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THE EFFECTS OF MATERNAL PROTEIN DEFICIENCY
ON THE DEVELOPMENT OF SYNAPTIC PLASMA
MEMBRANES IN RAT OFFSPRING

by

Duane M. Smith, Jr.

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

May

1982

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Finally, I would like to dedicate this dissertation to my loving wife Carole - her help and support are incalculable.

VITA

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LIST OF ABBREVIATIONS

Abs	Absorbance
2',3'-cAMP	2',3'-cyclic nucleotide (adenosine) monophosphate
3'-AMP	3'-adenosine monophosphate
5'-AMP	5'-adenosine monophosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate phosphohydrolase
CNPase	2',3'-cyclic nucleotide 3'- phosphohydrolase
CPM	counts per minute
DOC	deoxycholic acid
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
DPM	dissintegrations per minute
EDTA	ethylenediamine tetraacetic acid
4-HOQ	4-hydroxyquinoline
HPTLC	high performance thin layer chromatography
HSA	human serum albumin
KDHB	Kynuramine dihydrobromide
MAO	monoamine oxidase
NANA	N-acetylneuraminic acid
PAGE	Polyacrylamide gel electrophoresis
Pi	inorganic phosphate

POPOP	1,4-Bis (5-phenyloxazol 2-yl) benzene
PPO	2,5-diphenyloxazole
SDS	sodium dodecyl sulfate
SPM	synaptic plasma membrane
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TLC	thin layer chromatography
U.V.	ultra violet

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CHAPTER I

INTRODUCTION

Children who were undernourished early in life frequently have mental capabilities below normally accepted levels. Numerous studies of animal models of neonatal undernutrition have attempted to determine the neurological and/or neurochemical causes of undernutrition-associated mental retardation. Nonetheless, the exact mechanisms of how undernutrition affect intellectual development are not presently known. Recent studies have, however, suggested that the synapse is affected by neonatal undernutrition in terms of regional density and morphology.

Gangliosides and glycoproteins are carbohydrate-containing lipids and proteins, respectively, that are located in synaptic membranes. Gangliosides and glycoproteins are known to be involved in cell-cell recognition, contact and adhesion processes, and as components of several receptors for neurotransmitters. Thus, one would expect that an alteration in either the synthesis or structure of these components within the synaptic plasma membrane could result in altered synaptic connectivity and cause an alteration in normal interneuronal communication.

Thus, the present study was undertaken to examine the influences of neonatal undernutrition on synaptic development from a neurochemical standpoint. More specifically, this

dissertation assessed the quantitative accretion of synaptic membranes as well as the synthesis of synaptic proteins, gangliosides and glycoproteins. These studies were performed in age-matched control (C) rats and those that were undernourished (U) during lactation. In several experiments, the undernourished pups were nutritionally rehabilitated (U-RH), and it was hoped that any of the previous undernutrition-induced abnormalities might be reversed as a result of the nutritional rehabilitation. Protein synthesis was studied by following the in vivo incorporation of [^3H]-leucine into the SPM proteins in both control and undernourished animals. A double-label isotope technique was used to study the synthesis of SPM gangliosides and glycoproteins. An [^3H]-labeled compound (fucose or N-acetylmannosamine for glycoproteins or gangliosides, respectively) was injected intracerebrally into a control animal, and the [^{14}C]-labeled compound was injected into an undernourished animal. (In approximately half the samples, the order of the isotopes was reversed to eliminate any possible isotope effects.) The [^3H]- and [^{14}C]-labeled samples from control and undernourished animals, respectively, were combined and separated on a single polyacrylamide gel (glycoproteins) or on a single thin layer chromatography lane (gangliosides). This double-labeled radioisotope technique facilitates the recognition of small compositional differences. The experimental groups (U and U-RH) were

compared with control rats in order to assess the relative severity and reversibility of the nutritional stress.

CHAPTER II

REVIEW OF RELEVANT LITERATURE

Brain Development

In all mammals there is a period of rapid brain growth which precedes general body growth. This is called the brain growth "spurt" during which the brain is quite active metabolically. This brain growth spurt in man is a perinatal event. It begins about the third trimester of gestation and continues until about two years of age. This brain growth spurt in the rat is a postnatal event (Davison and Dobbing, 1966; Dobbing, 1968). The rat brain weight increases slowly during fetal growth. At birth the brain weight increases rapidly until weaning (about 21 days of age). Thereafter there is a slower growth throughout the life of the animal (Dobbing and Sands, 1971; Chevallier et al., 1975).

Biochemical Changes During Development

During the growth and development of the brain, there are many changes in the cellular and molecular levels. During the first two weeks after birth in the rat, brain DNA content increases in parallel to that of brain weight (Dobbing and Sands, 1971). Based on an assumption that the DNA content is constant in all diploid cells of any species, the total DNA content of an organ at a given time will

reflect its total cell number. Early brain development has been divided into three phases (Enesco and Leblond, 1962; Winick and Noble, 1965). Phase I consists of a period of hyperplastic growth in which cell division is quite active and cell number increases rapidly (increase in total DNA content). Phase II is a period of development characterized by both hyperplastic and hypertrophic growth in which there is an increase in both cell number and cell size (an increase in both DNA and protein content). Phase III consists of a period of hypertrophy in which the brain cells increase in size only, with no further increase in DNA content.

Although little information is known about cellular growth in the brain regions of man, it is known that post-natal cell division is comparable in the cerebrum and the cerebellum and ceases in both areas between 12 and 15 months of age (Winick et al., 1970). DNA synthesis continues in the rat cerebrum until the 21st day postnatally (Mandel et al., 1964). In the cerebellum, the synthesis of DNA continues until the 17th day postnatally. In the hippocampus, the rise in DNA content between the 14th and 15th day of life (Fish and Winick, 1969) corresponds not to an increase in cell division but to the migration of neurons from under the lateral ventricles into the hippocampus (Altman and Das, 1966). Although cell numbers can provide information about the general stage of development, the functional significance of altered cell numbers remains obscure. For example, an

alteration of total DNA content does not give an indication of which cell types are involved.

DNA polymerase activity in the developing rat brain parallels the rate of cell division and shows two peaks of activity (Brasel et al., 1970). The first peak of activity occurs prenatally, and the second occurs between 6 to 10 days postnatally. Although the exact cell types involved in active synthesis are not definitely known, it is believed that the first peak of DNA polymerase activity corresponds to the cell division of neurons while the second corresponds to glial cell division. Two peaks of DNA polymerase activity have also been found in man. The first peak occurs about 26 weeks of gestation, and the second occurs near birth (Dobbing and Sands, 1973).

There is a rapid decline in the rate of protein synthesis soon after birth with a more gradual decline thereafter (Schain et al., 1967). This is evidenced by results indicating that proteins of all subcellular fractions in the young animal are more highly labeled after an injection of a radiolabeled precursor than in the adult rat brain, thus indicating a decline in the rate of protein synthesis (Abdel-Latif and Abood, 1966; Oja, 1967). The average half-life of proteins in the rat brain progressively increases with the age of the animal due mainly to an increased synthesis of proteins with longer turnover rates (Lajtha et al., 1957). Total brain protein content and

concentration increase progressively in the cortex and white matter until about 30 days of age in the rat. A decrease in protein concentrations after 30 days of age is due to the accumulation of myelin lipids.

CNS glial cell proliferation has been shown to occur shortly after birth in both man and rat. In early brain development of all mammals, there is a large acquisition of oligodendroglial cells before any myelin can be isolated (Bunge, 1968). Myelination of axons by the oligodendroglia is primarily a postnatal event. Lipids are present in all cell membranes; however, the bulk of the brain lipids are present as a component of myelin membranes. Therefore, any developmental changes in brain lipids most often reflect a change in myelination.

Total brain lipids increase in content during development (Sperry, 1962). Brain lipids increase mainly within the white matter which reflects the area of heavy myelination, rather than in the grey matter (LeBaron, 1970). In man, there is no immediate increase in lipid deposition in the grey matter. Adult levels are reached by 3 months of age. In the white matter, there is a much less rapid increase in lipid deposition, which reaches 90% of its adult levels by 2 years of age.

Brain lipids consist of cholesterol, phospholipids, and glycolipids. Cholesterol is present in many cell membranes, but it is present in high concentrations within

myelin membranes. Although cholesterol is not uniquely located within these membranes, cholesterol content has been used as an index of brain myelin content (Dobbing and Sands, 1971). Brain cholesterol content and concentration increases during early postnatal development (Brante, 1949; Davison and Dobbing, 1968; Dobbing and Sands, 1971). There is also an increase in phospholipid content during early development, which parallels the development of membranes (Davison and Dobbing, 1968).

Glycolipids in the brain consist primarily of cerebrosides, sulfatides, and acidic glycosphingolipids (i.e., gangliosides). Cerebrosides and sulfatides contain long chain (22 to 26 carbon atoms) fatty acids attached to a sphingosine residue and D-galactose or sulfate ester. In the adult brain, 90% of the total cerebrosides are present within the myelin sheath. Therefore, changes in myelination might be better reflected by changes in cerebroside content.

Gangliosides are acidic glycosphingolipids which contain at least one or more residues of sialic acid in their oligosaccharide chains [See Figure 1]. The sialic acid residue found most commonly in human gangliosides is N-acetylneuraminic acid. Gangliosides are most abundant within the grey matter where they are found in neuronal membranes, although little is known about the distribution of individual ganglioside species within these membranes.

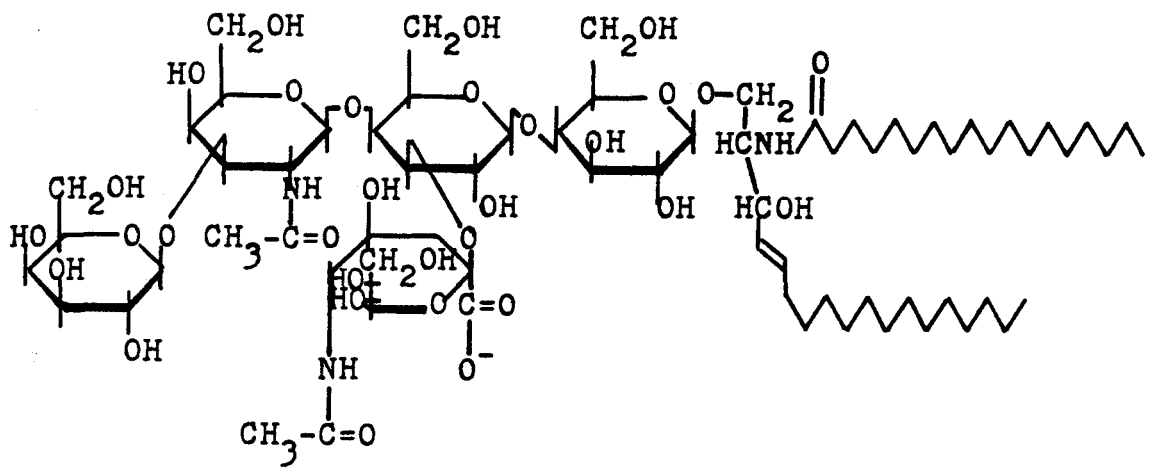


Figure 1. Structure of the monosialoganglioside G_{1M1}. Abbreviations of the gangliosides are those of Svennerholm (1963).

Gangliosides increase in content and concentration during brain development (Suzuki, 1965). During the first ten days of normal postnatal brain development in the rat, ganglioside content per cell greatly increases. In the normal rat cortex, ganglioside content peaks at about 20 days of age (Bass et al., 1969). This increase is correlated with the growth of dendritic processes and the formation of synaptic junctional complexes. Most of the evidence that the developmental peak in ganglioside concentration correlates with the formation of synapses has been obtained from observations that polysialogangliosides have been found to be particularly concentrated within the synaptic cleft region (Wiegandt, 1967; Bondareff and Sjostrand, 1969). Synaptic membranes are particularly rich in gangliosides (Tettamanti, 1971).

There is a large increase in $(\text{Na}^+ - \text{K}^+)$ -activated ATPase in the brain during neonatal maturation (Samson and Quinn, 1967). In the brain, $(\text{Na}^+ - \text{K}^+)$ -ATPase specific activity is highest in those subcellular fractions containing nerve endings (Abdel-Latif and Abood, 1964; Albers et al., 1965; Kurohawa et al., 1965; Whittaker, 1965). The early postnatal development of this ATPase parallels the development of electrical activity in the brain, suggesting a probable role in the transmission of impulses (Abdel-Latif et al., 1967; Zaheer et al., 1968).

In general, the postsynaptic terminal membrane specializes into a postsynaptic density after making connection with an appropriate presynaptic terminal membrane. Indeed, it has been shown that transient connections between dendrites and growing axons do not always result in the formation of synapses. However, it has also been demonstrated that differentiation of the postsynaptic membrane thickening is not entirely dependent upon making synaptic contact with a presynaptic terminal (Hinds and Hinds, 1976 a, b; Rees et al., 1976), although presynaptic membranes were shown to be present within the vicinity. During further maturation of the synapse, there is an accumulation of mitochondria and synaptic vesicles and the formation of a paramembraneous density ("Gray's apparatus") within the presynaptic terminal.

The synapse is a critical point of contact between nerve cells. The first detectable evidence of electrical activity has been correlated with the appearance of structurally mature synaptic components (Abdel-Latif et al., 1967). Interestingly, dogs, whose mothers were protein-malnourished during gestation, show evidence of marked variations in EEG recordings from normal tracings (Stewart, 1968). Since then, similar findings have been reported in children who had been previously malnourished (Stoch and Smythe, 1967; Taori and Pereira, 1974).

Synaptic Plasma Membranes

Intact synaptic terminals were first isolated by Gray and Whittaker (1960 and 1962) and DeRobertis et al. (1962). During homogenization of the central nervous system tissue, the nerve terminal region is sheared off and reseals while in isotonic solution forming artificial structures called "synaptosomes." Isolated synaptosomes have been shown to act as follows: 1) concentrate ions against a concentration gradient; 2) participate in uptake, release and metabolism of neurotransmitters; and 3) participate in limited in vitro protein synthesis. The synaptosome contains a self-limiting membrane. The synaptosome also contains the synaptic cleft, a postsynaptic thickening ("postsynaptic density"), affixed pre- and postsynaptic membranes, and presynaptically located vesicles and mitochondria.

