). Axolemma fractions isolated concomitantly were used in other studies.

Isolation of Axolemma-Enriched Fractions

An approximate 3% (w/v) homogenate was prepared

from fresh brains by the addition of 30-35 ml of 1.2 M (w/v) sucrose containing 0.15 M NaCl and 10 mM N-[2-hydroxy-1,1-bis(hydroxy-methyl)]ethyl taurine (TES) at pH 7.5 to the minced brain. After 10 up and down strokes with the glass Dounce hand homogenizer (loose fitting pestle), 1 ml was saved for analyses while the remaining homogenate was centrifuged at 82,500 x g (24,900 rpm on the SW-28 rotor) for 15 minutes. The resulting floating layer, containing myelinated axons, was removed from the top with a metal spoonula (Fisher Scientific, Itasca, Illinois) and resuspended in 30-35 ml of 1.0 M sucrose containing 0.15 M NaCl and 10 mM TES, pH 7.5, with three strokes of the loose fitting pestle of a Dounce hand homogenizer. After another 15 minute centrifugation at 24,900 rpm, the final floating layer was removed with the spoonula and resuspended in 30-35 ml of 0.85 M sucrose containing 0.15 M NaCl and 10 mM TES, pH 7.5. The suspension was centrifuged at 24,900 rpm for 15 minutes. The resulting floating layer was suspended in 35 ml of 10 mM TES with 10 up and down strokes of a tight fitting pestle of a Dounce homogenizer for the osmotic shock. The suspension of myelin (stripped from axons) was centrifuged for 30 minutes at 24,900 rpm. The resulting pellet was suspended in 8 ml of 0.75 M sucrose containing 1 mM TES and 1 mM ethyleneglycol-bis-(δ-amino ethyl ether) N,N'-tetra-acetic acid (EGTA), pH 7.5. This suspension was layered over a discontinuous gradient consisting

of 1.2 M, 1.0 M and 0.8 M sucrose solutions which contained 1 mM TES and 1 mM EGTA, pH 7.5. The gradient was centrifuged for 1 hour at 24,900 rpm. A Pasteur pipette was used to remove the 0.75 M sucrose layer for myelin purification and the 0.8 M/1.0 M and 1.0 M/1.2 M sucrose interfaces for purification of axolemma-enriched fractions. Each axolemma fraction was pelleted for 30 minutes at 24,900 rpm and pellets were resuspended in 0.8 M sucrose. The suspension was layered on a second gradient for further purification from contaminating membranes. The second gradient, consisting of 1.2 M sucrose, 1.0 M sucrose and suspended fraction in 0.8 M sucrose was centrifuged for an hour as described before. Interfaces at 0.8 M/1.0 M and 1.0 M/1.2 M sucrose were removed by Pasteur pipette and centrifuged in 10 mM TES, pH 7.5. Pellets were frozen at -20°C in 1 ml or less of 0.25 M sucrose containing 10 mM TES. Figure 9 is a schematic representation of this isolation procedure.

The purity of fractions from the gradients obtained from control and ethanol offspring (28-29 days old) was compared by assessing the distribution of protein, 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) and magnesium dependent, ouabain sensitive, sodium-potassium adenosine triphosphatase (Na^+-K^+-ATPase). Fractions from the first gradient that were analyzed included the floating myelin, the 0.75 M sucrose layer, the 0.75 M/0.85 M sucrose interface, the 0.85 M sucrose layer, the 0.85 M/1.0 M

Figure 9

Axolemma Isolation

Fresh brain

↓

Prepare 3% w/v homogenate in 1.2 M sucrose, with 0.15 M NaCl, 10 mM TES, pH 7.5

↓

Centrifuge at 82,500 x g (24,900 rpm) for 15 min.

discard pellet ← ↓

Remove floating layer, resuspend in 1.0 M sucrose with 0.15 M NaCl, 10 mM TES, pH 7.5

↓

Centrifuge at 24,900 rpm for 15 min.

discard pellet ← ↓

Remove floating layer, resuspend in 0.85 M sucrose with 0.15 M NaCl, 10 mM TES, pH 7.5

↓

Centrifuge at 24,900 rpm for 15 min.

discard pellet ← ↓

Remove floating layer, osmotically shock (strip myelin from axons) in 10 mM TES with tightly fitting hand homogenizer

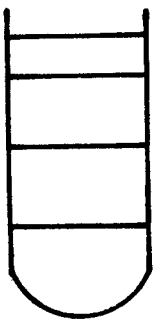
↓

Centrifuge at 24,900 rpm for 30 min.

↓

Suspend pellet in 0.75 M sucrose with 1 mM EGTA, 1 mM TES, pH 7.5. Layer onto gradient, centrifuge at 24,900 rpm for 60 min.

↓



← 0.75 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

← 0.8 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

← 1.0 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

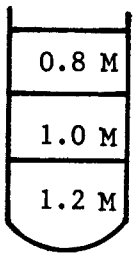
← 1.2 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

Remove
(1) Myelin from floating and 0.75 M layers
(2) 0.8/1.0 axolemma fraction
(3) 1.0/1.2 axolemma fraction

Centrifuge all fractions in 10 mM TES, pH 7.5 at 24,900 rpm for 30 min.

↓

Purify myelin on 0.32 M/0.85 M sucrose gradient ← → Resuspend axolemma fractions in 0.8 M sucrose, 1 mM EGTA, pH 7.5. Layer each fraction on another gradient



↓

← 0.8 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

← 1.0 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

← 1.2 M sucrose, 1 mM EGTA, 1 mM TES, pH 7.5

↓

Centrifuge for 60 min. at 24,900 rpm

↓

Remove axolemma fractions from 0.8 M/1.0 M and 1.0 M/1.2 M sucrose interfaces

↓

Dilute fractions in 10 mM TES, pH 7.5 and centrifuge for 30 min. at 24,900 rpm

↓

Suspend pellet in 0.25 M sucrose, 10 mM TES, pH 7.5 and store at -20°C

sucrose interface, the 1.0 M sucrose layer, the 1.0 M/1.2 M sucrose interface, the 1.2 M sucrose layer and the pellet. Fractions from the final gradient that were analyzed included the 0.8 M sucrose layer, the 0.8 M/1.0 M sucrose interface (0.8/1.0 axolemma), the 1.0 M sucrose layer, the 1.0 M/1.2 M sucrose interface (1.0/1.2 axolemma), the 1.2 M sucrose layer and the pellet. Homogenate and purified myelin samples were also analyzed. All fractions were centrifuged at 24,900 rpm for 30 minutes and suspended in small volumes of 0.25 M sucrose containing 10 mM TES at pH 7.5. Aliquots were removed for $Na^+ + K^+$-ATPase and CNPase analyses and were frozen at -20°C. Figures 10 and 11 are schematic representations of the fractions analyzed.

Assays and Analytical Procedures

Portions of the isolated membranes were used for the determination of protein, enzyme activity, and/or radioactivity. The remainder of each sample was lyophilized prior to extraction and separation of gangliosides, phospholipids or glycoproteins.

Protein

Protein determination was done by a modification of the procedure of Lowry et al. (1951). Human serum albumin (HSA) standard was prepared at a concentration of 1 mg/ml in d-water. The standard curve consisted of 0 to 60 micrograms (µg) protein in 5 or 10 µg increments. Standards and sample aliquots (in the 5-60 µg protein range) were ana-

Figure 10

Fractions Isolated from the Initial Gradient

for Enzymatic Analyses

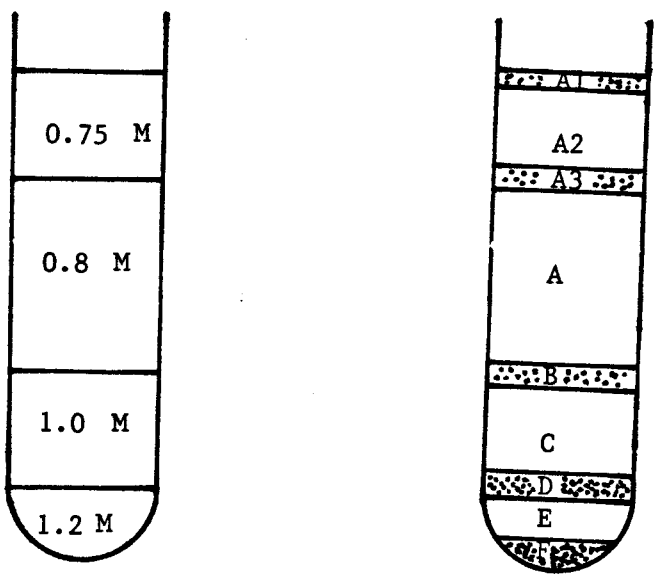


Figure 10. Osmotically shocked myelinated axons were suspended in 0.75 M sucrose and layered onto the 0.75/0.8/1.0/1.2 M sucrose gradient. After a 1 hour centrifugation at 24,900 rpm, subfractions were removed. Subfractions A1, A3, B and D corresponded to bands seen at the sucrose interfaces of different molarities. Subfractions A2, A, C and E were the layers of sucrose between the interfaces. Subfraction F was the pellet. Each subfraction was diluted with 1 mM TES buffer and centrifuged at 24,900 rpm for 30 minutes. Each pellet was suspended in a small volume of 0.25 M sucrose in 1 mM TES, pH 7.4, for enzyme marker assays.

Figure 11

Fractions Isolated from the Final Gradient

for Enzymatic Analyses

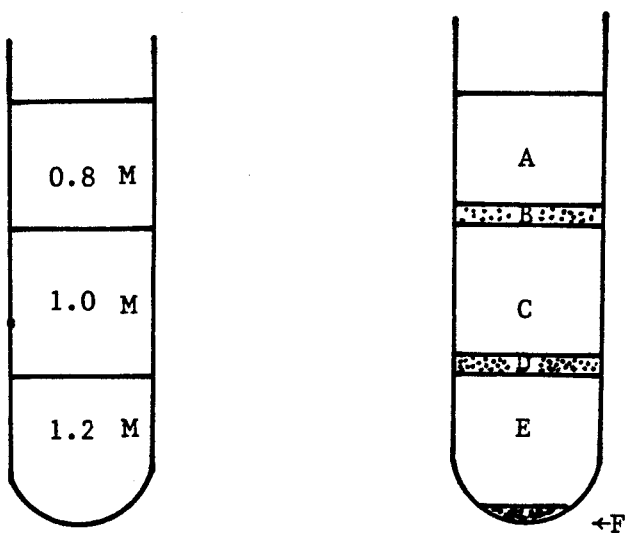


Figure 11. Interfacial material from both the 0.8 M/1.0 M and 1.0 M/1.2 M sucrose interfaces of the initial gradient was centrifuged as described in Figure 8., resuspended in 0.8 M sucrose and layered on the 0.8/1.0/1.2 M sucrose gradient. After a 1 hour centrifugation at 24,900 rpm, subfractions were removed. B and D subfractions were the 0.8 M/1.0 M and 1.0 M/1.2 M interfaces respectively while A, C and E were the layers of sucrose between the interfaces. Subfraction F was the pellet. Each subfraction was processed as described in Figure 10.

lyzed. To each tube was added 0.1 ml of 1 N NaOH, 1 ml of a 2% (w/v) Na^+-K^+-tartrate-1% (w/v) cupric sulfate-2% (w/v) sodium carbonate (0.1:0.1:10, v/v/v) solution and 0.1 ml of 1 N Folin-Phenol reagent. After 30 minutes, absorbance (O.D.) readings were taken at 700 nm.

Fiske-Subbarow Inorganic Phosphorous Assay

Inorganic phosphorous was determined by a modified procedure of Fiske and Subbarow (1925). A 0.04 mg/ml standard solution of KH_2PO_4 was made. Standard tubes contained 0 to 40 micrograms of phosphorus. Sample aliquots and standards were diluted to 1 ml with d-water. To each tube was added 0.25 ml 5 N H_2SO_4, 0.25% (w/v) ammonium molybdate and 0.1 ml reducing agent (3g Na_2SO_3, 60 g $NaHSO_3$ and 1 g 1-amino-2-naphthol-4-sulfonic acid in 500 ml of d-water). The final volume was adjusted to 2.6 ml with d-water. After 30 minutes the absorbance of each sample were determined at 625 nm.

Sodium-Potassium Dependent, Magnesium Stimulated, Ouabain-Sensitive Adenosine Triphosphate Phosphohydrolase (ATPase) Assay

Na^+-K^+-Adenosine triphosphate phosphohydrolase (EC 3.6.1.4) enzyme activity was measured by a modification of the procedure of Abdel-Latif et al. (1970). Reaction buffers were prepared with (10 mM) and without ouabain. Both buffers contained 150 mM NaCl, 20 mM KCl, 1.5 mM adenosine triphosphate (ATP), 3 mM $MgCl_2$ and 40 mM Tris

(Trizma base), pH 7.4. ATP solution was prepared fresh just prior to use. Optimal ranges of protein concentrations for the assay were 80-100 µg homogenate protein, 80-100 µg myelin protein, and 30-50 µg axolemma protein. Enzyme preparations were preincubated for 2 minutes in 0.02% (w/v) SDS (Sweadner, 1979). To each reaction tube was added 0.35 ml buffer, 0.05 ml preincubated enzyme and 0.05 ml d-water to give a final volume of 0.45 ml. The reaction was started with the addition of 0.05 ml of 30 mM ATP and was terminated 10 minutes later with the addition of 0.15 ml ice cold 50% (w/v) trichloroacetic acid (TCA). Tubes were cooled on ice, mixed on a vortex and centrifuged at 2,500 rpm in a table top centrifuge. Finally, 0.5 ml of the supernatant fraction was removed for assay of phosphate by the Fiske-Subbarow assay. Blanks included ATP and buffer (with and without ouabain) but no enzyme. The absorbance readings of these blanks gave a measurement of non-enzymatic hydrolysis of ATP. Other blanks, consisting of enzyme and buffer only, were also prepared. Specific activity was expressed as µmole P_i released /mg protein /hour.

2',3'-Cyclic Nucleotide 3'-Phosphohydrolase (CNPase) Assay

2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase) (EC 3.1.4.37) was assayed by a modification (Banik and Davison, 1969) of the method of Kurihara and Tsukada (1967). Samples were diluted in 1% Triton-X-100 to give a

final concentration of 5 µg/20 µl homogenate protein, 5-10 µg/20µl axolemma protein and 2-4 µg/20 µl myelin protein. Reaction tubes contained 0.05 ml of 0.03 M 2',3'-cAMP, 0.05 ml of 0.2 M Na_2HPO_4-0.1 M citric acid buffer, pH 6.2, and 0.08 ml of d-water. Blank tubes contained 2',3'-cAMP and buffer without enzyme. After a 2 minute preincubation period at 37°C, the reaction was initiated by the addition of diluted enzyme. After 20 minutes, the reaction was terminated by the addition of 0.02 ml of glacial acetic acid and the tubes were placed on ice. A sample aliquot of 0.02 ml was chromatographed on Whatman No. 1 filter paper (20cm x 20cm) for 3.5 hours in a solvent system containing isopropanol-ammonium hydroxide-d-water (7:2:1, v/v/v). After drying, substrate (cAMP) and product (2'-AMP) were visualized under shortwave UV light and were circled in pencil. Sample and blank spots were cut out. Nucleotides were eluted by shaking paper cuttings in 4.0 ml of 0.01 N HCl for 2 hours at room temperature. Paper was pelleted using a table top centrifuge. The clear solutions were used to determine absorbance at 260 nm. The absorbance for cAMP blank (no enzyme) was subtracted from the absorbance for sample products. Specific activity was expressed as µmole 2'-AMP released /mg protein /hour.

Solubilization of Sample

Aliquots of tissue fractions, including whole homogenate, were solubilized in 1 ml NCS tissue solubilizer and

0.1 ml of 0.1 N acetic acid. To this was added 10 ml of toluene based fluor. Samples were then mixed using a vortex, dark-adapted and counted in the liquid scintillation counter.

Radioactivity from gel slices was eluted with Protosol:d-water (9:1,v/v). The slices were incubated in 1 ml of solubilizer for 24 hours at room temperature prior to the addition of toluene based fluor.

Radiolabeled gangliosides and phospholipid TLC scrapings were eluted with 1 ml of d-water. Samples were sonicated (Branson Cleaning Equipment Company, Shelton, CT.) for 30-60 seconds. Scintillation counting was done after addition of 10 ml of Aquasol fluor.

Preparation of Toluene Based Fluor

To 4 liters of scintillation grade toluene was added 16 g PPO (2,5-diphenyloxazole), and 0.4 g POPOP (p-bis[2-(5-phenyloxazolyl)]-benzene. The solution was stirred overnight to ensure that PPO and POPOP were completely dissolved.

Liquid Scintillation Counting

The radioactivity of [^{3}H] and/or [^{14}C] in samples was measured in a Beckman LS 7500 (microprocessor controlled) liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA.). Different techniques were used to extract and solubilize radioactivity depending on the type of sample (e.g. tissue, gel slices, thin layer chromato-

graphy (TLC) scrapings). A toluene based fluor (described above) was used for all samples solubilized in NCS or protosol and Aquasol fluor was used for those solubilized in water.

Two quench correction curves were prepared in order to determine coefficients to be used in the programming of the computer assisted conversion of observed counts per minute (cpm) to disintegrations per minute (dpm). One quench curve computed values for the Aquasol fluor system and the other for NCS or Protosol in the toluene based fluor system. The LS 7500 counter uses a quench monitoring system called the H-number method which is based on the difference between the pulse height of the Compton edge of unquenched and quenched samples. Therefore an H number of 0 corresponds to an unquenched sample. The computer calculates a counting efficiency for the range of H numbers included in the quench curve. Many of the samples contained both [^{3}H] and [^{14}C]. Less than 1% of [^{3}H] activity was found in the [^{14}C] channel while approximately 10% of the [^{14}C] activity was found in the [^{3}H] channel. Based on the H number, the internalized quench curves for dual-labeled samples automatically corrected the counts for spectral shifts and counting efficiency.

In general, any counts obtained for a full gel or TLC lane that totaled less than 500 dpm ([^{3}H] or [^{14}C]) were discarded. This corresponded to single slices or

bands that were less than 4 times over background counts. The distribution of counts among separated proteins or gangliosides were similar whether the total cpm were high or at the lower acceptable limit of cpm.

Counting efficiencies in NCS and toluene were 40-45% for [^{3}H] and 78-80% for [^{14}C] while those in Protosol and toluene were 60-64% for [^{3}H] and 78-80% for [^{14}C]. Counting efficiencies of 28-32% for [^{3}H] and 71-75% for [^{14}C] were obtained for aqueous samples which used Aquasol fluor.

SDS Polyacrylamide Gel Electrophoresis of Proteins and Glycoproteins

Sample preparation for electrophoresis involved a delipidation step and a solubilization step. In the dual label myelin glycoprotein studies, a control sample and an ethanol sample (e.g. [^{3}H] control and [^{14}C] ethanol or vice versa) were combined. Lyophilized myelin sample pairs were delipidated by treating three times with chloroform-methanol (2:1, v/v). This procedure removes most myelin lipids and some non-MAG myelin proteins (i.e. myelin basic proteins and proteolipid protein) (Quarles et al., 1973a). The chloroform-methanol insoluble residue was extracted once with diethyl ether, pelleted and dried with nitrogen. Axolemma samples were delipidated with diethyl ether-ethanol (3:2) (Greenfield et al., 1971).

For cylindrical gels, the delipidated myelin pro-

teins were solubilized for a minimum of 1-2 hours at room temperature in a mixture of 2.5% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) sodium carbonate and 10% (w/v) mercaptoethanol (Quarles et al., 1973a). Samples were dialyzed overnight against a solution of 0.1% (w/v) SDS, 0.01 M sodium phosphate buffer (pH 7.2), 1.6 M urea and 0.05% (w/v) dithiothreitol. Samples were electrophoresed on 5% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS according to the procedure of Druse et al. (1974).

Cylindrical Gel Preparation

1. Solution A1 contained 0.2% (w/v) SDS in 0.04 M sodium phosphate buffer, pH 7.2. This solution was made monthly.

2. Solution A2 contained 22% (w/v) acrylamide and 0.6% (w/v) N,N'-methylene bisacrylamide (BIS). This solution was made monthly.

3. Solution B contained 90 mg ammonium persulfate and 0.05 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) made up to a total volume of 10 ml with d-water. This solution was made just prior to use.

4. Electrophoresis buffer solution contained 0.1% (w/v) SDS and 0.1 M phosphate buffer, pH 7.2.

Acrylamide gels (5%) were prepared by quickly mixing 22.5 ml of solution A1 with 11.25 ml of A2 and 11.25 ml of d-water and 5 ml of soltion B. The mixture was deaerated for 30-60 seconds. To each clean glass tube (internal

diameter, 6 mm) was added 2.5 ml of this solution to give a gel length of 5-6 cm. A few drops of d-water were layered over each gel and polymerization occurred within 30 minutes. Gels were used within 24 hours of preparation. To each dialyzed sample was added a small volume of bromophenol blue which acted as a tracking dye. Gels were loaded with 150-250 µg protein and were electrophoresed overnight at a voltage < 50 volts and an amperage < 10 mamp per tube. Electrophoresis was terminated when tracking dye was within 5 mm of the end of the gel.

Gels were removed from tubes and fixed in methanol: acetic acid:d-water (45:10:45) for a 24 hour period. Staining was done in 1% (w/v) Fast Green solution (prepared in fixing solution) for 2 hours at 37°C. After staining, gels were rinsed with d-water and placed in a diffusion destaining chamber until bands were optimally visualized.

Delipidated axolemma, myelin or SPM proteins that were to be separated on slab gels were solubilized in a solution containing 1% (w/v) SDS, 1.5% (w/v) dithiothreitol and 8% (w/v) sucrose. After addition of solubilizing solution, samples were tightly capped and heated at 100°C for 2 minutes. Bromophenol blue was added and used as a tracking dye.

Slab Gel Preparation

1. Solution A contained 30% (w/v) acrylamide and 0.8% (w/v) BIS.

2. Solution B contained 36.6% (w/v) Tris-OH buffer at pH 8.9.

3. Solution C contained 10% (w/v) SDS.

4. Solution D contained 5.89% (w/v) Tris-OH buffer at pH 6.7.

5. Chamber buffer contained 0.02% SDS, 1.4% (w/v) glycine and 0.3% Tris-OH buffer at pH 8.4.

Acrylamide gels (10%) were prepared by combining 33.3 ml solution A, 12.5 ml solution B, 1.0 ml solution C, 53.05 ml d-water, 100 mg ammonium persulfate and 0.15 ml of TEMED (diluted 1:10 with d-water). Isobutanol was layered over the freshly poured gel which was then allowed to polymerize. After polymerization the gel top was rinsed well with d-water and blotted with filter paper. Spacer gels were prepared by adding 1.0 ml solution A, 1.25 ml solution D, 0.1 ml solution C, 7.65 ml d-water, 10 mg ammonium persulfate and 0.05 ml TEMED (1:10 with d-water). The spacer gel was poured to within a few mm of the top of a comb used to create sample wells.

Spacer gels were allowed to polymerize for several hours before removal of comb and application of samples. Gels of 3 mm thickness were generally used. Voltage was set at 60 V until samples entered the stacking gel. The voltage was then increased to 120 V. Gels were removed when tracking dye had reached the end of the gel. Gels were fixed in acetic acid:methanol:d-water (1:5:4, v/v/v) then

stained with 0.1% (w/v) Coomassie Blue containing 50% (v/v) methanol and 10% (v/v) acetic acid. Destaining was done in a solution containing 7.5% (v/v) acetic acid and 7.5% (v/v) methanol.

Densitometric scanning of cylindrical gels was done using a Gilford 3400 spectrophotometer. Scanning of slab gels was done using a Schoeffel spectrodensitometer (Kratos Instruments, Westwood, N.J.) which was coupled to a Hewlett Packard 3390A integrator (Palo Alto, CA) for quantitation of peak areas. Scanning wavelength was 580 nm.

Low and high molecular weight standards (Pharmacia, Piscataway, N.J.) included Ferritin (220 and 18.5 K), Phosphorylase b (94 K), Bovine Serum Albumin (67 K), Catalase (60 K), Ovalbumin (43 K), Lactate Dehydrogenase (36 K), Carbonic Anhydrase (30 K), Soybean Trypsin Inhibitor (20.1 K) and Alpha-Lactalbumin (14.4 K). Molecular weights of unknowns were calculated from a standard curve of the known standards. The standard curve was generated from the R_f values of the standards calculated by the densitometer tracing measured by the Hewlett-Packard integrator.

Cylindrical gels were sliced using a Mickle Gel Slicer (Mickle Laboratory Engineering, Surrey, England). Slabs were sliced manually. The slicing protocol for cylindrical gels were as follows. The first 10 mm at the top were sliced in 2 mm sections. The next 20 mm (region of MAG) were sliced in 1 mm sections. The last portion of the

gel was sliced in 2 mm sections. Slab gels were sliced according to the stained bands. Slices were solubilized in protosol:d-water (9:1, v/v) at room temperature for 24 hours. A toluene based fluor was then added to each vial before counting.

Extraction of Gangliosides from Myelin

Myelin gangliosides were extracted according to the method of Irwin and Irwin (1979) with modifications (see below). Total lipids were extracted from each lyophilized myelin specimen with two 1 ml volumes of chloroform-methanol ($CHCl_3$-MeOH, 2:1), one 2 ml volume of $CHCl_3$-MeOH (1:2) and lastly with two 1 ml volumes of $CHCl_3$-MeOH (2:1). Each extract was sonicated and extensively mixed on a vortex for 30 minutes. Each extract was centrifuged for 15 minutes (2500 rpm) in a table top centrifuge and supernatants were removed with Pasteur pipettes. The lipid extracts from each step were combined and dried under nitrogen. The total lipid extract from each sample was reconstituted in 1 ml of $CHCl_3$-MeOH (2:1).

The lipid extract was applied to a 2 cm long column of silicic acid (Unisil) that rested on a base of glass wool in a Pasteur pipette (5 mm i.d.). Non-ganglioside lipids were separated from gangliosides on this column. Neutral lipids were first eluted by two 1 ml additions of $CHCl_3$-MeOH (2:1). The eluate was saved for further extraction. Gangliosides were eluted by the addition of two

1 ml portions of $CHCl_3$-MeOH-d-water (50:50:15). The column bed was stirred gently with the addition of the second ml and gentle pressure was applied to the column.

The neutral lipid fraction was dried under nitrogen and reconstituted in 0.5 ml of $CHCl_3$. This fraction was applied to a second (fresh) Unisil column prepared as described previously. Neutral glycolipids, cholesterol and phospholipids were eluted from the column using the method of Vance and Sweeley (1967). Neutral glycolipids were eluted first with the application of three 1 ml portions of $CHCl_3$. Remaining phospholipids were eluted with three 2 ml portions of acetone-MeOH (9:1). Neutral lipid fractions were discarded. Any gangliosides remaining on the column were eluted from the column by the addition of two 2 ml portions of $CHCl_3$-MeOH-d-water (50:50:15). These ganglioside extracts were then combined with the ganglioside eluants of the first Unisil column.

The procedure of Williams and McCluer (1980) was used to obtain the final purified ganglioside fraction. Sep-Pak C_{18} reverse phase cartridges (Waters Associates, Milford, MA.) were fitted with three way stopcocks and 30 ml syringes (Pharmseal Inc., Toa Alto, Puerto Rico) so that application of samples and solvents could be done in a controlled manner under slight pressure. Each cartridge was washed alternately with 10 ml MeOH and 20 ml $CHCl_3$-MeOH (2:1), three times. After the last MeOH wash the column

was washed with 10 ml of theoretical upper phase containing 0.1 M KCl ($CHCl_3$:MeOH:0.74% KCl, 3:48:47). Ganglioside eluants (from the two Unisil columns) were adjusted to 0.1 M KCl and applied to the pre-washed Sep-Pak columns with slight pressure. The eluate was reapplied to the column twice to ensure quantitative transfer of gangliosides. Salts and other contaminants were removed from the column with 10 ml of d-water. Purified gangliosides were eluted with 20 ml $CHCl_3$-MeOH (2:1) and were concentrated on a rotary evaporator under vacuum. Gangliosides were reconstituted in a small volume of $CHCl_3$-MeOH (2:1) and stored as a liquid at -20°C for further analyses.

In addition, myelin gangliosides from 20-day-old control animals were isolated by the method of Suzuki (1964) (described below) which involves a partitioning step. This was included to determine whether the method of ganglioside isolation significantly influences the composition of extracted gangliosides.

Ganglioside Extraction of Axolemma Membranes

Axolemma gangliosides were extracted using a modification of the procedure of Suzuki (1964). This procedure was chosen over the Unisil column procedure because of its efficiency at extracting complex gangliosides of which axolemma gangliosides are primarily composed (DeVries and Zmachinski, 1980). It is a particularly advantageous method over that using the Unisil columns in that con-

tamination from neutral lipids is significantly lower. Total lipids were extracted from lyophilized axolemma by the method of Folch et al. (1957). Each sample was extracted with 10 ml of $CHCl_3$-MeOH (2:1). Extraction was achieved with 1 minute sonications and extensive mixing during a 30 minute period. Centrifugation to obtain non-lipid pellets was done for 15 min at 2500 rpm in a table top centrifuge. Supernatants were removed with a Pasteur pipette and collected. Pellets were extracted again with 10 ml of $CHCl_3$-MeOH (2:1) then with 10 ml of $CHCl_3$-MeOH-d-water (1:2:.05). Supernatants (extracts) from each sample were combined and dried using a rotary evaporator (Buchi, Basel, Switzerland). The dried lipid extracts were reconstituted in 30 ml $CHCl_3$-MeOH (2:1) and transfered to 40 ml graduated glass conical tubes with stoppers.

Partitioning of lipids was begun with the addition of 6 ml of 0.1 N KCl. Tubes were stoppered and mixed extensively. After 10-20 minutes of centrifugation at 2000 rpm in a refrigerated centrifuge (International Centrifuge, Needham, MA.), upper phases were carefully removed with Pasteur pipettes and collected in 100 ml round bottom flasks. This was repeated three times with theoretical upper phase without KCl ($CHCl_3$-MeOH-d-water, 3:48:47) and once with theoretical upper phase with KCl ($CHCl_3$-MeOH-0.75% KCl, 3:48:47). Pooled upper phases were taken to dryness using the rotary evaporator under vacuum.

Samples were desalted by the method of Williams and McCluer (1980). Preparation and use of the Sep-Pak cartridges was the same as that described in the myelin gangliosides extraction procedure. Ganglioside extracts were stored as a liquid in a small volume of $CHCl_3$-MeOH (2:1) at 4°C for further analyses.

Ganglioside Separation by Thin Layer Chromatography

Separation of individual gangliosides was accomplished by thin layer chromatography (TLC). High-performance TLC (HP-TLC) precoated silica gel 60 plates (E. Merck, Darmstadt, West Germany) were used. To determine percent sialic acid distribution among the individual gangliosides, the ganglioside extracts were spotted in 1 cm lanes (separated by blank 1 cm lanes) using a Hamilton syringe. Ganglioside standards G_{M1}, G_{M2}, G_{D1a}, G_{T1} (Supelco, Bellefonte, PA.) and G_{M3}, G_{M4} (gifts from Drs. A. Rosenberg, Mariko Saito and Mitsuo Saito) were spotted on each plate to aid in the identification of individual ganglioside bands. The plates were developed in tanks saturated in the solvent system containing methyl acetate-n-propanol-$CHCl_3$-MeOH-0.25% (w/v) KCl (25:20:20:20:17) as described by Zanetta et al. (1980). Individual gangliosides were tentatively identified by comparison of their R_f values to those of the standards. Plates were sprayed with resorcinol-HCl (Svennerholm, 1957), covered with a glass plate and heated at 120°C for 20 minutes. Resorcinol

positive spots were quantitated densitometrically with a Schoeffel spectrodensitometer at 580 nm which was coupled to a Hewlett Packard 3390A integrator. For determination of radioactivity a portion of the ganglioside extract was applied across three 2 cm lanes. Plates were developed as previously described. One small part of the plate, spotted with standards and a small quantity of sample, was visualized with resorcinol (to aid in identification) while the remainder of the plate was visualized using iodine vapors. After the bands were lightly marked, the iodine was allowed to sublime and the individual bands were scraped into scintillation vials. Plates were periodically dampened with water spray to reduce the loss of silica gel. Gangliosides were eluted from the silica gel with 1 ml of d-water and brief sonication. Then 10 ml of Aquasol fluor was added to each vial and samples were counted in a liquid scintillation counter.

G_{M4} Ganglioside Identification

Radiolabeled gangliosides were extracted and separated into individual gangliosides as described above for the isolation of G_{M4}. This procedure was undertaken to establish that G_{M4} was present in myelin from young rats (previously unreported). Tentatively identified G_{M4} was subjected to neuraminidase treatment then chromatographed for identification of the products of degradation, that is, sialic acid and galactosylceramide.

Initially, radiolabeled G_{M4} bands from myelin gangliosides were separated on HPTLC plates. Iodine visulaized G_{M4} bands were scraped into conical centrifuge tubes and the silica scrapings were extracted with acetone to remove iodine stained non-ganglioside compounds. G_{M4} ganglioside was then extracted from the silica gel with the addition of $CHCl_3$:MeOH (2:1, v/v).

Myelin gangliosides, mixed bovine brain gangliosides and G_{M4} were dried under nitrogen and dissolved in 40 µl of sodium acetate buffer (0.1 M, containing 0.1% $CaCl_2 \cdot H_2O$) at pH 5.2. To the buffered gangliosides was added 40 µl of a 1 unit/ml neuraminidase solution (Clostridium perfringens, Type VIII, Sigma Chemical Co., St. Louis, MO.). The solution was mixed and heated at 37°C for 16 hours (Saito and Rosenberg, 1982a). After incubation, samples were dried under nitrogen and resuspended in $CHCl_3$:MeOH (2:1, v/v). Neuraminidase treated and untreated samples were co-chromatographed with ganglioside, glucosylceramide and galactosylceramide standards in the solvent system described previously. Spots were visualized with iodine, scraped, solubilized and counted in a scintillation counter as previously described.

Extraction and Analysis of Phospholipids

Lyophilized axolemma samples were delipidated by the method of Folch et al. (1957) and were partitioned by the method of Suzuki (1964) as previously described. Lower

phase lipids were taken to dryness on a rotary evaporator under vacuum. They were then dissolved in benzene and stored frozen at -20°C until further analysis.