Once a reasonably pure preparation of synaptosomes has been obtained, the synaptosomal constituents can be studied. The synaptosome can be subfractionated (usually by hypotonic lysis) and the external membranes, synaptic vesicles, cleft material, soluble cytoplasm and intraterminal mitochondria may be isolated. The isolated synaptic plasma membranes (SPM), as observed by electron microscopy, contain the synaptic cleft region, postsynaptic density, some presynaptic paramembraneous material, and fragments of affixed pre- and postsynaptic membranes.

There is an age-dependent change in buoyant density exhibited by presynaptic terminals (Gonatas et al., 1971) and by catecholamine-containing synaptosomes (Oberjat and Howard, 1973). In 1977, Norman and Howard demonstrated that isolated SPMs from the cerebral cortex of 5- to 6-day-old rat pups sediment as a broad peak between 0.9 and 1.1 M sucrose on a continuous gradient, whereas, SPMs from adult rats sediment to 1.2 M sucrose. Therefore, it is important to establish whether the densities of the selected membrane fractions from experimental rats are the same as those from age-matched control rats. It is also important to assess the purity of the SPMs from both developing control and undernourished pups via enzymatic markers and to assess the relative distribution of the marker enzymes on the gradient used to isolate the SPMs.

There are several methods (morphological and biochemical) of assessing the purity of the SPMs. One of these is through the use of electron microscopy. Another method of assessing the purity of the SPMs is through the use of marker enzymes. The objective in assaying marker enzymes is to assess the specific activity of enzymes that are reasonably specific for a particular membrane or organelle. In the present study ($\text{Na}^+ - \text{K}^+$)-activated, Mg^{+2} -dependent, ouabain-sensitive adenosine triphosphatase (ATPase), 5'-nucleotidase, alkaline phosphatase, acid phosphatase, 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNPase), monoamine oxidase

(MAO), and cytochrome c oxidase were used as enzymic markers.

($\text{Na}^+ - \text{K}^+$)-activated ATPase (EC 3.6.1.4.), 5'-nucleotidase (EC 3.1.3.5) and alkaline phosphatase (EC 3.1.3.1) are generally considered as enzymic markers of plasma membranes. The ($\text{Na}^+ - \text{K}^+$)-dependent, Mg^{+2} -stimulated, ouabain-sensitive ATPase has been shown to be located, though not exclusively, within neuronal membranes; being particularly concentrated within the synapse region (Abdel-Latif et al., 1967, 1970). The enzyme acid phosphatase (EC 3.1.3.2) may be used to assess the presence of lysosomal membranes (Verity et al., 1973). 2', 3'-Cyclic nucleotide 3'-phosphohydrolase, CNPase, (EC 3.1.4.16) has been shown to be localized in oligodendroglia and in myelin membranes (Kurihara and Tsukada, 1967; Banik and Davison, 1969). Any significant CNPase activity would indicate myelin contamination. Mitochondrial membrane contamination can be monitored using monoamine oxidase, MAO, (EC 1.4.3.4) as an enzymic marker for outer mitochondrial membranes (Schnaitman et al., 1967) and cytochrome c oxidase (EC 1.9.3.1) activity as an enzymic marker for inner mitochondrial membranes (Duncan and Mackler, 1966). Some of these enzymes may be found with lower specific activities in structures other than those for which they are used as markers. However, an assessment of a combination of markers can provide important information about the purity of the isolated SPMs.

The SPM components have been studied extensively by a number of investigators (Cotman et al., 1968; Morgan et al., 1971; Waehneltdt et al., 1971; McBride and Van Tassel, 1972; Morgan et al., 1973; Wang and Mahler, 1976; Yen et al., 1977; Kelly and Cotman, 1977; Smith and Loh, 1979; Mena et al., 1980). The SPM protein-polypeptide profile on polyacrylamide gels containing sodium dodecyl sulfate (SDS), is quite characteristic of SPMs and is distinguishable from that of whole brain, mitochondrial and myelin membranes. However, it is similar in some respects to microsomal and synaptic vesicular membranes (Mena et al., 1980). (It should be noted that microsomal fractions from the brain arise not only from endoplasmic reticulum but also from synaptosomal and neuronal plasma membranes.) Synaptic plasma membranes have been shown to contain at least some twenty or more polypeptides with three major polypeptides having apparent molecular weights of 93-110,000; 52-54,000; and 39-50,000 (Mena et al., 1980; Banker et al., 1972; Morgan et al., 1973).

Fibrous proteins have been identified in synaptosome fractions, which may function as a cytoskeleton of the synapse. Tubulin has been demonstrated to be a component of synaptic membranes (Babitch, 1981). One approach to identify the proteins at the synapse has been to prepare antibodies against specific proteins of the synaptic membrane fraction and to determine if they cross-react with the synapse region or influence normal synaptic function. Several neural

specific proteins such as antigens D_1 , D_2 , and D_3 are present in synaptosomal membranes but absent in synaptic vesicles and glial cells (Jørgensen and Bock, 1974; Bock and Jørgensen, 1975; Bock et al., 1975; Jørgensen, 1976; Reeber et al., 1978; Jørgensen, 1979; Jørgensen et al., 1980). The D_1 antigen was found to be composed of two polypeptide chains (apparent molecular weights of 50,300 and 116,000), D_2 of only one polypeptide chain (139,000) and D_3 of three polypeptides (molecular weights: 14,100; 23,500; and 34,400). Although tubulin has been demonstrated to be a major component of the synaptosomal polypeptides (apparent molecular weight \sim 54,000 (Wang and Mahler, 1976; Kornguth and Sunderland, 1975)), both Axelsen (1973) and Krøll (1973) demonstrated that tubulin was not a component of antigen D_1 . However, the polypeptide pattern of D_1 had certain similarities to that of $(Na^+ - K^+) - ATPase$ (Jørgensen, 1975, 1979).

The rate of CNS protein synthesis and the turnover of individual proteins can be examined metabolically by the use of isotopic precursors such as radiolabeled amino acids. Lisý and Lodin (1973) showed that the rate of incorporation of radiolabeled-leucine into proteins of neuronal perikarya and glial cells of the cerebral cortex decrease with age.

Gurd (1978) investigated the incorporation of [3H]-leucine into brain (homogenate), microsomal and synaptosomal proteins. He found that there are two peaks of incorporation

into synaptosomal proteins, i.e., one at 2-4 hours and another at 16 hours after injection. After the first wave of incorporation, there is a loss of labeled protein. After the second wave of incorporation, at 16 hours, the rate of turnover was slower.

Glycoproteins have been demonstrated to serve as recognition signals for a number of systems, for example, recognized attachment sites (Choppin and Scheid, 1980); cell-to-cell recognition (Steinemann et al., 1979; Muller et al., 1979; Geltosky et al., 1980); and other target-receptor functions (Morita et al., 1980). This is consistent with the external localization of membrane glycoproteins (for a review of this topic, see Sharon and Lis, 1981).

Cytochemical evidence also indicates that carbohydrates are present within the synapse region. Carbohydrate stains, such as ruthenium red and periodic acid-silver methenamine, are associated with synaptic cleft (Bondareff, 1967; Rambourg and Leblond, 1969). Using concanavalin A-ferritin conjugates, Cotman and Taylor (1974) and Bittiger and Schnebli (1974) both demonstrated the presence of α -D-mannosyl and glucopyranosyl residues on the external surface of the postsynaptic membranes. Using lactoperoxidase iodination, Wang and Mahler (1976) showed that a portion of the synaptosomal proteins are located externally. They confirmed that some of these externally located proteins contain oligosaccharide chains susceptible to labeling in

intact synaptosomes by oxidation with galactose oxidase, or periodate, followed by reduction with sodium boro- ^{3}H -hydrate (NaB^{3}H_4).

SPM glycoproteins are especially rich in glucosamine and galactosamine, in hexoses such as mannose, galactose and fucose, and in sialic acid (Churchill et al., 1976; Smith and Loh, 1979). Fucose-containing glycoproteins have been shown to be transported to the nerve terminal region within the fast component(s) of axoplasmic transport (Zatz and Barondes, 1971; Levin, 1977; Padilla and Morell, 1980). Membrane glycoconjugates may also be examined metabolically by the in vivo incorporation of appropriate carbohydrate precursors such as fucose (Buchsel et al., 1980; Webster and Klingman, 1980). Fucose is frequently used as a precursor for many membrane glycoproteins because fucose is not metabolized in the brain and because it is rapidly incorporated into the terminal position of oligosaccharide chains of many neural glycoproteins (Quarles and Brady, 1971; Margolis and Margolis, 1972).

Morgan and Routtenberg, 1979, have studied the apparent molecular weights, as well as the turnover, of fucosylated synaptosomal glycoproteins after intracranial injections of ^{3}H -fucose. They demonstrated at least 10 major ^{3}H -fucose containing glycoprotein peaks. The ^{3}H -fucose containing glycoprotein peaks were compared with coomassie blue stained synaptosomal protein bands separated

by polyacrylamide gel electrophoresis. The apparent molecular weights of the fucosylated glycoproteins ranged from 32,000 to 180,000 daltons. Tentative identification of several of the fucosylated glycoproteins were made by comparing their apparent molecular weights to the molecular weights of known proteins.

Using an [125 I]-labeled fucose-binding protein, Gurd (1979) showed there are a number of fucosyl-glycoproteins in synaptic membranes, synaptic junctions and postsynaptic density fractions, and the chief fractions do not have the same fucosyl-glycoproteins. The highest specific activity was found in synaptic membranes, followed in order by synaptic junctional complexes and postsynaptic densities. Also, using lectin affinity chromatography on Concanavalin A-Sepharose, Gurd showed that synaptic membrane glycoproteins could be separated into two fractions (a glycoprotein-rich and a glycoprotein-poor fraction). These two Con-A separated fractions had different electrophoretic patterns of [3 H]-fucosylated glycoproteins. Zanetta et al., (1975), also using Concanavalin A-Sepharose lectin affinity chromatography (under optimal binding conditions), demonstrated three glycoprotein-containing elution fractions. The first elution fraction (unabsorbed) contained most of the SPM protein (63%) but only 23% of the sugar (glycoprotein-poor). This fraction was rich in fucose, galactose and N-acetylneuraminic acid (NANA). The second elution fraction was mildly retained by

the column and therefore contained glycoproteins which reacted weakly with Concanavalin A-Sepharose. This fraction was especially rich in N-acetyl-glucosamine and NANA, relative to other sugars. The last fraction (absorbed) was especially glycoprotein rich, especially in mannose and N-acetyl-glucosamine. These three elution fractions had different electrophoretic patterns of coomassie-stained proteins and PAS-stained glycoproteins. In a later study using lectin affinity chromatography specific for L-fucose, Zanetta et al., (1977), showed that fucosyl-glycoproteins constitute a major proportion (85%) of the SPM glycoproteins. Polyacrylamide gel electrophoresis of the fucosyl-glycoprotein fractions revealed at least 28 major bands. These cited studies showed that there exist several different classes of fucosylated synaptic membrane glycoproteins.

Using [125 I]-labeled plant lectins, De Silva et al., (1979), showed developmentally related alterations of rat brain synaptic membrane glycoproteins. They demonstrated a 2-3 fold increase in synaptic membrane glycoprotein sialic acid between 5 and 60 days of age. Recently Fu et al., (1981), have also reported changes in the synthesis of synaptic membrane and synaptic junctional membrane fucosyl- and sialylglycoproteins with postnatal age.

In addition to age-dependent changes in glycoprotein synthesis, there appear to be environmental influences on glycoprotein synthesis. Burgoyne and Rose (1980) showed an

altered incorporation of [^3H]-fucose and [^3H]-lysine in the visual cortex of dark-reared rats upon first exposure to light. Additional support for environmentally related alterations of glycoprotein synthesis is found in studies of learning and memory (Routtenberg et al., 1974; Irwin et al., 1978; Sukumar et al., 1980).

Sialic acid, which is a sugar component present in many glycoproteins, is present in all gangliosides. The number and position of sialic acid residues on the ganglioside molecule determines the particular ganglioside species. There is some evidence that there is an enrichment of particular ganglioside species within various brain regions (Suzuki, 1965; Seyfried et al., 1979; Urban et al., 1980) and certain membranes, i.e., myelin (Suzuki et al., 1967, 1968; Ledeen et al., 1973). Little is known about the distribution of individual gangliosides within neuronal membranes. In a recent report, Seyfried et al., (1982) (using particular genetic mouse mutations that selectively destroy specific populations of neurons in the cerebellum) demonstrated a differential enrichment of ganglioside species into particular neuronal cell types. They found that GD_{1a} is more heavily concentrated in cerebellar granule cells than Purkinje cells, whereas GT_{1a} is more concentrated in the Purkinje cells.

SPM gangliosides may be studied metabolically by using radiolabeled sugars (Harzer et al., 1969; Panzetta

et al., 1980). Yohe et al., (1980), demonstrated that microsomal gangliosides initially have a higher specific radioactivity than synaptosomal gangliosides. However, both fractions reach similar specific radioactivities 18 hours after an interacerebral injection of the labeled precursor.

Malnutrition/Undernutrition

It has been estimated that at least half of the children of the world have suffered some type of malnutrition (Winick, 1969; World Health Organization, Scientific Publication No. 251, 1972). (Malnutrition is defined as an inadequate intake and/or utilization of protein, minerals or other essential nutrients as a result of inadequate supply, inappropriate amounts or ineffective use by the body.) Malnutrition early in life has been known to affect the physical and mental development of an individual (see Tizard, 1974). Malnutrition has been shown to cause a decrease in body and brain weight in both animal studies and human observations (see Dyson and Jones, 1976, for a review on this topic).

There have been numerous studies indicating that the mental functioning capabilities of children who had been malnourished early in life were below normally accepted standards (Stoch and Smythe, 1963 and 1967; Chase and Martin, 1980). In contrast, there are reports of Dutch army recruits that suffered through the Dutch potato famine during their early childhood (Stein et al., 1972 and 1975) and of adults

who were raised in Nazi concentration camps whose mental aptitude tests were within normal ranges. The effects of nutritional deprivation on the mental development of humans have been difficult to evaluate, for human malnutrition is seldom, if ever, an isolated event. For example, children who are subjected to malnutrition frequently come from lower socioeconomic environments (where sanitation and health care are lacking, disease is prevalent, educational facilities are substandard or not optimally used) or are the victims of neglect and abuse. Even when nutritional deficits have been indicated as the major factor, it is often difficult to obtain sufficient samples to do biochemical follow-up studies in order to pinpoint the mechanism of mental deficit. Therefore, animal models of malnutrition are especially useful since various experimental parameters can be carefully controlled.

When an adult animal is subjected to a nutritional deprivation, the animal will depend upon its metabolic reserves during the time of stress. If the period of stress is long enough, this may result in a decrease in the total body mass of the animal. Even in times of severe starvation, there may be considerable loss of body weight before there is any significant decrease in brain weight.

It is known that a nutritional deprivation during early brain development may lead to a decrease in brain weight. For example, undernutrition due to large litters

causes a decrease in brain weight that is not fully overcome in the 28-week-old adult rat (Dobbing and Sands, 1972). A deficit in brain weight has also been reported in humans (Stoch and Smythe, 1963 and 1967), the rat (Zamenhof et al., 1968; Dickerson, 1968; Adlard and Dobbing, 1972; Sobotka et al., 1974), dog and pig (Stewart, 1968) and rabbit (Schain and Wattanabe, 1973). It has also been reported (Lynch et al., 1975) that a direct deprivation of rat pups after weaning results in a deficit in brain weight which may eventually be overcome by subsequent nutritional rehabilitation. However, it is often difficult to correlate this deficit in brain weight to the decreased mental functioning capabilities, behavior alterations, or motor coordination abnormalities often seen associated with malnutrition. Possible explanation for these inconsistencies include: 1) the lack of any direct correlation between brain size and intelligence; and 2) the fact that brain weight does not accurately reflect cellular composition.

Ultrastructural Studies

There is ultrastructural evidence of synaptic abnormalities in undernourished rats. Yu et al., (1974, 1977) found that the most severely affected structure in the brains of undernourished rats was the presynaptic terminal. Many individual terminals contained lamellar whorls and showed aggregation of vesicles. Jones and Dyson (1976) demonstrated that both pre- and postnatal undernutrition

causes a decreased thickness of the pre- and postsynaptic membranes. They also showed a decreased number of synaptic terminals associated with the neurons. In addition, they showed that undernutrition during gestation and lactation results in a higher proportion of immature synaptic junctions in the undernourished animals than in controls. Nutritional rehabilitation resulted in a shift toward the more mature types (Dyson and Jones, 1976).

Pre- and postnatal malnutrition in the rat have been shown to cause a decrease in the thickness of the cerebral cortex (Bass et al., 1970; Cragg, 1972; Clark et al., 1973) and a poorly defined laminar layering (Bass et al., 1970). Assessment of neuronal density in 24- and 50-day-old malnourished rats reveals a normal but more compressed neuronal pattern (Cragg, 1972). The latter observation suggests that normal dendritic outgrowth has not occurred.

Since the majority of cerebellar neurons (excluding the Purkinje cells) are formed postnatally (Altman and Das, 1966), it is not surprising that postnatal malnutrition produces a marked decrease in cerebellar neuronal numbers that cannot be overcome by subsequent nutritional rehabilitation (Dobbing et al., 1971). According to Neville and Chase (1971), the decreased neuronal density is due to decreased number of cells of the internal granular and molecular layers. The latter cells have been reported to develop actively postnatally.

Eayrs and Horn (1955) demonstrated that the dendritic arborization of the cortex was impaired and the number of synapses was decreased in starved young rats. In a light microscopic study, Salas et al., (1974), reported a reduced number of dendritic spines and a reduction in the diameter of dendritic processes near their points of origin from the soma in postnatal nutritional deprivation. In an electron microscopic study, Cragg (1972) showed that the number of synaptic terminals associated with one neuron was decreased by as much as 38-41% in the undernourished rat. Jones and Dyson (1976) subsequently demonstrated a decreased number of synapses and fewer synapses per neuron. This is in agreement with observations of decreased dendritic spines per neuron as a result of undernutrition.

Postnatal malnutrition has also been shown to affect another cell type that proliferates postnatally. Postnatal undernutrition causes a decrease in glial cell numbers in the cortex (Bass et al., 1970; Dobbing et al., 1972; Siassi and Siassi, 1973) and in the cerebellum (Dobbing et al., 1971; Clos et al., 1973). Oligodendroglia are responsible for the deposition of myelin sheaths around axons within the CNS. With the probable decrease of axonal proliferation in undernourished animals, some decrease in myelin content is to be expected as the axonal surface area available for myelination is decreased. This, however, is not the total cause of the myelin lipid deficiency as it has been shown that

a restriction of the maternal dietary intake results in a decreased number of myelin lamellae in the offspring (Sima, 1974).

Biochemical Studies

Severe nutritional deprivation in the adult animal causes a decrease in brain protein content while the DNA content is unaffected. This indicates that there is only a decrease in cell size and not in cell number as a result of deprivation in the adult. It has been noticed that upon subsequent nutritional rehabilitation the loss in brain and body weights may be fully recovered (Hatai, 1905, 1907; Donaldson, 1911; Jackson, 1915; Dobbing and Widdowson, 1965; Dobbing, 1968). This is not always the case in the young, developing animal. Again, nutritional deprivation in the young will cause a decrease in body weight as in the adult; however, the brain is affected to a greater degree than in the adult. The brain is still the least affected organ compared with the rest of the body. There is a decrease in both DNA and protein content which suggests a decrease in both cell number and cell size within the brain (Winick and Noble, 1965; Winick and Noble, 1966; Dickerson and Walmsley, 1967; Cully and Lineberger, 1968; Guthrie and Brown, 1968; Zamenhof et al., 1968; Winick, 1969; Bass, 1970; Dobbing and Sands, 1971; Zamenhof, 1971; Krigman and Hogan, 1976; Subba Rao et al., 1980). As stated before, this decrease in DNA content does not give us an indication of the cell types

that are most susceptible to alteration by a nutritional insult. If the nutritional insult occurs during the period of hypertrophic growth, there is a decrease in protein content while there is no significant decrease in DNA content. Also, it has been noted that the decreased brain weight in the latter case may recover completely following a nutritional rehabilitation. Therefore, it can be seen that the time, length and severity of a nutritional insult and any subsequent nutritional rehabilitation are important as to the extent of any observed ill effects.

In the rat, neuronal cell division occurs prenatally in the cerebrum, but in the cerebellum there is a considerable increase in neuronal numbers postnatally (excluding the Purkinje cells which are formed prenatally). Therefore, a postnatal nutritional insult would more likely affect cerebellar neuronal numbers than cerebral neuronal numbers. Since glial cell proliferation is mainly a postnatal event, a postnatal nutritional insult would also affect glial cell numbers. Indeed, it has been shown that postnatal under-nutrition in the rat causes a decrease in glial cell numbers. Even though it has been shown that glial cells turnover throughout the life of the animal, the large deficit in glial cells due to early undernutrition is not restored following nutritionally rehabilitated after three weeks of age (Winick, et al., 1968). This same deficit in glial cell numbers has been found in undernourished children (Winick and

Rosso, 1969). Indeed, it is suggested from the above information that the brain region and cell types most susceptible to alteration by outside influences depend on which processes are most active during the time of insult.

Early postnatal malnutrition has been shown to cause a general decrease in total brain lipids (Davison and Dobbing, 1968; Culley and Lineberger, 1968; Chase, 1972). Undernutrition early in postnatal life has also been shown to cause a decreased total brain (and potentially myelin) cholesterol content and concentration (Dobbing and Widdowson, 1965; Davison and Dobbing, 1966; Dobbing and Sands, 1971). In addition, Culley and Mertz (1965) found that the deficit in cerebroside content (used as an index of CNS myelination) was most marked following an early postnatal nutritional deprivation, suggesting a retardation in myelination.

Malnutrition has been found to cause a decrease in brain ganglioside sialic acid content (Bass et al., 1970). Neonatal malnutrition also has been shown to cause a delay in the normal peak of ganglioside content. These findings have been interpreted as suggestive evidence for a decreased sprouting of dendrites as well as a diminished synapse formation during postnatal life as a result of the nutritional insult (Bass et al., 1970). Merat and Dickerson (1974) also demonstrated that neonatal undernutrition caused a transient to lasting decrease in ganglioside N-acetylneuraminic acid (NANA) content in the forebrain, cerebellum

and brainstem. Analysis of individual ganglioside species (nomenclature according to Svennerholm, 1963) showed decreases in the concentrations of GM_1 , GD_{1b} , and GT_1 (GD_{1a} was also decreased though not as significant) in the undernourished rat brains (Reddy and Sastry, 1978). The deficits in GD_{1b} and GT_1 were most pronounced when the nutritional insult was continued through 8 weeks of age. However, nutritional rehabilitation after the third week eventually reversed the cited ganglioside abnormalities. Yusuf and Dickerson (1978) found that the most severely affected ganglioside species varied within different brain regions (i.e., GD_{1a} in forebrain, GD_{1b} in the brain stem and GT_1 in the cerebellum).

Undernutrition and Synaptic Membranes

Gambetti et al., (1972) showed that although total brain protein content was decreased in undernourished animals, there was no decrease in synaptosomal protein content per cortex at 24 days of age. Rabié and Legrand (1973) showed that undernutrition caused a decrease in synaptosomal protein content per cerebellum but that synaptosomal protein content per mg of cerebellum was apparently unaffected.

Nutritional deprivation has been shown by several to have an effect on ATPase activity in the developing rat brain. Reddy and Sastry (1978) demonstrated that, in protein

malnourished rats, the specific activity of ouabain-insensitive ATPase was significantly reduced in 3-week-old rats which could be recovered by rehabilitation. The ouabain-sensitive ATPase specific activity, however, was not affected by protein malnutrition. Hernandez (1979) showed that there was a significantly increased specific activity of (Na^+-K^+) -ATPase in the brain cortex of rats malnourished either during gestation or by raising in large litters. In addition, Kissane and Hawrylewicz (1975, 1978) have investigated the effects of perinatal protein deficiency (during gestation and lactation) on ATPase activity in rat brain synaptosomes isolated from the cerebellum and cerebral cortex. They demonstrated that undernutrition during gestation caused a decrease in the ATPase activity that could be reversed by normal protein nutrition during lactation. In contrast, undernutrition only during lactation severely decreased both cerebral and cerebellar ATPase levels in young animals (14- and 20-day-old) almost as severely as those animals who were stressed during both gestation and lactation.

Experimental Models

Several methods of inducing neonatal undernutrition in experimental animals include the following: increasing the size of the suckling litter (Widdowson and McCance, 1960; Yu and Yu, 1977; and Crnic and Chase, 1978), decreasing the amount of the time spent nursing (i.e., by removing the

pups from the mother for part of the time) (Eayrs and Horn, 1955; Culley and Nertz, 1965; Crnic and Chase, 1978; Leuba and Rabinowicz, 1979), decreasing the quantity of food supplied to the mother (Venkatachalam and Ramanathan, 1964; Crnic and Chase, 1978; Hall et al., 1979; Leuba and Rabinowicz, 1979), or by some combination of these cited methods. Each of these methods also have their own special problems (Codo and Carlini, 1979; Hall et al., 1979; Smart, 1980); for example, 1) alteration of maternal care and behavior of the pups, especially in large litters; 2) competition between pups in large litters with the growth of some of the more aggressive pups at the expense of others, and 3) problems of keeping the pups warm during their separation from the mother or possible lack of maternal influences other than thermal or nutritional.

Normal, commercially prepared laboratory rat chow contains 20-28% protein, usually in the form of casein. It has been shown that, when laboratory rats are given a low protein (i.e., 7% w/w casein) diet, with the deficit in total calories being made up by an excess of carbohydrates, that the rats will consume less of their diet than the control fed counterparts. When a lactating female is fed a low protein diet, the quantity of milk is reportedly decreased without altering the proportion of its major constituents (Mueller and Cox, 1946; Miller, 1970). In contrast, there have been several reports (Crnic and Chase,

1978; Vangelder and Parent, 1981) that have found that a low protein diet during lactation will result in a lower nitrogen content and an elevated total fat content of the milk. The differences in findings may be due to recent advances which result in increased sensitivity of certain analytical procedures.

There are many problems (i.e., differences in the timing of certain developmental events) involved when trying to correlate different animal models with each other or with human experience. For example, in humans, the peak period of brain growth is during the last few months of gestation, continuing through the second year of life; whereas, many experimental animals (including rats) have their maximum brain growth spurt during early postnatal life (Davison and Dobbing, 1966; Dobbing, 1968). One possible solution to the problems inherent in using experimental models has been to correlate certain developmental events to developmental periods.

Since nutritional deprivation has been shown to cause both structural and biochemical abnormalities within the CNS nerve terminal region, it is quite possible that these abnormalities may cause an alteration in normal synaptic function. Therefore, it is of considerable interest to examine the effects of a neonatal undernutrition on the protein and lipid constituents on the synaptic plasma membranes and some of the enzymes involved in normal synaptic

function. Also, since gangliosides and glycoproteins have been reported to have an important role in intercellular adhesion and signal-receptor mediated functions, it would be of great interest to examine the effects of undernutrition on the metabolism of synaptic membrane gangliosides and glycoproteins as a possible mechanism of altered synaptic connectivity.

CHAPTER III

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats were obtained from Holtzman Supply Co. (Madison, Wisconsin). At birth, the litter sizes were adjusted to 9 pups and mothers of the experimental pups were placed on a low protein diet (ICN Nutritional Biochemicals, Cleveland, Ohio) ad libitum. Control mothers were maintained on normal Purina laboratory rat chow. The composition of the protein deficient diet was 8% casein, 78% starch, 10% vegetable oil and 4% salt mixture with vitamin fortification, while that of the control diet was 25-28% casein, 59% starch, 10% vegetable oil and 4% salt mixture with vitamin fortification. All rat mothers and offspring were fed standard lab chow after the 20th postnatal day.