Lower phase lipids were analyzed by TLC using the method of Selivonchick et al. (1980). Lipid samples were thawed and dried under nitrogen. For radioactivity analyses, a portion of each sample was spotted on a 1 cm lane in one corner of a 20cm x 20cm, 500 micron, silica gel H plate (Analabs, North Haven, CT.) that had previously been activated at 120 °C for one hour. Plates were developed in two dimensions for resolution of major phospholipid classes. The solvent used in the first dimension was $CHCl_3$-MeOH-ammonium hydroxide (65:35:10) and the solvent used in the second direction was $CHCl_3$-acetone-MeOH-acetic acid-d-water (40:20:20:20:10). After the first dimension the plates were thoroughly dried under nitrogen for 15-30 minutes. Tentative identification of phospholipid classes was accomplished by comparison of the migration of standard phospholipids to samples. The positions of phospholipids were marked on plates that had been exposed to iodine vapors. After sublimation of iodine, portions of the silica gel corresponding to the positions of the phospholipids were scraped into vials. One ml of d-water was added and samples were sonicated. Ten ml of Aquasol fluor was added prior to liquid scintillation counting.

Remaining sample aliquots were used to determine

total phospholipid phosphorus by a modification of the Bartlett (1959) assay. To each sample was added 1 ml of 70% perchloric acid (PCA). Samples were digested in a 190°C sand bath for 1 hour to hydrolyze the phosphate from the phospholipid. After cooling, 4.5 ml molybdate solution (2.2 g ammonium molybdate and 7 ml concentrated sulfuric acid in 500 ml) and 0.2 ml 1-amino-2-naphthol-4-sulfonic acid (ANSA) solution (0.5 g ANSA, 30 g $NaHSO_3$ and 6 g Na_2SO_3 in 250 ml) were added to each tube. All tubes were mixed on a vortex and heated in a 100°C heating block for 10 minutes. The total volume was adjusted to 9 ml with d-water after the samples had cooled. After mixing on a vortex the absorbance of the samples was determined at 830 nm.

Statistical Analyses

Each reported value represents the mean +/- the standard deviation of six animals unless otherwise specified. Students 't' test for statistical significance was used. P values of <0.05 represent significant differences.

Each assay requiring a linear analysis of standards was analyzed according to a linear regression analysis by the method of least squares.

A non-parametric statistical test was used to evaluate significance of differences between proportions. The non-parametric test was used in certain cases since it does not assume a Gaussion distribution of values. The

formula for this test is

$$z = \frac{P_1 - P_2}{\sqrt{\frac{p(1-p)}{N_1} \quad \frac{p(1-p)}{N_2}}}$$

where P_1 is one proportion and P_2 is the other,

$$p = \frac{N_1P_1 + N_2P_2}{N_1 + N_2}$$

N_1 = number of animals studied to give P_1

N_2 = number of animals studied to give P_2

CHEMICALS

Casein (high nitrogen)	--Dyets, Bethlehem, PA.
AIN diet salt mix	--Dyets
AIN-76A vitamin mix	--Dyets
liquid diet suspending agent	--Dyets
L-Methionine	--ICN Pharaceuticals, Cleveland, OH.
L-Cysteine	--ICN Pharmaceuticals
Maltose-dextrins	--B & R Sugar, Harvey, IL.
Iodine	--Mallinckrodt Inc., St. Louis, MO.
Resorcinol	--Mallinckrodt Inc.
Ammonium molybdate	--Mallinckrodt Inc.
Organic Solvents	--Mallinckrodt Inc.
Acids	--Mallinckrodt Inc.
Bases	--Mallinckrodt Inc.
Mercaptoethanol	--Eastman Kodak Co., Rochester, NY.
Acrylamide	--Eastman
N,N-methylene bis-acrylamide	--Eastman
N,N,N',N'-tetramethylethylene-diamine (TEMED)	--Eastman
Bromophenol Blue	--Eastman
N-[2-Hydroxy-1,1-bis(hydroxy-methyl)]ethyl taurine (TES)	--Eastman
Methyl acetate	--Eastman
Human Serum Albumin	--Sigma Chemical Co., St. Louis, MO.
Folin-Phenol reagent	--Sigma

Ethyleneglycol-bis-(δ-amino ethyl ether)N,N'-tetraacetic acid (EGTA)	--Sigma
Tris (Trizma base)	--Sigma
Triton-x-100	--Sigma
Dithiothreitol	--Sigma
Ammonium persulfate	--Sigma
Fast Green dye	--Sigma
Coomassie Brilliant Blue dye	--Sigma
Adenosine triphosphate	--Sigma
Adenosine 2':3'-cyclic monophosphoric acid	--Sigma
Aquasol	--New England Nuclear, Boston, MA.
Protosol	--New England Nuclear
2,5-Diphenyloxazole (PPO)	--Amersham Corp., Arlington Heights, IL.
p-bis[2-(5-Phenyloxazolyl)]-benzene (POPOP)	--Amersham
NCS solubilizer	--Amersham
Sucrose	--J. T. Baker Co., Phillipsburg, N. J.
Toluene (scintillation grade)	--Baker
Sodium Dodecyl Sulfate (SDS)	--Matheson, Coleman & Bell, Norwood, PA.
1-Amino-2-naphthol-4-sulfonic acid (ANSA)	--Matheson, Coleman & Bell
Unisil (silicic acid)	--Clarkson Chemical Co., Williamsport, PA.
95% Ethanol	--Pharmacy, LUMC, Maywood, IL.

CHAPTER III

RESULTS

This study examined the offspring of female rats that were pair-fed a control or ethanol liquid diet prior to and during gestation. In the control and ethanol diets protein accounted for either 18% (original) or 21% (revised) of the calories. Suckling neonates were generally maintained with their biological mothers; however, a small number were cross-fostered to control chow fed surrogate mothers.

Diet Consumption and Maternal Weight Gain

Diet consumption of females fed the original diet was 65-80 ml/day which corresponds to 140-170 Kcal/Kg body weight, 6-7.5 g protein/Kg and 8-10 g ethanol/Kg. Consumption of the revised 21% protein diet was 100-120 ml/day, corresponding to 210-250 Kcal/Kg body weight, 11-13 g protein/Kg and 12-14 g ethanol/Kg. The difference in dietary consumption may be due to the fact that the revised diet was made more palatable to the animals by the addition of chocolate Sego.

C and E mothers gained 50-60 grams during the 6

week period prior to breeding and 40-60 grams during gestation while C* and E* mothers gained 90-150 grams and 90-120 grams, respectively, during the same time periods. Although the weight gains of control and ethanol mothers within each dietary protocol were comparable, the mothers fed the revised diet (C* and E*) were better nourished than those fed the original diet (C and E) since their weight gains were comparable to normal chow fed mothers.

Blood ethanol levels of chronically fed mothers (E*-E*) ranged between 50-100 mg/100 ml several hours after diet consumption. Ethanol levels were undetectable in control (C*-C*) animals.

Brain and Body Weights of Offspring

Body weights of C-C and E-E offspring were comparable at all ages examined. The weights of the C*-Ch and E*-Ch offspring were also comparable. However, the body weights of offspring of mothers fed the original diets (both C and E) were significantly lower than the cross-fostered offspring of mothers fed the revised diet (both C*-Ch and E*-Ch) at the 14 and 18 day age periods. (See Figure 12 and Table V.)

Brain weights of control and ethanol offspring were generally comparable within each diet paradigm. There were; however, significant differences between the brain weights of animals fed the original and revised diets. At 14, 18 and 23 days of age C and E animals had lower brain weights

Figure 12. Body Weights of Offspring

Body weights from original (C-C and E-E), revised (C*-C* and E*-E*) and revised cross-foster (C*-Ch and E*-Ch) offspring are shown. The data shown are representative of approximately 50 litters. Each point represents a mean of a minimum of 6 animals. A 'b' indicates that values from C-C and E-E pups differ from those of age-matched C*-Ch and E*-Ch pups, respectively, at $p < 0.05$.

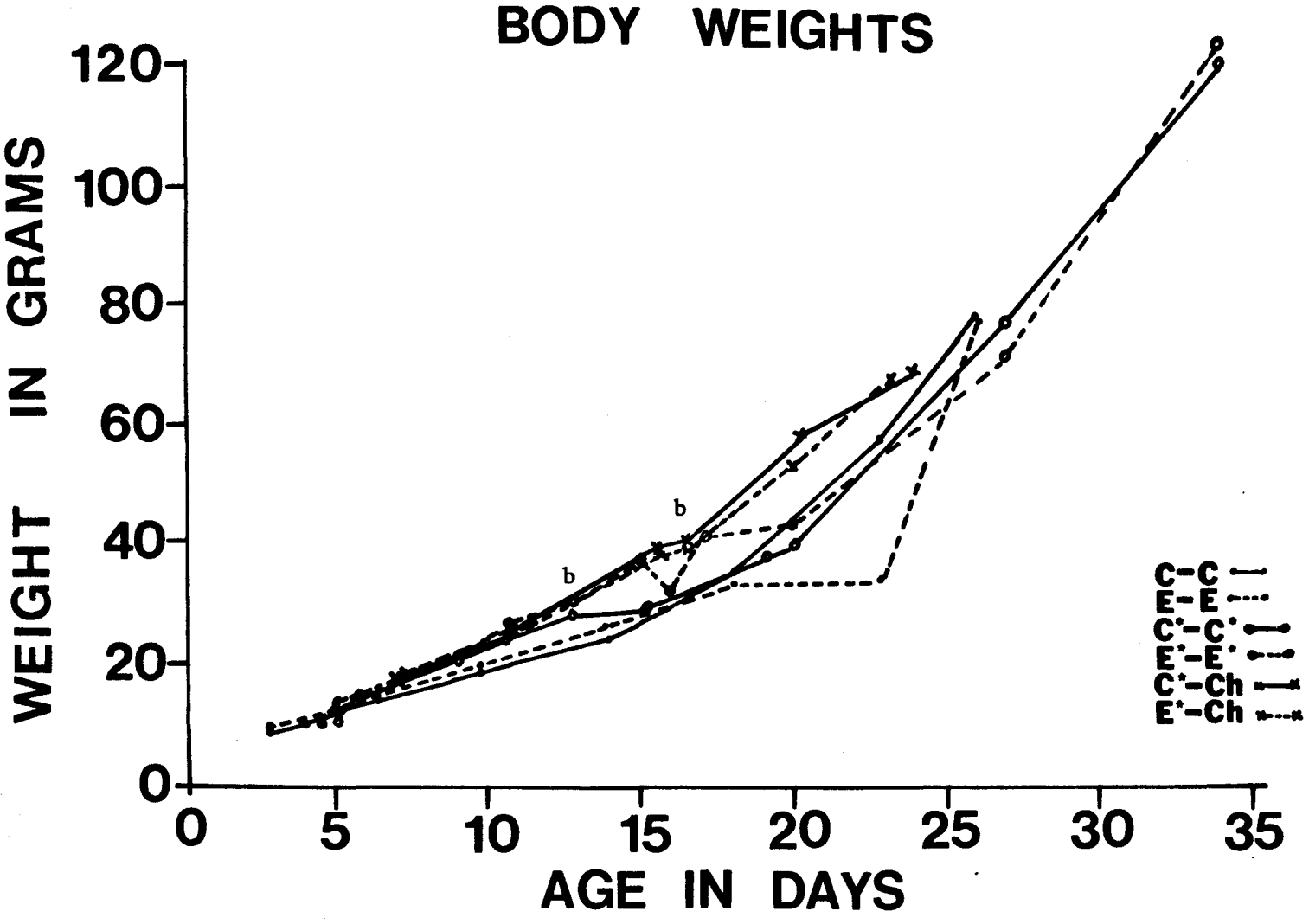

BODY WEIGHTS
WEIGHT IN GRAMS
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
AGE IN DAYS
b
b
C-C
E-E
C'-C'
E'-E'
C'-Ch
E'-Ch

TABLE V

Brain and Body Weights (Grams)

Animal-Age	Brain Weight		Body Weight	
C-C-14 Days	1.12+/-0.11		24+/-4	
E-E-14 Days	1.16+/-0.04		26+/-4	
C*-Ch-14 Days	1.28+/-0.07	b	31+/-3	b
E*-Ch-14 Days	1.21+/-0.06		31+/-2	b
C-C-18 Days	1.28+/-0.09		35+/-7	
E-E-18 Days	1.28+/-0.08		34+/-2	
C*-Ch-18 Days	1.43+/-0.11	b	43+/-3	b
E*-Ch-18 Days	1.43+/-0.05	b	43+/-2	b
C*-C*-20 Days	1.43+/-0.05		40+/-6	
E*-E*-20 Days	1.41+/-0.06		42+/-3	
C-C-23 Days	1.52+/-0.07		68+/-11	
E-E-23 Days	1.35+/-0.15	a	55+/-24	
C*-Ch-24 Days	1.55+/-0.06		70+/-8	
E*-Ch-24 Days	1.54+/-0.08	b	67+/-7	
C*-C*-27 Days	1.57+/-0.08		77+/-12	
E*-E*-27 Days	1.51+/-0.07		71+/-7	
C*-C*-34 Days	1.75+/-0.10		120+/-17	
E*-E*-34 Days	1.66+/-0.09		123+/-14	

Each value represents the mean of 6 values +/- the standard deviation. C & E and C* & E* are abbreviations for the control and ethanol offspring of rats that were fed the original (C & E) or revised (C* & E*) diets, respectively. The C*-Ch and E*-Ch rats were cross-fostered to rats that were chow-fed throughout the study, while the C-C & E-E rats were maintained with their biological mothers. An 'a' indicates that values from age- and diet-matched control and ethanol pups differ at $p < 0.05$. A 'b' indicates that values from C-C & E-E pups (original diets) differ from those of age-matched revised diet-cross-fostered rats at $p < 0.05$.

than C*-Ch and E*-Ch offspring (Table V).

In summary, control and ethanol offspring within each diet paradigm were comparably nourished. However, offspring from C*-C*, C*-Ch, E*-E* and E*-Ch mothers were better nourished than offspring of C and E mothers in comparison to brain and body weights of chow fed controls.

Myelin Protein

In agreement with previous studies from this laboratory (Druse and Hofteig, 1977), myelin protein content was increased in ethanol offspring at the beginning of myelination (day 23 in Table VI). This increase was significant in the E-E offspring but not in the E*-Ch offspring. At the latter age period (31 days), myelin protein content of E*-E* offspring was near normal.

Fucosylated Myelin Glycoproteins

The double label isotope protocol, used to study the incorporation of fucose into myelin glycoproteins (particularly MAG), is useful in detecting differences in molecular weights of major fucosylated proteins or in recognizing differences in the relative incorporation of fucose into proteins from two animals. Since ethanol and control samples were electrophoresed on the same gel, one would expect a comparable distribution of radioactivity on the gel if the incorporation of label into glycoproteins in the two animals was comparable. Similarly, one would expect that the value for the ratio, (% dpm in a given fraction

Table VI

Myelin Protein Content (mg./brain)

Age (days)	C-C	E-E	C*-Ch	E*-Ch
		(Norton and Poduslo isolation)		
14	0.68+/-0.19	1.70+/-0.30a	1.18+/-0.27	N.A.
18	2.19+/-0.20	2.26+/-0.19	2.32+/-1.21	2.66+/-0.33
23	4.37+/-0.29	5.13+/-0.52a	4.60+/-0.88	4.62+/-0.39

	C*-C*	E*-E*
	(DeVries isolation)	
20	1.68+/-0.15	1.77+/-0.15
27	4.38+/-0.70	4.37+/-0.55
34	5.40+/-0.56	4.47+/-0.42

Each value represents the mean value obtained from 6 rats +/- the standard deviation. An 'a' indicates that values from age- and diet-matched control and ethanol pups differ at $p < 0.05$. N. A. indicates that data is not available. The abbreviations are explained in Table V.

from one pup)/(% dpm in a given fraction from another pup) would be ~1 if the two samples were similar. In agreement with this hypothesis, previous work in this laboratory (Druse et al., 1981) demonstrated that the observed ratio of the % dpm in fractions of a gel loaded with membrane proteins from 2 unaffected animals was 1.0 +/- 0.05 (mean +/- standard deviation) for n=36. Since previous developmental studies showed that the MW shift of MAG can be detected by comparing the positions of the peaks of radioactivity on polyacrylamide gels, the position of MAG peaks from control and ethanol pups was also assessed.

Figure 13a shows the typical distribution of fucose derived radioactivity (% of total gel dpm) found on a gel that was loaded with myelin proteins from 14-day-old control (C-C) and ethanol (E-E) pups (original diet). Figure 13a resembles Figure 13b (distribution of radioactivity). Figures 13c and 13d are graphical representations of the ratios of % dpm in a given fraction from an ethanol pup/% dpm in a given fraction from a control pup and the ratio of dpm in a given fraction from an ethanol pup/dpm in a given fraction from a control pup, for the region of the MAG peak (e.g. fractions 16-21). A comparison of Figures 13c and 13d demonstrates the similarity in form between actual dpm ratios and percent dpm ratios. Both 13c and 13d relate E-E counts to C-C counts along the gel. However, only 13c was used for statistical analysis since the ratio of %dpm

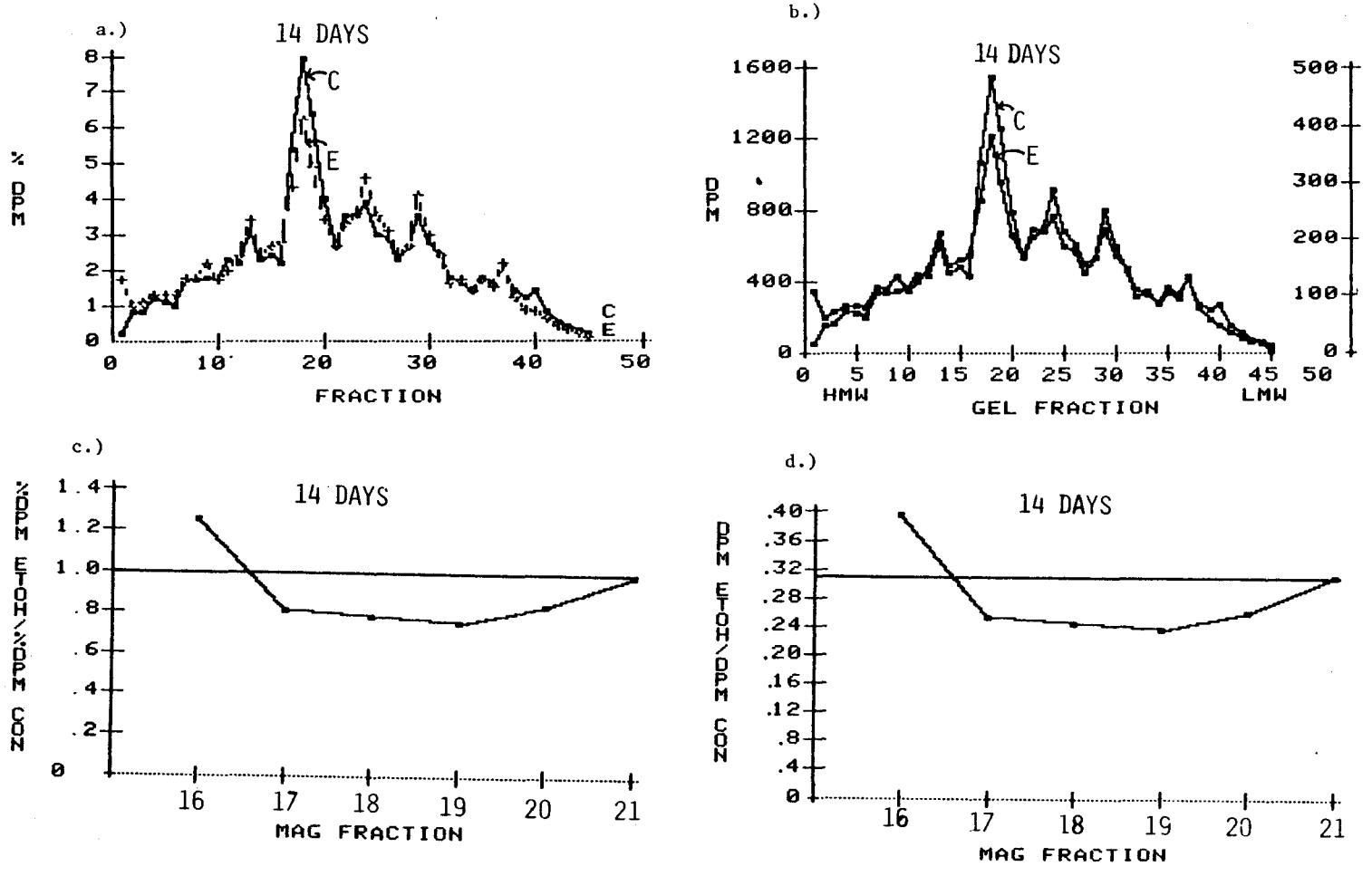
Figure 13. Radiolabeled Myelin Glycoproteins from 14-Day-Old (C-C) and (E-E) Offspring.

a). Typical distribution of radioactive fucose among myelin-associated glycoproteins from 14-day-old control (•) and ethanol (+) pups (original diet). This graph depicts the relative distribution of radioactivity (% of total gel dpm recovered in a given fraction). A total of 20,000 [^{3}H] dpm (control) and 6200 [^{14}C] dpm (ethanol) were recovered from this gel. Fraction #1 is the high molecular weight end of the gel.

b). This graph depicts the distribution of radioactivity along the same gel in terms of dpm/fraction. The scales for the left and right ordinates in b represent the [^{3}H] and [^{14}C] dpm, respectively.

c). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of the MAG peak from the same gel.

d). Ratio of (dpm in a given fraction from an ethanol pup)/(dpm in a given fraction from a control pup) in the region of the MAG peak of the same gel. The line drawn at 0.31 represents the total dpm recovered from E-E gangliosides/total dpm recovered from C-C gangliosides.


a.)
14 DAYS
DPM X
C
E
FRACTION
b.)
14 DAYS
DPM
C
E
HMW
GEL FRACTION
LMW
c.)
14 DAYS
DPM ETOH\DPM CON
MAG FRACTION
d.)
14 DAYS
DPM ETOH\DPM CON
MAG FRACTION

E/%dpm C is independent of the injected dose and data from several gels can be compared (see previous paragraph). In contrast, the ratio of dpm E/dpm C is dependent on the consistency of injections.

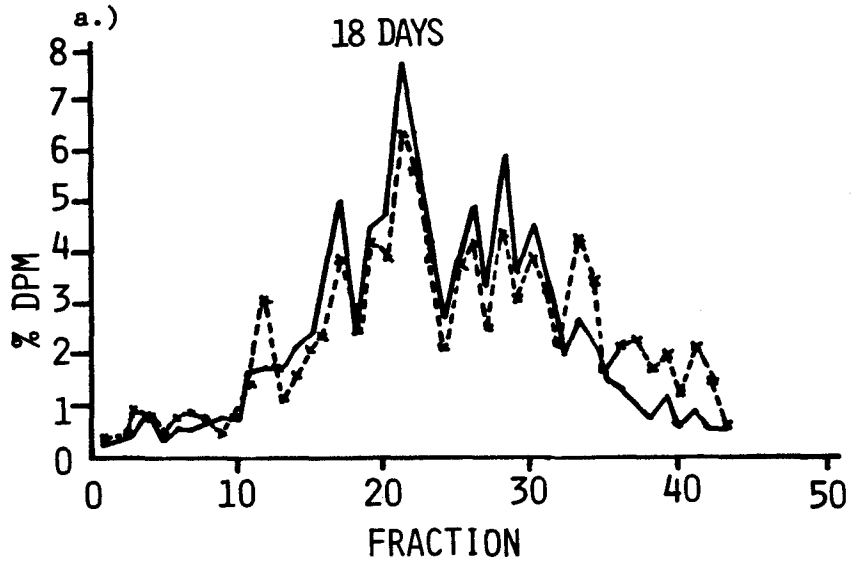
These figures demonstrate that the peak of radioactivity associated with MAG coincides in the control and ethanol offspring. Since the ethanol pups did not demonstrate an altered shift in the MW of MAG, it would appear that myelin maturation was normal. Although the maturation of MAG did not appear to be altered in the ethanol pups, we did observe a consistently decreased incorporation of label into the MAG peak of ethanol offspring.

Figures 14-16 represent the typical distribution of radioactivity and %dpm ratios from the other ages examined; 18 days, 23 days and 26 days. Again, each figure demonstrates that the MAG peak from C offspring coincides with that from E offspring. Each graph also demonstrates the decreased incorporation of radiolabeled fucose into the major fucosylated myelin associated glycoprotein. Although only one gel (1 pair of C and E pups) is represented for each age in Figures 13-16, a decreased net incorporation into MAG of ethanol pups (represented by ethanol/control ratios <0.90) was found in 88% of the pairs from 14-day-old ethanol pups. In the 18- and 23- to 26-day-old pups, 60% and 75%, respectively, of the ethanol pups showed this alteration. A minimum of 6 pairs (C & E) of animals were

Figure 14. Radiolabeled Myelin Glycoproteins from 18-Day-Old (C-C) and (E-E) Offspring.

a). Distribution of fucose-derived radioactivity in myelin from 18-day-old control and ethanol (original diet) offspring. A total of 30,000 [^{3}H] dpm (ethanol) and 3,000 [^{14}C] dpm (control) were recovered from this gel.

b). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of MAG for the above gel.


a.)
18 DAYS
% DPM
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
FRACTION

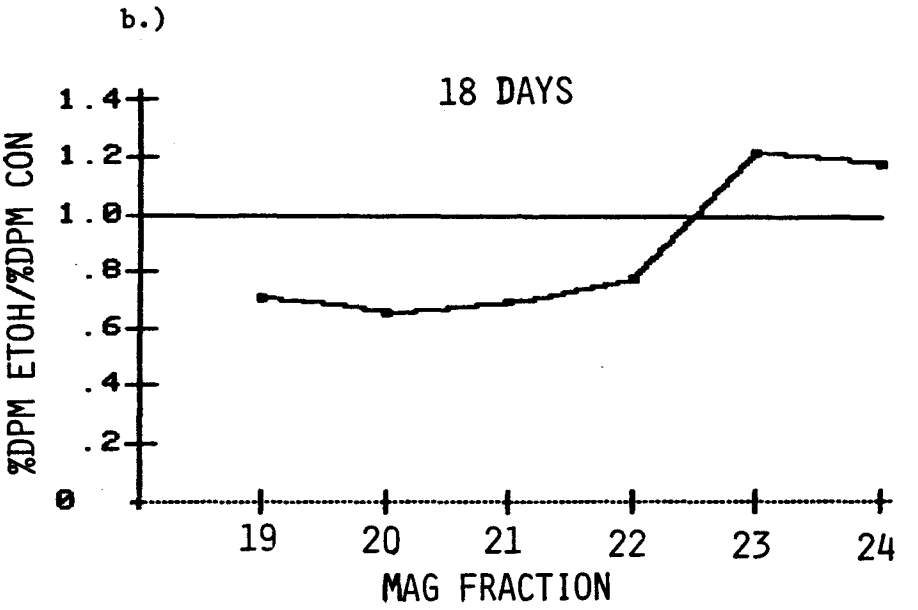
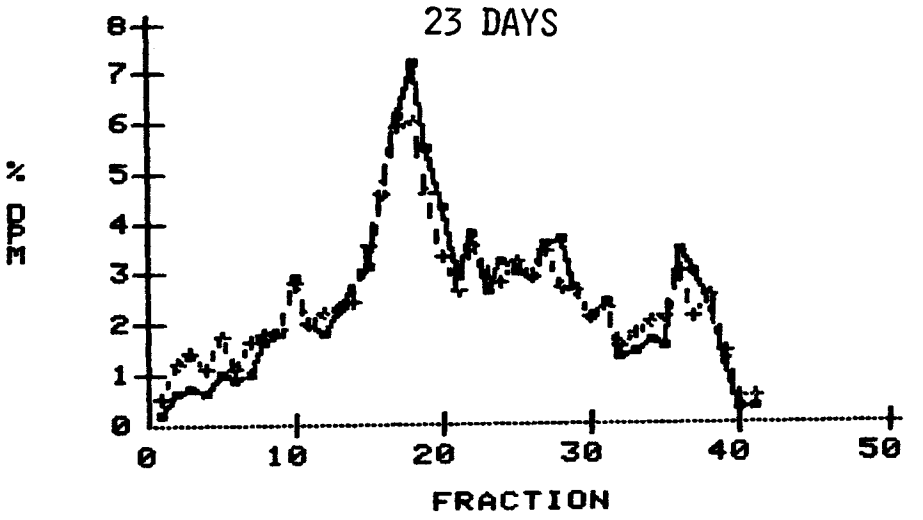

b.)
18 DAYS
%DPM ETOH/%DPM CON
0
.2
.4
.6
.8
1.0
1.2
1.4
19
20
21
22
23
24
MAG FRACTION

Figure 15. Radiolabeled Myelin Glycoproteins from 23-Day-Old (C-C) and (E-E) Offspring.

a). Distribution of fucose-derived radioactivity in myelin from 23-day-old control and ethanol (original diet) offspring. A total of 10,000 [^{3}H] dpm (ethanol) and 2,000 [^{14}C] dpm (control) were recovered from this gel.

b). Ratio of (% dpm in a given fraction the the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of MAG for the above gel.

a.)


23 DAYS
% DOM
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
FRACTION

b.)

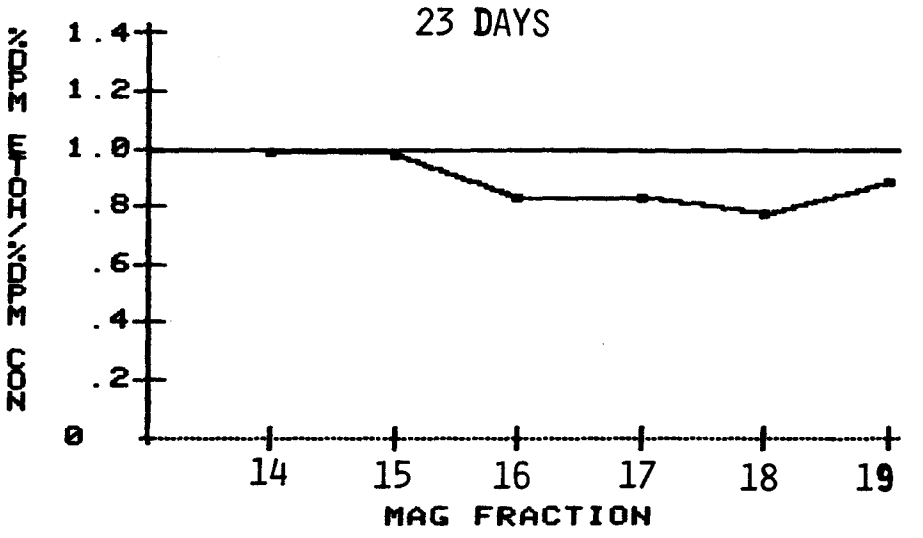
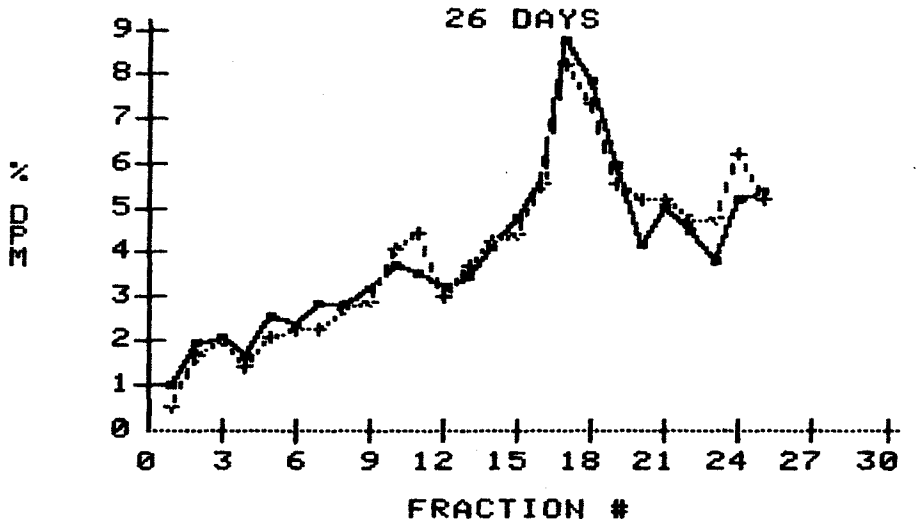
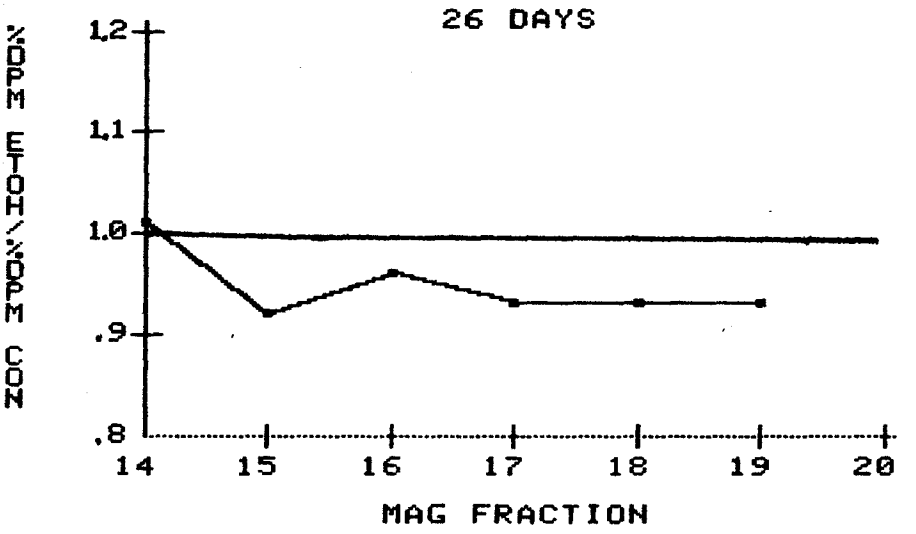

23 DAYS
% DOM EFOH\%DOM COX
0
.2
.4
.6
.8
1.0
1.2
1.4
14
15
16
17
18
19
MAG FRACTION

Figure 16. Radiolabeled Myelin Glycoproteins from 26-Day-Old (C-C) and (E-E) Offspring.

a). Distribution of fucose-derived radioactivity in myelin from 26-day-old control and ethanol (original diet) offspring. A total of 96,000 [^{3}H] dpm (control) and 48,000 [^{14}C] dpm (ethanol) were recovered from this gel.

b). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of MAG for the above gel.