Chemicals

L-[4,5-³H]-Leucine (6i Ci/mmole), L-[1-¹⁴C] fucose (59 mCi/mmole), L-[1-³H]fucose (5.4 Ci/mmole), N-[³H]-acetyl-D-mannosamine (500 mCi/mmole) and N-acetyl-D-[U-¹⁴C]-mannosamine (18 mCi/mmole) were purchased from Amersham (Arlington Heights, Ill.). Analytical grade organic solvents, concentrated acids and bases, as well as ammonium molybdate and sucrose were purchased from Mallinckrodt, Inc.

(St. Louis, Mo.). Scintillation grade toluene was purchased from J. T. Baker Chemical Company (Phillipsburg, N. J.). Aquasol cocktail fluor, protosol gel solubilizer and 1,4-bis(5-phenyloxazol-2-yl) benzene (POPOP) were obtained from New England Nuclear (Boston, Ma.). NCS tissue solubilizer and 2,5-diphenyloxazole (PPO) were purchased from Amersham (Arlington Heights, Ill.). Eastman Kodak was the source of acrylamide, N,N-methylene bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), 2-mercaptoethanol and 1-amino-2-naphthol-4-sulfonic acid. Sephadex G-10 and Ficoll 400 were purchased from Pharmacia Pharmaceuticals, Inc. (Piscataway, N. J.). Sodium dodecyl sulfate (SDS) was obtained from Matheson, Coleman and Bell (Norwood, Oh.). All other analytical reagent grade chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Precoated 20 x 20 cm² gel 60 thin layer chromatography (TLC) and 10 x 20 cm² silica gel 60 high performance (HPTLC) plates were purchased from E. M. Laboratories, MC/B Manufacturing Chemists, Inc. (Cincinnati, Ohio).

Experimental Procedures

Preparation and Injection of Isotopes

The specific activity of L-[1-³H]-fucose was adjusted to 200 mCi/mmole with non-radioactive fucose and the specific activity of N-[³H]-acetyl-D-mannosamine adjusted to 200 mCi/mmole with non-radioactive N-acetylmannosamine prior to injection. Under mild ether anesthesia, control and undernourished pups were given a single 10 μ l

intracerebral injection (to the right of the midline of the skull) of either L-[4,5-³H]-leucine, L-[1-³H]- or L-[1-¹⁴C]-fucose, or N-[³H]-acetyl-D- or N-acetyl-D-[U-¹⁴C]-mannosamine in physiological saline.

[³H]-Leucine was used as a precursor for proteins while [³H]- and [¹⁴C]-fucose and [³H]- and [¹⁴C]-N-acetyl-mannosamine was used respectively as the precursors of glycoproteins and of the sialic acid moiety of gangliosides and glycoproteins. The amount of each isotope that was injected is: 1) 100 μ Ci of [³H]-leucine; 2) 30 μ Ci of [³H]-fucose of [³H]-N-acetylmannosamine; or 3) 9 μ Ci of [¹⁴C]-fucose or [¹⁴C]-N-acetylmannosamine. In glycoprotein and ganglioside studies, approximately half of each group of animals (control and experimental) received the [³H]-labeled compound and approximately half received the [¹⁴C]-labeled compound. Animals were sacrificed by decapitation either at 16 hours (protein study) or 18 hours (glycoprotein and ganglioside studies) after injection. These times were selected for convenience and because they had been shown by others to be optimal for uniform distribution of label into proteins or glycoproteins and gangliosides, respectively.

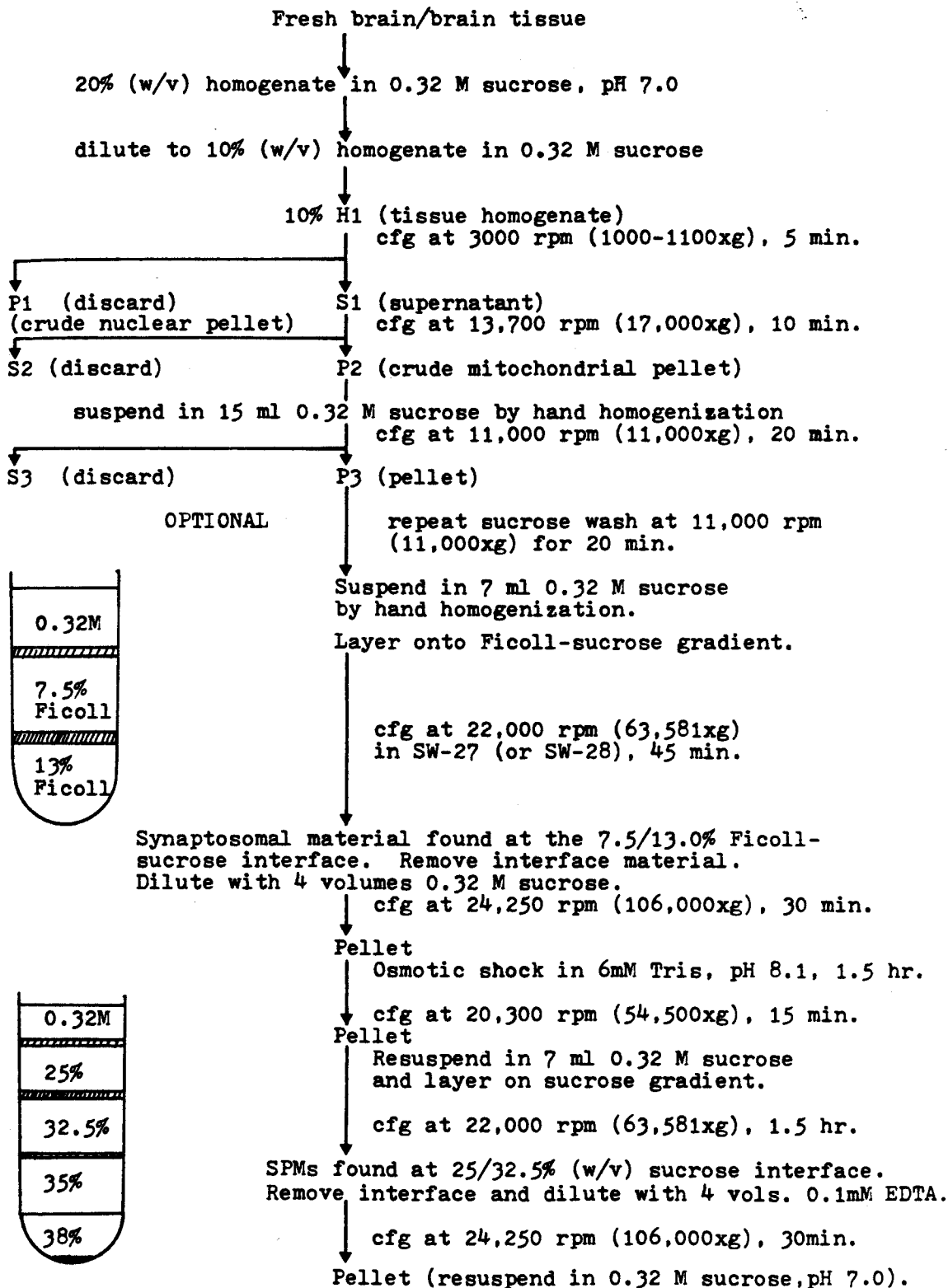
Synaptic Plasma Membranes

During mechanical homogenization of nervous tissue, the nerve terminals are sheared off. In isotonic solution, the nerve terminals can seal and form "synaptosomes." Differential centrifugation techniques can be used to

separate synaptosomes from cell nuclei, myelin, microsomes, mitochondria and other subcellular components. Synaptosomes contain a characteristic self-limiting membrane, the synaptic cleft region, presynaptic mitochondria and vesicles, and other substructures. After synaptosomes are hypotonically lysed, the synaptic plasma membranes (SPM) can be separated from remnant myelin fragments, presynaptic mitochondria and vesicles by differential centrifugation techniques.

In the present study, synaptic plasma membranes were prepared following a procedure of Cotman and Matthews (1971) [See Figure 2]. Animals were sacrificed by decapitation with a guillotine. Pups 20 days and older were lightly anesthetized with ether before sacrifice. Brains (including the brain stem) were quickly removed, cooled on ice and weighed. Brains were homogenized, 20% (w/v), in cold, 0.32 M sucrose (pH 7.0) in a glass homogenizer using a motor-driven teflon coated pestle. The homogenizer and pestle were rinsed with an equal volume of 0.32 M sucrose (pH 7.0). The rinse and homogenate were combined so that the final concentration of the homogenate was 10% (w/v). All subsequent steps were done at 0-4°C. An aliquot (0.5-1.0 ml) was then removed for protein determination. The homogenate was centrifuged in a refrigerated (0-4°C) DuPont Sorvall RC-5B at 3,000 rpm (1,000-1,100 x g) for 5 minutes using a Sorvall SS-34 rotor. This resulted in a crude nuclear pellet (P_1),

SYNAPTOSOMAL PLASMA MEMBRANES



containing cell bodies and nuclei, and a supernatant (S_1). The supernatant (S_1) was decanted from the nuclear pellet and centrifuged at 13,700 rpm (17,000 x g) for 10 minutes in the Sorvall. After this centrifugation, the supernatant (S_2) was decanted. The crude mitochondrial pellet (P_2) was resuspended in 15 ml of cold 0.32 M sucrose (pH 7.0) by hand homogenization using a smooth, round bottom, hand-held homogenizer. The suspension was centrifuged at 11,000 rpm (11,000 x g) for 20 minutes in the Sorvall. The resulting supernatant (S_3) was decanted and discarded, and the pellet (P_3) was resuspended in 7.0 ml of 0.32 M sucrose (pH 7.0) by hand homogenization. An additional, optional rinse was omitted. The suspension was layered on top of a Ficoll gradient consisting of 13.5 ml of 13.0% (w/v) Ficoll in 0.32 M sucrose (pH 7.0) which had been previously overlaid with 13.5 ml of a 7.5% (w/v) Ficoll-sucrose solution (pH 7.0). The gradient was centrifuged at 22,000 rpm (63,500 x g) for 45 minutes. This and all subsequent centrifugations were performed in a Beckman L 5-65 ultracentrifuge and utilized either the SW-27 or SW-28 rotor. All centrifugations were stopped by an electrical braking device. Crude myelin was found at the 0.32 M sucrose/7.5% Ficoll-sucrose interface. Synaptosomal material was found at the 7.5/13.0% Ficoll-sucrose interface. The synaptosomal material was removed, diluted with 4 volumes of 0.32 M sucrose (pH 7.0) and centrifuged at 24,250 rpm (106,000 x g) for 30 minutes. The

supernatant was decanted and the pellet resuspended in a small volume of 0.32 M sucrose. The pellet was then osmotically shocked for 1.5 hours (0-4°C) by resuspending it in 6 mM Tris (pH 8.1). It was shown by Cotman and Matthews (1971) that osmotic shock at alkaline pH (pH 8.1) for 1.5 hours is necessary for the subsequent resolution of synaptosomal mitochondria from the synaptic plasma membrane. The suspension was centrifuged at 20,300 rpm (54,500 x g) for 15 minutes. The pellet was resuspended in 7 ml of 0.32 M sucrose and layered onto a sucrose gradient consisting of the following sucrose solutions in order of decreasing density (heaviest on the bottom): 5 ml of 38% (w/v); 7.5 ml of 35.0% (w/v); 7.5 ml of 32.5% (w/v); and 7.5 ml of 25.0% (w/v) sucrose. The gradient was centrifuged at 22,000 rpm (63,500 x g) for 1.5 hours. Synaptic plasma membrane (SPM) fragments were found at the 25/32.5% (w/v) sucrose interface. The SPM bands were removed with clean Pasteur pipets and diluted with 0.1 mM EDTA. (In the sucrose gradient subfraction study, the subfractions A-D were removed in 8 ml aliquots starting from the top of the gradient to the bottom, using a graduated 10 ml pipet. The last subfraction, E, included the pellet and the remaining 2.5 ml of 38% (w/v) sucrose. The subfractions A-E were also diluted with 0.1 mM EDTA.) This suspension was centrifuged at 24,250 rpm (106,000 x g) for 30 minutes. The supernatant was decanted and the pellet was resuspended in a small volume of 0.32 M sucrose (pH 7.0) for

enzyme assays or in deionized H₂O for protein, glycoprotein or ganglioside studies.

The relative purity of the SPMs was assessed by determining the activities of monoamine oxidase, MAO, (EC 1.4.3.4) (Youdin, 1975), 2',3'-cyclic nucleotide 3'-phosphohydrolase, CNPase, (EC 3.1.4.16) (Kurihara and Tsukada, 1967), and Mg⁺²-dependent, ouabain-sensitive (Na⁺-K⁺)-ATPase (Sweadner, 1979) in brain homogenates and in SPMs. MAO, CNPase and (Na⁺-K⁺)-ATPase were used as marker enzymes for outer mitochondrial membranes, myelin and plasma membranes, respectively. In addition, the relative distribution of several other 'marker' enzymes was assessed in several subfractions of the discontinuous sucrose gradient used to isolate SPMs from lysed synaptosomes from control and undernourished rats. The activities of the plasma membrane-associated enzymes: 5'-nucleotidase (EC 3.1.3.5) (Emmelot et al., 1964) and alkaline phosphatase (EC 3.1.3.1) (Linhardt and Walter, 1963), and the activities of lysosomal acid phosphatase (EC 3.1.3.2) (Linhardt and Walter, 1963) and inner mitochondrial membrane cytochrome c oxidase (EC 1.9.3.1) (Duncan and Mackler, 1966) were also determined. The activity of these enzymes was assayed in the 5 subfractions (A-E) of the 10%/25%/32.5%/35%/38% sucrose gradient [See Figure 3]. The subfractions were suspended in 0.1 mM EDTA, pelleted by centrifugation at 24,250 rpm (106,000 x g) and resuspended in 0.32 M sucrose (pH 7.0) for

<u>MOLARITY</u>	<u>VOLUME</u>	<u>SUCROSE GRADIENT</u> (w/v)	<u>GRADIENT SUBFRACTIONS</u>	<u>VOLUME</u>
0.32 M	7.0 ml	10 %	A	8.0 ml
0.73 M	7.5 ml	25 %	B	8.0 ml
0.95 M	7.5 ml	32.5 %	C	8.0 ml
1.02 M	7.5 ml	35 %	D	8.0 ml
1.11 M	5.0 ml	38 %	E	2.5 ml

Figure 3. Osmotically lysed synaptosomal material was resuspended in 7.0 ml of 0.32 M sucrose, pH 7.0, and layered onto the 25/32.5/35/38% sucrose gradient. (All sucrose solutions also contained 5 mM Trizma base.) The gradient was centrifuged at 22,000 rpm (63,581xg, average), in an SW-27 (or SW-28) rotor) for 1.5 hr. The sucrose gradient subfractions (A-D) were removed in 8.0 ml aliquots. The final 2.5 ml, including the pellet (subfraction E), were resuspended with an additional 5.5 ml of 0.32 M sucrose, pH 7.0. Each subfraction was diluted with 4 volumes of 0.1 mM EDTA and centrifuged at 24,250 rpm (106,000xg, maximum) for 30 mins. Each pellet was resuspended in a small volume of 0.32 M sucrose, 5 mM Trizma, pH 7.0 for enzyme marker assays.

enzyme assays. The "myelin" band at the 10%/25% sucrose interface was included in fraction B, and the "SPM" band at the 25%/32.5% sucrose interface was included in fraction C.

Brain Region Study

A study was also undertaken to examine the effects of neonatal undernutrition on the SPM protein concentration within different brain regions in order to evaluate the sensitivity of the different brain regions to a nutritional insult. The SPMs were isolated from various brain regions in both control and undernourished rat pups at several ages: 10, 14, 20 and 34 days of age. The brain regions selected were as follows: prefrontal lobes, hippocampus, cerebral cortex and cerebellum. At some of the younger age periods, several (6-9) brain regions (i.e., prefrontal lobes and hippocampus) were combined in order to obtain sufficient material to isolate the SPMs.

Assays

PROTEIN

Protein determination was done following the procedure of Lowry et al. (1951). Crystalline human serum albumin (HSA) was used as the protein standard. A standard 1 mg HSA protein/ml solution was prepared in deionized H₂O. Protein standards contained 0 µg (reagent blank) - 50 µg HSA. Absorbance (Abs) were measured at 700 nm in a Gilford 2400 spectrophotometer.

FISKE - SUBBA ROW INORGANIC PHOSPHATE ASSAY

Inorganic phosphate was determined following a modified procedure of Fiske and Subba Row (1925). A standard solution of inorganic phosphate (KH_2PO_4) was made that contained 5 - 20 $\mu\text{g P}_1$ per 0.25-1.0 ml. The volumes of the standards and sample aliquots were adjusted to 1.0 ml. The following solutions were added to the samples and standards: 1) 0.25 ml 5 N H_2SO_4 ; 2) 0.25 ml 2.5% (w/v) ammonium molybdate; 3) 0.1 ml of reducing reagent. (The reducing reagent was prepared by dissolving 3 grams anhydrous Na_2SO_3 and 60 grams of NaHSO_3 in 300 ml of deionized water, after which 1.0 gram of 1-amino-2-naphthol-4-sulfonic acid was added. The final volume was adjusted to 500 ml with deionized water. The reducing agent was stored at 0-4°C in a brown bottle.) The final assay volume was adjusted to 2.6 ml with deionized water. After 30 minutes, the absorbance was measured at 625 nm in a Gilford 2400 spectrophotometer.

Sodium-Potassium (Na^+ - K^+)-dependent,
Magnesium (Mg^{+2})-stimulated, ouabain-sensitive
Adenosine Triphosphate Phosphhydrolase

(Na^+ - K^+)-dependent, Mg^{+2} -stimulated, ouabain-sensitive ATPase (EC 3.6.1.4) enzyme activity was assessed by a modification of the procedure of Abdel-Latif et al. (1970). The final reaction buffer contained 150 mM NaCl, 20 mM KCL, 3 mM ATP, 3 mM MgCl_2 and 40 mM Tris, pH 7.4. The final reaction volume was 0.5 ml. Ouabain sensitivity was measured using the above reaction mixture plus 10 mM ouabain. The

enzyme was preincubated in the buffer (minus ATP) for 10 minutes at 37°C. It was found that optimal protein enzyme concentrations were as follows: a) 30-50 µg SPM protein; b) 50-80 µg synaptosomal protein; or c) 90-100 µg of protein from a 10% (w/v) brain homogenate. It was also discovered that maximal ATPase enzyme stimulation occurred when the protein samples were preincubated for two minutes at room temperature in 0.02% (w/v) sodium dodecyl sulfate (SDS) prior to addition to the reaction mixture (Sweadner, 1979). The reaction was initiated by the addition of 50 µl of an ATP solution (18.0 mg/ml deionized H₂O). After an additional 10 minutes, the reaction was terminated by the addition of 150 µl of ice-cold 50% (w/v) trichloroacetic acid (TCA) and placing the reaction tubes in an ice bath. The reaction tubes were centrifuged at low speed and a 0.5 ml aliquot of the supernatant was removed. The amount of inorganic phosphate released from the ATP was then measured by the Fiske-Subba Row assay. Corresponding controls included ATP and reaction buffer (with or without ouabain) but contained no enzyme, to assess nonspecific hydrolysis of ATP, or reaction buffer (with or without ouabain) plus enzyme but no ATP, to assess for background levels of substrate and inorganic phosphate.

5'-NUCLEOTIDASE

5'-Nucleotidase (EC 3.1.3.5) was assayed following the procedure of Emmelot et al. (1964). A 1.9 ml volume of a

buffer-substrate solution containing 100 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl and 3 mM 5'-AMP (pH 7.2) was preincubated for 5 minutes at 37°C. The reaction was initiated by the addition of 0.1 ml of enzyme solution (approximately 200 - 300 µg homogenate protein or 50 - 100 µg of SPM protein). The reaction was incubated for 15 minutes at 37°C and terminated by cooling in an ice bath. The cooled mixture was immediately centrifuged at low speed and an aliquot was removed. The released inorganic phosphate was measured using the Fiske-Subba Row inorganic phosphate assay. The blank contained buffer-substrate solution without added enzyme.

ALKALINE PHOSPHATASE

Alkaline phosphatase (EC 3.1.3.1) activity was measured by the p-nitrophenyl phosphate method of Linhardt and Walter (1963). A 5.0 ml aliquot of an alkaline buffer-substrate solution containing 0.05 M glycine, 5.5 mM p-nitrophenyl phosphate, pH 10.5, was preincubated 5-10 minutes at 37°C. The reaction was initiated by the addition of 0.05 ml of tissue homogenate or sample. After incubating for 30 minutes at 37°C, the reaction was terminated by the addition of 5.0 ml of 0.02 N NaOH. Blanks consisted of no added enzyme after the 0.02 N NaOH. The p-nitrophenol liberated by the phosphatase forms a yellow anion and was quantitated by measuring the absorbance at 405 nm. Standards containing 2.5×10^{-5} mmole to 2.5×10^{-4}

mmole p-nitrophenol were made up in 5.0 ml 0.02 N NaOH, 50 μ l deionized H₂O and 0.5 ml substrate-buffer solution. One phosphatase unit is described as the amount of enzyme contained in 1000 ml of serum which liberates 1 mmole p-nitrophenol at 37°C (mmole units). With an assay volume of 11.1 ml, 0.1 ml sample and an incubation period of 30 minutes, a unit corresponds to an absorbance of 0.0833 at 405 nm. Therefore, the enzyme units were calculated as follows:

$$\frac{\text{Abs.}_{405}}{0.0833} = \text{Abs.}_{405} \times 12.00 = \text{alkaline phosphatase units (mmole)}.$$

Alkaline phosphatase specific activity was expressed as mmole/time/mg protein.

ACID PHOSPHATASE

Acid phosphatase (EC 3.1.3.2) activity was determined according to the p-nitrophenyl phosphate method described by Linhardt and Walter (1963). The acid buffer-substrate solution contained 0.05 M citrate buffer, 5.5×10^{-3} M p-nitrophenyl phosphate, pH 4.8. A 0.5 ml aliquot of buffer-substrate solution was preincubated for 5-10 minutes at 37°C. The reaction was initiated by the addition of 0.05 ml of tissue homogenate (100-500 μ g protein) or sample (10-50 μ g protein). After incubating for 30 minutes at 37°C, the reaction was terminated by the addition of 2.0 ml of 0.1 N NaOH. Blanks were prepared by adding the enzyme solution after the addition of the 0.1 N NaOH and a reagent blank was

prepared which contained no enzyme. The tubes were vortexed and the absorbance of the sample was measured at 405 nm on a Guilford 2400 spectrophotometer. Standards containing 2.5-10 (10^{-5}) mmole p-nitrophenol in 2.0 ml 0.1 N NaOH, 50 μ l deionized H₂O and 0.5 ml of the substrate-buffer solution. Calculations were as follows:

$$\frac{\text{Abs.}_{405}}{0.362} = \text{Abs.}_{405} \times 2.81 = \text{mmole acid phosphatase units.}$$

Acid phosphatase specific activity was expressed as mmole/time/mg protein.

CYCLIC NUCLEOTIDE PHOSPHOHYDROLASE

2',3'-Cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.16), CNPase, was assayed according to the method by Kurihara and Tsukada (1967) except that the chromatogram was developed in a tank containing isopropahol: concentrated ammonia: water, 7:1:2 (v/v/v) as described by Banik and Davison (1969). Samples, containing enzyme, were prepared in 1% (v/v) Triton X-100 so that 20 μ l would contain approximately 5 μ grams of brain homogenate, 2-4 μ grams of myelin protein or 10 μ grams of SPM protein. The reaction tubes containing substrate, 50 μ l of 0.03 M 2',3'-cAMP, 50 μ l of 0.2 M Na₂HPO₄-0.1 M citric acid buffer, pH 6.2 and 80 μ l of deionized H₂O were preincubated 5 minutes at 37°C. The reaction was initiated by the addition of 20 μ l of the enzyme solution in 1% Triton X-100. After 20 minutes, the reaction was terminated by the addition of 20 μ l of glacial acetic acid

and the tubes were placed in an ice bath. The tubes were centrifuged at low speed. A 20 μ l aliquot of the supernatant was spotted onto 20 x 20 cm^2 Whatman paper. The paper was then chromatographed in isopropanol: ammonia: water, 7:1:2 (v/v/v) for 3½-4 hours until the solvent reached the top of the paper. The paper was allowed to dry and the nucleotide spots were visualized using an ultraviolet (U.V.) lamp. The location of the visualized spots were marked. A control sample, containing the reaction mixture without enzyme, was included. Portions of the paper chromatogram which corresponded to product, substrate and paper blank (background) were cut into small pieces. The substrate and product (and blank) were eluted with 4 ml of 0.01 N HCl by shaking the paper-HCl mixture for 2 hours at room temperature. The paper was pelleted by low speed centrifugation. The optical density of the clear solution was determined at 260 nm in a Gilford 2400 spectrophotometer. Calculations of values were as follows:

$$S_1 = \text{Absorbance of substrate} - \text{Absorbance of paper blank}$$

$$P_1 = \text{Absorbance of product} - \text{Absorbance of paper blank}$$

$$X = S_1/1.5 = \text{Absorbance per } \mu\text{mole per assay}$$

$$Y = 3(P_1/X) = \mu\text{mole per } 20 \mu\text{l of assay per hour}$$

$$Z = Y/\text{mg protein in } 20 \mu\text{l} = \mu\text{mole per hour per mg protein}$$

$$\text{Total activity} = Z \times \text{total (mg) protein.}$$

MONOAMINE OXIDASE

The activity of monoamine oxidase (EC 1.4.3.4), MAO, was determined by the fluorometric assay by Youdin (1975), which is based on the fact that when kynuramine dihydrobromide (KDHB) is oxidized by monoamine oxidase it spontaneously cyclizes to form the fluorescent 4-hydroxyquinoline (4-HOQ). A 0.10 ml aliquot of a 10% (w/v) mitochondrial preparation, or other enzyme sample, was added to 0.5 ml of a 0.2 M sodium phosphate buffer, pH 7.4, containing 1.0 mole kynuramine dihydrobromide (KDHB). The final volume was adjusted to 1.0 ml with deionized water. The reaction mixture was incubated for 5-10 minutes at 30°C. The reaction was terminated with the addition of 0.50 ml of 20% perchloric acid. The mixture was centrifuged at low speed and a 0.5 ml of the supernatant was removed and added to 1.0 ml of 1N NaOH in a quartz fluoremetry cuvette. The product, 4-hydroxyquinoline (4-HOQ), will fluoresce at 380 nm when excited at 315 nm. This fluorescence was measured on a Aminco-Bowman spectrophotofluorometer. Concentrations were calculated from a standard curve of the relative fluorescence intensity of 4-hydroxyquinoline. A standard solution of 4-HOQ (22 nmoles per ml) was prepared. Standards were made containing 2.2-11 nmoles 4-HOQ in 1.5 ml 1 N NaOH.

CYTOCHROME C OXIDASE

Cytochrome c oxidase (EC 1.9.3.1) was assayed by the procedure of Duncan and Mackler (1966) which measures the

rate of decrease in absorbance at 550 nm accompanying the cytochrome oxidase-catalyzed conversion of ferrocytochrome c (Fe^{+2}) to ferricytochrome c (Fe^{+3}). Ferrocytochrome c was prepared by the addition of sodium dithionite to ferricytochrome c. Excess dithionite was removed by aeration. The assay system consists of 0.2 ml of 0.2 M phosphate buffer, pH 7.5, 0.1 ml of 1% (w/v) ferrocytochrome c and deionized water in a volume of 1.0 ml. When the absorbance at 550 nm stabilized, the recorder pen was set to 100% which corresponded to an absorbance of 2.0 (full scale). The reaction was initiated by the addition of 105 μ grams of enzyme protein. The rate of reaction was calculated from the slope of the linear decrease in absorbance as a function of time.