26 DAYS
MPD %
0
1
2
3
4
5
6
7
8
9
0 3 6 9 12 15 18 21 24 27 30
FRACTION #


26 DAYS
MPD/MPD MEOH CON
.8
.9
1.0
1.1
1.2
14 15 16 17 18 19 20
MAG FRACTION

assessed at each age.

Figure 17 is a set of graphs similar to those in Figure 13 and depicts the relative distribution of radioactivity and the E/C ratios of a pair of 14-day-old control and ethanol cross-fostered offspring of mothers fed the revised diets (C*-Ch and E*-Ch). The peaks of MAG from C*-Ch and E*-Ch rats coincided. Thus, it would appear that the molecular weight of MAG was apparently normal in the E*-Ch offspring at all ages examined indicating that myelin maturation was normal in E*-Ch pups. Decreased fucose incorporation into MAG (ethanol/control ratios <0.95) was found at all ages in these offspring (see Figures 17-19). However, this decrease was not as great as that seen in the E-E offspring (original study). In contrast to the E-E animals, the proportion of cross-fostered animals that demonstrated a decreased incorporation of fucose radioactivity in MAG was age dependent. At 14, 18 and 24 days the percentages of cross-fostered animals affected were 80%, 60% and 33%, respectively. A non-parametric statistical comparison of the proportion of age-matched E-E and E*-Ch animals affected (decreased incorporation into MAG) indicated that a similar proportion of 14- and 18-day-old offspring from both groups demonstrated the decrease while a statistically ($p<0.05$) greater proportion of 23- to 26-day-old E-E animals showed the decrease. The distribution (% dpm) of radioactivity was not consistently altered in re-

Figure 17. Radiolabeled Myelin Glycoproteins from 14-Day-Old (C*-Ch) and (E*-Ch) Offspring.

a). Typical distribution of radioactive fucose among myelin-associated glycoproteins from 14-day-old control (•) and ethanol (+) pups (revised diet-cross-fostered). A total of 31,500 [^{3}H] dpm (ethanol) and 3,500 [^{14}C] dpm (control) were recovered from this gel.

b). This graph depicts the distribution of radioactivity along the same gel in dpm/fraction. The scales for the left and right ordinates in b represent [^{3}H] and [^{14}C] dpm, respectively.

c). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of the MAG peak from the same gel.

d). Ratio of (dpm in a given fraction from the ethanol pup)/(dpm in a given fraction from the control pup) in the region of the MAG peak of the same gel. The line drawn at 9.0 represents the total dpm recovered from E*-Ch gangliosides/total dpm recovered from C*-Ch gangliosides.

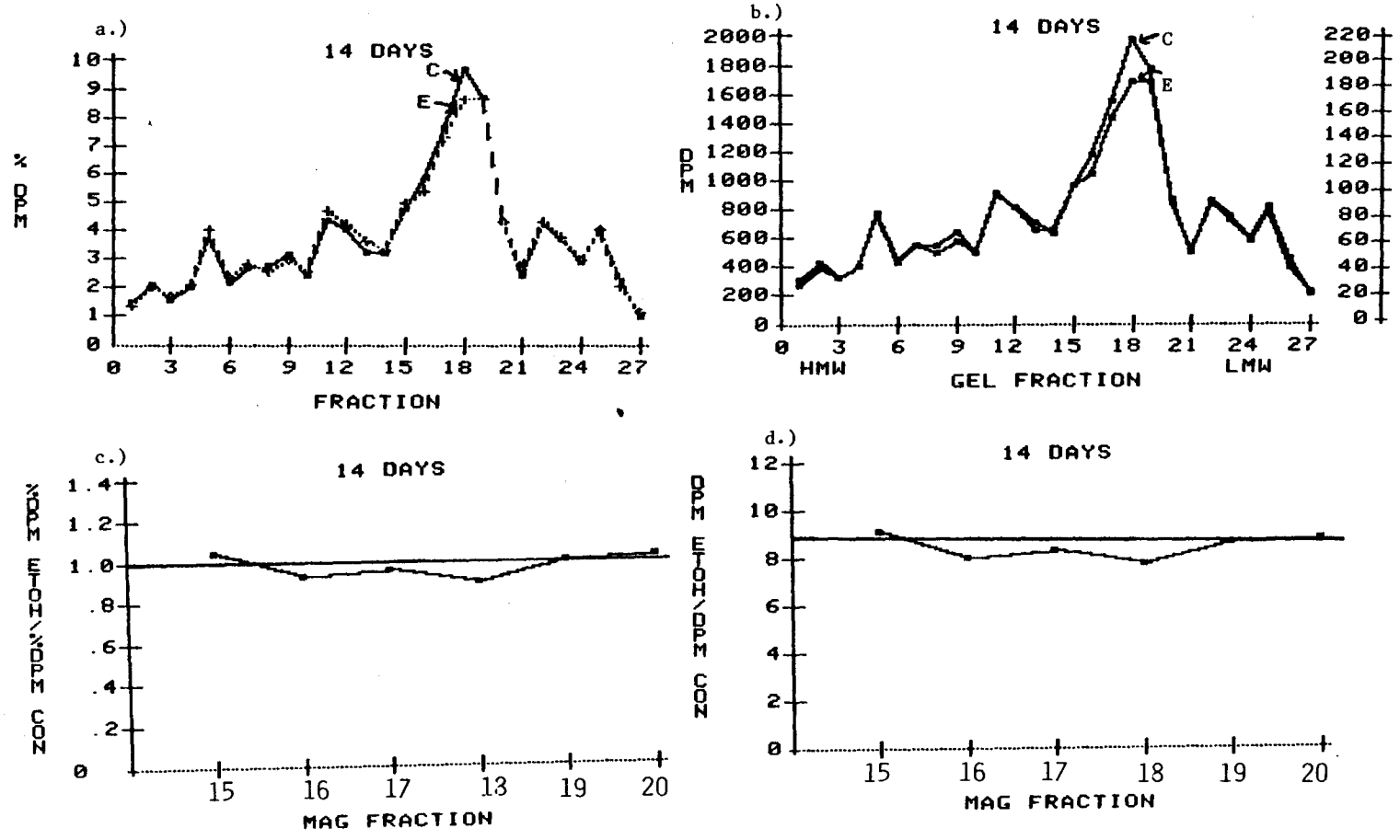
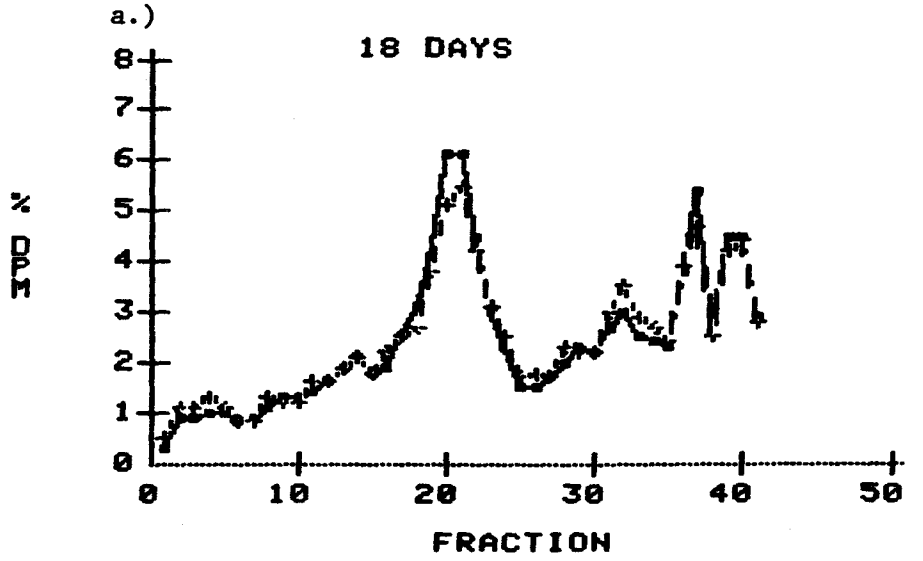

a.)
14 DAYS
C
E
% DPM
FRACTION
b.)
14 DAYS
C
E
DPM
HMW
GEL FRACTION
LMW
c.)
14 DAYS
MOM ETOH\MOM CON
MAG FRACTION
d.)
14 DAYS
DPM ETOH\DPM CON
MAG FRACTION

Figure 18. Radiolabeled Myelin Glycoproteins from 18-Day-Old (C*-Ch) and (E*-Ch) Offspring.

a). Distribution of fucose-derived radioactivity in myelin from 18-day-old control and ethanol (revised diet-cross-fostered) offspring. A total of 20,500 [^{3}H] dpm (control) and 3,500 [^{14}C] dpm (ethanol) were recovered from this gel.

b). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of MAG for the above gel.


a.)
18 DAYS
% DAM
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
FRACTION

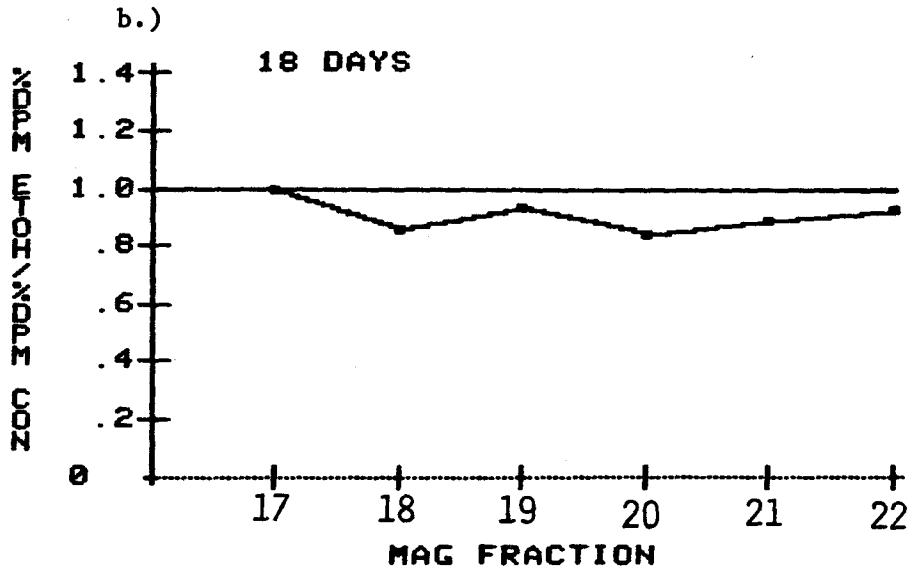
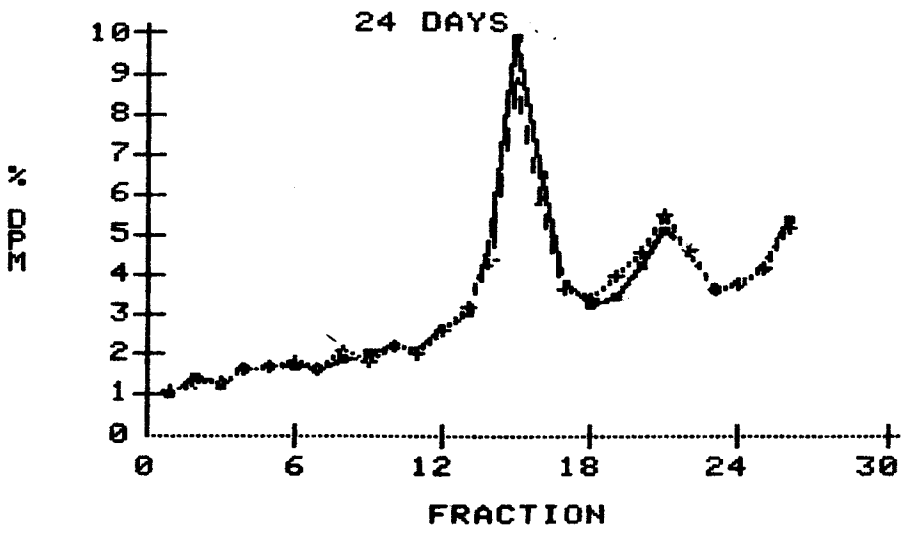

b.)
18 DAYS
NORM ETOH\NORM CON
1.4
1.2
1.0
.8
.6
.4
.2
0
17
18
19
20
21
22
MAG FRACTION

Figure 19. Radiolabeled Myelin Glycoproteins from 24-Day-Old (C*-Ch) and (E*-Ch) Offspring.

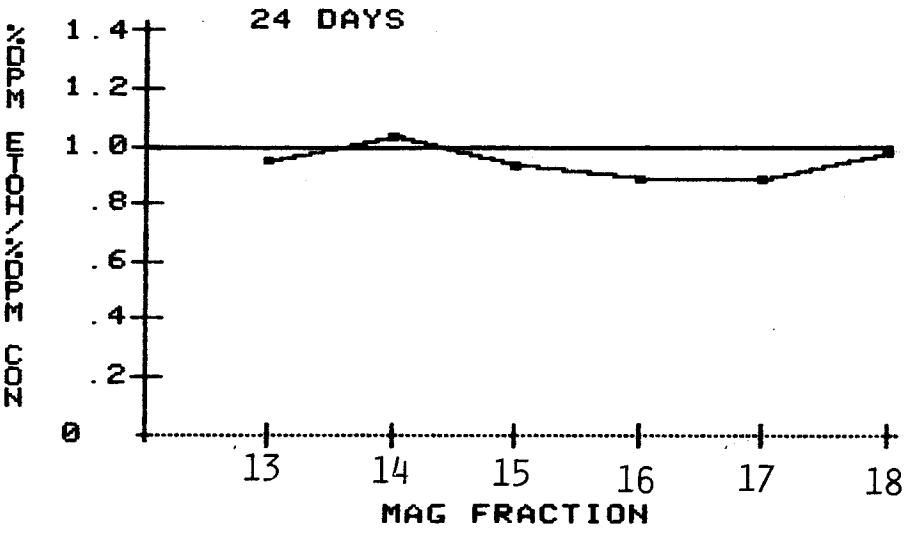
a). Distribution of fucose-derived radioactivity in myelin from 24-day-old control and ethanol (revised diet-cross-fostered) offspring. A total of 23,000 [^{3}H] dpm (control) and 4,500 [^{14}C] dpm (ethanol) were recovered from the gel.

b). Ratio of (% dpm in a given fraction from the ethanol pup)/(% dpm in a given fraction from the control pup) in the region of MAG for the above gel.

a.)


24 DAYS
MPD %
10
9
8
7
6
5
4
3
2
1
0
0
6
12
18
24
30
FRACTION

b.)


24 DAYS
ROI MPD%\HIGHEST MPD%
1.4
1.2
1.0
.8
.6
.4
.2
0
13
14
15
16
17
18
MAG FRACTION

gions of the gel containing glycoproteins other than MAG.

(Total fucose radioactivity incorporated into myelin fractions did not differ between C-C and E-E or between C*-Ch and E*-Ch offspring.)

MYELIN GANGLIOSIDES

Gangliosides were extracted from myelin of 17-, 24- and 31-day-old offspring. Separated gangliosides were assessed in terms of relative composition and incorporation of radioactivity labeled N-acetylmannosamine by scanning densitometry (individual samples) and analysis of radioactivity (combined [^{3}H] and [^{14}C] samples), respectively.

There were significant developmental differences in the distribution of sialic acid and radioactivity in the gangliosides from both control (C*-C*) and ethanol (E*-E*) rats (Figure 20). The most dramatic changes were seen in G_{T1b} and G_{M1}. The proportion of ganglioside sialic acid in G_{T1b} decreased from 26% at 17 days to 4% at 31 days and that in G_{M1} increased from 15% to 38% during the same time interval. Although the proportion of G_{D1a} and G_{D1b} remained constant, there were small but significant developmental changes in G_{D3} (which increased from 2% to 6%) and in G_{T1a} (which decreased from 4% to 2%) during the 17 to 31 day interval. Developmental changes in G_{M3} and G_{M4} could not be evaluated since the peaks could not be reliably quantitated due to contaminants in that region. The proportion of sialic acid in G_{M3} and G_{M4} (and occasionally

Figure 20. Developmental Trends in % Sialic Acid Distribution and % Distribution of Radioactivity in Myelin Gangliosides.

a). Developmental changes in % sialic acid distribution among gangliosides from 17-, 24- and 31-day-old animals. Only significant trends ($p<0.05$ between 17 and 31 day values) are shown.

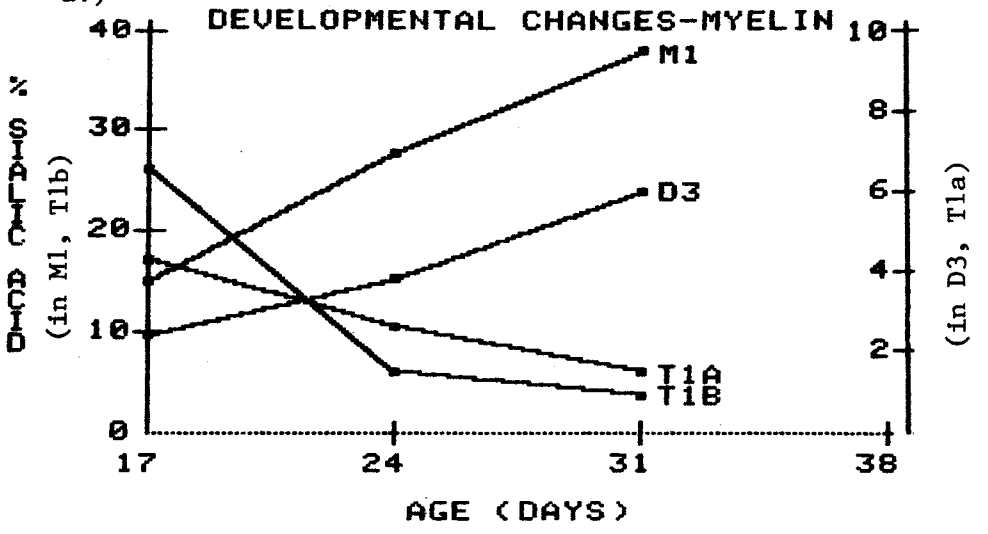
b). Developmental changes in % of the total dpm incorporated into individual gangliosides from 17-, 24- and 31-day-old animals. Only significant trends ($p<0.05$ between 17 and 31 day values) are shown.

The left hand scales apply to gangliosides G_{T1b} and G_{M1} while the right hand scales apply to G_{D2}, G_{D3}, G_{T1a} and G_{M4}.

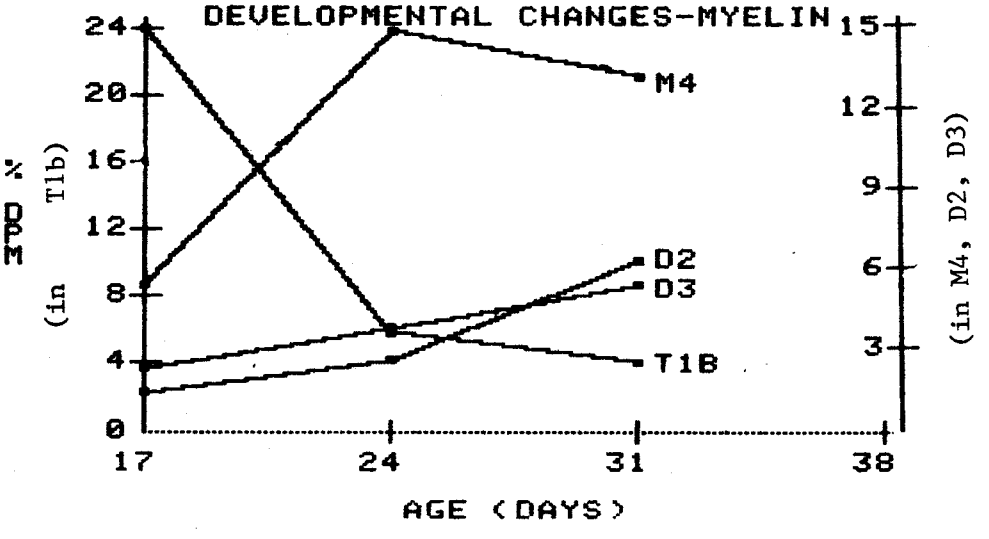
Only control values are shown though ethanol results were similar.

This data is presented with standard deviations in tabular form in Tables A-1 and A-2 in the Appendix.

a.)


DEVELOPMENTAL CHANGES-MYELIN
M1
D3
T1A
T1B
40
30
20
10
0
10
8
6
4
2
17
24
31
38
AGE (DAYS)
(in M1, T1b)
(in D3, T1a)

b.)


DEVELOPMENTAL CHANGES-MYELIN
M4
D2
D3
T1B
24
20
16
12
8
4
0
15
12
9
6
3
17
24
31
38
AGE (DAYS)
(in T1b)
(in M4, D2, D3)

G_{M2}) were therefore combined. However, in those samples in which G_{M3} and G_{M4} could be separated, G_{M4} accounted for at least 4.5% of the resorcinol positive material and G_{M3} for 1.5%.

It is interesting to note that although percentages of total sialic acid and total radioactivity incorporation among gangliosides differed, similar significant developmental trends were seen using these two different methods of analysis. The proportion of radioactivity associated with G_{T1b} decreased from 24% at 17 days to 4% at 31 days, while G_{M1} increased from 16% to 22% during the same time period (non-significantly). Small, but significant ($p < 0.05$) developmental differences were found in the proportion of radioactivity associated with G_{D2} (increased from 1% to 6%), G_{D3} (increased from 2% to 5%) and G_{M4} (increased from 5 to 13%).

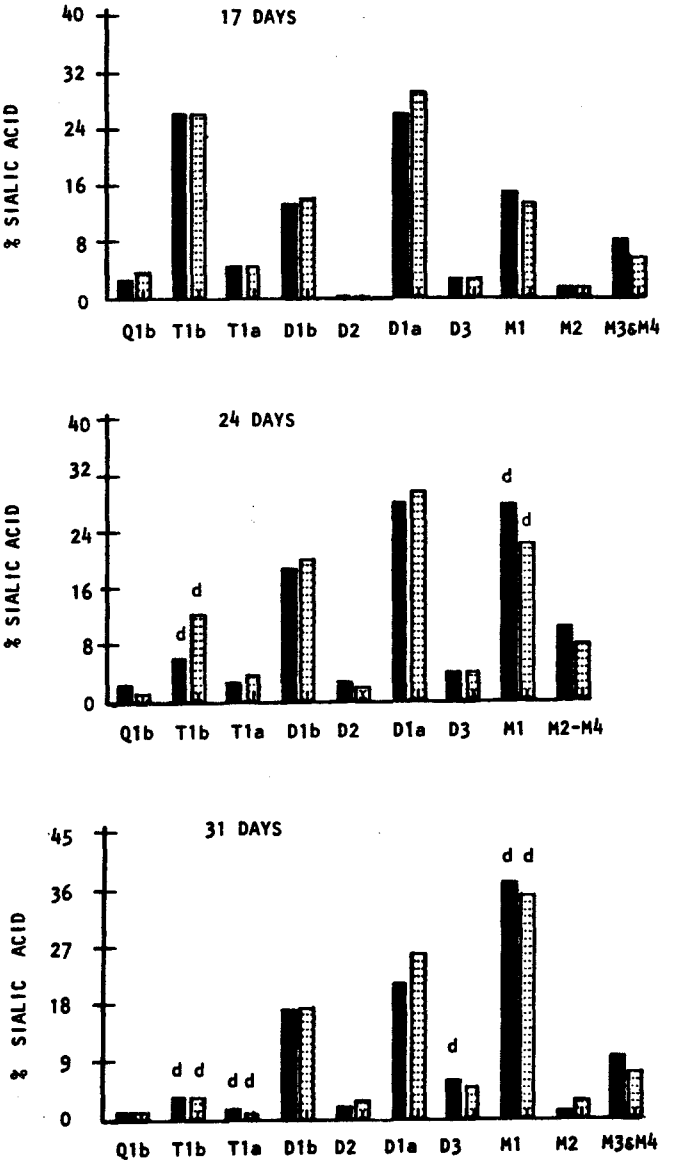
Figure 21 is a graphical representation of the distribution of sialic acid among individual gangliosides. The data is presented in tabular form with standard deviations in Table A-1 (Appendix). There were no statistically significant differences between the distribution of myelin gangliosides from control and ethanol offspring. However, there were some consistent, though non-significant, trends. These include a decreased amount of monosialylated gangliosides and an elevated amount of G_{D1a} in ethanol pups at all ages. There was also a non-significant transient increase

Figure 21. Percent Distribution of Sialic Acid Among Myelin Gangliosides from 17- to 31-Day-Old Control (C*-C*) and Ethanol (E*-E*) Offspring (Revised Diet).

Ganglioside extracts were isolated, chromatographed on HPTLC plates, sprayed with resorcinol reagent, densitometrically scanned and identified as described in the Materials and Methods. Nomenclature of gangliosides is that described by Svennerholm.

Solid and hatched bars represent control and ethanol offspring, respectively.

Each value represents the mean of 5-6 control and 5-6 ethanol animals. No statistical differences were seen between control and ethanol offspring.
A 'd' indicates that the noted value is significantly ($p<0.05$) different from the value at 17 days.
See Table A-1 (Appendix) for standard deviations.


17 DAYS
% SIALIC ACID
40
32
24
16
8
0
Q1b T1b T1a D1b D2 D1a D3 M1 M2 M3&M4
24 DAYS
% SIALIC ACID
40
32
24
16
8
0
d
d
d
d
Q1b T1b T1a D1b D2 D1a D3 M1 M2-M4
31 DAYS
% SIALIC ACID
45
36
27
18
9
0
d d
d d
d
d d
Q1b T1b T1a D1b D2 D1a D3 M1 M2 M3&M4

in G_{T1b} content in ethanol offspring at 24 days.

Figure 22 is the graphical depiction of the distribution of radioactivity among myelin gangliosides from [^{3}H]- and [^{14}C]-N-acetylmannosamine labeled control and ethanol pups. Table A-2 (Appendix) presents the data in tabular form with standard deviations. There were no significant differences between control and ethanol offspring although there was a decreased proportion of label associated with G_{M1} ganglioside of ethanol offspring at all ages. There were also other consistent non-significant trends. The incorporation of label into other monosialogangliosides (G_{M2}, G_{M3} and G_{M4}) of ethanol offspring was decreased at all ages. There was also an increased incorporation into G_{T1b}, G_{T1a} and G_{D1b} in ethanol offspring.

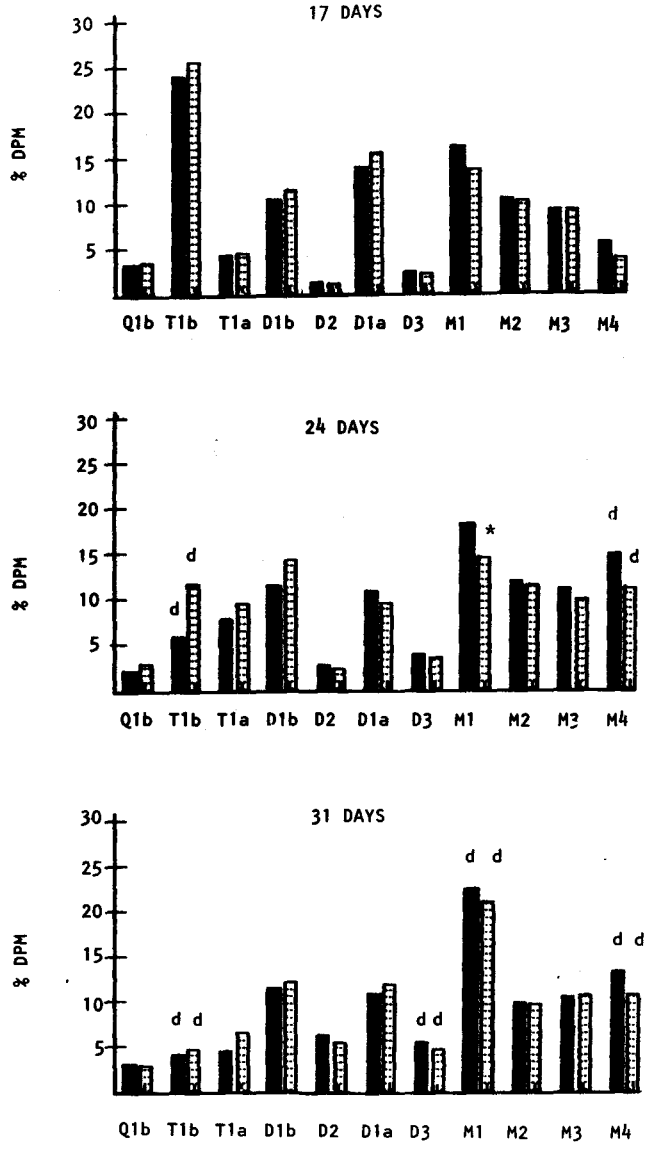
Further studies were conducted to confirm the identity of the band which co-migrated with the G_{M4} standard. In these studies G_{M4} was isolated from radiolabeled myelin gangliosides of 20-day-old rats as described in the Materials and Methods. Before neuraminidase treatment, there was no radioactivity associated with the region of sialic acid and the majority was found in the region of the G_{M4} standard. After neuraminidase treatment, there was loss of radioactivity from the G_{M4} band and a quantitative appearance of radioactivity in the region associated with sialic acid.

Figure 22. Percent Distribution of Radioactivity Among Individual Myelin Gangliosides from Control and Ethanol Offspring at 17, 24 and 31 days of age.

Solid and hatched bars represent control and ethanol offspring respectively.

At all ages, total counts from a single lane ranged between 1,300-5,400 [^{3}H] dpm and 350-2,290 [^{14}C] dpm.

Each value represents the mean of 5-6 control or ethanol animals. Percent dpm is calculated by the formula [(dpm in a ganglioside/dpm recovered from a TLC lane) X 100]. A 'd' indicates that the noted value is significantly different ($p<0.05$) from the value at 17 days. (Standard deviations are given in Table A-2 in the Appendix.)


17 DAYS
% DPM
30
25
20
15
10
5
Q1b T1b T1a D1b D2 D1a D3 M1 M2 M3 M4
24 DAYS
% DPM
30
25
20
15
10
5
d
d
*
d
d
Q1b T1b T1a D1b D2 D1a D3 M1 M2 M3 M4
31 DAYS
% DPM
30
25
20
15
10
5
d d
d d
d d
d d
Q1b T1b T1a D1b D2 D1a D3 M1 M2 M3 M4

Purity of Axolemma

The relative purity of axolemma membrane was assessed by determining the enzymatic activities of two "marker" enzymes and the protein patterns on stained SDS polyacrylamide gels. Axolemma protein patterns were compared to proteins of myelin, synaptic plasma membrane (SPM) and intermediate axolemma (0.8/1.0) membrane fractions. The relative distribution of the "marker" enzymes and protein was also assessed in fractions from the two sucrose gradients (initial and final) used to isolate axolemma-enriched fractions to determine whether axolemma from control (C*-C*) and ethanol (E*-E*) offspring migrated to the same region on the sucrose gradient (i.e. whether the densities of the fractions were similar).

Enzymatic Activities

The source of the fractions depicted in Tables VII and VIII are described in Figures 10 and 11 in the Materials and Methods. Fractions A1, A2 and A3 of the initial gradient are representative of myelin of slightly different densities (A3 > A2 > A1). These fractions were removed prior to centrifugation of the final gradient (thus the final axolemma fractions should have little myelin contamination). Fractions B and D represent the intermediate axolemma and axolemma-enriched fractions, respectively, for both the initial and final gradients.

In comparison with brain homogenates, the specific

Table VII

Distribution of Protein and Enzyme Activity on the Initial Axolemma Subfraction Gradient

Specific Activity (μmole/mg protein/hour)	Fraction		
	Brain	Myelin	Axolemma
CNPase			
Control (C*-C*)	159	1951	172
Ethanol (E*-E*)	140	2483	220*
ATPase			
Control (C*-C*)	2.17	2.67	8.1
Ethanol (E*-E*)	2.34	4.63	8.7

	% Total Protein	RSA		Total Activity			
		CNPase	ATPase	CNPase	%CNPase	ATPase	%ATPase
A1							
C*-C*	67.9	0.98	0.56	12,909	67	17.8	38.5
E*-E*	61.8	1.05	0.84	13,643	65	25.5	52.5
A2							
C*-C*	4.9	2.81	2.08	2,637	13.7	4.7	10.2
E*-E*	4.0*	1.66	1.13	1,389	6.6	2.2*	4.5
A3							
C*-C*	7.4	1.81	2.98	2,582	13.4	10.2	22.0
E*-E*	12.2	1.78	0.99	4,603*	21.9	5.9	12.2
A							
C*-C*	4.6	0.72	1.82	646	3.4	3.9	8.4
E*-E*	4.8	0.74	1.23	754	3.6	2.9	6.0
B							
C*-C*	4.4	0.38	2.58	330	1.7	5.3	11.4
E*-E*	4.9	0.34	2.49	354	1.7	5.9	12.2
C							
C*-C*	1.4	0.13	0.86	37	0.2	0.56	1.2
E*-E*	1.7	0.17	2.1	64*	0.3	1.8*	3.7
D							
C*-C*	4.9	0.08	1.67	82	0.4	3.8	8.2
E*-E*	5.1	0.09	1.72	107*	0.5	4.3	8.8
E							
C*-C*	1.2	0.05	0.03	13	0.1	0.02	0.04
E*-E*	1.4	0.08	0.07	27*	0.1	0.05	0.1
F							
C*-C*	3.3	0.04	0	26	0.1	0	0
E*-E*	3.9	0.05	0	48*	0.2	0	0

An * indicates that the values for (C*-C*) and (E*-E*) for that particular fraction are significantly different at $p < 0.05$. Relative Specific Activity (RSA) is defined as the % of the total enzyme activity recovered in that fraction divided by the % of the total protein recovered in that fraction. Statistics were not performed on RSA values. Each value is the mean of six 29-day-old animals. (C*-C*) and (E*-E*) are abbreviations for control and ethanol respectively. Refer to Figure 10 for identity of fractions.