PROTEINS/GLYCOPROTEINS

Individual [^3H]-leucine-labeled brain homogenate and SPM samples from control and undernourished pups were lyophilized separately. Combined (dual-label) [^3H]-fucosylated control and [^{14}C]-fucosylated undernourished (or [^{14}C]-fucosylated control and [^3H]-fucosylated undernourished) homogenates and SPM samples were also lyophilized. Protein/glycoprotein samples were treated following the procedure of Druse and Krett (1979). Lipids were extracted twice with ether: ethanol, 3:2 (w/v) and discarded. The delipidated protein residue was dried with N_2 . The protein pellet was solubilized in a small volume of

a 2.5% (w/v) sodium dodecyl sulfate (SDS), 1.0% (w/v) Na_2CO_3 , 10% (w/v) 2-mercaptoethanol solution. The volume of solution was chosen so that the concentration of protein would be approximately 1-2 mg/ml. The solubilized protein was dialyzed against a solution containing 0.1% (w/v) SDS, 1.6 M urea, 0.05% (w/v) dithiothreitol, 0.01 M sodium phosphate buffer (pH 7.2) at room temperature for a period of 16-24 hours.

SDS-7.5% polyacrylamide gels were prepared as follows: one hundred and eighty milliliters of solution A_1 were prepared by mixing 40 ml of 1% (w/v) SDS with 80 ml of a 0.5 M sodium phosphate buffer (pH 7.2) and deionized H_2O (remaining volume). Solution A_2 (180 mls) contained 40 grams of acrylamide, 1.040 grams of N,N'-methylene-bis-acrylamide and deionized water. Solutions A_1 and A_2 were stored at room temperature for periods of less than a month. Solution B was prepared immediately prior to use. Solution B contained 90 mg of ammonium persulfate, 50 μl of TEMED and 10 ml of deionized H_2O . The solution necessary to prepare 12 polyacrylamide gels in glass cylinders (i.d. 5mm) was prepared from 22.5 mls of solution A_1 , 16.9 ml of solution A_2 , 5.6 ml of deionized H_2O and 5.0 ml of solution B. The solutions were mixed and deaerated using a vacuum, for 30 to 60 seconds. A volume of 2.5 ml of the gel solution was layered into clean glass gel tubes. The gel solution was carefully overlaid with deionized H_2O . Polymerization of

the gel occurred in about 30 minutes. Gels were generally made 2-4 hours before use. Electrophoresis buffer contained 0.1% SDS in 0.1 M phosphate buffer, pH 7.2. Bromophenol blue was added to the protein samples as a tracking dye. Gels were electrophoresed at a maximum of 50 volts or 10 mamps per tube. Electrophoresis was terminated when the tracking dye reached the end of the gel.

After electrophoresis, the gels were removed from the gel tubes and fixed in a solution containing MeOH:HAc:H₂O, 45:10:45 (v/v/v). After a minimum of 18 hours in the fixing solution, the protein bands on the gels were stained with a solution of 1% (w/v) Fast Green dye (prepared in the fixing solution) for 2 hours at 37°C. The stained gels were placed in a diffusion destainer containing fixing solution. Gels were destained until portions of the gel with no protein (i.e., in the region of the tracking dye) achieved a light or colorless background.

On gels where the control and experimental samples were electrophoresed separately, the gels were scanned densitometrically at 600 nm in a recording Gilford 2400 spectrophotometer. Individual protein bands were quantitated (based on their dye binding capacity) by measuring the peak areas from densitometric scans. Protein molecular weight standards were co-electrophoresed on polyacrylamide gels.

Samples of [³H]-leucine labeled control and undernourished SPMs and brain homogenates were electrophoresed

at the same time and under identical conditions as molecular weight standards. These single labeled gels were sliced in 1mm or 2mm sections using a Mickle gel slicer from Mickle Laboratory Engineering, Brinkman Instruments. The gel slices were solubilized in capped scintillation vials which contained 1 ml Protosol:water, 10:1 (v/v) for 3 days at room temperature. Prior to scintillation counting, 10 ml of a PPO:POPOP: toluene (16.0 gms: 0.4 gms: 4.0 liters), scintillation cocktail was added to each vial. The vials were dark-adapted for minimum of 18 hours prior to counting in a Beckman LS 7500 liquid scintillation counter. Counts per minute (CPM) were automatically converted to disintegrations per minutes (DPM) by the LS 7500 which had previously been standardized with quenched standards.

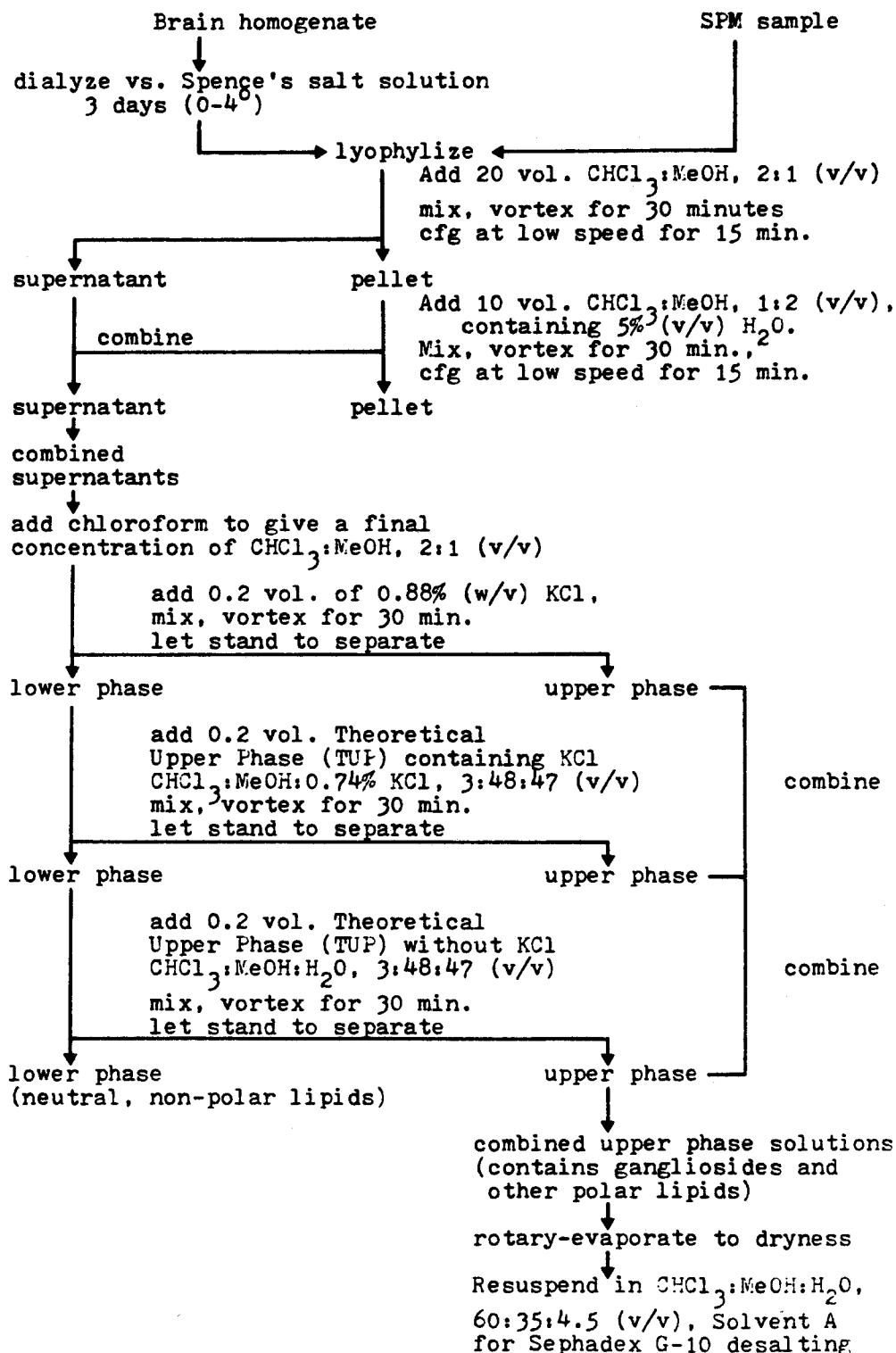
In the dual-isotope labeled glycoprotein study, a [^3H]-fucosylated SPM sample from a control pup was combined with a [^{14}C]-fucosylated SPM sample from an undernourished pup. Similarly, a [^{14}C]-fucosylated SPM sample from a control pup was combined with a [^3H]-fucosylated SPM sample from an undernourished pup. In the sialylated glycoprotein study, appropriate labeled SPM samples from control and undernourished pups were combined as described in the dual-isotope labeled fucosylated glycoprotein study. The combined control and undernourished samples were then lyophilized. The dual-labeled lyophilized samples were delipidated, solubilized and electrophoresed as described for the

[³H]-leucine SPM protein samples. The dual-labeled [³H]-/¹⁴C]-fucosylated glycoproteins or [³H]-/¹⁴C]-sialylated glycoproteins were sliced from the acrylamide gels according to individual protein bands. The gel slices were, similarly, solubilized and counted by liquid scintillation counting as described for the [³H]-leucine SPM proteins.

EXTRACTION OF GANGLIOSIDES

Brain homogenates (in sucrose) were dialyzed against Spence's salt solution (Spence and Wolfe, 1967) for two days at 4°C before lyophilization. Brain homogenate and SPM samples were lyophilized and lipids were extracted in 20 volumes of CHCl₃:MeOH, 2:1 (v/v). Gangliosides were extracted following the procedure of Folch, Lees and Stanley (1957) as modified by Suzuki (1964) [See Figure 4]. The samples were vortexed extensively and centrifuged at low speed. The chloroform-methanol solution was decanted from the CHCl₃-MeOH insoluble pellet and saved. The residue was re-extracted with 10 volumes of CHCl₃:MeOH, 1:2 (v/v) containing 5% (v/v) water. The extracts were combined and adjusted to a final concentration of CHCl₃:MeOH, 2:1 (v/v). Using 50 ml conical tubes, with ground glass stoppers, 0.2 volumes of 0.88% (w/v) KCl were added to the chloroform:methanol, 2:1 (v/v) lipid extract. The conical tubes were vortexed extensively during a 30-minute interval. The aqueous and organic phases were allowed to separate. Polar gangliosides partitioned into the aqueous upper phase.

GANGLIOSIDE EXTRACTION



The upper phase was removed and saved. The lower phase was re-extracted with 0.2 volumes of a theoretical upper phase (MeOH:H₂O:CHCl₃, 48:47:3 (v/v/v)) containing 0.74% KCl. The resulting upper phase was removed and combined with the first upper phase. The lower phase was re-extracted with 0.2 volumes of theoretical upper phase (without KCl). The resulting upper phase was removed and combined with the others. The combined upper phases were evaporated to dryness using a rotary evaporator. The dried residue was suspended in Solution A (CHCl₃:MeOH:H₂O, 60:30:4.5, (v/v/v)) and samples were desalted on Sephadex G-10 column by the procedure of Wells and Dittmer (1963). Sephadex G-10 was allowed to swell overnight in deionized H₂O. Water was decanted the following day. In a series of steps, the Sephadex was resuspended in a solution overnight and removed from the solution by decanting the liquid the following day. The steps utilized the following solutions in sequence:

- 1) methanol;
- 2) CHCl₃:MeOH, 1:1 (v/v);
- 3) CHCl₃:MeOH, 2:1 (v/v) and
- 4) Solution A.

A slurry of the Sephadex G-10 in Solution A was poured into a pasteur pipet containing a glass wool plug. The Sephadex column height was adjusted to that a void volume of 1.0 ml was obtained. The Sephadex column was washed with 20 mls of Solvent A. Samples were suspended in 1.0 ml of Solvent A and sonicated. The sample was applied to the Sephadex bed. An additional 0.5 ml of Solvent A was used to rinse the round bottom flask and added to the Sephadex

column. In order to standardize the column, an SPM ganglioside sample which had been labeled with [³H]-N-acetylmannosamine was applied to a Sephadex column and aliquots of the effluent removed and the radioactivity determined. Radioactive gangliosides appeared to come off in a volume equivalent to 1½ times the void volume. The first 1.5 ml of effluent (containing the gangliosides) was collected, dried under a stream of N₂ and resuspended in chloroform: methanol, 1:1 (v/v).

GANGLIOSIDES

The ganglioside samples were dried under a stream of N₂ prior to separation of ganglioside species by thin layer chromatography (tlc). The dried samples were resuspended in a small volume of CHCl₃:MeOH, 1:1 (v/v). Aliquots of whole brain gangliosides (containing 4-5 µg NANA) and SPM gangliosides (1-2 µg NANA) were spotted on a 20 x 20 cm² silica gel G-60, without fluorescent indicator, 0.25 mm thin layer chromatography plates. The plate was developed in a saturated tank containing CHCl₃:MeOH: 0.25% (w/v) CaCl₂, 60:35:8 (v/v/v) Solvent System I (Irwin and Irwin, 1979; Williams and McCluer, 1980). Alternatively, gangliosides were separated on 10 cm x 20 cm HPTLC plates in a methylacetate: n-propanol: chloroform: methanol: 0.25% aqueous KCl, 25:20:20:20:17 (v/v/v/v/v) Solvent System II. After chromatography, the plates were allowed to dry. They were sprayed lightly, via atomizer, with resorcinol-HCl

reagent (Svennerhlo, 1957). The tlc plate was covered with a clean glass plate and heated in an oven at 100-110°C for 15 minutes (Irwin et al., 1980). During this heating, the ganglioside bands turned purple. The tlc plate was densitometrically scanned at 580 nm using a Kratos Schoeffel SD 3000 densitometer. Individual ganglioside species were quantitated by measuring the peak areas from a densitometric scan.