Table VIII

Distribution of Protein and Enzyme Activity on the Final Axolemma Subfraction Gradient

Specific Activity (μmole/mg protein/hour)	Fraction		
	Brain	Myelin	Axolemma
CNPase			
Control (C*-C*)	212	1524	95
Ethanol (E*-E*)	132	1473	118
ATPase			
Control (C*-C*)	1.91	4.23	6.9
Ethanol (E*-E*)	0.51	3.07	5.8

	% Total Protein	RSA		Total Activity			
		CNPase	ATPase	CNPase	%CNPase	ATPase	%ATPase
A							
C*-C*	7.9	3.47	1.43	65	27.5	0.8	11.3
E*-E*	9.6	3.25	1.71	60	29.6	0.9	16.4
B							
C*-C*	33.1	1.48	1.20	116	49.0	2.8	39.7
E*-E*	29.8*	1.54	1.54	88	43.0	2.5	45.7
C							
C*-C*	19.1	0.70	1.48	32	13.5	2.0	28.4
E*-E*	14.1*	0.62	1.16	17*	8.4	0.9	16.4
D							
C*-C*	18.9	0.40	0.97	18	7.6	1.3	18.4
E*-E*	28.1	0.40	0.71	22	10.8	1.1	20.1
E							
C*-C*	12.8	0.13	0.16	4	1.7	0.15	2.1
E*-E*	12.4*	0.12	0.05	3	1.5	0.04	0.7
F							
C*-C*	8.2	0.08	0	1.7	0.7	0	0
E*-E*	6.0*	0.11	0	1.3	6.0	0.029	0.5

An * indicates that the values for (C*-C*) and (E*-E*) for that particular fraction are significantly different at $p < 0.05$. Relative Specific Activity (RSA) is defined as in Table VII. Statistics were not performed of RSA values. Each value is the mean of six 28-day-old animals. (C*-C*) and (E*-E*) are abbreviations for control and ethanol respectively. Refer to Figure 11 for description of the fractions.

activity of Na^+-K^+-ATPase was increased 4 times (significant at $p < 0.05$) in the axolemma-enriched fractions from the initial gradient (fraction D, Table VII) and CNPase activity was increased 1 to 2 times (significant at $p < 0.05$ for E*-E* only). In the final gradient (Table VIII), the specific activity of Na^+-K^+-ATPase remained high (3.6 times) ($p < 0.05$ compared to homogenate) in the purified axolemma fraction while CNPase specific activity was less than that in whole brain homogenate ($p < 0.05$ for C*-C* only). The CNPase data suggests that there was very little myelin contamination in the final axolemma-enriched fraction.

Myelin isolated from the initial gradient (primarily in fraction A1, Table VII) demonstrated a 12-18 fold enrichment in specific activity of CNPase over that of whole brain ($p < 0.01$) and a minor (1.2-2.0 fold) enrichment in ATPase (non-significant). Purified myelin from C*-C* and E*-E* pups demonstrated similar activities (Table VIII) on the final gradient with the exception of ATPase enrichment in myelin of E*-E* offspring.

There were some differences in the total activities of both enzymes and distribution of protein in fractions from the gradients from C*-C* and E*-E* pups, but these did not appear to affect the axolemma fraction of the final gradient. On the initial gradient, however, the total CNPase activity was increased in fraction D, the axolemma-

enriched fraction, from the ethanol offspring. Normal CNPase activity was seen in this fraction on the final gradient.

It is interesting that the E*-E* pups had an increase in the total activity of CNPase in fraction A3 (4,600 +/- 800 units in E*-E* compared to 2,600 +/- 550 units in C*-C*) which corresponds to a "heavier" myelin fraction. This correlates well with previous work from our laboratory demonstrating that myelin from developing ethanol pups has a greater amount of a heavier immature myelin fraction (Druse and Hofteig, 1977).

Protein Patterns

Proteins from ether-ethanol (3:2, v/v) delipidated myelin, axolemma-enriched fraction, intermediate (0.8/1.0) axolemma, and synaptic plasma membranes were electrophoresed on 7.5% acrylamide, 0.1% SDS slab gels. Gels were stained with 0.1% Coomassie Blue and densitometrically scanned.

Figure 23 is a photograph of a gel that was loaded with proteins from various membrane fractions. Molecular weight estimates were determined as described in the Materials and Methods. The low molecular weight basic proteins distinguish the lanes loaded with myelin (lanes a1,a2). The intensely staining band at ~52 K MW on the myelin lanes corresponds to the Wolfgram proteins. (Protein patterns of myelin obtained as a byproduct from the

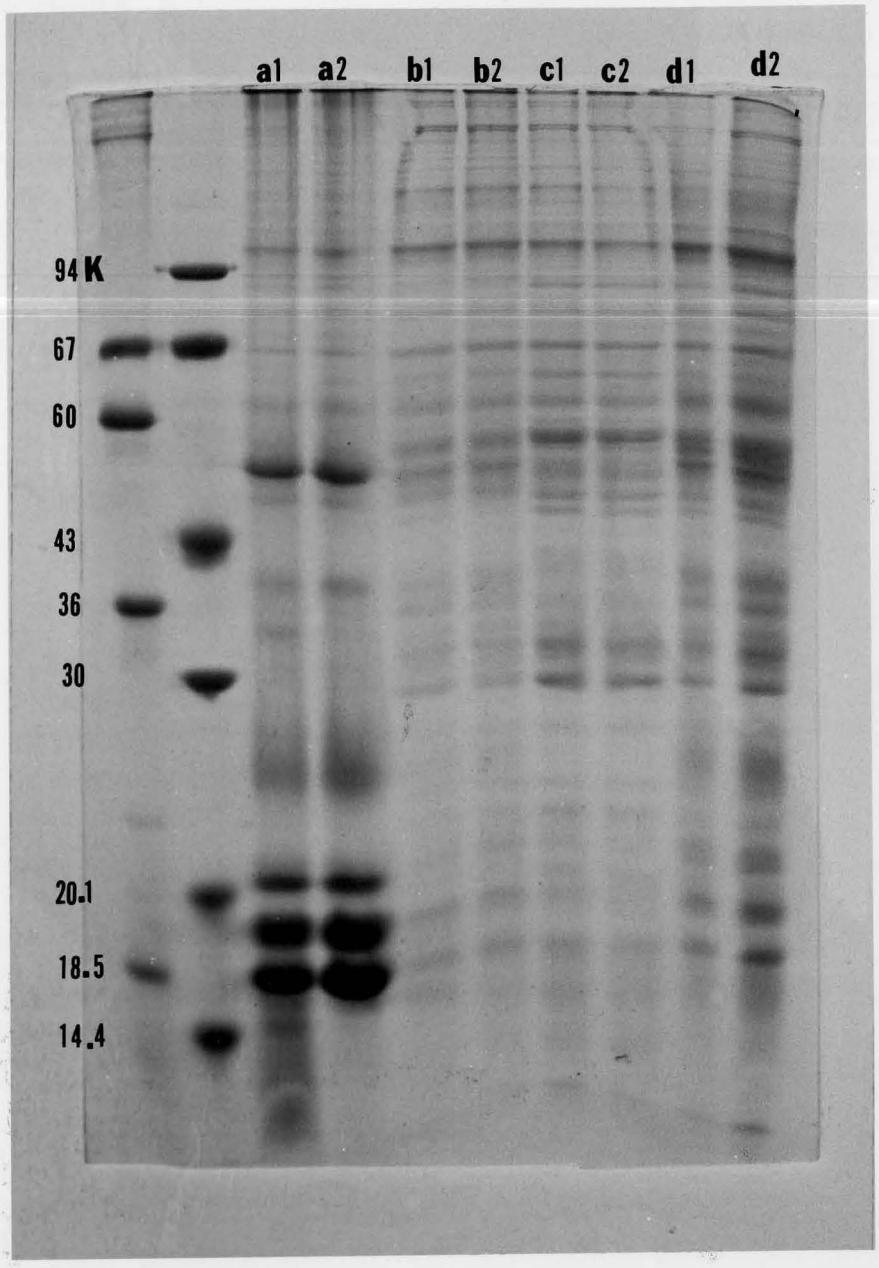


Figure 23. SDS-Polyacrylamide Gel (7.5%) of Myelin (a1,2), 0.8/1.0 Axolemma (b1,2), 1.0/1.2 Axolemma (c1,2), SPM (d1,2) and High and Low Molecular Weight Standard Proteins (lanes 1 and 2).

Axolemma, SPM and myelin lanes contain 100 µg, 150 µg and 400 µg respectively of protein. SPM was isolated from 39-day-old rats while myelin and axolemma were isolated from 21-day-old animals.

axolemma procedure showed similar patterns to those found in myelin isolated by Norton and Poduslo (1973b).) The lower MW Wolfgram protein is minor in myelin but a prominent component in the other fractions. Figure 24 is a set of densitometric tracings from the various lanes. Whereas the myelin proteins (proteolipid, small and large basic proteins) accounted for 69% of the total dye binding capactiy in myelin (lanes a1,a2), proteins of comparable R_f values in the 0.8/1.0 axolemma (lanes b1,b2), 1.0/1.2 axolemma (lanes c1,c2) and SPM (lanes d1,d2) accounted for 12%, 6.5% and 11.6% of the total proteins, respectively. Wolfgram proteins were not included in this analysis since one of the proteins in the Wolfgram doublet is not thought to be specific to myelin. The lower band of the Wolfgram doublet corresponds to tubulin or a tubulin-like protein (51-53 K) (Reig et al., 1982) which would be expected to be enriched in membrane fractions. Major bands in the axolemma-enriched (1.0/1.2) fraction include a triplet of proteins of ~55 K, ~52 K and ~49 K MW that account for 15%, 9%, and 8%, respectively, of the total dye binding capacity, and a doublet of proteins of MW ~35 K and ~32 K that account for 12% and 8%, respectively, of dye binding capacity. The less dense axolemma (0.8/1.0) fraction also contains two major bands at ~55 K and ~52 K MW corresponding to 12% and 17%, respectively, of the total. The 0.8/1.0 fraction also has a major band at ~100,000 MW (10%

Figure 24. Densitometric Tracings of the Slab Gel of Figure 23.

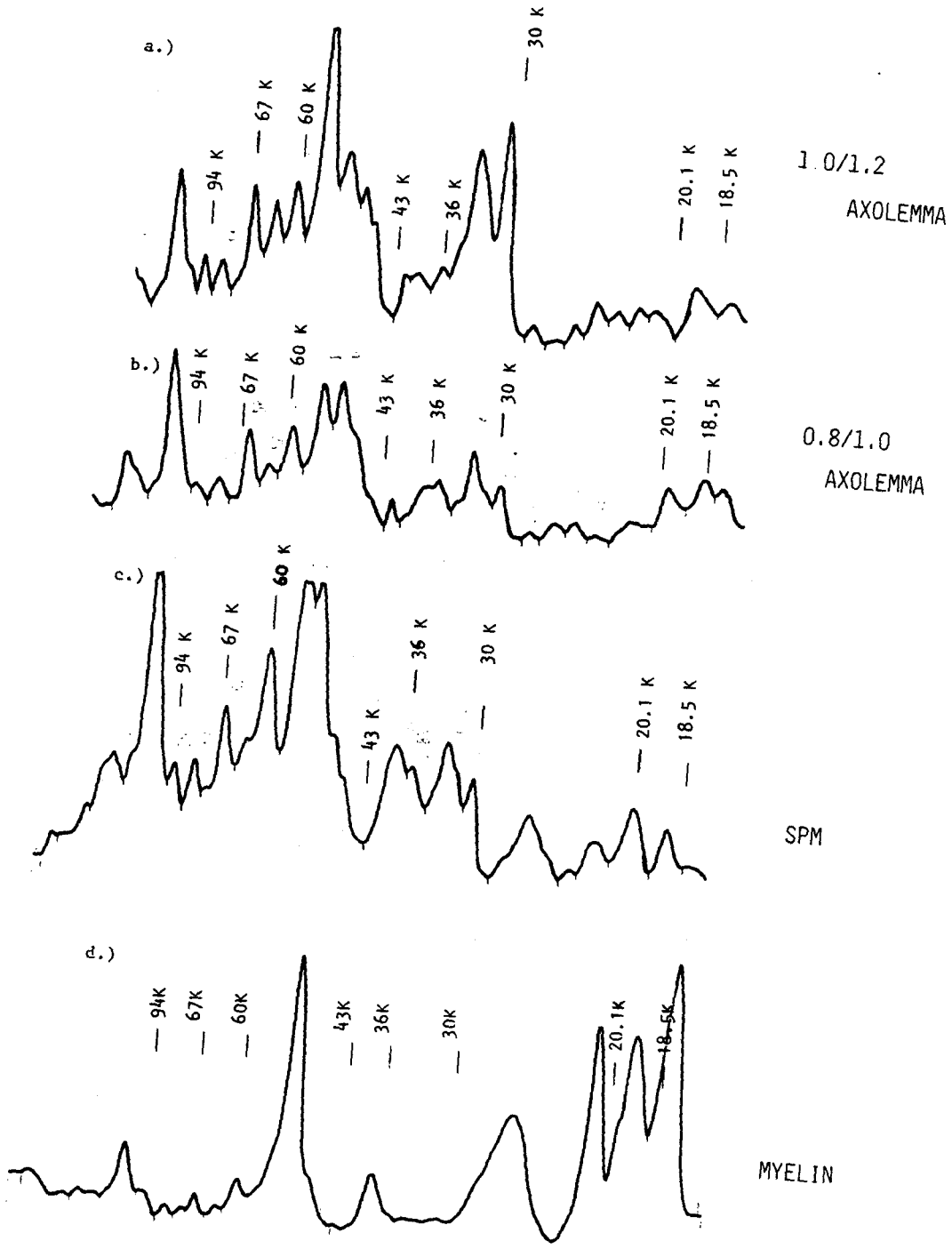
Coomassie blue stained gels were scanned at a wavelength of 520 nm.

a). Scan of lane loaded with 100 µg of 1.0/1.2 axolemma protein from a 21-day-old rat (lane c-1).

b). Scan of lane loaded with 100 µg of 0.8/1.0 axolemma protein from 21-day-old rat (lane b-1).

c). Scan of lane loaded with 150 µg of SPM protein from a 39-day-old animal (lane d-1).

d). Scan of lane loaded with 400 µg of myelin protein from a 21-day-old rat (lane a-1).


a.)
94 K
67 K
60 K
30 K
43 K
36 K
20.1 K
18.5 K
1.0/1.2
AXOLEMMA
b.)
94 K
67 K
60 K
43 K
36 K
30 K
20.1 K
18.5 K
0.8/1.0
AXOLEMMA
c.)
94 K
67 K
60 K
43 K
36 K
30 K
20.1 K
18.5 K
SPM
d.)
94K
67K
60K
43K
36K
30K
20.1K
18.5K
MYELIN

of total) and two bands at ~40 K and ~35 K (7% and 8%, respectively, of total areas).

Major bands in SPM are found at the same molecular weights as those seen in axolemma. However, the distribution of proteins is distinct in the two fractions. There are major bands are at ~100 K (12%), ~60 K (10%), ~55 K (12%) and ~52 K (12%). These data are summarized in Table IX.

Axolemma Proteins

The yield of axolemma-enriched fractions (i.e. protein) is presented in Figure 25. With the exception of an increased yield of the 1.0/1.2 fraction in E*-E* pups at 20 days of age, the yield of axolemma from C*-C* and E*-E* pups was comparable. It should be noted that the axolemma isolation procedure was not designed for a quantitative recovery but rather for purity. However, similar yields at the latter ages in the 1.0/1.2 fraction suggest comparable gross axolemma development.

Delipidated proteins from the axolemma-enriched fraction (1.0/1.2) from 22-, 31- and 32-day-old animals were loaded onto 3 mm thick, 7.5% acrylamide, 0.1% SDS slab gels. After electrophoresis and staining with Coomassie Blue dye, gels were scanned using a Schoeffel spectrodensitometer to quantitate the % dye binding capacity of individual protein bands. As has been previously noted, five major bands in axolemma include three in the molecular

Table IX

% Dye Binding Capacity of Proteins in Myelin, Axolemma-Enriched, 0.8/1.0 Axolemma, and SPM Membranes

Apparent Molecular Weight (dalton)	Fraction			
	Myelin	0.8/1.0 Axo	1.0/1.2 Axo	SPM
100,000	3.0	10.0	6.0	12.5
70,000	--	5.4	5.6	7.0
60,000	1.9	8.0	6.0	10.0
55,000	--	12.0	15.2	12.5
52,000	19.1	16.7	8.6	12.5
49,000	--	--	7.9	--
42,000	--	7.4	4.8	3.9
35,000	--	7.6	12.6	7.3
32,000	1.2	3.1	8.3	2.9
26,000	16.3	1.2	0.5	2.6
20,000	10.8	2.7	2.0	2.8
18,000	18.2	4.9	3.2	2.6
16,000	23.6	1.5	1.5	0.5

Apparent molecular weights of proteins were determined by comparison to protein standards of known molecular weights. The values were determined from the gel in Figure 23, of 21-day-old offspring. Statistical analysis was not performed (the mean of two values is shown). Dashes indicate there was no detection of that MW band.

Figure 25

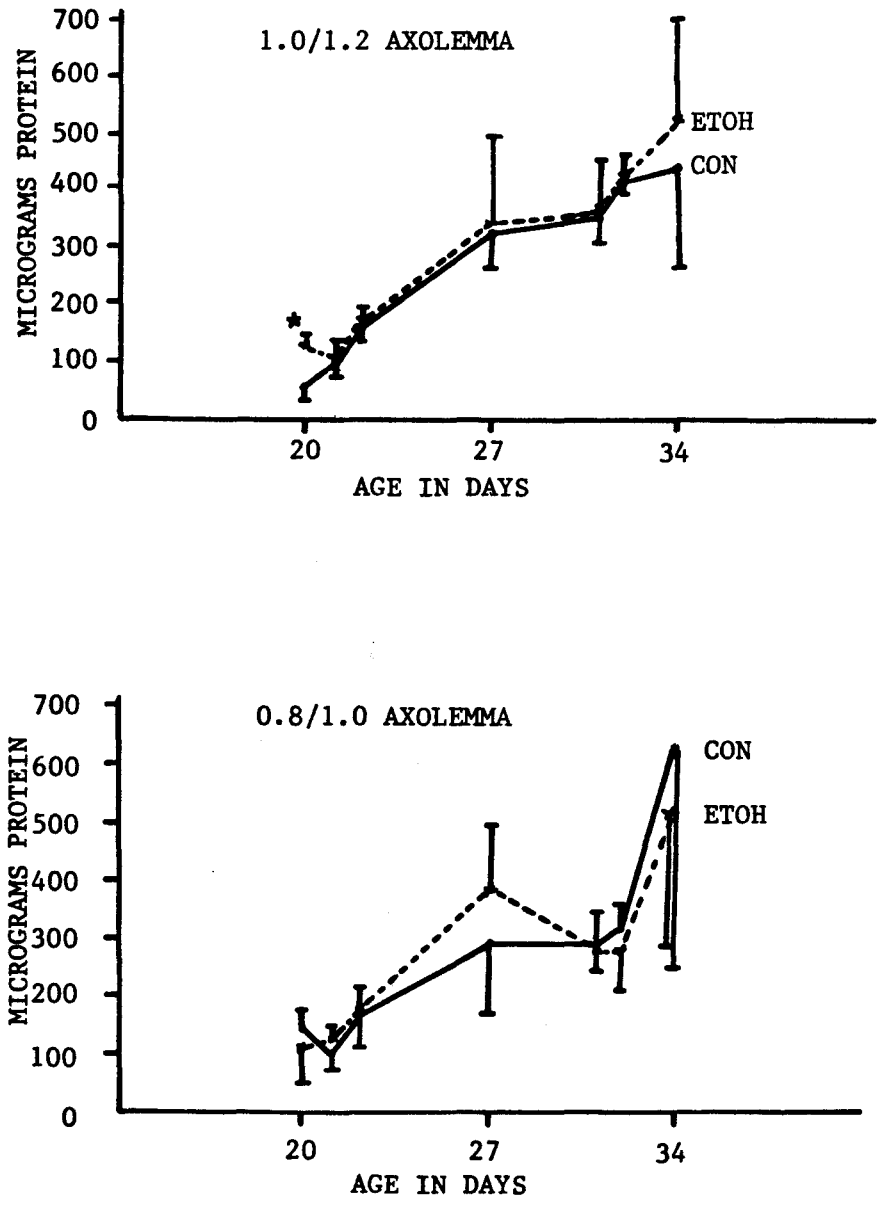


Figure 25. Total Protein Recovered from Isolated Axolemma Fractions from Control (C*-C*) and Ethanol (E*-E*) Offspring Between 20 and 34 Days of Age.

Each point represents the average of 3 to 6 animals from 1 to 2 litters.

A * indicates a statistically significant difference ($p<.05$) between C*-C* and E*-E*.

weight range between 48 K and 58 K, and two in the region between 30 K and 37 K. There are approximately 50 bands visible on the gel, however, only 20 to 30 bands were resolved by the scanning densitometer. The 15 major bands were quantitated by the Hewlett-Packard integrator. The relative distribution of the dye binding capacity of the individual bands is depicted in Figure 24a. Figures 26, 27 and 28 are pictures of the slab gels that were used to quantitate axolemma proteins. Table X is a tabular summary of the densitometric data from these gels.

When the distribution of axolemma proteins from C*-C* and E*-E* rats was compared, it appeared that there was a consistent decrease in the proportion of the 150 K protein (significant at 22 and 31 days) in the E offspring. In addition, there were transient alterations in the 100 K protein (decreased at 22 day). No changes were seen in the major protein band at ~55 K (possibly corresponding to tubulin, Reig et al., 1982).

Several significant developmental changes were seen in the distribution of axolemma proteins. There was a significant decrease in the amount of the 150 K and 100 K proteins between 22 and 31 days (8.2% to 4.4% and 11.3% to 7.3%, respectively). In addition, the proportion of the 65 K band protein decreased between 22 and 32 days.

Axolemma Glycoproteins

The gels in Figures 23, 26, 27 and 28 were sliced

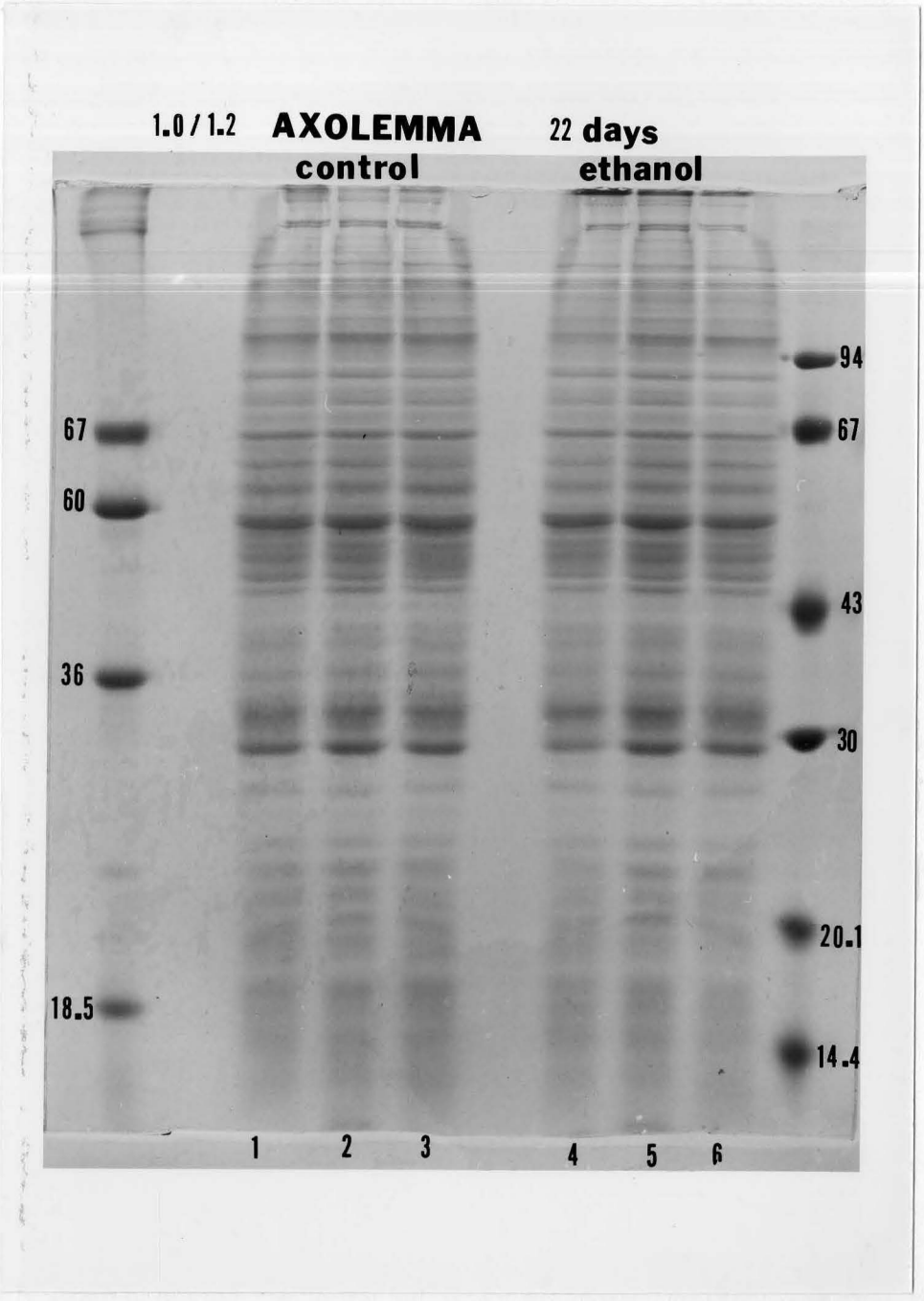


Figure 26. Axolemma Proteins from 22-Day-Old Animals.

Sample lanes 1, 2 and 3 were loaded with samples from control offspring while lanes 4, 5 and 6 were loaded with proteins from ethanol offspring. Approximately 160 µg of protein was loaded on each lane.

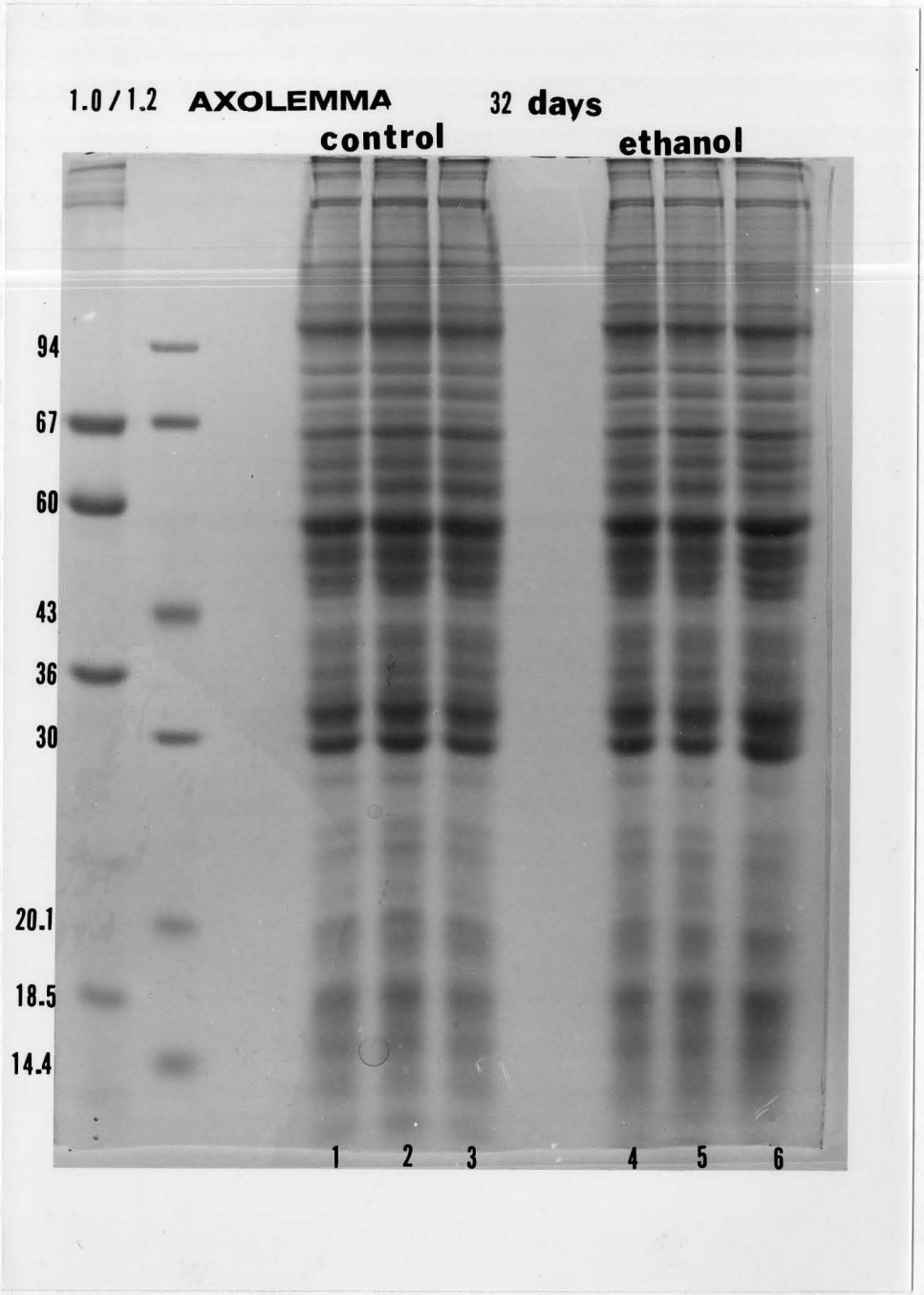


Figure 27. Axolemma Proteins from 32-Day-Old Animals.

Sample lanes 1, 2 and 3 were loaded with samples from control offspring while lanes 4, 5 and 6 were loaded with proteins from ethanol offspring. Approximately 300 µg of protein was loaded on each lane.

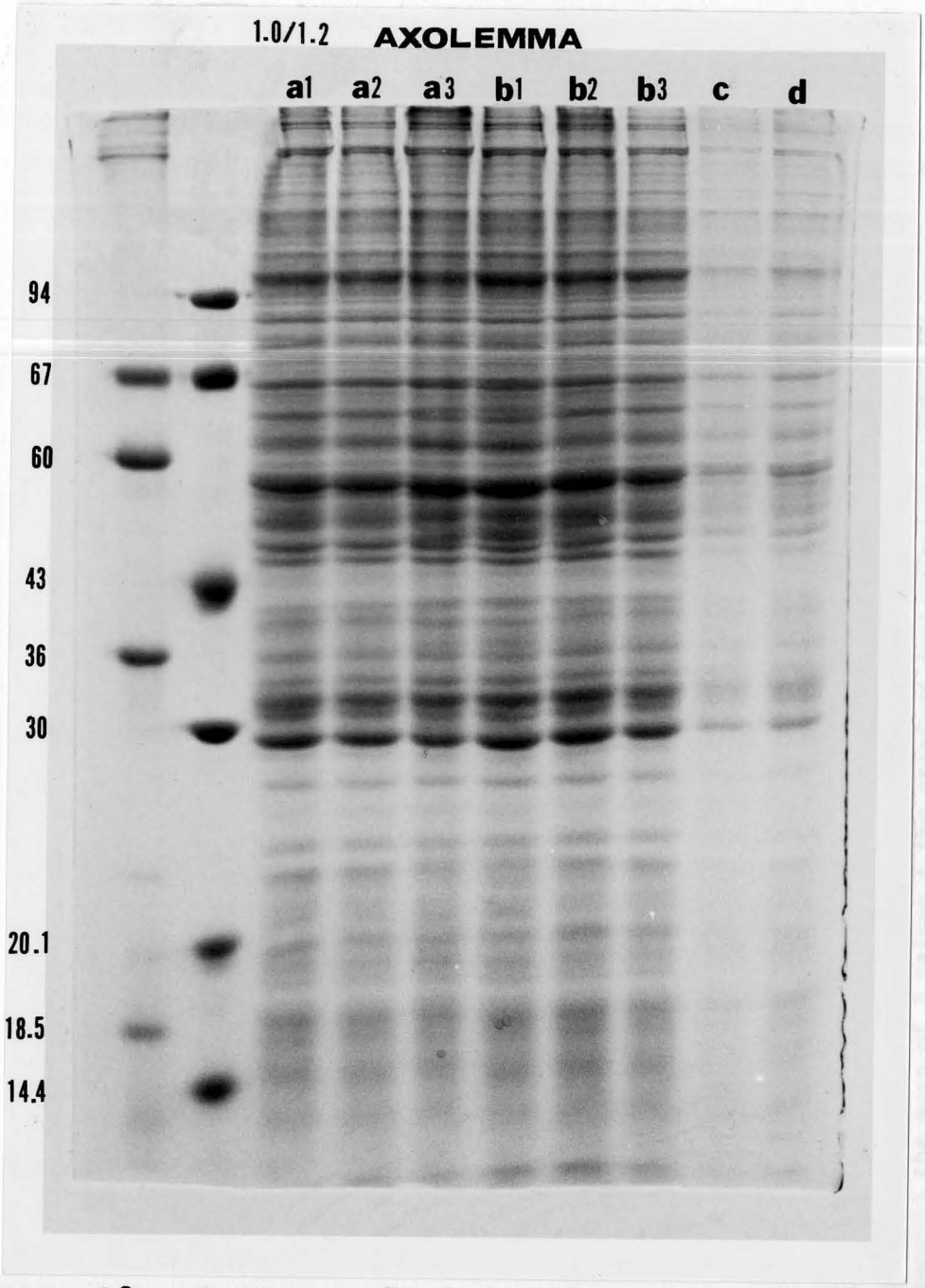


Figure 28. Axolemma Proteins from 21- and 31-Day-Old Animals.

Sample lanes a1, a2 and a3 were loaded with samples from 31-day-old control while lanes b1, b2 and b3 were loaded with samples from 31-day-old ethanol. Lane c was loaded with a sample from a 21-day-old control and lane d was loaded with a sample from a 21-day-old ethanol. Approximately 350 µg of protein were loaded on lanes a1-3 and b1-3 while 100 µg were applied to lanes c and d.

Table X

Percent Dye Binding Capacity of 1.0/1.2 Axolemma Proteins

Apparent Molecular Weight (kDalton)	22 d		31 d		32 d	
	C *-C*	E *-E*	C *-C*	E *-E*	C *-C*	E*-E *
>200	2.0±0.2	1.3 #	3.4±1.8	1.8±0.6	2.4±0.2	1.6±0.5
150	8.2±0.6	3.4±1.2*	4.4±0.2†	3.0±0.5*	4.3±0.6°	3.5±0.6
100	11.3±1.1	8.4±1.0*	7.3±0.8†	7.0±0.9	9.4±0.1°	8.2±1.1
85	3.0±0.5	2.6±0.5	2.0±0.2	1.9±0.1	2.1±0.2	1.8±0.3
76	3.5±0.3	3.0±0.6	2.4±0.2	2.3±0.3	2.4±0.2	2.2±0.5
70	7.8±1.7	6.7±1.7	5.9±1.1	6.4±0.1	6.0±0.3	6.2±0.5
65	5.7±0.7	4.8±0.7	4.1±0.3°	4.5±0.4	4.3±0.1°	4.2±0.5
60	6.8±1.0	5.9±0.8	5.7±0.8	5.6±1.2	5.2±0.8	4.7±1.0
55	15.4±1.9	17.0±1.9	12.5±0.4	12.8±1.4	12.8±0.2	12.0±0.8
52			6.8±0.5	7.3±1.8	6.9±0.8	8.0±0.6
49	[13.6±2.2]	[16.9±0.3]	7.7±0.5	8.7±1.4	6.9±0.2	6.9±0.5
42	4.0±0.1	3.8±0.8	3.6±1.2	3.3±1.3	4.2±0.2	4.3±1.0
38	3.2±0.2	3.7±0.8	3.2±0.3	3.7±0.2	3.2±0.1	3.2±0.6
35	8.6±2.8	10.7±1.7	11.5±0.4	12.5±0.7	10.3±0.4	10.7±1.4
32	4.6±1.8	6.3±2.4	6.7±0.9	7.7±0.9	6.9±0.8	7.9±1.6

Each value represents the mean of 3 values ± the standard deviation. A # indicates there were not enough samples to determine standard deviations. An * indicates that control (C*-C*) and ethanol (E*-E*) values were statistically different ($p < 0.05$) A ° indicates that there was a significant ($p < 0.05$) developmental change (between 22 days and 31/32 days). A † indicates that the developmental change was significant at $p < 0.01$.

according to bands for quantitation of [^{3}H]-fucose radioactivity in axolemma (1.0/1.2) glycoproteins. It is important to note that this study utilized one isotope only, not the dual label isotope methodology previously used.

Figure 29 depicts the distribution of radioactivity among fucosylated axolemma glycoproteins from 21-day-old control (C*-C*) and ethanol (E*-E*) offspring. Each graph represents one pair of animals (both labeled with [^{3}H]). At this particular age a single C and E pair was separated in separate lanes on a single slab gel. Therefore, results from each gel are shown. Data for each band, from separate gels, were not averaged due to slight differences in the migration of proteins on the different gels. However the distribution of radioactivity in molecular weight regions from different gels was averaged (Table XI). There were no apparent significant differences in the distribution of fucose radioactivity among axolemma glycoproteins from C*-C* and E*-E* pups. Data from each fraction is shown in Table A-3 of the Appendix.

Figure 30 depicts the distribution of radioactivity among fucosylated glycoproteins at 22, 31 and 32 days of age. Each graph represents the mean of three control and three ethanol offspring. All samples from one age were run on one gel. Tabular representation of this data can be found in Table A-4 (Appendix).

Developmental changes in incorporation of radio-

Figure 29. Distribution of Radioactivity Among Fucosylated Axolemma Glycoproteins from 21-Day-Old Control (C*-C*) and Ethanol (E*-E*) Pups.

a). Total counts for control (C*-C*) and ethanol (E*-E*) lanes were 4,400 dpm and 4,000 dpm, respectively.

b). Total counts for C*-C* and E*-E* were 2,200 dpm and 3,600 dpm, respectively.

c). Total counts for C*-C* and E*-E* were 3,900 dpm and 5,900 dpm, respectively.

Each graph represents one pair of animals from one gel.

See Table A-3 (Appendix) for tabular representation of this data.

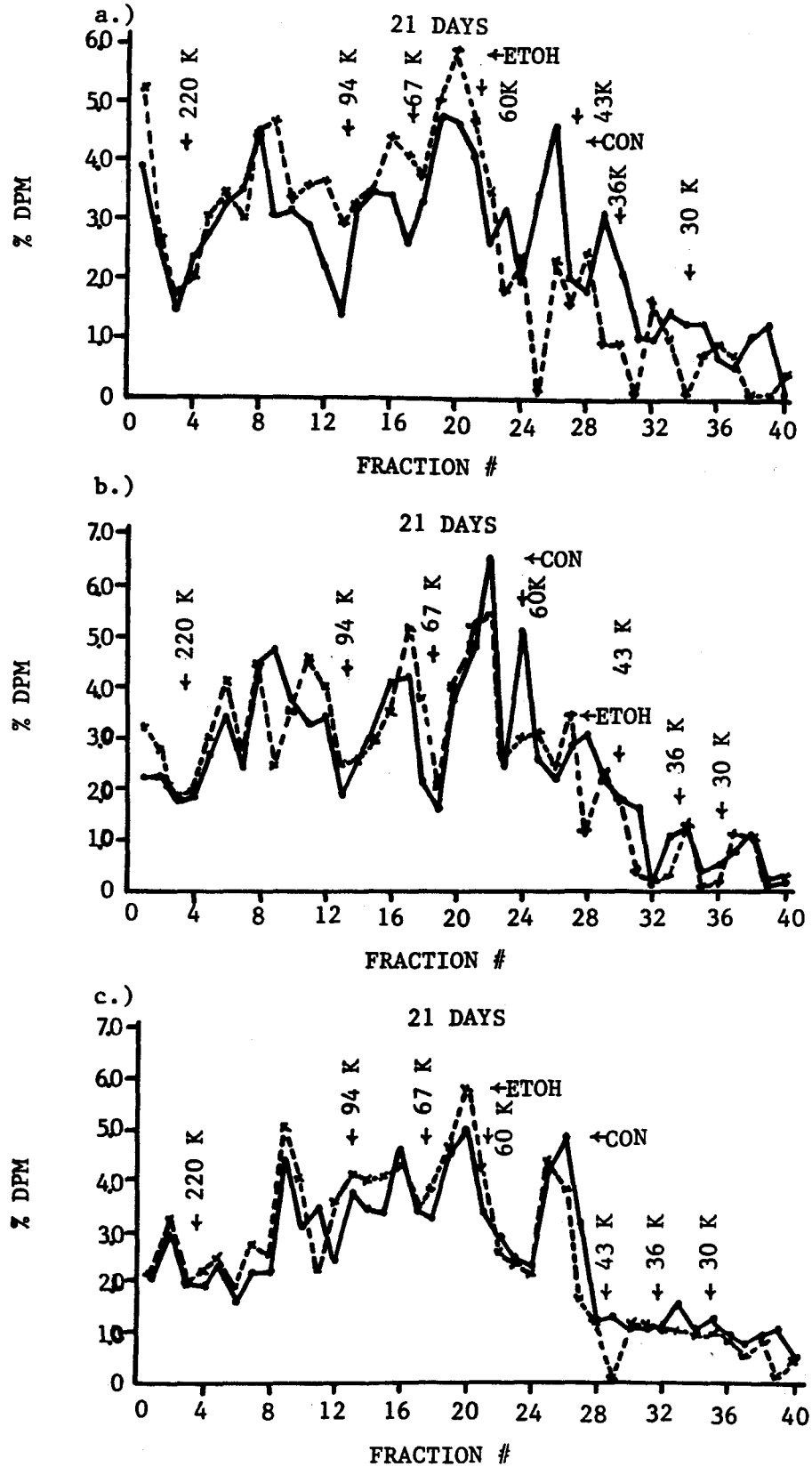

a.)
21 DAYS
+ 220 K
+ 94 K
+67 K
+ETOH
+ 60K
+ 43K
+CON
+36K
+ 30 K
% DPM
FRACTION #
b.)
21 DAYS
+ 220 K
+ 94 K
+ 67 K
+CON
60K
43 K
+ETOH
+ 36 K
+ 30 K
% DPM
FRACTION #
c.)
21 DAYS
+ 220 K
+ 94 K
+ 67 K
+ETOH
60 K
+CON
+ 43 K
+ 36 K
+ 30 K
% DPM
FRACTION #

Table XI

% Distribution of Radioactivity Among Fucosylated Axolemma Glycoproteins from Molecular Weight Regions

21 Days	Molecular Weight Region (in Kdalton)						
	> 220	100-220	67-100	60-67	43-60	30-43	< 30
C*-C*							
Mean	9.0	22.3	18.8	18.2	19.5	8.5	3.7
S.D.	1.1	2.6	2.5	3.0	1.7	3.2	1.4
E*-E*							
Mean	10.2	23.8	22.0	20.3	15.5	5.1	2.6
S.D.	1.2	2.5	1.6	2.4	3.5	2.6	.2

The mean +/- the standard deviation from the regions of three gels are shown. The boundaries of each region were determined by the migration of molecular weight markers on each gel (see Table A-3 in the Appendix). The values for each region represent the sum of each slice in that region. There were no significant differences between mean C*-C* and mean E*-E* values.

Figure 30. Distribution of Radioactivity Among Fucosylated Axolemma Glycoproteins from 22-, 31- and 32-Day-Old Control (C*-C*) and Ethanol (E*-E*) Offspring.

Each graph represents the mean of 3 control and 3 ethanol offspring, electrophoresed on individual lanes of the same gel.

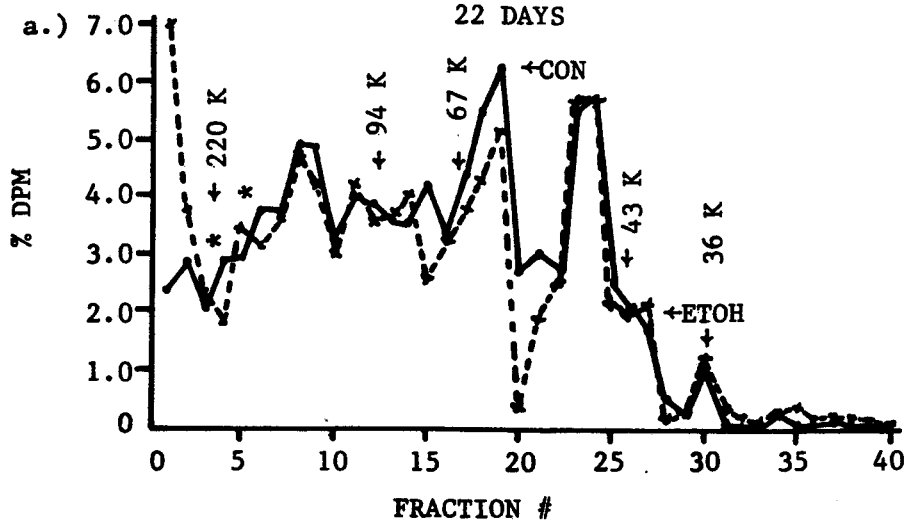
a). Average total counts found in a control lane was 7,300 dpm while in an ethanol lane was 6,100 dpm.

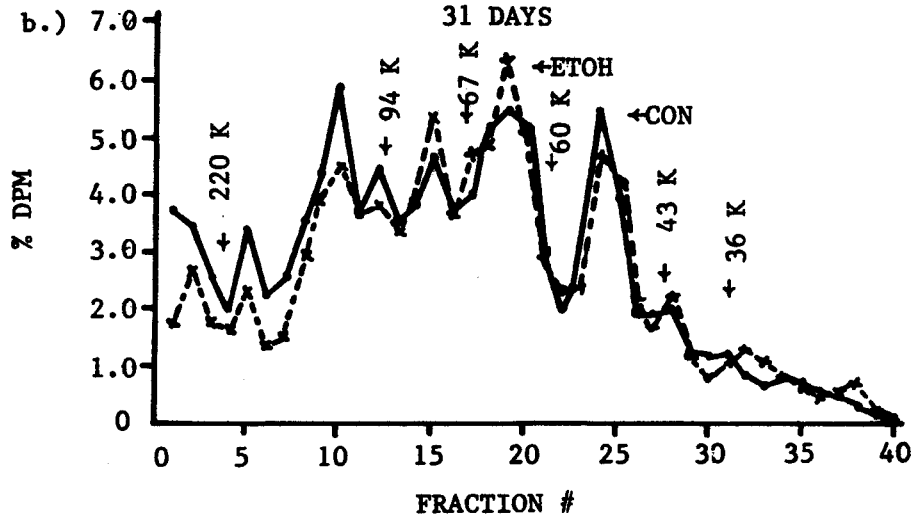
b). Average total counts found in both control and ethanol lanes was 3,900 dpm.

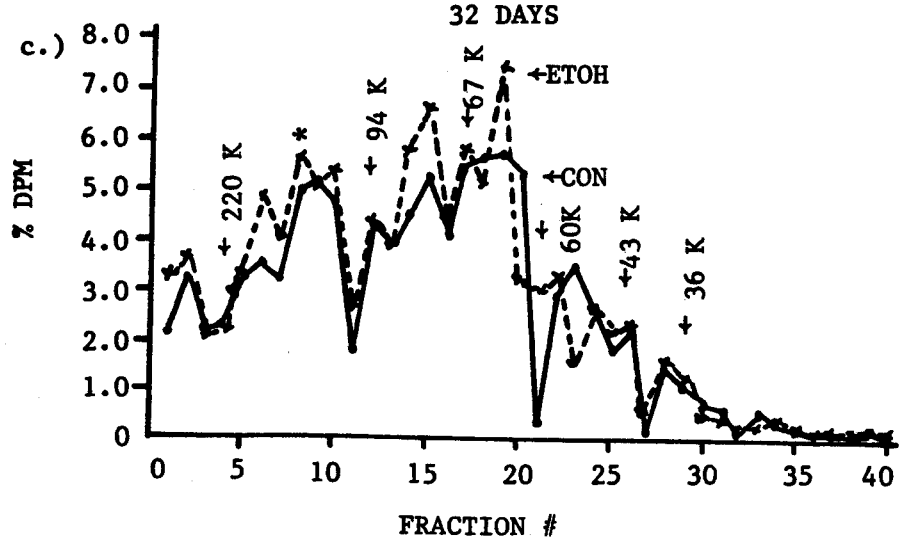
c). Average total counts found in a control lane was 7,500 dpm while in an ethanol lane was 3,100 dpm.

Statistically significant differences between C*-C* and E*-E* are marked with an *.

See Table A-4 (Appendix) for standard deviations.


a.)
22 DAYS
% DPM
FRACTION #
+ 220 K
+ 94 K
+ 67 K
+CON
+ 43 K
36 K
+ETOH


b.)
31 DAYS
% DPM
FRACTION #
+ 220 K
+ 94 K
+67 K
+ETOH
+60 K
+CON
+ 43 K
+ 36 K


c.)
32 DAYS
% DPM
FRACTION #
+ 220 K
+ 94 K
+67 K
+ETOH
+CON
+ 60K
+43 K
+ 36 K

labeled fucose between 22 and 31/32 days of age include reductions in glycoproteins of 110 K, 55 K and 52 K and increases in those of 120 K, 85 K, 67 K, 62 K and 61 K. There were differences between C*-C* and E*-E* pups in incorporation of label into several glycoproteins. At 22 days of age, the E*-E* pups had a decreased proportion of radioactivity associated with glycoproteins of molecular weights ~220 K, ~180 K and ~61 K. Although there were no significant differences between 31 day C*-C* and E*-E* offspring, there were minor alterations detected in 32 day E*-E* pups (increased incorporation into ~150 K, ~85 K and ~76 K glycoproteins and decreased incorporation into a ~62 K glycoprotein).

Axolemma Gangliosides

Gangliosides were isolated from N-acetylmannosamine radiolabeled axolemma-enriched fractions. The relative distribution of radioactivity among axolemma gangliosides is shown in Figures 31 and 32.

There were statistically significant ($p<0.05$) normal developmental changes seen in offspring between 20 and 34 days of age. In both fractions, (0.8/1.0 and 1.0/1.2 axolemma), G_{D1a} decreased from 30-33% to 21-25% and G_{T1b} increased from 20-26% to 33-34%. The proportion of G_{D1b} decreased during development, but the change was significant ($p < 0.05$) in the 0.8/1.0 fraction only. There was also an apparent increase in G_{Q1b} (from ~5% to ~10%) in

Figure 31. Distribution of Radioactivity Among Radiolabeled 0.8/1.0 Axolemma Ganglioside Fractions in 20-, 27- and 34-Day-Old Control (C*-C*) and Ethanol (E*-E*) Offspring.

Solid bars represent the mean of 6 control animals, while hatched bars are the mean of 6 ethanol animals.

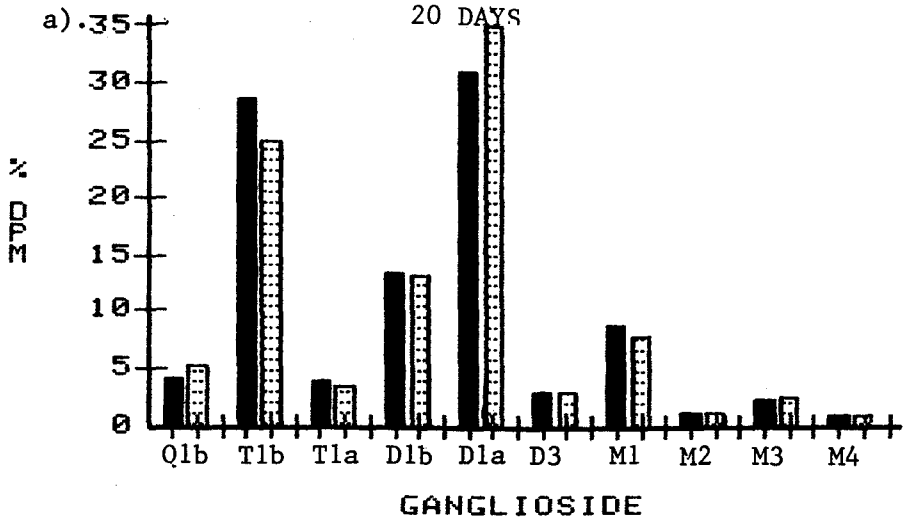
There were no significant differences seen between control and ethanol at any age. Significant ($p < 0.05$) developmental trends (compared to 20 days) are noted by a 'd'.

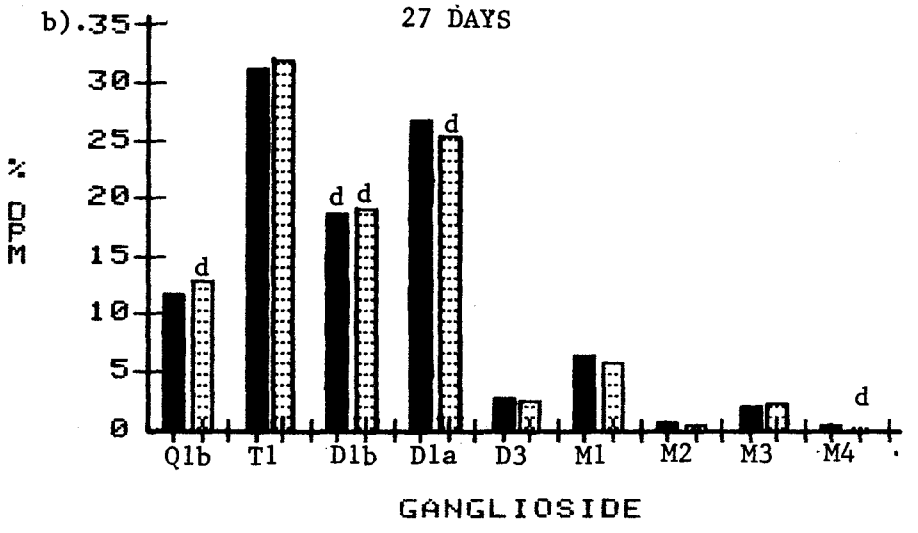
a). Total average dpm recovered from a TLC lane was 800 ([^{3}H]) and 300 ([^{14}C]) for 20-day-old animals.

b). Total average dpm recovered from a TLC lane was 2500 ([^{3}H]) and 700 ([^{14}C]) for 27-day-old animals.

c). Total average dpm recovered from a TLC lane was 2600 ([^{3}H]) and 1700 ([^{14}C]) for 34-day-old animals.

See Table A-5 (Appendix) for standard deviations.


a).35
20 DAYS
MPD %
30
25
20
15
10
5
0
Q1b
T1b
T1a
D1b
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE


b).35
27 DAYS
MPD %
30
25
20
15
10
5
0
d
d d
d
d
Q1b
T1
D1b
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE

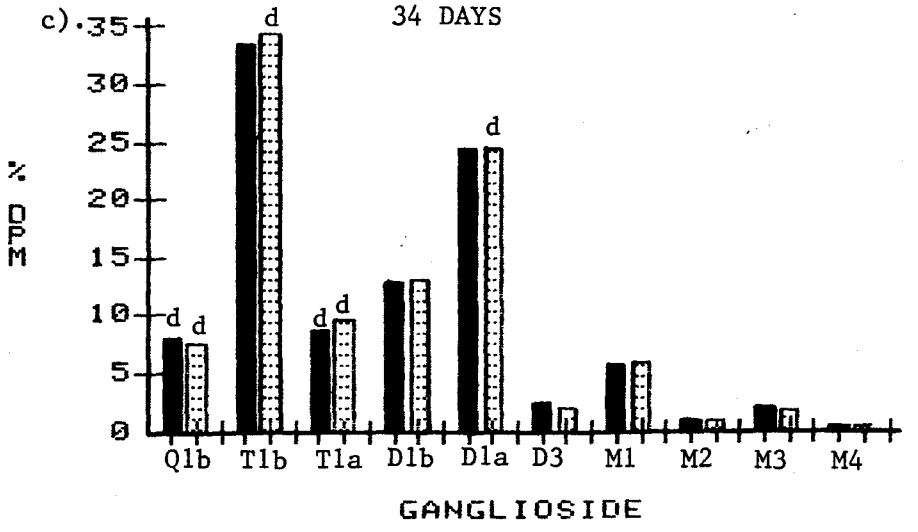

c).35
34 DAYS
MPD %
30
25
20
15
10
5
0
d
d d
d d
d
d
Q1b
T1b
T1a
D1b
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE

Figure 32. Distribution of Radioactivity Among Radiolabeled 1.0/1.2 Axolemma Gangliosides from 20-, 27- and 34-Day-Old Control (C*-C*) and Ethanol (E*-E*) Offspring.

Solid bars represent the mean of 6 control animals, while hatched bars represent the mean of 6 ethanol animals.

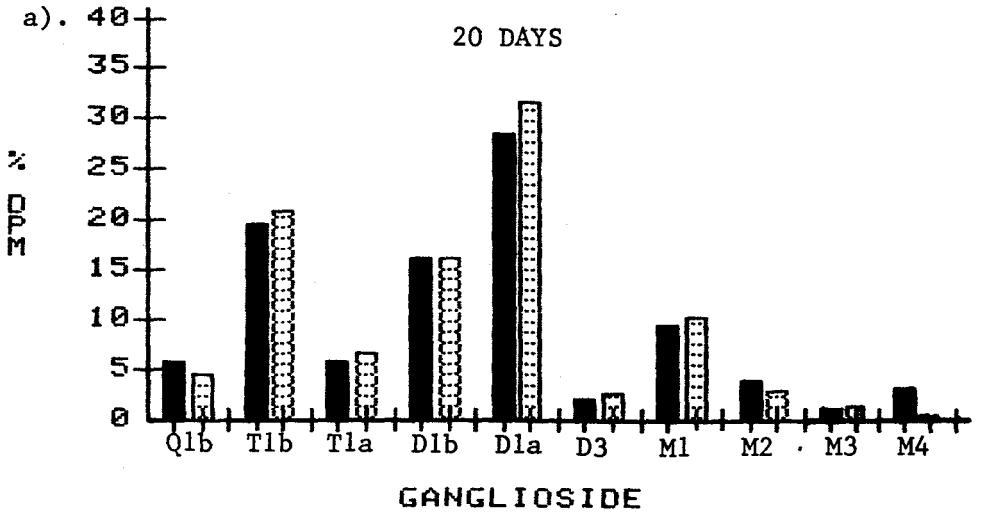
There were no significant differences seen between control and ethanol animals. Developmental differences ($p < 0.05$ when compared to 20 days) are noted by a 'd'.

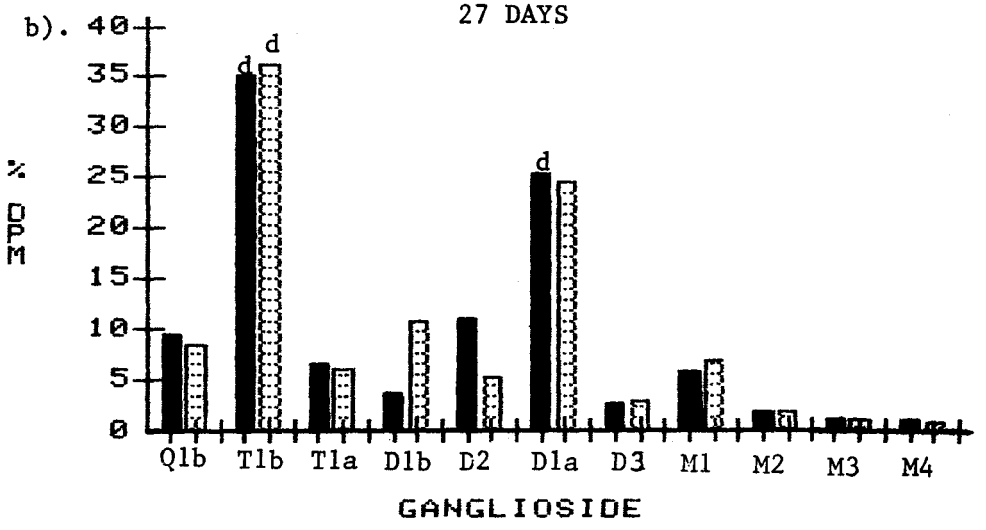
a). Total average dpm recovered from a TLC lane was 310 ([^{3}H]) and 210 ([^{14}C]) 20-day-old animals.

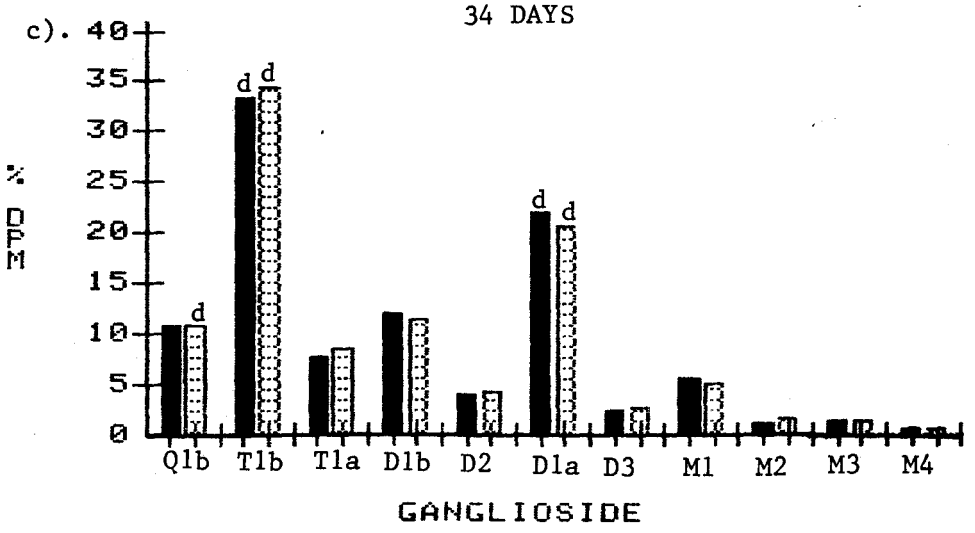
b). Total average dpm recovered from a TLC lane was 1300 ([^{3}H]) and 500 ([^{14}C]) for 27-day-old animals.

c). Total average dpm recovered from a TLC lane was 1900 ([^{3}H]) and 600 ([^{14}C]) for 34-day-old animals.

See Table A-6 (Appendix) for standard deviations.


a).
20 DAYS
% DPM
40
35
30
25
20
15
10
5
0
Q1b
T1b
T1a
D1b
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE


b).
27 DAYS
% DPM
40
35
30
25
20
15
10
5
0
d
Q1b
T1b
T1a
D1b
D2
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE


c).
34 DAYS
% DPM
40
35
30
25
20
15
10
5
0
d
Q1b
T1b
T1a
D1b
D2
D1a
D3
M1
M2
M3
M4
GANGLIOSIDE

both fractions; however, the change was not statistically significant in the 1.0/1.2 fraction from controls. A significant developmental change was noted in the minor monosialoganglioside G_{M4} (a decrease) in the 0.8/1.0 axolemma fraction from ethanol offspring only. Figure 33 shows these developmental trends in 0.8/1.0 axolemma fractions from control offspring while Figure 34 depicts developmental trends in 1.0/1.2 axolemma of control offspring. In general, ethanol and control offspring exhibited similar trends. Figure 35 is a photograph of 0.8/1.0 axolemma gangliosides and standard gangliosides.

No significant differences between C*-C* and E*-E* were found in the distribution of radioactivity among axolemma gangliosides from either the 0.8/1.0 or 1.0/1.2 axolemma fractions.

Axolemma Phospholipids

Phospholipids from control (C*-C*) and ethanol (E*-E*) offspring were extracted from 1.0/1.2 axolemma fractions radiolabeled with [^{3}H]- and [^{14}C]-glycerol.

Samples were analyzed for total phospholipid and distribution of radioactivity among the phospholipid classes. Table XII summarizes the total phospholipid data. Highest values for μg phospholipid/μg protein were found at the earliest age point (20 days). At 27 and 34 days of age, values were comparable and both were significantly lower ($p<0.05$) than the 20 day values. At all ages, the

Figure 33

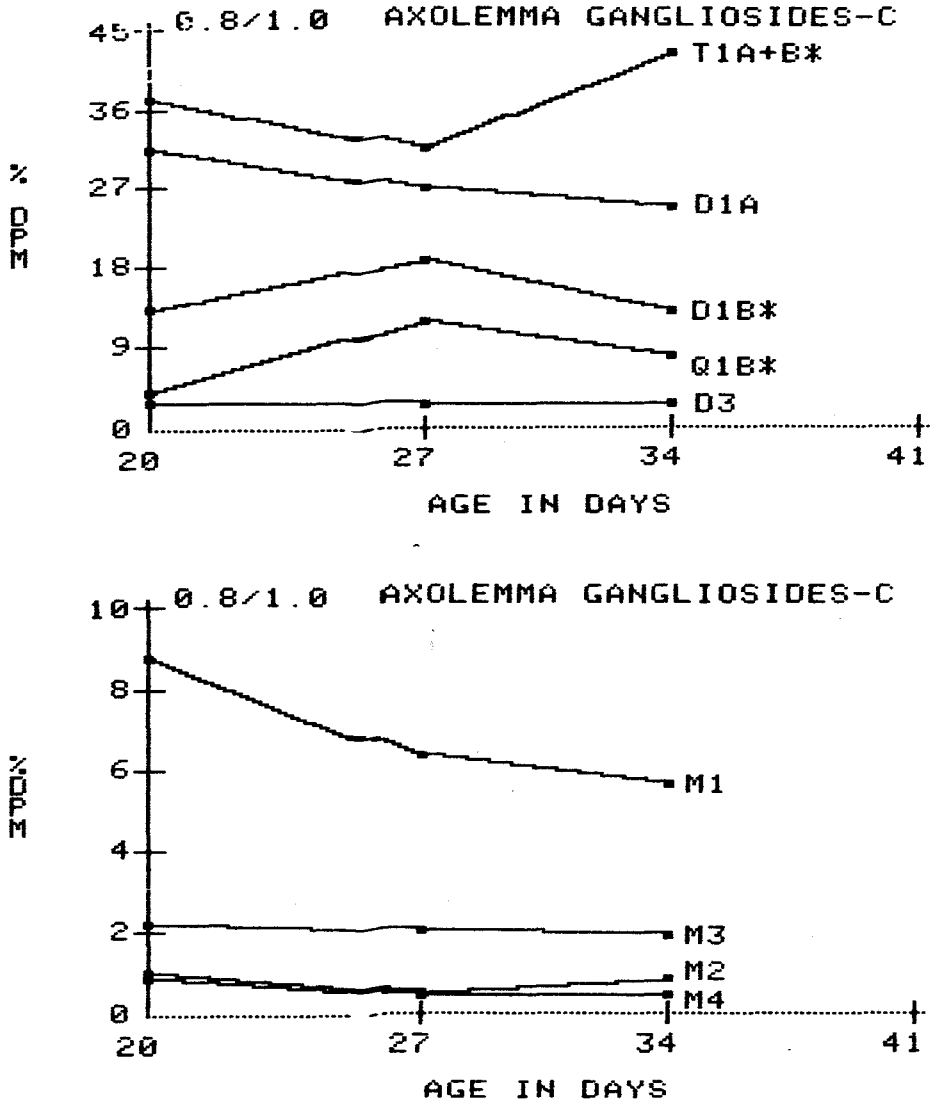


Figure 33. Developmental Trends in 0.8/1.0 Axolemma Ganglioside Patterns from 20-, 27- and 34-Day-Old Control (C*-C*) Offspring.

Each point represents the mean of 6 control offspring. An * indicates a statistically significant developmental trend ($p < 0.05$ when compared to 20 days). See Table A-5 (Appendix) for standard deviations.

Note the two different Y-axis scales used in representing the abundant complex gangliosides (0-45% of the total dpm) and the minor monosialogangliosides (0-10% of the total dpm).