For radioactive studies, using a [^3H]- or [^{14}C]-N-acetylmannosamine, an [^3H]-labeled SPM sample from a control animal was combined with a [^{14}C]-labeled SPM sample from an undernourished animal before lyophilization. (The assignment of the isotopes was reversed in approximately half of the animals.) The combined dual-label samples were simultaneously extracted, purified, desalted and chromatographed (using System I). The chromatographed gangliosides were visualized using iodine (I_2) vapors. The separated ganglioside species were scraped from the tlc plates and placed into scintillation vials. One ml of deionized H_2O was added to each vial. Vials were sonicated prior to the addition of 10 ml of Aquasol cocktail fluor (New England Nuclear). The samples were dark-adapted for a minimum of 18 hours and counted by liquid scintillation in a Beckman LS 7500.

STATISTICAL DETERMINATIONS

Numerical calculations were generally expressed as the mean \pm the standard deviation of several determinations (where N = the number of determinations). Statistics on the differences between two related groups (control and undernourished) were performed using the Student's t-test of probability.

In the double-labeled fucosylated glycoprotein or sialylated ganglioside studies, a [^3H]-labeled (fucose or N-acetylmannosamine, respectively) sample from a control animal was combined with a [^{14}C]-labeled (fucose or N-acetylmannosamine, respectively) sample also from an age-matched control animal. The combined samples were extracted and separated either by SDS-polyacrylamide gel electrophoresis (fucosylated glycoprotein study) or by thin layer chromatography (sialylated ganglioside study). The individual protein/glycoprotein or ganglioside bands were excised (as described previously) and counted by liquid scintillation. The results for each determination were expressed as a ratio of the [^3H] %DPM to [^{14}C] %DPM. This was done to determine the normal range of variance between control samples. A ratio of 1.0 represents perfect correlation. Values between several samples of control animals ranged from 0.95 to 1.05. A ratio of the %DPM in a sample from an undernourished animal to the %DPM in a sample from a control animal of less than 0.95 or greater than 1.05

was therefore considered to be abnormal. If this same abnormality was consistently observed in three quarter similarly paired samples, then the abnormality was considered to be significant.

CHAPTER IV

RESULTS

Brain and Body Weights

One of the most pronounced effects of maternal undernutrition was the decreased body weights of the offspring [Table I]. The body weights of the undernourished pups were 50, 42 and 32% of control levels at 10, 14 and 20 days of age, respectively. The decreased body weights persisted for at least two weeks after nutritional rehabilitation was initiated.

The brain weights of the undernourished rat pups were also significantly reduced (85, 78, 71 and 76% of control values at 10, 14, 20 and 34 days of age, respectively) [Table I] although to a lesser degree than the body weights. These findings support the previously recognized phenomenon of "brain sparing" in nutritional deprivation (Hatai, 1904; Donaldson, 1911; Jackson, 1915; Dobbing and Widdowson, 1965; Dickerson and Walmsley, 1967; Krigman and Hogan, 1976).

Brain and SPM Protein

The whole brain protein content (mg protein/brain) was significantly reduced in the undernourished pups at all ages examined (84, 78, 70 and 78% of control values at 10, 14, 20 and 34 days postnatal, respectively) [Table II].

Table I

BRAIN AND BODY WEIGHTS IN CONTROL (C) AND UNDERNOURISHED (U) RATS

		Age (Days)			
		10d	14d	20d	34d
Body weight (grams)	C	24 ± 1	31 ± 2	47 ± 3	138 ± 15
	U	12 ± 2*	13 ± 0.3*	15 ± 2*	58 ± 9*
U as % of C		50%	42%	32%	42%
Brain wet weight (grams)	C	1.06 ± 0.02	1.29 ± 0.06	1.58 ± 0.05	1.85 ± 0.07*
	U	0.90 ± 0.06*	1.00 ± 0.03*	1.12 ± 0.10*	1.40 ± 0.07*
U as % of C		85%	78%	71%	76%

Each value represents the mean of 12 samples ± the standard deviation. An * indicates that the values from control (C) and undernourished (U) rats are different at $p < 0.05$.

Table II

BRAIN AND SPM PROTEIN IN CONTROL (C) AND UNDERNOURISHED (U) RATS

		Age (Days)			
		10d	14d	20d	34d
Brain protein content (mg/brain)	C	88 ± 5	135 ± 13	188 ± 16	220 ± 12
	U	74 ± 5*	105 ± 7*	132 ± 25*	171 ± 24*
	U as % of C	84%	78%	70%	78%
Brain protein concentration (mg/gram wet weight of brain)	C	83 ± 5	104 ± 7	119 ± 9	119 ± 6
	U	83 ± 2	105 ± 7	118 ± 18	122 ± 12
	U as % of C	100%	101%	99%	103%
SPM protein content (µg/brain)	C	206 ± 45	316 ± 23	474 ± 59	758 ± 164
	U	115 ± 59*	126 ± 9*	264 ± 40*	429 ± 84*
	U as % of C	56%	40%	56%	57%
SPM protein concentration (µg/gram wet weight of brain)	C	193 ± 42	245 ± 18	300 ± 43	352 ± 118
	U	126 ± 60*	127 ± 12*	229 ± 43*	307 ± 54
	U as % of C	65%	52%	76%	87%

Brain and SPM protein in control (C) and undernourished (U) rats. Values represent the mean ± the standard deviation of two separate experiments (n=12). An * represents that the difference between the controls and the undernourished animals is significant at $p < 0.05$.

However, the whole brain protein concentration (mg protein/gram of brain wet weight) was not reduced; this indicated that the decreased whole brain protein content was directly related to the decrease in brain weight.

The SPM protein content (μg protein/brain) was significantly decreased (56, 40, 56 and 57% of control levels at 10, 14, 20 and 34 days of age, respectively) in the undernourished pups [Table II]. The SPM protein concentration (μg protein/gram wet weight of brain) was also significantly decreased (65, 52 and 76% at 10, 14 and 20 days, respectively). The size of the deficiency of SPM protein was greater than that which could be accounted for solely by a decrease in the brain weight in the undernourished pups. At 34 days of age, the SPM protein concentration (but not content) of the undernourished pups had returned to normal.

Assessment of Purity of SPMs

The relative purity of the SPMs was assessed by determining the activities of several "marker" enzymes in brain homogenates and in SPMs. In addition, the relative distribution of these "marker" enzymes was assessed in several fractions of the sucrose gradient used to isolate SPMs from lysed synaptosomes [Table III].

There was approximately a 5-fold enrichment in the specific activity of the plasma membrane marker Mg^{+2} -dependent, ouabain-sensitive $(\text{Na}^{+}-\text{K}^{+})$ -ATPase in the SPMs over that in the whole brain homogenate of 20-day-old control

Table III

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITY
ON SYNAPTOSOMAL SUBFRACTIONATION GRADIENT

				Fractions				
				A	B	C	D	E
% Protein recovered from gradient								
CONTROL (C)				0	14	17	14	53
UNDERNOURISHED (U)				0	10	13*	14	63
Specific Activity		Brain		Relative Specific Activity				
Enzyme		Brain	SPM					
(Na ⁺ K ⁺)-ATPase	C	5.6	26.7	0	2.5	2.6	0.9	0.1
	U	5.3	42.4	0	3.2	2.8	0.7	0.3
Alkaline Phosphatase	C	68	385	0	1.4	2.0	1.8	0.4
	U	52	683	0	1.7	3.6	1.2	0.3
5'-Nucleotidase	C	2.7	3.5	0	1.7	1.4	0.6	0.8
	U	2.4	5.3	0	0.8	4.0	1.0	0.4
Acid Phosphatase	C	960	2013	0	1.5	2.2	1.5	0.4
	U	842	2300	0	2.2	2.7	1.2	0.4
Monoamine Oxidase	C	8.0	0	0	0	0	0	1.9
	U	6.9	0	0	0	0	0.8	1.4
Cytochrome c Oxidase (x10 ⁻⁴)	C	16	7.9	0	0.04	0.6	3.9	0.7
	U	17	10.7	0	0.08	1.3	3.0	0.6
2',3'-cyclic nucleotide phosphorylase	C	96	81	0	4.8	1.5	0.3	0.02
	U	77	130	0	4.1	1.2	0.8	0.5

Distribution of protein and enzyme activity on synaptosomal subfractionation gradient. The 5 fractions (A-E) correspond roughly to the 5 sucrose densities (10%, 25%, 32%, 35% and 38%) in the gradient. The "myelin" interface is included in fraction B and the SPM interface is included in fraction C. Protein content was determined for each of the fractions. The specific activity for each of the enzymes is defined in units of μ moles of substrate hydrolyzed (or μ moles of product produced) per hour per mg protein, in whole brain, purified SPMs and sucrose gradient subfractions of control and undernourished pups at 20 days of age. Relative Specific Activity (RSA) is defined as the % of the protein recovered in a fraction divided by the % of the enzyme activity recovered in that fraction. The specific activity and RSA of each enzyme was determined in 3 samples. An * indicates that the values between control and undernourished animals are significant at $p < 0.05$. Statistical analysis was not performed on the RSAs. C and U are abbreviations for control and undernourished animals, respectively.

animals. The relative specific activity (RSA) (% recovered enzyme activity/% recovered protein) of $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$ was highest in fractions B and C. (An RSA value greater than 1.0 indicates a particular enrichment in marker enzyme activity within that fraction.)

There was also a 6-fold enrichment in the specific activity of alkaline phosphatase in the SPMs over that in whole brain in control animals. The relative specific activity of alkaline phosphatase ranged from 1.4 to 2.0 in fractions B, C and D, which indicated the relative abundance of plasma membranes with fraction C having the highest (2.0 RSA).

5'-Nucleotidase is another reported "marker" enzyme for plasma membranes. There was, roughly, a 1.4-fold increase in the specific activity of 5'-nucleotidase in the SPMs over the whole brain. In control animals it was observed that the relative specific activity of 5'-nucleotidase was higher (RSA = 1.7) in a lighter fraction of the gradient (Fraction B) than in the SPM-containing fraction (Fraction C). This is consistent with the report of Cotman and Matthews (1971).

There was approximately a 2-fold increase in specific activity of acid phosphatase in the SPMs over the whole brain in control animals. The relative specific activity for acid phosphatase ranged from 1.5 to 2.2 in fractions B, C and D, with the highest (RSA 2.2) in fraction C.

There was no enrichment in the specific activity of the membrane marker (CNPase) in the SPMs over that in the whole brain. The relative specific activity of CNPase was highest (4.8) in fraction B, whereas there was also some activity (RSA 1.5) in fraction C. This may indicate that there is some contamination of the SPMs by myelin membranes. Assuming there is some degree of contamination of the SPMs with myelin membranes, it was calculated that the SPMs contain from 0.1 to 0.4% of the total whole brain CNPase activity. With the amount of SPM protein recovered at 20 days of age, in either control or undernourished animals, this would amount to less than 5 μ grams of myelin membranes present in the SPM fraction (or less than 1% contamination by myelin membranes).

There was no detectable contamination of the SPMs by outer mitochondrial membranes, as indicated by the lack of described monoamine oxidase (MAO) activity. Most, if not all, of the MAO activity was located in the pellet (fraction E) of the sucrose gradient.

There was also no enrichment in the specific activity of cytochrome c oxidase in the SPMs over that in the whole brain homogenates. Most of the cytochrome c oxidase relative specific activity was found in sucrose fraction D above the pellet-containing fraction (fraction E). Although there was shown to be relatively no contamination of the SPMs by outer mitochondrial membranes (as measured by the MAO activity),

there may be slight contamination by inner mitochondrial membranes (as measured by cytochrome c oxidase activity).

This experiment was also performed in order to determine whether the SPMs from the undernourished rats (20 day old) would be distributed in the same fraction as those from age-matched control rats [Table III]. In general, the undernourished rats had a lower proportion of protein in fractions B and C, which would be expected to contain myelin and SPMs, and a larger proportion of protein in the pellet (fraction E).

There was approximately an 8-fold enrichment in the specific activity of Mg^{+2} -dependent, ouabain-sensitive ($Na^{+}-K^{+}$)-ATPase in the SPMs over that in the whole brain homogenate in the undernourished animals, compared to the 5-fold enrichment observed in control animals. In addition, the undernourished animals had a higher RSA of ($Na^{+}-K^{+}$)-ATPase in fraction C was very similar in both control and undernourished animals.

There was a 13-fold enrichment in the specific activity of alkaline phosphatase in the SPMs over that in whole brain in undernourished animals. This compared to only approximately a 6-fold enrichment in the SPMs over whole brain in control animals. In addition, there was an increase in the specific activity and relative specific activity of alkaline phosphatase in the SPM gradient fraction B when undernourished animals are compared with age-matched controls.

The specific activity of 5'-nucleotidase in whole brain homogenates was comparable in control and undernourished pups. There was, roughly, a 2-fold increase in the specific activity of 5'-nucleotidase in the SPMs over the whole brain in both control and undernourished animals. However, there was an increase in the relative specific activity of 5'-nucleotidase in the sucrose fraction C in the undernourished animals.

There was no difference in the enrichment of acid phosphatase specific activity in the SPMs of control or undernourished rats. There was also no altered distribution of this enzyme-containing membrane fraction within the sucrose gradient of either control or undernourished animals as measured by the relative specific activity of acid phosphatase.

There were comparable amounts of the myelin membrane marker (CNPase) in the SPMs of both undernourished and control animals. There was a slight enrichment in specific activity of CNPase in the SPMs from undernourished animals although there was no altered distribution of the relative specific activity of CNPase within the sucrose subfractions in the undernourished animals.

There was no detectable contamination of the SPMs by outer mitochondrial membranes in either control or undernourished animals as measured by the absence of any detectable monoamine oxidase activity. There was, however, a

slight increase in the specific activity of inner membrane cytochrome c oxidase activity in the purified SPMs of the undernourished animals when compared to that in the controls, although the distribution (relative specific activity) within the sucrose gradient subfractions were nearly comparable in control and undernourished animals. Assuming that there was some degree of contamination of the SPMs by inner mitochondrial membranes, then it can be calculated that from 0.1 to 0.2% of the total cytochrome c oxidase activity was present in the SPMs from control and undernourished animals, respectively. This amount of cytochrome c oxidase activity, even though slightly higher in SPMs from undernourished animals, still amounts to considerably less than 1% contamination of the SPMs by inner mitochondrial membranes.