Figure 34

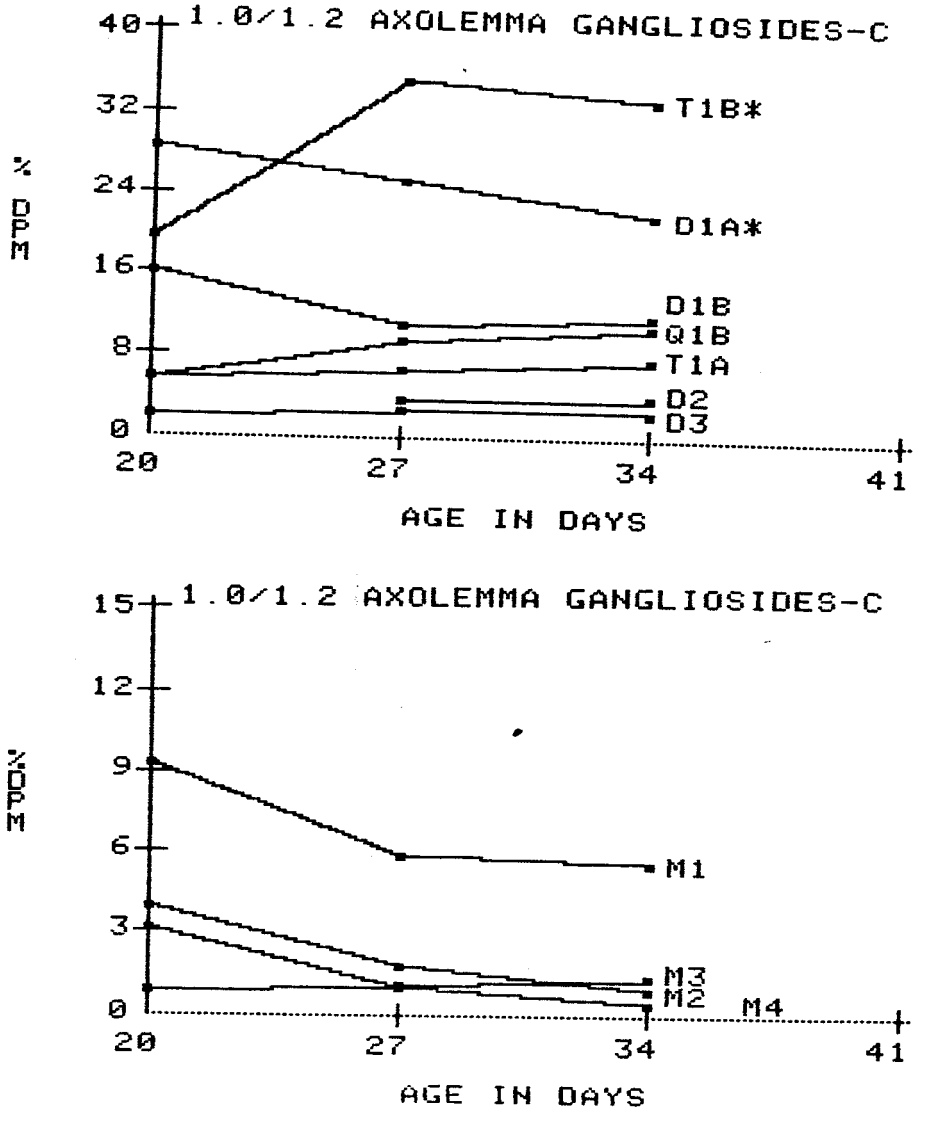


Figure 34. Developmental Trends in 1.0/1.2 Axolemma Ganglioside Patterns from 20-, 27- and 34-Day-Old Control (C*-C*) Offspring.

Each point represents the mean of 6 control offspring. An * indicates a statistically significant developmental trend ($p < 0.05$ when compared to 20 days). See Table A-6 (Appendix) for standard deviations.

Figure 35

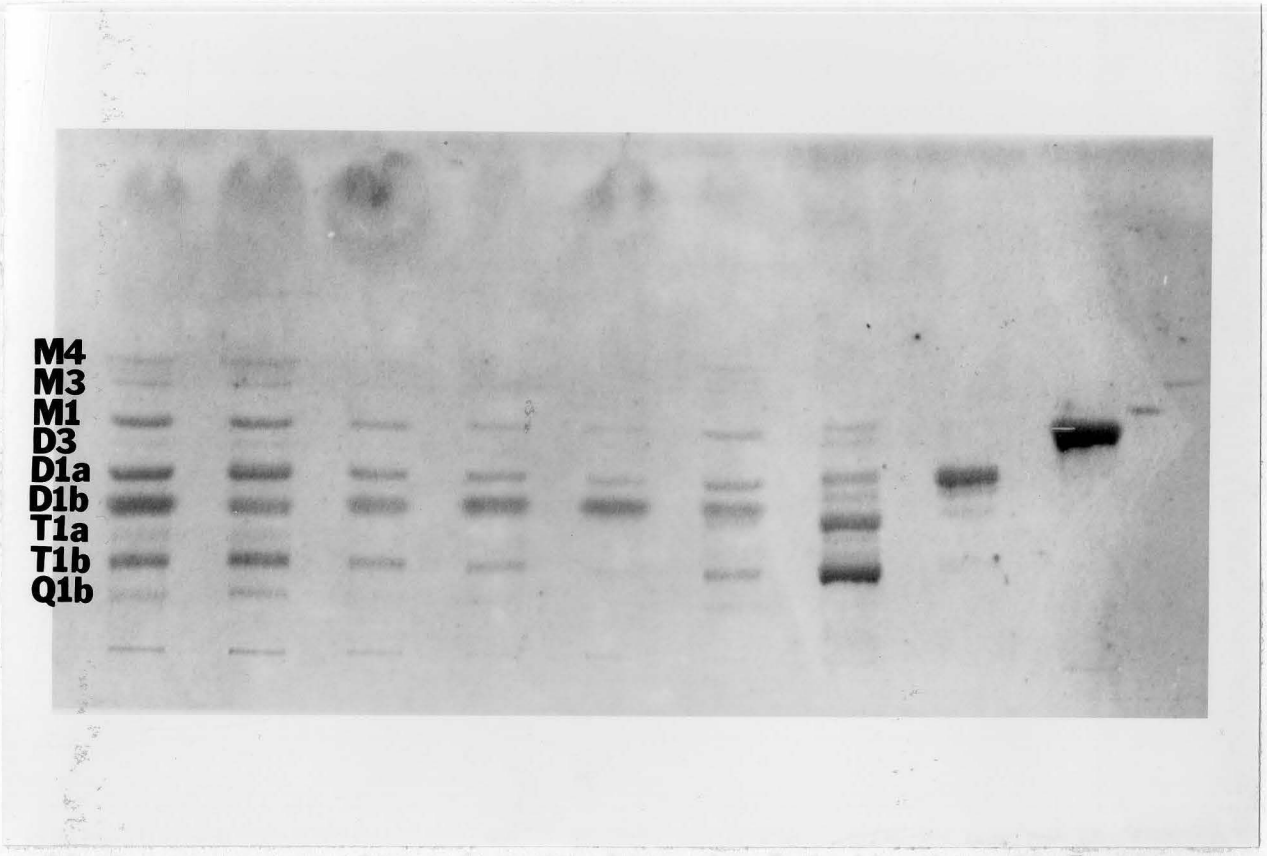


Figure 35. Gangliosides from the 0.8/1.0 M Sucrose Fraction from 34-Day-Old Animals.

Lanes 1-3 were loaded with proteins from control animals while lanes 4-6 were loaded with proteins from ethanol animals. Lanes 7-11 were loaded with G_{T1}, G_{D1a}, G_{M1}, G_{M2} and G_{M3} standards, respectively.

Table XII

Phospholipid Content of 1.0/1.2 Axolemma Membrane

Age in Days	µg phospholipid per µg protein	
	Control	Ethanol
20	1.23+/-0.45	2.38+/-1.42
27	0.59+/-0.13	0.93+/-0.14*
34	0.57+/-0.12	0.81+/-0.37

Each value for the 20 and 27 day ages represents the mean +/- standard deviation of 4 animals while the means for the 34 day age are from 6 animals. An * indicates that the control and ethanol values are different at $p<0.05$.

ethanol offspring had higher phospholipid/protein ratios than controls. However, this was statistically significant ($p<0.01$) at the 27 day age only.

Figure 36 is the graphic representation of the distribution of radioactivity among the glycerol-labeled phospholipid classes. The most significant level of incor-(PC)(~55-58%) while phosphatidyl ethanolamine (PE) accounted for second highest at ~25% of the total dpm incorporated. Phosphatic acid (PA), phosphatidyl serine (PE) and phosphatidyl inositol (PI) accounted for less than 10% each of the total radioactivity. There was a minor level of incorporation (<10% of total activity) found in a non-phospholipid, neutral lipid class (containing cholesterol) that migrated with the solvent front (see Table A-7 in the Appendix for data).

There were no differences seen in the incorporation of the glycerol label into control (C*-C*) and ethanol (E*-E*) offspring.

Figure 36. Distribution of Radioactivity Among Glycerol Radiolabeled 1.0/1.2 Axolemma Phospholipids.

The abbreviations for the phospholipid classes are as follows; PA (phosphatidic acid), PS (phosphatidyl serine), PI (phosphatitdyl inositol), PC (phosphatidyl choline) and PE (phosphatidyl ethanolamine).

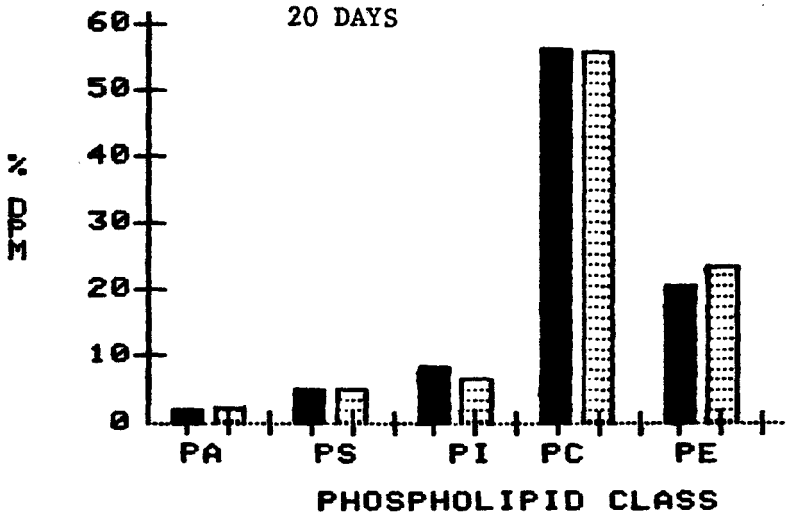
Solid bars represent the mean of control (C*-C*) animals while hatched bars represent the mean of ethanol (E*-E*) animals.

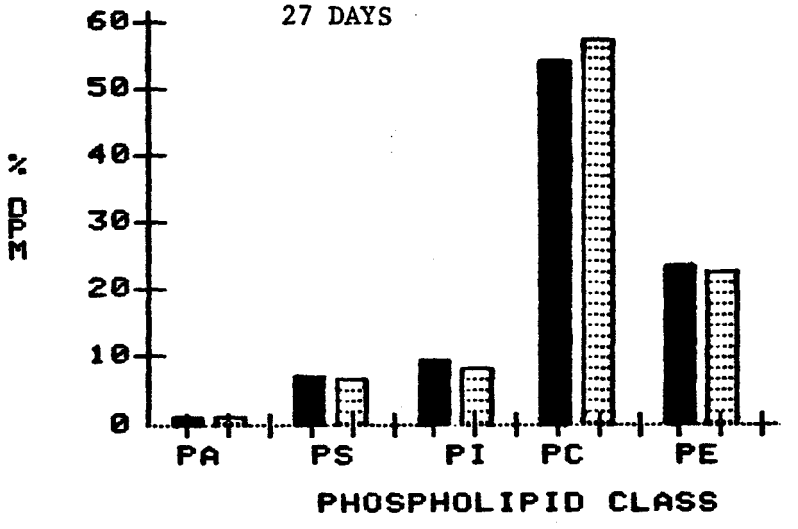
a). Distribution of label at 20 days. Each value represents the mean of 3 animals. Average dpm for [^{3}H] was 400 while for [^{14}C] was 385.

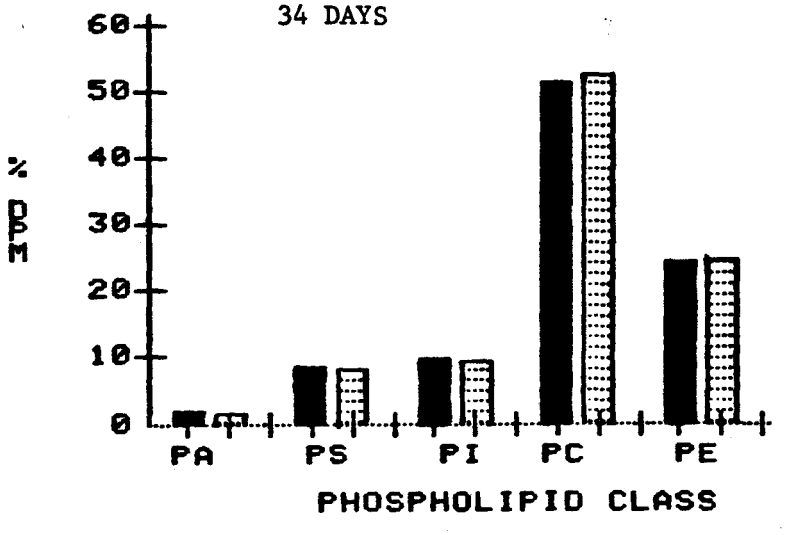
b). Distribution of label at 27 days. Each value represents the mean of 6 animals. Average dpm for [^{3}H] was 2140 while for [^{14}C] was 765.

c). Distribution of label at 34 days. Each value represents the mean of 6 animals. Average dpm for [^{3}H] was 4150 while for [^{14}C] was 1870.

See Table A-7 (Appendix) for standard deviations.


20 DAYS
60
50
40
30
20
10
0
% DPM
PA
PS
PI
PC
PE
PHOSPHOLIPID CLASS


27 DAYS
60
50
40
30
20
10
0
% DPM
PA
PS
PI
PC
PE
PHOSPHOLIPID CLASS


34 DAYS
60
50
40
30
20
10
0
% DPM
PA
PS
PI
PC
PE
PHOSPHOLIPID CLASS

CHAPTER IV

DISCUSSION

Maternal Weight Gain and Diet Consumption

Based on consumption of diet and weight gain both before and during gestation, it appears that mothers fed the revised control and ethanol diets were better nourished than the mothers fed the original diets (when compared to chow-fed controls). However, within each dietary protocol, control and ethanol mothers received a comparable amount of protein and calories. Thus differences seen between the control and ethanol pups within a given protocol should be due primarily to the presence of ethanol. Differences could also be related to the interaction of nutrition (marginally protein deficient in the original diet) and ethanol consumption.

Brain and Body Weights of Offspring

Body and brain weights of offspring reflect the different consumption of nutrients by mothers fed the original and revised diets. In general, brain and body weights of C*-Ch and E*-Ch pups were significantly greater than those of age-matched C-C and E-E pups, respectively. The

normal to near-normal body and brain weights of age- and diet-matched C-C and E-E animals ($\leq$ 14 days of age) in these studies is consistent with previous studies from this laboratory (Druse and Hofteig, 1977; Hofteig and Druse, 1978; Druse *et al.*, 1981; Noronha and Druse, 1982). However, these results conflict with reports of reduced fetal and newborn body weights in offspring that were prenatally exposed to ethanol (Lochry *et al.*, 1980; Riley *et al.*, 1979c; Able, 1982). Newborn weights were not recorded in this study as early handling of pups was considered detrimental. It is possible that brain weights are affected in the present study at younger ages and at a time closer to the last exposure to ethanol. Our observation of normal body (at $\leq$3 days of age) and brain weights (at $\leq$14 days of age) may relate to the fact that the ethanol mothers had adjusted to the diet (chronic) and consumed more than those that are fed during gestation only. Postnatal administration of ethanol to pups via gastric intubation or inhalation chambers results in significantly reduced brain weights (Diaz and Samson, 1980; Bauer-Moffet and Altman, 1975; 1977; Stibler *et al.*, 1983). However, the conditions of stress (intubations) and malnutrition (decreased consumption of food by lethargic animals in inhalation chambers) may contribute to the decreased brain weights seen in the latter studies. The present investigation did not attempt to administer ethanol postnatally because of the

mentioned potentially confounding factors. Therefore any abnormalities found in the present study are presumably due to effects of ethanol on the prenatal development of oligodendroglial cells which produce myelin.

Myelin Yield

Myelin protein content was significantly increased in 14- and 23-day-old E-E pups. This is in agreement with this laboratory's previous report of increased myelin protein early in development (increased myelin protein in E-E pups until 25 days, normal to decreased myelin protein at 54 days) (Druse and Hofteig, 1977). Since there were no similar significant differences in the revised cross-foster animals, it would appear that the increased myelin in E-E pups may relate to differences in the biological and cross-fostered lactating dams or to differences in the originial and revised liquid diets. It is likely that there are hormonal, metabolic, nutritional and/or nurturing differences between the foster mothers (lactating dams that were fed laboratory chow ad libitum throughout the study) and those that were withdrawn from alcohol after parturition.

This laboratory's observed normal to increased myelin protein content (present study; Druse and Hofteig, 1977; Hofteig and Druse, 1978) in ethanol pups early in myelination contrasts with reports of delayed myelination (Rosman and Malone, 1976; 1979; Jacobson et al., 1979;

Lancaster et al., 1982). However, in the studies of Jacobson et al. and Rosman and Malone, ethanol and control mothers were not pair-fed and the ethanol pups were nursed by dams that consumed ethanol during lactation. Since ethanol is known to inhibit oxytocin release (Fuchs and Wagner, 1963) (and hence lactation) and since ethanol and control mothers were not comparably nourished, the observation of delayed myelination could be explained by nutritional deficits (such as reported by Figlewicz et al., 1978). Lancaster et al. (1982) pair-fed their animals; however, their report of delayed myelination is based on a a 30% decrease in the relative synthesis of myelin protein in 16-day-old ethanol pups rather than on yield of myelin (not assessed at 16 days). In addition, their animals may have suffered from the combined stress of ethanol (or ethanol metabolite) exposure and low diet consumption of the liquid diets during gestation (50 ml/day versus 110 ml/day). At 30 days, Lancaster et al. (1982) reported comparable yields of myelin protein from control and ethanol pups.

Myelin Gangliosides

In the present study we utilized non-partitioning methods of ganglioside extraction to study myelin gangliosides in developing control (C*-C*) and ethanol (E*-E*) rats.

We observed a developmentally related increase in

the relative proportion and distribution of N-acetylmannosamine derived radioactivity in two gangliosides (G_{M1} and G_{M4}) which are enriched in CNS myelin (Suzuki *et al.*, 1967; Ledeen *et al.*, 1973; Yu and Yen, 1975; Yu and Iqbal, 1979; Cochran *et al.*, 1981; 1982; 1983). In contrast there was a developmentally related decrease in G_{T1b} which is found in high concentrations in synaptic membranes (Noronha and Druse, unpublished observations). Previous studies have also shown a developmentally related increase in G_{M1} and a decrease in polysialogangliosides in rodent myelin (Suzuki *et al.*, 1967; Yu and Yen, 1975). We also observed developmental changes in G_{D2} (increased), G_{D3} (increased) and G_{T1a} (decreased) which have not been previously reported.

As mentioned, the present study detected resorcinol positive (sialic acid) material and N-acetylmannosamine-derived radioactivity in the vicinity of the G_{M4} standard in rats aged 17 days. After the neuraminidase treatment, the radiolabeled portion of this compound migrated predominantly with sialic acid. G_{M4} was not previously detected in myelin from rodents (mice) less than 35 days of age (Yu and Yen, 1975), although it has been found in the myelin of mature humans (Ledeen *et al.*, 1973; Yu and Iqbal, 1979), birds (Cochran *et al.*, 1981; 1982; 1983), rodents and other mammalian and non-mammalian species (Cochran *et al.*, 1982). Detection of G_{M4} in rats as early as 17 days in the present study appears to relate to the use of a method for ganglio-

side extraction which did not involve solvent partitioning.

The proportion of G_{M1} in the present study is lower than that reported previously (Suzuki et al., 1967; Cochran et al., 1982). The difference was partly because of the younger age of the animals in the present study (the proportion of G_{M1} increases more than 50% between 15 days and 425 days in the rat (Suzuki et al., 1967)) and because of the non-partitioning extraction procedure.

The composition and distribution of radioactivity among myelin gangliosides was nearly comparable in ethanol and control rats. These results suggest that myelin ganglioside maturation is unaffected in the ethanol pups.

Myelin Associated Glycoprotein

In the present study the developmental shift in the molecular weight of the major myelin associated glycoprotein (MAG) appears to be normal in E-E and E*-Ch pups when compared to age-matched C-C and C*-Ch pups, respectively, since the peaks of fucose incorporation into MAG coincided on SDS gels. Since other studies have shown that the developmental timing of the shift in the MW of MAG is a sensitive indication of myelin maturation (Zimmerman et al., 1976; Druse and Krett, 1979; Matthieu et al., 1975b), it appears that MAG maturation was not delayed by prenatal consumption of alcohol. However, we did find a significantly decreased net incorporation of radiolabeled fucose into MAG in the majority of ethanol pups. Since this

decrease was found both in the E-E offspring that were raised by their biological mothers and in the E*-Ch cross-fostered offspring, it would appear that this abnormality is due to a prenatal influence of ethanol (or ethanol metabolite) exposure. (In utero ethanol (or ethanol metabolite) exposure has also been shown to alter the synthesis of certain synaptic membrane glycoproteins (Noronha and Druse, 1982).) At present, neither the functional significance or cause of the decreased synthesis of MAG are known. It is possible that decreased synthesis of the polypeptide backbone of MAG, decreased synthesis of any of the sugars in the oligosaccharide portion of MAG, or altered activity of a fucosyl transferase or fucosidase could contribute to the present findings. It is interesting that ethanol administration directly to neonates (6-16 days) significantly reduced glycoprotein and glycolipid-bound sialic acid concentrations in synaptosomal fractions and decreased ecto-sialyltransferase (membrane bound sialyltransferase) activity, although total protein and lipid concentrations of the synaptosomes did not differ between control and ethanol treated pups (Stibler et al., 1983). Alternatively, there may also be compartmentalized pools of fucose in brain myelin that are depleted under conditions of previous exposure to ethanol or an ethanol metabolite. Whatever the cause(s) of the decreased MAG synthesis, it would appear that cross-fostering eventually reverses the MAG abnormali-

ty since the frequency of the decreased incorporation into MAG was statistically less in the 24-day-old E*-Ch (compared to that in the E-E rats.)

Decreased MAG synthesis was also seen in 'Quaking' mice (Matthieu et al., 1978a) and in copper deficient rats (this author's interpretation of the work of Zimmerman et al., 1976). However, the results of the present study differ from the two mentioned studies since the animals in the other studies exhibited a deficit of myelin and a delay in myelin maturation (as shown by a delayed shift in molecular weight of MAG).

Both the myelin ganglioside and MAG studies suggest that myelin maturation is not affected in the developing offspring of rats that were given ethanol on a chronic basis prior to parturition. This conclusion conflicts with the work of Rosman and Malone (1976; 1979), Jacobson et al. (1979) and Lancaster et al. (1982) which suggest that in utero exposure to ethanol delays CNS myelination. As mentioned earlier, near-normal myelination in the present studies presumably relates to the high protein diet which was pair-fed on a chronic basis prior to parturition.

The results of CNS myelination studies using rats cannot be directly extrapolated to humans since it is a totally postnatal event in rats but partly prenatal in humans. As myelination begins prenatally in humans, one might expect there to be a greater possibility that abnor-

malities would occur with *in utero* ethanol exposure. It is clear that postnatal ethanol administration to developing rat offspring results in more severe alterations than those that were prenatally exposed (e.g. demonstrating reduced brain and body weights). In addition to the difference in timing of myelination there is the difficulty in comparing the well-nourished (revised diet) ethanol fed rat mothers of the present study with the poorly nourished alcoholic human mother. It may be that the original study was a more accurate model for the human condition.

Axolemma

In order to determine whether ethanol affected the development of axolemma, the axolemma proteins, glycoproteins, gangliosides and phospholipids were examined.

Protein Yield

The yield of axolemma protein (although the preparation procedure is not necessarily quantitative) was increased at 21 days in E*-E* pups. Although myelin yield was not significantly increased in the E*-E* pups, the increased axolemma protein content correlates with our findings of increased myelin until ~25 days in E-E offspring. The protein yields from axolemma of C*-C* and E*-E* pups were comparable at all other ages.

Proteins

There appear to be developmentally related changes in axolemma proteins between 22 days and 31/32 days. The

significant alterations include a developmentally related decrease in two high molecular weight components (~150 and ~100 K) and a decreases in one protein of MW ~65 K. There are non-significant decreases in proteins of MW ~60 and ~55 K also. The ~100 K protein is thought to be the catalytic subunit of Na^+-K^+-ATPase (Sweadner, 1979) and the 65 K protein may be acetlycholinesterase. Tubulin and actin would be present in the 50-60 K region (Reig *et al.*, 1982). Tubulin is reportedly present on the surface of neuronal membranes (Estridge, 1977) while actin filaments have been reported in a subaxolemma network (Chang and Goldman, 1973). A developmentally related decrease in these proteins may reflect an increase in other axolemma proteins (e.g. ~32 and ~35 K).

Although the 1.0/1.2 axolemma fraction was clearly distinct (in its proportion of proteins) from SPM, the 0.8/1.0 axolemma fraction, and myelin (most pronounced), each fraction did contain proteins having the same molecular weight. One would expect similarities in the proteins of axolemma and SPM since these fractions are extensions of the same membrane (i.e. neuronal membrane). Major protein bands in SPM are those of MW 95-100, 52-60 and 40-49 K (Druse *et al.*, 1982; present study) whereas in the 1.0/1.2 axolemma fraction the major bands are of MW 100, 49-55 and 32-35 K. As mentioned, the ~100 K protein is most likely the catalytic subunit of Na^+-K^+-ATPase and would be ex-

pected to be a large percentage of total SPM proteins (an energetically active membrane) while perhaps a smaller percentage of the total in axolemma from myelinated axons (since this protein is concentrated at nodal regions only). The two subunits of tubulin (~52-56 K), a contractile protein, would be expected to be a major protein in both membranes also. In all fractions there is a relatively large percentage of protein in the ~52 K band(s). This is apparently a region of heterogeneity between fractions. In myelin, the ~52 K bands (19% total dye binding capacity) are known as the Wolfgram doublet, containing CNPase and tubulin. The major protein in the postsynaptic density (a subfraction of SPM) is also in the ~52 K region (Kelly and Cotman, 1978). (In the present study, SPM protein of ~52 K accounted for 12% of the total.) In axolemma (which contained the smallest proportion of the ~52 K protein of all the fractions examined), the protein most likely is tubulin (~8% total). In myelin, the major proteins were found in MW regions at <26 K (~69%). In all other fractions this low MW area accounted for < 13% of total dye binding capacity. The cluster of proteins in the 50 K region of 1.0/1.2 axolemma is similar to that found by DeVries (1981) in adult animals. Leucine incorporation into axolemma proteins also contrasts sharply with myelin (Matthieu et al., 1978b) since there is a large amount of radioactivity in high molecular weight regions (100-250 K) in axolemma

and only a small amount of radioactivity in this region in myelin.

Abnormalities in the E*-E* offspring occurred in the ~150 and ~100 K bands of axolemma and also in two lower MW bands of ~38 and ~35 K at 22 and 31/32 days of age. E*-E* animals had a significantly lower proportion of the 150 and 100 K bands and higher proportions (not significant) of the two lower bands. The abnormalities may reflect transient changes in the maturation of specific membrane proteins. In particular, since the 150 and 100 K proteins normally demonstrate a developmentally related decrease, there may be a premature or accelerated decrease of these proteins in the E*-E* offspring.

DeVries and Lazdunski (1982) have recently demonstrated that two classes of neurotoxins bind to axolemma. (One class includes saxitoxin and tetrodotoxin and the other class includes polypeptide neurotoxins). Both classes are directly involved in the blocking of sodium channels in the membrane. It would be interesting to see whether the ionic channels that appear to be heavily concentrated in axolemma are affected in E*-E* offspring in terms of activity or concentration.

Glycoproteins

Fucosylated axolemma glycoproteins were shown in this study to contain a heterogeneous population of proteins ranging from ~36 to 330 K. Major peaks of incor-

poration (>4% of total per band) were found at 150, 67, 65, 55 and 52 K in 22-day-old offspring, while at 31/32 days of age they were found at 130-150, 100, 85, 76, 62-66 and 52 K. Developmentally related changes in incorporation of fucose (22 days of age versus 31/32 days of age) included an increase in glycoproteins of MW 150, 130, 85, and 67 K and a decrease in those of MW 110, 62, 61, 55 and 52 K . It is difficult to make conclusions regarding these changes as the identity of these glycoproteins can only be postulated via molecular weight comparisons and previous knowledge regarding carbohydrate content of known constituents. However, it is interesting to note the developmentally related decrease in the major glycoproteins in the region of 50-55 K (a MW which is associated with the tubulin protein as previously mentioned) which correlates with the developmental decrease (though non-significant) in the dye binding capacity of the protein in that area.

The present studies which examined developmentally related changes in axolemma glycoproteins considerably expand previous studies of radiolabeled axolemma glycoproteins from adult rats (DeVries, et al., 1978) and 30-day-old rabbits (Matthieu et al., 1978b) and PAS stained axolemma glycoproteins from adult bovine corpus callosum (DeVries et al., 1981) by improving the resolution of axolemma glycoproteins and by examining young, developing rats.

The number and complexity of glycoproteins found in the axolemma preparation may relate to the degree of contact between neurons and glia as proposed by Fischer et al. (1970). Mammalian CNS axolemma have a large amount of complex glycoproteins (DeVries et al., 1981) and these membranes have close contact with oligodendroglia. In contrast, the axons of the garfish olfactory bulb and lobster leg nerve have only a limited number of glycoproteins, and limited contact with glial cells (a bundle of a hundred or more axons making contact with a single glial cell). Since contact relationships between cells are thought to be mediated by carbohydrate containing cells, the presence of complex carbohydrates in axolemma confirms this correlation.

The distribution of fucose-derived radioactivity (in MW regions defined by protein MW 'markers') along SDS gels did not significantly differ between 21-day-old C*-C* and E*-E* offspring. However, changes were found at the 22, 31 and 32 day ages since statistics could be performed on each band. It is interesting to note that in E*-E* offspring there were significant reductions in the incorporation of label into two high MW (220 and 180 K) glycoproteins at the younger 22 day age while at 32 days the radiolabeling of these glycoproteins was normal. There were; however, significant increases at 32 days in a slightly lower MW band (150 K) and in major bands of ~85

and ~76 K. There were significant reductions at both 22 and 32 days in the incorporation of label into bands of ~61 and ~62 K. These abnormalities may be due to factors involving the synthesis and breakdown of fucosylated proteins or to alterations in the timing of the axolemma development or maturation of the brain. Earlier studies indicate that there are developmentally related changes in the carbohydrate portions of glycoproteins of parallel axolemma fibers in rat cerebellar tissue during development (Zanetta et al., 1978; Reeber et al., 1980) which appear to be related to early recognition phenomenon between axon fibers and their postsynaptic cells. Although the present study assessed myelinated axolemma from later ages, the abnormalities seen in the glycoproteins may be related to select abnormalities in the normal maturational phenomenon.

Gangliosides

Ganglioside content of axolemma fractions from C*-C* and E*-E* animals was assessed during the 20 to 34 day time period. The ganglioside patterns of axolemma-enriched fractions resembled, somewhat, those of sialic acid (content) patterns of neuronal membranes (Ledeen, 1978) since the major gangliosides in both studies were the complex gangliosides. Smaller amounts of monosialylated gangliosides were also detected in both studies. The present quantitative radiolabeling studies extend the only previous study of axolemma gangliosides (DeVries and Zmachinski,

1980). The previous study lacked the sensitive radioactive developmental analyses. The present study demonstrated a developmentally related increase in G_{T1b} in both axolemma fractions (0.8/1.0 and 1.0/1.2). G_{T1b} also increased in microsomes, synaptosomes and synaptic plasma membranes (Noronha and Druse, submitted for publication). We also found a developmentally related increase in the proportion of radiolabel associated with G_{Q1b} and decrease in the proportion in G_{D1a} and G_{M2} which have not been previously reported. In SPM, a similar decrease was seen in G_{D1a} while G_{M2} and G_{Q1b} did not change developmentally. There was a developmentally related decrease in radiolabel incorporation into G_{M1} of SPMs which was not seen in axolemma of the present study.

There were no differences detected in incorporation of label into axolemma gangliosides of E*-E* pups.

Phospholipids

The present developmental and radiolabel studies of axolemma phospholipids extends the work of DeVries and Zmachinski (1980) which was limited to quantitative analysis of axolemma phospholipids from older rats.

The present and previous studies both found PC and PE to be the major phospholipids. It is interesting that there were no developmental changes in the composition of membrane phospholipids during the 20 to 34 day age period. Developmental changes have been reported in synaptic mem-

branes when younger animals were included in the study (age range was 7 days to adult, Hitzemann and Johnson, 1983). Hitzemann and Johnson found a developmentally related decrease in PC and a developmentally related increase in PE and sphingomyelin. The composition of phospholipids from axolemma and SPM are similar. Similarities include high phospholipid to cholesterol ratios and low levels of sphingomyelin. The present study demonstrated that axolemma, like SPM, contains large percentages of PC and PE, significant levels of PS and very small amounts of PI (Simon et al., 1971).

Only one significant alteration was seen in the phospholipid study. At 27 days of age, E*-E* offsping had a significantly greater content of phospholipid (expressed as μg phospholipid/ μg protein) in axolemma membrane.

In summary, it appears that axolemma phospholipid classes are not significantly altered in E*-E* pups nor are there developmentally related changes within the age range studied. However, it appears there is a general increase in the phospholipid to protein ratio. Abnormal phospholipid content in 1.0/1.2 axolemma contrasts with the normal synthesis of myelin phospholipids in offspring of chronically alcohol fed mothers (Druse and Hofteig, 1977).

SUMMARY

The objective of the research described in this dissertation was to examine specific biochemical constituents, the glycoproteins, gangliosides and phospholipids, in central nervous system (CNS) myelin and/or axolemma-enriched fractions during the early development of offspring of Sprague-Dawley rats that were pair-fed a high protein ethanol-containing liquid diet on a chronic basis prior to parturition.

During normal development there was a decrease in G_{T1b} and an increase in G_{M1} in myelin in terms of the relative proportions of resorcinol-positive material (ganglioside sialic acid) and of N-acetylmannosamine-derived radioactivity. In addition, there was an increase in the proportion of the minor myelin gangliosides G_{D2} and G_{D3} and a decrease in G_{T1a} in myelin. N-Acetylmannosamine-derived radioactivity was detected in the vicinity of G_{M4} ganglioside standard in myelin from rat offspring as young as 17 days of age and in addition demonstrated a developmental increase between 17 and 31 days of age.

There were also developmentally-related changes associated with axolemma proteins. The proportion of two high molecular weight proteins (~150 and ~100 K) and a ~65 K protein decreased between 22 and 32 days of age. The ~100 K protein is thought to be the catalytic subunit of Na^+-K^+-ATPase and the ~65 K protein may be acetylcholin-

esterase. Axolemma protein profiles were distinct from those of myelin. In addition, although proteins with similar MW were found in synaptic plasma membrane (SPM) and axolemma, the proportion of the proteins in 1.0/1.2 axolemma was distinct from that of SPM. The proportion of three fucosylated axolemma glycoproteins (120, 85 and 67 K) increased between 22 and 32 days of age while the proportion of another three (110, 55 and 52 K) decreased during the same time period. The present studies provide significant new information about axolemma glycoproteins from developing animals.

During early development (i.e. between 20 and 34 days of age) there was an increase in G_{T1b} in axolemma and a decrease in axolemma G_{D1a}. These changes are similar to developmental changes found in microsomes, synaptosomes and SPM. There were also developmentally-related changes in the proportion of radiolabel associated with G_{Q1b} (increased), and with G_{D1a} and G_{M2} (decreased).

Clinical studies have shown that the development of the central nervous system is affected in the offspring of alcoholic women (Fetal Alcohol Syndrome, FAS). Indeed, CNS abnormalities may be the most common outcome of FAS. There has been a controversy as to whether or not myelination was delayed in animal models of FAS. The inability to resolve the ethanol-related effects appears to be primarily due to the variability in the nutritional

status of the animals and in the timing and duration of the ethanol feeding in the various studies. The present investigation studied the offspring of rat mothers that were pair-fed control control or 6.6% (v/v) ethanol liquid diets prior to parturition.

There was no apparent delay in myelination in offspring of chronically-treated, well-fed mothers, as assessed by myelin protein content, the major myelin-associated glycoprotein (MAG) development and myelin ganglioside developmental patterns. However, an abnormalitiy (decrease) in the proportion of radioactive fucose associated with MAG was found in ethanol-treated offspring. The cause of the decrease in the proportion of radiactivity associated with MAG in the ethanol-treated offspring is as yet unknown, but may be due to an alteration of enzymes involved with the synthesis or degradation of the oligosaccharide or polypeptide of the glycoprotein. There were no significant abnormalities in the content of myelin gangliosides or in the incorporation of radioactively-labeled N-acetylmannosamine into myelin gangliosides from ethanol-treated offspring. However, there was a consistent decrease in the proportion of G_{M1} in myelin from the ethanol-treated rats.

There were specific and transient abnormalities in the protein profiles and fucosylated glycoproteins in axolemma from ethanol-treated offspring; however, there

were no abnormalities in axolemma gangliosides. Although the identity of the proteins and glycoproteins are not known it is possible that functionally important ion pumps/channels or recognition molecules were altered at a critical time of axolemma development. Axolemma phospholipid classes were not affected in ethanol-treated rats; however, the ratio of axolemma phospholipid to protein was increased at all ages.

The research reported represents the first account of the effects of chronic maternal ethanol consumption on gangliosides and glycoproteins from both myelin and axolemma membranes. It also represents the first report of the development of glycoproteins, gangliosides and phospholipids from axolemma.

BIBLIOGRAPHY

Abel, E., (1982) Consumption of alcohol during pregnancy: A review of effects on growth and development of offspring. Hum. Biol. 54:421-453.

Abel, E., (1980) The fetal alcohol syndrome: behavioral teratology. Psych. Bull. 87:29-50.

Abel, E., (1979) Prenatal effects of alcohol on adult learning in rats. Pharmacol. Biochem. Behav. 10:239-243.

Abel, E., (1978) Effects of ethanol on pregnant rats and their offspring. Psychopharmacol. 57:5-11.

Abel, E., and Dintcheff, B., (1978) Effects of prenatal alcohol exposure on growth and development in rats. J. Pharmacol. Exp. Ther. 207:916-921.

Abdel-Latif, A., and Akhtar, R., (1982) Cations and the acetylcholine-stimulated ^{32}P-labelling of phosphoinositides in the rabbit iris. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G Ansell and G Porcellati, Eds. pp. 251-264. Raven Press, New York.

Abdel-Latif, A., Smith, J., and Ellington, E., (1970) Subcellular distribution of sodium-potassium adenosine triphosphatase, acetylcholine and acetylcholinesterase in developing rat brain. Brain Res. 18:441-450.

Agnati, L., Benfenati, F., Battistini, N., Cavicchioli, L., Fuxe, K., and Loffano, G., (1983) Selective modulation of ^{3}H-spiperone labeled 5-HT receptors by subchromic treatment with the ganglioside G_{M1} in the rat. Acta Phys. Scan. 117:311-314.

Aguayo, A. G., Epps, J., Charron, L., and Bray, G., (1976) Multipotentiality of schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: Qauntitative microscopy and autoradiography. Brain Res. 104:1-20.

Banik, N., and Davison, A., (1969) Enzyme activity and composition of myelin and subcellular fractions in the developing rat brain. Biochem. J. 115:1051-1062.

Barnes, D., and Walker, D., (1981) Prenatal ethanol exposure permanently reduces the number of pyramidal neurons in rat hippocampus. Dev. Brain Res. 1:3-24.

Barondes, S., (1970) Brain glycomacromolecules and interneuronal recognition. In The Neurosciences. F. Schmitt, Ed. pp. 747-769. Rockefeller University Press, New York.

Bartlett, G., (1959) Colorimetric assay methods for free and phosphorylated glyceric acids. J. Biol. Chem. 234:466-468.

Bauer-Moffett, C., and Altman J., (1975) Ethanol-induced reductions in cerebellar growth of infant rats. Exp. Neurol. 48:378-382.

Bauer-Moffett, C., and Altman, J., (1977) The effect of ethanol chronically administered to pre-weanling rats on cerebellar development: A morphological study. Brain Res. 119:249-268.

Benjamins, J., and McKhann, G., (1973) [2-^{3}H]Glycerol as a precursor of phospholipids in rat brain: evidence for lack of recycling. J. Neurochem. 20:1111-1120.

Benjamins, J., and McKhann, G., (1981) Development, regeneration, and aging of the brain. In Basic Neurochemistry. G. Seigel, R. Albers, B. Agranoff, R. Katzman, Eds. pp. 445-469. Little, Brown and Company, Boston.

Blakemore, W., (1975) Remyelination by Schwann cells of axons demyelinated by intraspinal injection of 6-aminonicotinamide in the rat. J. Neurocytol. 4:745-757.

Bond, N., and Digiusto, E., (1976) Effects of prenatal alcohol consumption on open-field behavior and alcohol preference in rats. Psychopharmacol. 46:163-168.

Bond, N., and Digiusto, E., (1977) Effects of prenatal alcohol consumption on shock avoidance learning in rats. Psychol. Rpts. 41:1269-1270.

Bonnichsen, R., and Theorell, H., (1951) An enzymatic method for the micro-determination of ethanol. Scand. J. Clin Lab Invest. 3:58-62.

Branchey, L., and Friedhoff, A., (1976) Biochemical and behavioral changes in rats exposed to ethanol in utero. Ann N. Y. Acad. Sci. 273:328-330.

Braun, P., (1977) Molecular architecture of myelin. In Myelin. P. Morell, Ed. pp. 91-115. Plenum Press, New York.

Braun, P., Pereyra, P., and Greenfield, S., (1980) Myelin organization and development: A biochemical perspective. In Myelin: Chemistry and biology. G. Hashim, Ed. pp. 1-17. Alan R. Liss Inc., New York.

Brostoff, S., (1977) Immunological responses to myelin and myelin components. In Myelin. P. Morell, Ed. pp. 415-445. Plenum Press, New York.

Brunngraber. E., (1969) Possible role of glycoproteins in neural functions. Perspect. Biol. Med. 12:467-470.

Brunngraber, E., (1979) Function of Gangliosides, Function of glycoproteins. In Neurochemistry of Aminosugars. Neurochemistry and Neuropathology of the Complex Carbohydrates. pp. 90-127,491-535. Charles C. Thomas, Springfield, Illinois.

Chang, C.-M., and Goldman, R., (1973) The localization of actin-like fibers in cultured neuroblastoma cells as revealed by heavy meromysin binding. J. Cell Biol. 57:867-874.

Chernoff, G., (1977) The fetal alcohol syndrome in mice: An animal model. Teratol. 15:223-229.

Chernoff, G., (1980) The fetal alcohol syndrome in mice: Maternal variables. Teratol. 22:71-75.

Christoffel, K., and Salafsky, I., (1975) Fetal alcohol syndrome in dizygotic twins. J. Pediatr. 87:963-967.

Clarren, S., Alvord, E., Sumi, M., Streissguth, A., and Smith, D. W., (1978) Brain malformations related to prenatal exposure to ethanol. J. Pediatr. 92:64-67.

Clarren, S., and Smith, D. W., (1978) The fetal alcohol syndrome: A review of world literature. N. Engl. J. Med. 298:1063-1067.

Cochran, F., Ledeen, R., and Yu., R., (1983) Gangliosides and proteins in developing chicken brain myelin. Dev. Brain Res. 6:27-32.

Cochran, F., Yu, R., and Ledeen, R., (1982) Myelin gangliosides in vertebrates. J. Neurochem. 39:773-779.

Cochran, F., Yu, R., Susumo, A., and Ledeen, R., (1981) Myelin gangliosides: An unusual pattern in the avian central nervous system. J. Neurochem. 36:696-702.

Costantino-Ceccarini, E., Cestelli, A., and DeVries, G., (1979) Characterization and developmental changes of UDP-galactose-ceramide galactosyl transferase in a rat CNS axolemma-enriched fraction. Differences and similarities of the enzyme associated with the microsomal and myelin fractions. J. Neurochem. 32:1175-1182.

Cotman, C., and Matthews, D., (1971) Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterization. Biochim. Biophys. Acta 249:380-394.

Crews, F., Camacho, A., Phillips, I., Willink, E., Calderini, G., Hirata, F., Axelrod, J., McGivney, and Siraganian, R., (1982) Effects of membrane fluidity on mast cell and nerve cell function. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell and G. Porcellati, Eds. pp. 21-35. Raven Press.

Detering, N., Edwards, E., Ozand, P., and Karahasan, A., (1980) Comparative effects of ethanol and malnutrition on the development of catecholamine neurons: Changes in specific activities of enzymes. J. Neurochem. 34:297-304.

DeVries, G., (1981) Isolation of axolemma-enriched fractions from mammalian CNS. In Research Methods in Neurochemistry. Vol. 5, N. Marks and R. Rodnight, Eds. pp. 3-38. Plenum Press, New York.

DeVries, G., and Lazdunski, M., (1982) The binding of two classes of neurotoxins to axolemma of mammalian brain. J. Biol. Chem. 257:11684-11688.

DeVries, G., Matthieu, J.-M., Beny, M., Chicheportiche, R., Lazdunski, M., and Dolivo, M., (1978) Isolation and partial characterization of rat CNS axolemma-enriched fractions. Brain Res. 147:339-352.

DeVries, G., Norton, W., and Raine, C., (1972) Axons: Isolation from mammalian central nervous system. Science 175:1370-1372.

DeVries, G., Payne, W., and Saul, R., (1981) Composition of axolemma-enriched fractions isolated from bovine CNS myelinated axons. Neurochem. Res. 6:521-537.

DeVries, G., and Zmachinski, C., (1980) The lipid composition of rat CNS axolemma-enriched fractions. J. Neurochem. 34:424-430.

Diaz, J., and Samson, H., (1980) Impaired brain growth in neonatal rats exposed to ethanol. Science 208:751-753.

Drummond, R., and Dean, G., (1980) Comparison of 2',3'-cyclic nucleotide 3'-phosphodiesterase and the major component of Wolfgram protein W1. J. Neurochem. 35:1155-1165.

Druse, M., Brady, R., and Quarles, R., (1974) Metabolism of a myelin-associated glycoprotein in developing rat brain. Brain Res. 76:423-434.

Druse, M., and Hofteig, J., (1977) The effect of chronic maternal alcohol consumption on the development of central nervous system myelin subfractions in rat offspring. Drug Alcohol Depend. 2:421-429.

Druse, M., and Krett, N., (1979) CNS myelin-associated glycoproteins in the offspring of protein deficient rats. J. Neurochem. 32:665-667.

Druse, M., Tonetti, D., and Smith, D.M., (1982) Effects of maternal nutritional stress on synaptic plsama membrane proteins and glycoproteins in offspring. Exp. Neurol. 78:99-111.

Druse, M., Waddell, C., and Haas, G., (1981) Maternal ethanol consumption during the third trimester of pregnancy: Synaptic plasma membrane glycoproteins

and gangliosides in offspring. Sub. Alc. Actions/Misuse 2:359-368.

Elis, J., Krisiak, M., and Poschlova, N., (1978) Effect of alcohol given at different periods of gestation on brain serotonin in offspring. Activitas nerv. sup. 20:287-288.

Elis, J., Krisiak, M., Poschlova, N., and Masek, K., (1976) The effect of alcohol administration on the concentration of noradrenaline, dopamine and 5-hydroxytryptamine in the brain of offspring of mice. Activitas. nerv. sup. 18:220-221.

Eichberg, J., Bell, M., and Peterson, R., (1982) Metabolism of polyphosphoinositides and other phospholipids in peripheral nerve of normal and streptozotocin diabetic rats. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell, and G. Porcellati, Eds. pp. 271-282. Raven Press, New York.

Estridge, M., (1977) Polypeptides similar to the a and b subunits of tubulin are exposed on the neuronal surface. Nature 268:60-63.

Evans, W., (1974) Nucleotide pyrophosphatase, a sialoglycoprotein located on the hepatocyte surface.Nature 250:391-394.

Figlewicz, D., Hofteig, J., and Druse, M., (1978) Maternal deficiency of protein or protein and calories during lactation: Effect upon CNS myelin subfraction formation in rat offspring. Life Sci. 23:2163-2172.

Figlewicz, D., Quarles, R., Johnson, D., Barbarash, G., and Sternberger, N., (1981) Biochemical demonstration of the myelin-associated glycoprotein in the peripheral nervous system. J. Neurochem. 37:749-758.

Fischer, S., Cellino, M., Zambrano, F., Sampighi, G., Tellez-Nagel, M., Marcus, D., and Canessa-Fischer, M., (1970) Isolation and characterization of plasma membranes from the retinal axons of the squid: An axolemma-rich preparation. Arch. Biochim. Biophys. 138:1-15.

Fiske, C., and Subbarow, Y., (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

Folch, J., Lees, M., and Sloane-Stanley, G., (1957) A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226:497-509.

Fong, J., Ledeen, R., Kundu, S., and Brostoff, S., (1976) Gangliosides of peripheral nerve myelin. J. Neurochem. 26:157-162.

Freysz, L., Dreyfus, H., Vincendon, G., Binaglia, L., Roberti, R., and Porcellati, G., (1982) Asymmetry of brain microsomal membranes: Correlation between the asymmetric distribution of phospholipids and the enzymes involved in their synthesis. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell and G. Porcellati, Eds. 37-47. Raven Press, New York.

Fuchs, A.-R., and Wagner, G., (1963) The effect of ethyl alcohol on the release of oxytocin in rabbits. Acta Endocrinol. (Copenh.) 44:593-605.

Garber, B., and Moscona, A., (1972) Reconstruction of brain tissue from cell suspensions. I. Aggregation patterns of cells dissociated from different regions of the developing brain. Dev. Biol. 27:217-234.

Gatt, S., (1980) Introductory remarks on ganglioside metabolism. In Structure and function of gangliosides. L. Svennerholm, P. Mandel, H. Dreyfus, P.-F. Urban, Eds. pp. 209-212. Plenum Press, New York.

Giesing, M., and Gerken, U., (1982) The effects of carbamylcholine on extrasynaptic phosphatidylcholine biosynthesis in grey matter of cerebral cortex. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell, and G. Porcellati, Eds. pp. 145-164. Raven Press, New York.

Gombos, G., Ghandour, M., Vincendon, G., Ruber, A., and Zanetta, J., (1977) Formation of neuronal circuitry of rat cerebellum: Plasma membrane modification. In Maturation of Neurotransmission. A. Verraclakis, E. Giacolini, and G. Filagamo, Eds., pp. 16-22. S. Karger, Basel.

Greenfield, S., Norton, W., and Morrell, P., (1971) Quaking mouse: Isolation and characterization of myelin proteins. J. Neurochem. 18:2119-2128.

Haley, J., Samuels, F. and Ledeen, R., (1981) Study of myelin purity in relation to axonal contaminants. Cell. Mol. Neurobiol. 1 :175-187.

Hammer, R., and Scheibel, A., (1981) Morphologic evidence for a delay of neuronal maturation in fetal alcohol exposure. Exp. Neurol. 74:587-597.

Hanson, J., Jones K. L., and Smith D. W., (1976) Fetal alcohol syndrome: Experience with 41 patients. JAMA 235 :1458-1460.

Harford, J., Waechter, C., Saul, R., and DeVries, G., (1979) Evidence for the biosynthesis of mannosylphosphoryldolichol and N-acetylglucosaminylpyrophosphoryldolichol by an axolemma enriched membrane preparation from bovine white matter. J. Neurochem. 32 :91-98.

Harlap, S., and Shiono, P., (1980) Alcohol, smoking and incidence of spontaneous abortions in the first and second trimester. Lancet 2:173-176.

Hausman, R., and Moscona, A., (1975) Purification and characterization of the retina specific cell aggregating factor. Proc. Natl. Acad. Sci. USA 72:916-920.

Havlicek, V., and Childaeva, R., (1976) EEG component of fetal alcohol syndrome. Lancet 2 :477.

Hawthorne, J., and Kai, M., (1970) Metabolism of phosphoinositides. In Handbook of neurochemistry. Vol.III. A. Lajtha, Ed. pp. 491-508. Plenum Press, New York.

Hawthorne, J., and Pickard, M., (1979) Phospholipids in synaptic function. J. Neurochem. 32 :5-14.

Henderson, G., and Schenker, S., (1977) The effects of maternal alcohol consumption on the viability and visceral development of the newborn rat. Res. Comm. Chem. Path. Pharmacol. 16:15-32.

Hess, H., and Pope, A., (1972) Quantitative neurochemical histology. In Handbook of Neurochemistry. Vol II. A. Lajtha, Ed. pp. 289-329. Plenum Press, New York.

Hitzemann, R., and Johnson, D., (1983) Developmental changes in synaptic membrane lipid composition and fluidity. Neurochem. Res. 8 :121-131.

Hoffman, S., Sorkin, B., White, P.,Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B., and Edelman, G., (1982) Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. J. Biol. Chem. 257:7720-7729.

Hofteig, J., and Druse, M., (1978) Central nervous system myelination in rats exposed to ethanol in utero. Drug Alcohol Depend. 3:429-434.

Holm, M., and Svennerholm, R., (1972) Synthesis and biodegradation of rat brain gangliosides in vivo. J. Neurochem. 19:609-622.

Holmgren, J., Lonnroth, I., Mansson, J.-E., and Svennerholm, L., (1975) Interaction of cholera toxin and membrane GM_1 ganglioside of small intestine. Proc. Natl. Acad. Sci. USA 72:2520-2524.

Hukkanen, V., (1982) Lectin-reactive components in white matter membranes from normal and multiple sclerosis brains. J. Neurochem. 38 :1537-1541.

Ilyas, A., and Davison, A., (1983) Cellular hypersensitivity to gangliosides and myelin basic protein in multiple sclerosis. J. Neurol. Sci. 59:85-95.

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

Jacobson, M., (1978) Differentiation, growth and maturation of neurons. In Developmental neurobiology. pp. 166-180. Plenum Press, New York.

Jacobson, S., Rich, J., and Tovsky, N., (1979) Delayed myelination and lamination in the cerebral cortex of the albino rat as a result of the fetal alcohol syndrome. Curr. Alc. Vol V:123-133.

Jones, K. L., Smith, D. W., (1973) Recognition of the fetal alcohol syndrome in early infancy. Lancet 2:999-1001.

Jones, K. L., Smith, D. W., and Hanson, J., (1976) The fetal alcohol syndrome: Clinical deliniation. Ann. N.Y. Acad. Sci. 273:130-137.

Jones, K. L., Smith, D. W., Streissguth, A., and Myrianthopoulos, N., (1974) Outcome of offspring of alcoholic women. Lancet 1:1076-1078.

Jones, K. L., Smith D. W., Ulleland, C., and Streissguth, A., (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1:1267-1271.

Kaminski, M., Franc, M., Lebouvier, M., DuMazaubrun, C., Rumeau-rouquette, C., (1981) Moderate alcohol use and pregnancy outcome. Neurobehav. Toxicol. Teratol. 3:173-181.

Kaminski, M., Rumeau-rouquette, C., and Schwartz, D., (1976) Consummation d'alcool chez les femmes encientes et issue de la grossesse. Rev. Epidem. Med. Soc. Sante Pub. 24:27-40

Kao, C., Chang, L., and Bloodworth, J., (1977) Axonal regeneration across transected mammalian spinal cords: An electron microscopic study of delayed microsurgical nerve grafting. Exp. Neurol. 54:591-615.

Karsarskis, E., Karpiak, S., Rappport, M., and Bass, N., (1979) Abnormal cortical maturation after neonatal anti-GM_1 ganglioside exposure. Trans. Am. Soc. Neurochem. 10:233.

Karsarskis, E., Karpiak, S., Rapport, M., Yu, R., and Bass, N., (1981) Abnormal maturation of cerebral cortex and behavioral deficit in adult rats after neonatal administration of antibodies to ganglioside. Brain Res. 227 :25-35.

Kasai, N., Pachner, A., and Yu, R., (1983) Anti-glycolipid antibodies in CSF of Multiple Sclerosis patients. Trans. Am. Soc. Neurochem. 14:182.

Kelly, P., and Cotman, C., (1978) Synaptic proteins. Characterization of tubulin and actin and identification of a distinct postsynaptic density polypeptide. J. Cell Biol. 79:173-183.

Kornguth, S., Rutledge, J., Sunderland, E., Siegel, F., Carlson, I., Smollen, J., Juhl, V., and Yound B., (1979) Impeded cerebellar development and reduced

serum thyroxine levels associated with fetal alcohol intoxication. Brain Res. 177:347-360.

Kornick, J., (1976) Teratogenic effects of ethyl alcohol administered to pregnant mice. Am. J. Obstet. Gynecol. 124:676-680.

Krigman, M., and Hogan, E.. (1976) Undernutrition in the developing rat: Effect upon myelination. Brain Res. 107:239-255.

Krisiak, M., Elis, J., Poschlova, N., and Masek, K., (1977) Increased aggressiveness and lower brain serotonin levels in offspring of mice given alcohol during gestation. J. Stud. Alcohol 38:1696-1704.

Kurihara, T., and Tsukada, Y., (1967) The regional and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphohydrolase in the central nervous system. J. Neurochem. 14:1167-1174.

Lancaster, F., Mayur, B., Patsalos, P., Samorajski, T., and Wiggins, R., (1982) The synthesis of myelin and brain subcellular membrane proteins in the offspring of rats fed ethanol during pregnancy. Brain Res. 235:105-113.

Langley, O., (1971) Sialic acid and membrane contact relationship in peripheral nerve. Exp. Cell. Res. 68:97 -105.

Lau, C., Thadani, P., Schanberg, S., and Slotkin, T., (1976) Effects of maternal ethanol ingestion on development of adrenal catecholamines and dopamine beta- hydroxylase in the offspring. Neuropharmacology 15:505-507.

Ledeen, R., (1978) Ganglioside structures and distribution: Are they localized in the nerve ending? J. Supramolec. Struc. 8:1-17.

Ledeen, R., Cochran, F., Yu, R., Samuels, F., and Haley, J., (1981) Gangliosides of the CNS myelin membrane. In Structure and function of gangliosides. L. Svennerholm, P. Mandel, H. Dreyfus, P.-F. Urban, Eds. pp. 167-176. Plenum Press, New York.

Ledeen, R., Yu, R., and Eng, L., (1973) Gangliosides of human myelin: Sialosylgalactoslyceramide (G_7) as a major component. J. Neurochem. 21: 829-839.

Leichter, J., and Lee, M., (1982) Method of ethanol administration as a confounding factor in studies of fetal alcohol syndrome. Life Sci. 31:221-227.

Lemoine, P., Harousseau, H., Borteyru, J., and Menuet, J., (1968) Les enfants de parents alcoholiques. Anomalies observees. A propos de 127 cas. Ouest Med. 25:477-482.

Lieber, C., and DeCarli, L., (1974) An experimental model of alcohol feeding and liver injury in the baboon. J. Med. Prim. 3:153-163.

Lieber, C., and DeCarli, L., (1982) The feeding of alcohol in liquid diets: Two decades of applications and 1982 update. Alcoholism 6:523-531.

Little, R., (1977) Moderate alcohol abuse during pregnancy and decreased infant birth weight. Am. J. Publ. Health 67:1154-1156.

Lochry, E., Shapiro, N., and Riley, E., (1980) Growth deficits in rats exposed to alcohol in utero. J. Stud. Alcohol 41:1031-1039

Lowry, O., Rosenbrough, N., Farr, A., and Randall, R., (1951) Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-275.

Majewski, F., and Bierich, J., (1979) Auxologic and clinical findings in the Alcohol embryopathy. Experience with 76 patients. Proceedings of the first International Congress of Auxology. Acta Med. Scan. 206:413-423.

Majewski, F., Bierich, J., Loser, H., Michaelis, R., Lieber, B., and Bethecken, F., (1976) Zur Klinik and Pathogenese der alkohol-embryopathie: Bericht uber 68 Fulle. Munch. Med. Wochenschr. 118:1635-1642.

Margolis, R. K., (1967) Acid mucopolysaccharides and proteins of bovine whole brain, white matter and myelin. Biochim. Biophys. Acta 141:91-102.

Margolis, R. K., and Margolis, R. U., (1979a) Structure and distribution of glycoproteins and glycosaminoglycans. In Complex carbohydrates of nervous tissue. R. K. Margolis and R. U. Margolis, Eds. pp. 45-55. Plenum Press, New York.

Margolis, R. U., and Margolis, R. K., (1979b) Perspectives and functional implications. In Complex carbohydrates of nervous tissue. R. U. Margolis and R.K. Margolis, Eds. pp. 377-384. Plenum Press, New York.

Margolis, R.U., Margolis, R.K., Chang, L., and Preti, C., (1975) Distribution and metabolism of glycoproteins and glycosaminoglycans in subcellular fractions of brain. Biochemistry 14:4797-4804.

Martin, J. C., Martin, D. C., Sigman, P., and Radow, B., (1978) Maternal ethanol consumption and hyperactivity in cross-fostered offspring. Physiol. Psychol. 6:362-365.

Matthieu, J.-M., Brady, R., and Quarles, R., (1974) Anomalies of myelin associated glycoproteins in Quaking mice. J. Neurochem. 22:291-296.

Matthieu, J.-M., Brady, R., and Quarles, R., (1975a) Change in a myelin-associated glycoprotein in rat brain during development: Metabolic aspects. Brain Res. 86:55-65.

Matthieu, J.-M., Koellreutter, B., and Joyet, M., (1978a) Protein and glycoprotein composition of myelin and myelin subfractions from brains of Quaking mice. J. Neurochem. 30:783-790.

Matthieu, J.-M., Quarles, R., Brady, R., and Webster, H., (1973) Variation of proteins, enzyme markers and gangliosides in myelin subfractions. Biochim. Biophys. Acta 329:305-317.

Matthieu, J.-M., Quarles, R., Poduslo, J., and Brady, R., (1975c) [^{35}S]Sulfate incorporation into myelin glycoproteins I. Central Nervous System. Biochim. Biophys. Acta. 392:159-165.

Matthieu, J.-M., Reier, P., and Sawchack, J., (1975b) Proteins of rat brain myelin in neonatal hypothyroidism. Brain Res. 84:443-451.

Matthieu, J.-M., Webster, H., DeVreis, G., Corthay, S., and Koellreuter, B., (1978b) Glial versus neuronal origin of myelin proteins and glycoproteins studies by combined intraocular and intracranial labeling. J. Neurochem. 31:93-102.

McIlwain, H., (1961) Characterization of naturally occurring materials which restore excitability to isolated cerebral tissues. Biochem. J. 78:24-32.

McIntyre, L., Quarles, R., and Brady, R., (1978a) the effect of trypsin on myelin-associated glycoprotein. Trans. Am. Soc. Neurochem. 9:106.

McIntyre, L., Quarles, R., and Brady, R., (1979) Lectin binding proteins in central nervous system myelin. Detection of glycoproteins of purified myelin on polyacrylamide gels by [^{3}H]Concanavalin A binding. Biochem. J. 183:205-212.

Michaelis, E., Michaelis, M., Chang, H., Grubbs, R., Kuonen, D., (1981) Molecular characteristics of glutamate receptors in the mammalian brain. Mol. Cell. Biochem. 38:163-179.

Michell, R., (1982) Perturbed lipid metabolism in the brain: Not so much a summing-up, more a provocation. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell and G. Porcellati, Eds. pp. 315-325. Raven Press, New York.

Mitchell, R., Allan, D., and Finean, J., (1975) Significance of minor glycerolipids in membrane structure and function. In Function and metabolism of phopholipids in the central and peripheral nervous systems. G. Porcellati, L. Amaducci and C. Galli, Eds. pp. 3-14. Plenum Press, New York.

Morell, P., Bornstein, M., and Raine,C., (1981) Diseases involving myelin. In Basic Neurochemistry. G. Siegel, R. Albers, B. Agranoff, R. Katzman, Eds., pp. 641-659 Little, Brown and Company, Boston.

Morgan, B., and Winick, M., (1980a) Effects of administration of N-acetylneuraminic acid (NANA) on brain NANA content and behavior. J. Nutr. 110:416-424.

Morgan, B., and Winick, M., (1980b) Effects of environmental stimulation on brain N-acetylneuraminic acid content and behavior. J. Nutr. 110:425-432.

Mozzi, R., Goracci, G., Siepi, D., Francescangeli, E., Andreoli, V., Horrocks, L., and Porcellati, G., (1982) Phospholipid synthesis by interconversion reactions in brain tissue. In Phospholipids in the nervous system. Vol. 1. L. Horrocks, G. Ansell, and G. Porcellati, Eds. pp. 1-12. Raven Press,

New York.

Mullin, B., Decandis, F., Montanoro, J., and Reid, J., (1981) Myelin basic protein interacts with the myelin specific ganglioside G_{M4}. Brain Res. 222:218-221.

Mullin, B., Montanaro, A., Reid, J., and Nishimura, R., (1980) Interaction of multiple sclerosis serum with liposomes containing ganglioside G_{M1}. Ann. Neurol. 7:587-590.

Mulvihill, J., Klimas, J., Stokes, D., and Risemberg, H., (1976) Fetal alcohol syndrome: Seven new cases. Am. J. Obstet. Gynecol. 125:937-941.

Mulvihill, J., and Yeager, A., (1976) Fetal alcohol syndrome. Teratol. 13:345-348.

Nagai, Y., and Iwamori, M., (1980) Brain and thymus gangliosides: Their molecular diversity and its biological implications and a dynamic annular model for their function in cell surface membranes. Mol. Cell. Biochem. 29:81-90.

Nagai, Y., Momoi, T., Saito, M., Mitsuzawa, E., and Ohtani, S., (1976) Ganglioside syndrome, a new autoimmune neurologic disorder, experimentally induced with brain ganglioside.Neurosci. Let. 2:107-111.

Nielsen, L., Pitts, M., Grady, S., and McGuire, E., (1981) Cell-cell adhesion in the embryonic chick: partial purification of liver adhesion molecules from liver membranes. Dev. Biol. 86:315-326.

Noronha, A., and Druse, M., (1982) Maternal ethanol consumption and synaptic membrane glycoproteins in offspring. J. Neurosci. Res. 8:83-97.

Norton, W., (1981) Formation, structure and biochemistry of myelin. In Basic neurochemistry, third edition. G. Siegel, R. Albers, B. Agranoff, and R. Katzman, Eds. pp. 63-92. Little, Brown and Co., Boston.

Norton, W., and Autilio, L., (1966) The lipid composition of purified bovine brain myelin. J. Neurochem. 13:213-222.

Norton, W., and Poduslo, S., (1973a) Myelination in rat brain: method of myelin isolation. J. Neurochem. 21:749-757.

Norton, W., and Poduslo, S., (1973b) Myelination in rat brain: Changes in myelin composition during brain maturation. J. Neurochem. 21:759-773.

Ocklind, C., Forsum, U., ad Obrink, B., (1983) Cell surface localization and tissue distribution of a hepatocyte cell-cell adhesion glycoprotein (Cell-CAM 105). J. Cell Biol. 96:1168-1171.

Ocklind, C., and Obrink, B., (1982) Intercellular adhesion of rat hepatocytes. Identification of a cell surface glycoprotein involved in the initial adhesion process. J. Biol. Chem. 257:6788-6795.

Olegard, R., Sabel, K.-G., Aaronsson, M., Sandin, B., Johansson, P., Carlsson, C., Kyllerman, M., Iversen, K., and Drbek, A., (1979) Effects on the child of alcohol abuse during pregnancy: Retrospective and prospective studies. Acta Paediatr. Scand. Supplement No. 275:112-212.

Osborne, G., Caul, W., and Fernandez, K., (1980) Behavioral effects of prenatal ethanol exposure and differential early experience in rats. Pharmacol. Biochem. Behav. 12:393-401.

Ouellette, E., Rosett, H., Rosman, N., and Weiner, L., (1977) Adverse effects on offspring of maternal alcohol abuse during pregnancy. N. Engl. J. Med. 297:528-530.

Palmer, H., Ouellette, E., Warner, L., and Leichtman, S., (1974) Congenital malformation in offspring of a chronic alcoholic mother. Pediatrics 53:490-494.

Peiffer, J., Majewski, F., Fischbach, H., Bierich, J., and Volk, B., (1979) Alcohol embryo- and fetopathy. Neuropathology of 3 children and 3 fetuses. J. Neurol. Sci. 41:125-137.

Pierog, S., Chandavasu, O., and Wexler, I., (1977) Withdrawal symptoms in infants with the fetal alcohol syndrome. J. Pediatr. 90:630-633.

Poduslo, J., Harman, J., and McFarlin, D., (1980) Lectin receptors in central nervous system myelin. J. Neurochem. 34:1733-1744.

Poser, C., (1978) Dysmyelination Revisited. Arch. Neurol. 35:401-408.

Quarles, R., Barbarash, G., and MacIntosh, T., (1983) Methods for the identification and characterization of glycoproteins in central and perpheral myelin. In Research Methods in Neurochemistry. N. Marks and R. Rodnight, Eds. Plenum Press, New York, (in press).

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

Quarles, R., and Everly, J., (1977) Glycopeptide fractions prepared from purified contral and peripheral rat myelin. Biochim. Biophys. Acta 466:176-186.

Quarles, R., Everly, J., and Brady, R., (1972) Demonstration of a glycoprotein which is associated with a purified myelin fraction from rat brain. Biochem. Biophys. Res. Commun. 47:491-497.

Quarles, R., Everly, J., and Brady, R., (1973a) Evidence for the close association of a glycoprotein with myelin in rat brain. J. Neurochem. 21:1177-1191.

Quarles, R., Everly, J., and Brady, R., (1973b) Myelin-associated glycoprotein: A developmental change. Brain Res. 58:506-509.

Quarles, R., Foreman, C., Poduslo, J., and McIntyre, L., (1977) Interactions of myelin-associated glycoproteins with immobilized lectins. Trans. Am. Soc. Neurochem. 8:201.

Quarles, R., McIntyre, L., and Sternberger, N., (1979) Glycoproteins and cell surface interactions during myelinogenesis. Society for Neuroscience Symposia IV:322-343.

Raine, C., (1977) Morphological aspects of myelin and myelination. In Myelin. P. Morell., Ed. pp. 1-49. Plenum Press, New York.

Randall, C., and Taylor, W., (1979) Prenatal ethanol exposure in mice: Teratogenic effects. Teratol. 19:305-312.

Randall, C., Taylor, W., Tabakoff, B., and Walker, D., (1977) Ethanol as a teratogen. In Alcohol and aldehyde metabolizing systems. R. Thurmon, J. Williamson, H., Drott, and B. Chance, Eds. pp. 659-670. Academic Press, New York.

Rappoport, D., Fritz, R., and Yamagami, S., (1971) Development. In Handbook of Neurochemistry Vol V. Part B, A. Lajtha, Ed. pp. 439-471. Plenum Press, New York.

Rapport, M., Karpiak, S., Mahadik, S., (1980) Perturbation of CNS functions by antibodies to gangliosides. Speculations on biological roles of ganglioside receptors. In Structure and function of gangliosides. L. Svennerholm, P. Mandel, H. Dreyfus, P.-F. Urban, Eds. pp. 335-338. Plenum Press, New York.

Rawat, A., (1975) Ribosomal protein synthesis in the fetal and neonatal rat brain as influenced by maternal ethanol consumption. Res. Comm. Chem. Path. Pharmacol. 12:723-732.

Rawat, A., (1977) Developmental changes in the brain levels of neurotransmitters as influenced by maternal ethanol consumption in the rat. J. Neurochem. 28:1175-1182.

Rawat, A., (1980) Development of histaminergic pathways in brain as influenced by maternal alcoholism. Res. Comm. Chem. Path. Pharmacol. 27:91-103.

Reeber, A., Vincendon, G., and Zanetta, J.-P., (1980) Transient Concanavalin A-binding glycoproteins of the parallel fibers of the developing rat cerebellum: Evidence for the destruction of their glycans. J. Neurochem. 35:1273-1277.

Reig, J., Ramos, J., Cozar, M., Aguilar, J., Criado, M., and Monreal, J., (1982) Purification and chemical characterization of a W2 protein from brain myelin. J. Neurochem 39:507-511.

Reiss, D., Lees, M., and Sapirstein, V., (1981) Is Na + K ATPase a myelin-associated enzyme? J. Neurochem. 36:1418-1426.

Rick, J., Gregson, A., Liebowitz, S., and Adinolfi, M., (1980) Behavioural changes in adult rats following administration of antibodies against brain gangliosides.Develop. Med. Child Neurol. 22:719-724

Riley, E., Lochry, E., and Shapiro, N., (1979a) Lack of response inhibition in rats prenatally exposed to alcohol. Psychopharmacol. 62:47-52.

Riley, E., Lochry, E., Shapiro, N., and Baldwin, J., (1979b) Response perseveration in rats exposed to alcohol prenatally. Pharmacol. Biochem. Behav. 10:255-259.

Riley, E., Lochry, E., and Shapiro, N., (1979c) Response perseveration in rats exposed to alcohol prenatally. Pharmacol. Biochem. Behav. 10:255-259.

Rogart, R., and Ritchie, J., (1977) Physiological basis of conduction in myelinated nerve fibers. In Myelin. P. Morell, Ed. pp. 117-159. Plenum Press, New York.

Roseman, S., (1970) Synthesis of complex carbohydrates by multi-glycosyltransferase systems and their potential functions in intercellular adhesion. Chem. Phys. Lipids 5:270-297.

Rosman, N., and Malone, M., (1976) An experimental study of the fetal alcohol syndrome. Neurology 26:365.

Rosman, N., and Malone, M., (1979) Spontaneous reversal of delayed myelinogenesis in experimental fetal alcohol syndrome. Neurology 29:543.

Rossiter, R., and Strickland K., (1970) Metabolism of phosphoglycerides. In Handbook of Neurochemistry. Vol. III. A. Lajtha, Ed. pp. 467-489. Plenum Press, New York.

Root, A., Reiter, E., Andriola, M., and Duckett, G., (1975) Hypothalamic-pituitary function in the fetal alcohol syndrome. J. Pediatr. 87:585-588.

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

Saito, A., and Rosenberg, A., (1982b) Sialosylgalactosylceramide (G_{M4}) is a major ganglioside in chicken embryonic liver. J. Lipid Res. 23:9-13.

Salvetera, P., and Matthews, D., (1980) Isolation of rat brain subcellular fraction enriched in putative neurotransmitter receptors and synaptic junctions. Neurochem. Res. 5:181-195.

Santolaya, J., Martinez, G., Gorostiza, E., Aizpiri, J., and Hernandez, M., (1978) Alcoholismo fetal. Drogalcohol 3:183-192.

Sato, S., Quarles, R., and Brady, R., (1982) Susceptibility of the myelin-associated glycoprotein and basic protein to a neutral protease in highly purified myelin from human and rat brain. J. Neurochem. 39:97-105.

Schachter, H., and Roseman, S., (1980) Mammalian glycosyltransferases: Their role in the synthesis and function of complex carbohydrates and proteoglycans. In The biochemistry of glycoproteins and proteoglycans. W. Lennarz, Ed. pp. 85-160. Plenum Press, New York.

Selivonchick, D., Schmid, P., Natarajan, V., and Schmid, H., (1980) Structure and metabolism of phospholipids in bovine epididymal spermatozoa. Biochim. Biophys. Acta 618:242-254.

Shaywitz, S., Cohen, D., and Shaywitz, B., (1980) Behavior and learning deficits in children of normal intelligence born to alcoholic mothers. J. Pediatr. 96:978-982.

Shaywitz, B., Klopper, J., and Gordon, J., (1976) A syndrome resembling minimal brain dysfunction (MBD) in rat pups born to alcoholic mothers. J. Pediatr. 96:978-982.

Sherwin, B., Jacobson, S., and Zagorski, D., (1981) A rat model of the fetal alcohol syndrome: Preliminary histological findings. Curr. Alc. 8:485-510.

Shichi, H., (1981) Molecular biology of the visual process. In Basic Neurochemistry. G. Siegel, R. Albers, B. Agranoff, and R. Katzman, Eds. pp. 145-158. Little, Brown and Co., Boston.

Shruygin, G., (1974) Characteristics of the mental development of children of alcoholic mothers. Pediatriya Mosk. 11:71-73.

Siegel, G., Stahl, W., and Swanson, P., (1981) Ion transport. In Basic Neurochemistry. G. Siegel, R. Albers, B. Agranoff, and R., Katzman, Eds. pp. 107-143. Little, Brown and Co., Boston.

Simon, R., Gill, M., Lemkey-Johnston, N., and Larramendi, L., (1971) Cholesterol and phospholipid composition of cerebellar fractions enriched in unmyelinated axons and synaptosomes. Brain Res. 13:504-513.

Simpson, D., Thorne, D., and Loh. H., (1976) Sulfated glycoproteins, glycolipids, and glycosaminoglycans from synaptic plasma and myelin membranes: Isolation and characterization of sulfated glycopeptides. Biochemistry 15:5449-5457.

Slotkin, T., Schanberg, S., and Kuhn, C., (1980) Synaptic development in brains of rats exposed perinatally to ethanol. Experientia 36:1005-1007.

Smith, D. W., (1980) Alcohol effects on the fetus. In Psychopharmacology of alcohol, M. Sandler, Ed. pp. 257-263. Raven Press, New York.

Smith, R., (1977) Non-covalent cross-linking of lipid bilayers by myelin basic protein. A possible role in myelin formation. Biochem. Biophys. Acta 470:170-184.

Smith, R., (1980) Sedimentation analysis of the self-association of bovine myelin basic protein. Biochemistry 19:1826-1831.

Sokol, R., Miller, S. I., and Reed, G., (1980) Alcohol abuse during pregnancy: An epidemiologic study. Alc. Clin. Exp. Res. 4:135-145.

Soto, E., deBohner, L., and Calvino, M., (1966) Chemical composition of myelin and other subcellular fractions isolated from bovine white matter. J. Neurochem. 13:989-998.

Stanley, J., Saul, R., Hadfield, M., and DeVries, G., (1979) Mammalian central nervous system axolemma: Histochemical evidence for axonal plasma membrane origin of bovine and rat axolemma-enriched membrane fractions. Neuroscience 4:155-167.

Sternberger, N., Quarles, R., Itoyama, Y., and Webster, H., (1979) Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rat. Proc. Natl. Acad. Sci., USA 76:1510-1514.

Stibler, H., Burns, E., Kruckeberg, T., Gaetano, P., Cerven, E., Borg,S., Tabakoff, B., (1983) Effect of ethanol on synaptosomal sialic acid metabolism in the developing rat brain. J. Neurol. Sci., 59:21-35.

Streissguth, A., (1976) Psychologic handicaps in children with the fetal alcohol syndrome. Ann. N. Y. Acad. Sci. 273:140-145.

Streissguth, A., Barr, H., Martin, D., and Herman, C., (1980) Effects of maternal alcohol, nicotine and caffeine used during pregnancy on infant mental and motor development at eight months. Alcoholism 4:152-164.

Streissguth, A., Herman, C., and Smith, D. W., (1978) Intelligence, behavior and dysmorphogenesis in the fetal alcohol syndrome. A report of 20 pateints. J. Pediatr. 92:363-367.

Streissguth, A., Martin, D., Martin, J., and Barr, H., (1981) The Seattle longitudinal prospective study on alcohol and pregnancy. Neurobehav. Toxicol. Teratol. 3:223-233.

Struck, D., and Lennarz, W., (1976) Utilization of exogenous GDP-mannose for the synthesis of mannose-containing lipids and glycoproteins by oviduct cells.J. Biol. Chem. 251:2511-2519.

Sulik, K., Johnston, M., and Welb, M., (1981) Fetal alcohol syndrome: Embryogenesis in a mouse model. Science 214:936-938.

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

Suzuki, K., (1967) Formation and turnover of the major brain gangliosides during development. J. Neurochem. 14:917-925.

Suzuki, K., (1970) Formation and turnover of myelin ganglioside. J. Neurochem. 17:209-213.

Suzuki, K., Poduslo, S., and Norton, W., (1967) Ganglioside in the myelin fraction of developing rats. Biochim. Biophys. Acta 144:375-381.

Suzuki, K., Poduslo, J., and Poduslo, S., (1968) Further evidence for a specific ganglioside fraction closely associated with myelin. Biochim. Biophys. Acta 152:576-586.

Svennerholm, L., (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta 24:604-611.

Sweadner, K., (1979) Two molecular forms of $(Na^+ + K^+)$-stimulated ATPase in brain . Separation and difference in affinity for strophanthidin. J. Biol. Chem. 254:6060-6067.

Tewari, S., Do, K., and Medina, R., (1983) Brain protein metabolism and in utero drug exposure. Trans. Am. Soc. Neurochem. 14:117.

Thadani, P., Lau, C., Slotkin, T., and Schanberg, S., (1977) Effects of maternal ethanol ingestion on amine uptake into synaptosomes of fetal and neonatal rat brain. J. Pharmacol. Exp. Ther. 200:292-297.

Townsend, R., Li, Y., and Li, S., (1979) Brain glycosidases. In Complex carbohydrates of nervous tissue. R.U. Margolis and R.K. Margolis, Eds. pp. 127-137. Plenum Press, New York.

Tze, W., and Lee, M., (1975) Adverse effects of maternal alcohol consumption on pregnancy and fetal growth in rats. Nature 257:479-480.

Ulleland, C., (1972) The offspring of alcoholic mothers. Ann. N. Y. Acad. Sci. 197:167-169.

Van den Bosch, H., (1974) Phoshophoglyceride metabolism. Ann. Rev. Biochem. 43:243-277.

Van Heyningen, W., (1974) Cholera toxin: Interaction of its subunits with ganglioside G_{M1}. Science 183:656-657.

Vance, D., and Sweeley, C., (1967) Quantitative determination of the neutral glycosyl ceramides in human blood. J. Lipid Res. 8:621-630.

Volk, B., (1977) Delayed cerebellar histogenesis in embryofetal alcohol syndrome. Acta Neuropathol. (Berlin) 39:157-163.

Volk, B., Maletz, J., Tiedemann, M., Nall, G., Klein, C., and Berlet, H., (1981) Impaired maturation of Purkinjie cells in the fetal alcohol syndrome of the rat. Acta Neuropathol. (Berlin) 54:19-29.

Waehneldt, T., Matthieu, J.-M., and Neuhoff, V., (1977) Characterization of a myelin-related fraction (SN 4) isolated from rat forebrain at two developmental stages. Brain Res. 138:29-43.

Weinberg, H., and Spencer, P., (1976) Studies on the control of myelinogenesis. II. Evidence for neuronal regulation of myelin production. Brain Res. 113:363-378.

Weinberg, E., and Spencer, P., (1979) Studies on the control of myelinogenesis. III. Signalling of oligodendrocyte myelination by regenerating peripheral axons. Brain Res. 162:273-279.

Weiner, S., (1980) Nutritional considerations in the design of animal models of fetal alcohol syndrome. Neurobehav. Toxicol. 2:175.

Weiner, S., Shoemaker, W., Koda, L., and Bloom, F., (1981) Interaction of ethanol and nutrition during gestation: Influence on maternal and offspring development in the rat. J. Pharmacol. Exp. Ther. 216:572-579.

West, J., Hodges, C., and Black, A., (1981) Prenatal exposure to ethanol alters the organization of hippocampal mossy fibers in rats. Science 211:957-959.

West, J., and Hodges-Savola, C., (1983) Permanent hippocampal mossy fiber hyperdevelopment following prenatal ethanol exposure. Neurobeh. Toxicol. Teratol. 5:139-150.

Wiggins, R., and Fuller, G., (1978) Early postnatal starvation causes lasting brain hypomyelination. J. Neurochem. 30:1231-1237.

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

Wood, J., Jean, D., Whitaker, J., McLaughlin, B., and Albers, R., (1977) Immunocytochemical localization of the sodium, potassium activated ATPase in knifefish brain. J. Neurocytol. 6:571-581.

Yamada, K., Critchley, D., Fishman, P., and Moss, J., (1983) Exogenous gangliosides enhance the interaction of fibronectin with ganglioside-deficient

cells. Exp. Cell Res. 143:295-302.

Yamakawa, T., and Nagai, Y., (1978) Glycolipids at the cell surface and their biological functions. Trends in Biochem. Sci. 3:128-131.

Yohe, H., Ueno, K., Chang, N., Glaser, G., and Yu, R., (1980) Incorporation of N-acetylmannosamine into rat brain subcellular gangliosides: Effect of pentylenetetrazol-induced convulsions on brain gangliosides. J. Neurochem. 34:560-568.

Yohe, H., and Yu, R., (1982) Myelin basic protein protects gangliosides from neuraminidase action. Trans. Am. Soc. Neurchem. 10:93.

Yu, R., and Iqbal, K., (1979) Sialosylgalactosylceramide as a specific marker for human myelin and oligodendroglial perikarya: Gangliosides of human myelin, oligodendroglia and neurons. J. Neurochem. 32:293-300.

Yu, R., and Lee, S., (1976) In vitro biosynthesis of sialosylgalactosylceramide (G_7) by mouse brain microsomes. J. Biol. Chem. 251:198-203.

Yu, R., and Yen, S., (1975) Gangliosides in developing mouse brain myelin. J. Neurochem. 25:229-232.

Zanetta, J.-P., Roussels, G., Ghandour, M., Vincendon, G., and Gombos, G., (1978) Postnatal development of rat cerebellum: Massive and transient accumulation of Concanavalin A binding glycoproteins in parallel fiber axolemma. Brain Res. 142:301-319.

Zanetta, J.-P., Vitiello, F., and Vincendon, G., (1980) Gangliosides from rat cerebellum: Demonstration of considerable heterogeneity using a new solvent system for thin layer chromatography. Lipids 15:1055-1061.

Zatz, M., and Barondes, S., (1971) Rapid transport of fucosyl glycoproteins to nerve endings in mouse brain. J. Neurochem. 18:1125-1133.

Zetusky, W., Calabrese, V., and DeVries, G., (1978) Sphingolipid fatty acid compisition of human CNS myelin and axolemma. Trans. Am. Soc. Neurochem. 9:180.

Zetusky, W., Calabrese, V., Zetusky, Z., Anderson M. Cullen, M., and DeVries, G., (1979) Isolation and partial characterization of human CNS axolemma-enriched fractions. J.Neurochem. 32:1103-1109.

Zimmerman, A., Matthieu, J.-M., Quarles, R., Brady, R., and Hsu, J., (1976) Hypomyelination in copper deficient rats. Arch. Neurol. 33:111-119.

APPENDIX

Table A-1

% Distribution of Sialic Acid

Among Individual Myelin Gangliosides of Control and Ethanol Offspring

Ganglioside	Control	Ethanol	Control	Ethanol	Control	Ethanol
	17 Days		24 Days *		31 Days *	
G_{Q1b}	2.6±1.3	3.5±1.6	2.2±1.8	1.0±0.8	1.1±1.0	0.9±0.3
G_{T1b}	26.1±5.0	26.2±4.0	6.0±4.6 d	12.2±8.9 d	3.5±4.3 d	3.3±3.0 d
G_{T1a}	4.3±0.4	4.4±0.5	2.6±2.6	3.6±2.1	1.5±0.7 d	0.7±1.4 d
G_{D1b}	13.3±1.1	14.2±3.1	18.6±3.8	20.0±1.8	17.4±7.4	17.7±5.0
G_{D2}	--	--	2.5±1.0	1.7±0.6	1.9±0.3	2.7±1.6
G_{D1a}	26.0±3.0	29.2±2.6	28.0±5.7	29.5±3.8	21.56.6	26.3±7.9
G_{D3}	2.4±1.2	2.4±1.6	3.8±2.0	3.9±1.9	6.0±3.2 d	4.9±3.7
G_{M1}	15.0±4.3	13.4±3.2	27.7±6.0 d	22.0±4.9 d	38.0±9.9 d	35.9±8.0 d
G_{M2}	1.1±1.3	1.2±0.7			0.9±0.6	2.9±2.4
G_{M3} G_{M4}	[8.3±3.6]	[5.3±2.6]	[10.3±6.1]	[7.8±2.1]	[9.9±5.7]	[7.3±2.8]

Each value represents the mean of values from 5 (24 and 31d) to 6 (17d) C and E pups ± the standard deviation. Values in brackets represent combined G_{M3} and G_{M4} (17 and 31d) and combined G_{M2}, G_{M3} and G_{M4} (24d). A 'd' indicates a statistically significant ($p < 0.05$) developmental change (compared to 17 days).

Table A-2

% Distribution of Radioactivity

Among Individual Myelin Gangliosides of Control and Ethanol Offspring

Ganglioside	Control	Ethanol	Control	Ethanol	Control	Ethanol
	17 Days		24 Days		31 Days	
G_{Q1b}	3.2±1.3	3.5±1.6	2.0±1.0	2.8±1.0	3.1±1.4	2.9±1.4
G_{T1b}	23.9±8.1	25.6±5.0	5.8±4.3 d	11.7±10.8 d	4.0±2.4 d	4.6±3.3 d
G_{T1a}	4.2±3.1	4.5±3.7	7.6±2.3	9.4±2.9	4.5±3.6	6.4±6.6
G_{D1b}	10.5±3.9	11.4±4.1	11.4±3.5	14.3±6.4	11.5±7.3	12.0±4.9
G_{D2}	1.4±0.8	1.3±0.9	2.6±1.2	2.3±1.6	6.3±4.2 d	5.4±2.7 d
G_{D1a}	13.8±4.3	15.4±5.1	10.9±3.6	9.5±3.8	10.8±2.2	11.9±7.6
G_{D3}	2.3±1.0	2.0±1.0	3.8±0.7	3.5±2.4	5.4±1.5 d	4.7±2.0 d
G_{M1}	16.0±4.1	13.5±4.0	18.2±4.0	14.4±2.3	22.4±6.3	21.0±5.6 d
G_{M2}	10.3±4.5	10.0±5.1	11.9±3.0	11.4±3.3	9.8±2.7	9.6±3.1
G_{M3}	9.0±2.3	9.0±2.4	11.1±1.4	9.8±4.4	10.4±3.7	10.7±4.3
G_{M4}	5.4±3.1	3.6±2.6	14.9±4.0 d	11.0±5.7 d	13.2±5.3 d	10.7±6.3 d

Each value represents the mean ± the standard deviation from 6 samples. A 'd' indicates a statistically significant ($p < 0.05$) developmental change (compared to 17 days).

Table A-3

% Distribution of Radioactivity in Fucosylated Glycoproteins of Individual Coomassie Blue Stained Bands in 1.0/1.2 Axolemma from 21 Day-Old Offspring

Band	MW region	Gels from which data is taken: Figure 23-Gel A C	Figure 23-Gel A E	Gel D- Not pictured C	Gel D- Not pictured E	Figure 28-Gel E C	Figure 28-Gel E E
1		3.9	5.2	2.2	3.2	2.0	2.1
2	220 K	2.5	2.6	2.2	2.8	3.0	3.2
3	and up	1.4	1.7	1.7	1.7	2.0	1.9
4		2.4	2.0	1.8	2.0	1.9	2.2
5		2.8	3.0	2.6	3.0	2.3	2.5
6		3.2	3.4	3.4	4.1	1.5	1.9
7	100-	3.5	3.0	2.4	2.9	2.2	2.7
8	220 K	4.5	4.4	4.5	4.4	2.2	2.5
9		3.0	4.6	4.7	2.4	4.7	5.1
10		3.1	3.3	3.6	3.6	3.1	4.0
11		2.9	3.6	3.2	4.6	3.5	2.2
12		2.2	3.6	3.4	3.9	2.4	3.6
13		1.4	2.9	1.8	2.4	3.8	4.1
14	67-	3.2	3.2	2.5	2.6	3.45	4.0
15	100 K	3.4	3.4	3.2	3.0	3.3	4.1
16		3.4	4.4	4.1	3.5	4.7	4.4
17		2.6	4.1	4.2	5.2	3.4	3.5
18		3.3	3.7	2.1	3.7	3.2	3.9
19	60-	4.8	5.1	1.6	2.0	4.5	4.7
20	67 K	4.6	5.9	4.2	4.0	5.0	5.8
21		4.1	4.6	4.7	5.2	3.4	4.1
22		2.6	3.4	6.6	5.5	2.9	2.5
23		3.15	1.8	2.4	2.6	2.4	2.3
24	43-	1.9	2.4	5.1	3.0	2.3	2.1
25	60 K	3.4	0.1	2.5	3.1	4.2	4.4
26		4.6	2.4	2.1	2.4	4.9	3.8
27		2.0	1.5	2.8	3.4	3.2	1.6
28		1.8	2.5	3.1	1.1	1.2	1.2
29		1.4	0.9	2.1	2.3	1.3	0.01
30	30-	2.1	0.9	1.8	1.7	1.0	1.1
31	43 K	1.0	0.05	1.6	0.3	1.0	1.1
32		1.0	1.7	0.04	0.1	1.1	1.0
33		1.5	1.0	1.0	0.3	1.6	1.0
34		1.3	0.02	1.2	1.3	1.0	0.9
35		1.3	0.7	0.4	0.02	1.2	0.9
36	<30 K	0.7	0.9	0.5	0.2	1.0	0.8
37		0.5	0.7	0.8	1.1	0.8	0.5
38		1.0	0.02	1.1	1.0	0.9	0.8
39		1.2	0.02	0.04	0.08	1.0	0.06
40		0.02	0.4	0.2	0.3	0.5	0.4

All values represent a single sample. Molecular weight regions analyzed in Table XI are marked off for each gel.

Table A-4

Distribution of Radioactivity in Fucosylated Glycoproteins of Individual Coomassie Blue Stained Bands in 1.0/1.2 Axolemma

Band	~MW	22 Days C*-C*	22 Days E*-E*	31 Days C*-C*	31 Days E*-E*	32 Days C*-C*	32 Days E*-E*
1	330 K	2.9±1.2	9.5±7.4	3.7±2.2	1.7±1.0	3.1±0.5	3.2±1.1
2		2.8±0.9	3.7±1.2	3.4±1.4	2.7±0.2	3.2±0.4	3.6±0.8
3		2.0±0.7	2.2±0.3	2.5±1.1	1.76±0.05	2.2±0.4	2.0±0.7
4	220 K	2.8±0.5	1.8±0.2 *	2.0±1.0	1.6±0.3	2.4±0.3	2.2±0.2
5		2.9±0.7	3.4±0.7	3.4±1.4	2.3±0.2	3.2±0.1	3.2±0.5
6	180 K	3.8±0.3	3.1±0.2 *	2.2±0.7 d	1.4±0.4 dd	3.5±0.6	4.8±0.7 d
7		3.7±0.5	3.6±0.4	2.5±0.8	1.5±0.1	3.1±0.2	4.0±0.7
8	150 K	4.9±0.7	4.7±0.3	3.5±0.9	2.9±0.2	4.9±0.4	5.6±0.1 *
9		4.8±0.4	4.1±0.5	4.3±0.2	4.0±0.3	5.1±0.3	5.0±1.4
10		3.2±0.3	3.0±0.4	5.9±1.2 d	4.5±0.5 d	4.7±0.5 d	5.3±0.1 dd
11		4.0±0.8	4.2±0.1	3.7±0.8	3.7±0.1	1.7±0.1 dd	2.7±0.9 d
12	100 K	3.9±0.6	3.6±0.5	4.5±1.1	3.8±0.5	4.2±0.2	4.2±0.3
13	94 K	3.5±0.3	3.6±0.4	3.6±1.1	3.3±0.4	3.9±0.4	3.8±0.6
14	85 K	3.6±0.7	4.0±0.1	3.8±0.3	3.9±0.3	4.4±0.3	5.7±0.1 dd**
15	76 K	4.2±2.2	2.5±2.6	4.7±0.3	5.4±0.5	5.2±0.3	6.6±0.8 *
16	70 K	3.4±0.7	3.2±0.4	3.7±0.6	3.6±0.5	4.1±0.4	4.4±1.2
17	67 K	4.4±0.3	3.8±0.9	4.0±0.2	4.7±1.0	5.5±0.3 d	5.8±0.9
18		5.4±0.1	4.3±1.3	5.1±1.5	4.9±0.7	5.6±0.3	5.0±1.5
19	65 K	6.3±0.7	5.2±1.8	5.5±0.8	6.4±1.3	5.7±0.3	7.4±1.6
20	62 K	2.7±2.5	0.4±0.4	5.2±2.5	5.0±0.1 dd	5.3±0.2	2.2±1.7 *
21		3.1±0.6	1.9±0.3 *	3.0±0.5	2.9±0.4 d	2.5±0.2	2.9±0.5 d
22	60 K	2.8±1.0	2.6±1.3	1.9±0.8	1.4±1.1	3.0±0.6	2.2±2.0
23	55 K	5.6±0.7	5.7±0.8	3.0±1.0 d	2.3±0.6 dd	3.5±1.5	1.0±1.0 dd
24	52 K	5.8±0.3	5.7±1.0	5.5±0.7	4.7±0.6	2.6±0.1 dd	2.6±1.0 d
25	49 K	2.5±0.3	2.2±0.2	3.9±1.2	4.2±0.7	1.74±0.01	2.1±0.4
26		2.1±0.2	2.0±0.1	1.9±0.4	2.2±0.5	2.1±0.1	2.3±0.9
27	43 K	1.7±0.7	2.2±0.1	1.9±0.9	1.7±0.3	0.1±0.1	0.1±0.1
28	42 K	0.6±0.6	0.1±0.2	2.0±0.6	2.2±0.2	1.4±0.1	1.6±0.2
29	38 K	0.3±0.4	0.3±0.4	1.2±0.2	1.2±0.3	1.0±0.2	1.2±0.1
30	35 K	1.1±0.3	1.3±0.2	1.2±0.4	0.8±0.1	0.7±0.6	0.4±0.6

Each value represents the mean of 3 animals ± the standard deviation. An * indicates a statistically significant difference ($p < 0.05$) between C*-C* and E*-E*. A ** indicates a significant difference at $p < 0.01$. A 'd' indicates a significant ($p < 0.05$) developmental change (compared to 22 days)and a 'dd' indicates the change is significant at $p < 0.01$.

Table A-5

Distribution of Radioactivity in Gangliosides from 0.8/1.0 Axolemma

Ganglioside	20 Days		27 Days		34 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
Q1b	4.0±1.4	5.1±1.6	11.7±6.8 d	12.8±5.9 d	7.9±0.7 dd	7.6±1.4 d
T1b	28.6±8.3	25.0±6.5	[31.2±5.1	31.8±5.3]	33.6±5.3	34.5±3.0 dd
T1a	3.8±2.2	3.3±1.3			8.7±2.5 d	9.6±3.5 dd
D1b	13.4±1.4	13.0±1.2	18.6±3.7 d	19.2±3.5 dd	12.9±1.0	13.0±1.5
D2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
D1a	31.0±7.6	34.9±3.9	26.8±6.0	25.3±5.1 dd	24.5±1.5	24.5±3.0 dd
D3	2.9±0.8	2.8±1.9	2.6±0.8	2.5±0.8	2.4±1.6	2.0±0.9
M1	8.8±4.3	7.8±1.9	6.3±1.6	5.7±1.1	5.6±0.8	5.8±0.7
M2	1.0±0.8	1.1±0.6	0.5±0.4	0.4±0.3	0.8±0.9	0.9±0.9
M3	2.2±0.6	2.5±0.7	2.0±0.9	2.2±0.7	1.9±1.2	1.8±1.1
M4	0.9±0.8	0.9±0.6	0.4±0.6	0.1±0.1 d	0.4±0.4	0.4±0.4

Each value represents the mean of six animals except for the 20d values (mean of 4). A 'd' represents a significant ($p < 0.05$) developmental change (compared to 20 days) while a 'dd' represents a developmental change at $p < 0.01$. Values in [] are T_{1b} and T_{1a} combined.

Table A-6

Distribution of Radioactivity in Gangliosides from 1.0/1.2 Axolemma

Ganglioside	20 Days		27 Days		34 Days	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
Q1b	5.6±3.7	4.4±0.9	9.4±3.9	8.4±4.4	10.8±4.6	10.8±4.7 d
T1b	19.5±2.9	20.9±3.5	35.0±3.1 dd	36.1±2.2 dd	33.2±4.3 dd	34.2±2.7 dd
T1a	5.7±3.5	6.4±3.3	6.4±1.8	6.0±2.1	7.6±3.1	8.3±2.5
D1b	15.9±7.8	16.0±7.9	10.9±2.3	10.6±2.1	11.9±1.5	11.3±1.1
D2	N.D.	N.D.	3.7±1.0	5.2±0.4	3.9±1.7	4.0±1.5
D1a	28.4±1.8	31.6±7.0	25.3±1.7 d	25.6±3.5	21.9±2.3 dd	20.5±2.3 d
D3	1.9±0.7	2.4±0.4	2.5±0.8	2.8±0.7	2.3±0.5	2.4±0.4
M1	9.3±3.0	10.2±0.5	5.8±0.9	6.7±1.3	5.5±1.3	5.0±0.5
M2	3.9±3.3	2.8±2.2	1.7±1.3	1.6±0.5	0.8±0.3	1.4±0.8
M3	0.8±1.4	1.1±1.1	1.0±0.8	1.0±0.5	1.3±1.2	1.3±0.9
M4	3.1±3.5	0.4±0.5	0.9±0.8	0.6±0.8	0.3±0.2	0.3±0.3

Each value represents the mean of 6 animals except for the 20d values (mean of 4). A 'd' signifies a significant ($p < 0.05$) developmental difference (compared to 20 days). A 'dd' represents a significant developmental change at $p < 0.01$ (compared to 20 days).

Table A-7

Distribution of Radioactivity in Phospholipids from 1.0/1.2 Axolemma

	20 Days		27 Days		34 Days	
Phospholipid	Control	Ethanol	Control	Ethanol	Control	Ethanol
PA	1.7±0.7	2.2±0.3	1.1±0.5	0.9±0.5	1.4±0.3	1.4±0.5
PS	5.1±2.0	5.0±1.0	7.0±1.1	6.7±1.2	8.4±1.8	8.3±0.9
PI	8.7±2.3	6.6±2.8	9.2±2.5	8.3±0.7	9.2±1.4	9.2±2.6
PC	56.2±2.6	55.8±2.7	54.0±3.6	56.9±1.6	52.9±0.8	52.2±2.8
PE	20.8±2.4	22.6±3.7	23.1±1.1	22.5±1.3	24.7±2.0	24.5±1.4
NL*	7.6±0.9	7.8±0.7	5.6±2.0	5.1±1.3	3.4±1.3	4.3±0.7

Each 20 day value represents the mean of three animals while 27 and 34 day values are the means of 5-6 animals. The * indicates that the neutral lipid fraction is not a phospholipid. No statistically significant differences were seen between C*-C* and E*-E* or developmentally (compared to 20 days).

APPROVAL SHEET

The dissertation submitted by Jean Mary Gnaedinger has been approved by the following committee:

Mary Druse-Manteuffel, Ph.D., Director
Associate Professor, Biochemistry and Biophysics, Loyola

Michael A. Collins, Ph.D.
Professor, Biochemistry and Biophysics, Loyola

Irene Held, Ph.D.
Associate Professor, Pharmacology and Biochemistry and Biophysics, Loyola
and Chief, Neuroscience Laboratory, Veterans Administration Hospital, Hines

Faith W. LaVelle, Ph.D.
Associate Professor, Anatomy
Loyola

Ruthann P. Sturtevant, Ph.D.
Associate Professor, Anatomy and Surgery, Loyola

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

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

August 26, 1983 — Mary Druse-Manteuffel

Date — Mary Druse-Manteuffel, Ph.D., Director