Brain Region Study

There were no significant differences between control and undernourished animals at any of the ages examined (14, 20 and 34 days) [Table IV] for brain region tissue protein concentration in any of the selected brain regions (prefrontal lobes, hippocampus, cerebral cortex and cerebellum). This is in agreement with the findings (above) of normal whole brain protein concentration in undernourished animals [Table II]. When SPMs were isolated from each of these brain regions, it was found that there was a significant decrease in SPM protein concentration (μg SPM protein/gram wet weight of brain region tissue) only in the hippocampus region (61 and 31% of control

Table IV
BRAIN REGION TISSUE PROTEIN CONCENTRATION

		Age (Days)		
		14d	20d	34d
Prefrontal lobes	C	85.1 ± 20.5	110.8 ± 13.2	103.3 ± 1.4
	U	74.2 ± 11.7	86.9 ± 4.2	91.8 ± 5.1
Hippocampus	C	68.9 ± 11.4	89.5 ± 19.4	105.9 ± 9.3
	U	61.7 ± 13.5	83.9 ± 4.9	84.1 ± 21.4
Cortex	C	95.5 ± 6.5	101.3 ± 9.2	100.5 ± 1.0
	U	83.9 ± 5.4	97.2 ± 13.1	108.5 ± 9.3
Cerebellum	C	99.5 ± 5.7	112.5 ± 9.9	115.1 ± 6.3
	U	93.8 ± 13.1	104.2 ± 3.0	111.6 ± 6.0

Units represent mg protein per gram wet weight of tissue. Values represent the mean ± standard deviation of three experiments. Brain region tissue was obtained from 6-9 animals to obtain sufficient material from both control (C) and undernourished (U) rat pups.

values at 14 and 20 days of age, respectively) of the undernourished pups [Table V].

(Na⁺-K⁺)-ATPase ACTIVITY

The activity of (Na⁺-K⁺)-ATPase in the synaptic plasma membranes (SPMs) was investigated at several stages of development in control and undernourished rats. In order to optimize the assay conditions, we performed the following experiments. 1) We measured the effects of various cations in the incubation buffer on ATPase activity [See Figure 5]. It can be seen from the graph that a large part of the ATPase activity is dependent on the presence of Mg⁺² (3mM) ions. There is only a low amount of activity when Na⁺ and K⁺ (150 mM and 20 mM, respectively) are present in the medium and when Mg⁺² is lacking. Optimal activity is achieved in the presence of all three (Na⁺, K⁺ and Mg⁺²) cations in the incubation buffer (150 mM NaCl, 20 mM KCl and 3 mM MgCl₂). 2) Since ATPase is a membrane-bound enzyme, the effects of such membrane solubilizing detergents as sodium dodecyl sulfate (SDS), Triton X-100 and sodium deoxycholate (DOC) on ATPase activity were determined. It can be seen from Figure 6 that SDS concentrations from 0.01-0.04% (w/v) had a stimulatory effect on the membrane ATPase activity, while concentrations of SDS greater than 0.04% (w/v) reduced the activity of the enzyme even below control (no SDS) levels. Concentrations of less than 0.01% (w/v) SDS had little or no effect on stimulating the membrane ATPase activity. While

Table V
BRAIN REGION SPM PROTEIN CONCENTRATION

		Age (Days)		
		14d	20d	34d
Prefrontal lobes	C	359 ± 96	356 ± 73	267 ± 169
	U	185 ± 17	157 ± 118	265 ± 116
Hippocampus	C	503 ± 39	483 ± 58	256 ± 124
	U	307 ± 70*	152 ± 52*	356 ± 104
Cortex	C	299 ± 37	421 ± 183	222 ± 38
	U	220 ± 28	231 ± 80	278 ± 85
Cerebellum	C	282 ± 68	215 ± 50	282 ± 58
	U	252 ± 9	285 ± 202	290 ± 55

Units represent μg SPM protein per gram of tissue wet weight. Values represent the mean \pm standard deviation of three experiments. An * indicates that values from control (C) and undernourished (U) rats are different at $p < 0.05$. SPMs were isolated from brain region tissue isolated from 6-9 animals to obtain sufficient material.

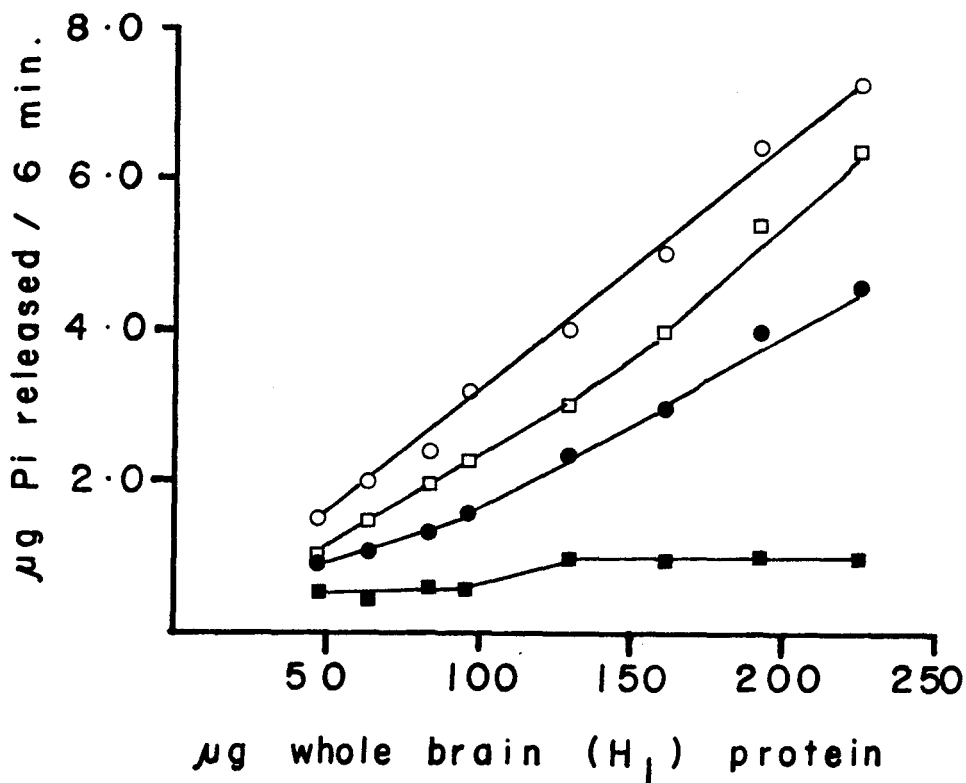


Figure 5. Effect of various cations on ATPase activity in whole brain homogenates. The reaction buffers contained: (■) 150 mM NaCl and 20 mM KCl; (◻) 3 mM MgCl₂; (○) 150 mM NaCl, 20 mM KCl and 3 mM MgCl₂; or (●) 150 mM NaCl, 20 mM KCl, 3 mM MgCl₂ and 10 mM ouabain. All reaction buffers contained 40 mM Tris, pH 7.4. Reactions were initiated by the addition of 3 mM ATP. The reactions were stopped by the addition of 150 µl of ice-cold trichloroacetic acid. The reaction tubes were centrifuged at low speed for 10 minutes, and the amount of inorganic phosphate in the supernatant was determined using the Fiske-Subba Row inorganic phosphate assay. Values represent the average of a single assay done in duplicates.

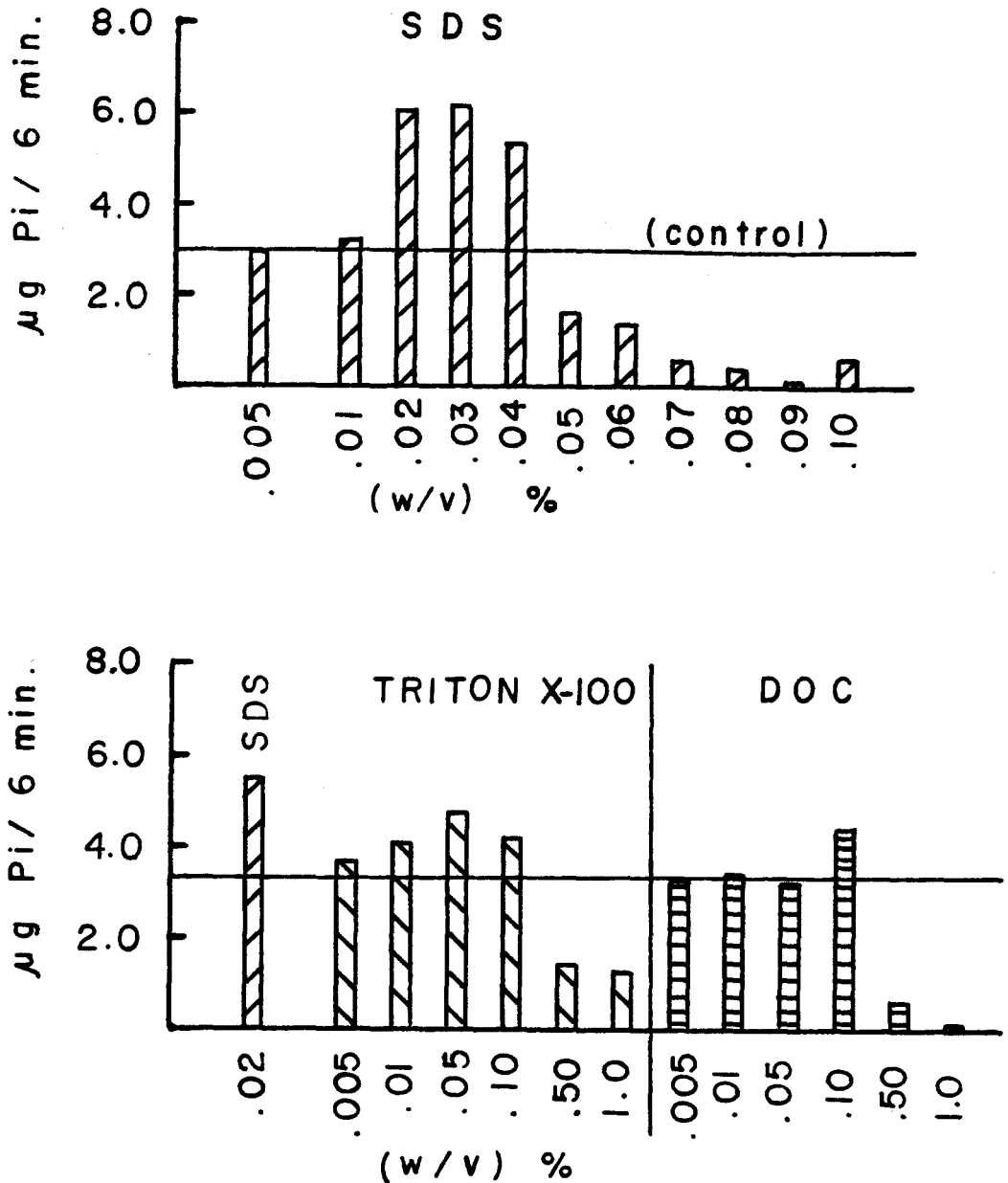


Figure 6. Effect of several membrane solubilizing agents on brain ATPase activity. Samples were preincubated with the appropriate concentration of detergents for 2 minutes at room temperature before initiation of enzyme activity. Control levels represent samples that were preincubated in the absence of detergent. Enzyme activities were determined from the amount of inorganic phosphate released from ATP during a six minute incubation at 37°C. Values represent the average of a single experiment done in duplicates. SDS and DOC are abbreviations for sodium dodecyl sulfate and deoxycholate, respectively.

0.005-0.10% (w/v) Triton X-100 and 0.10% (w/v) DOC had a stimulatory effect on (Na^+-K^+) -ATPase activity [Figure 6]; neither detergent achieved the level of stimulation as that of 0.02% SDS. Thus, 0.2% (w/v) SDS was included in the pre-incubation step of all subsequent ATPase assays.

The Mg^{+2} -dependent (Na^+-K^+) -stimulated ATPase activity was determined in the absence and presence of ouabain. The enzyme activity was expressed as micromoles of inorganic phosphate released per hour per milligram of protein ($\mu\text{mole Pi/hr/mg protein}$). Mg^{+2} -dependent, ouabain-sensitive (Na^+-K^+) -ATPase was determined in whole brain and SPMs at 10, 14, 20 and 34 days of age in both control and undernourished rat pups. Total ATPase activity in whole brain was decreased to 63, 48, 54 and 63% of control levels in the undernourished pups at 10, 14, 20 and 34 days of age, respectively [Table VI]. This decrease was statistically significant (using the Student's t-test) at 14, 20 and 34 days of age. The specific activity of (Na^+-K^+) -ATPase in brain homogenates was also significantly decreased in undernourished rats at 14 days of age. Although the ATPase specific activity in the SPMs was significantly decreased to 33% of control levels by 10 days in the undernourished pups, it was increased to 171 and 194% of control levels at 20 and 34 days, respectively. This is in contrast to observations by Kissane and Hawrylewicz (1975, 1978) who found that (Na^+-K^+) -ATPase specific activity was decreased

Table VI

(Na⁺-K⁺)-ATPase ACTIVITY IN DEVELOPING CONTROL (C) AND UNDERNOURISHED (U) RATS

		Age (Days)			
		10d	14d	20d	34d
Brain Total Activity (μ mole Pi/hr)	C	353 \pm 191	915 \pm 183	1651 \pm 575	3067 \pm 418
	U	224 \pm 65	438 \pm 159*	887 \pm 234*	1927 \pm 113*
	U as % of C	63%	48%	54%	63%
Brain Specific Activity (μ mole Pi/hr/mg prot.)	C	3.9 \pm 2.0	6.8 \pm 1.5	9.0 \pm 3.7	13.8 \pm 2.2
	U	3.1 \pm 1.0	4.2 \pm 1.4*	6.6 \pm 2.7	11.5 \pm 2.0
	U as % of C	79%	62%	73%	82%
SPM Total Activity (μ mole Pi/hr)	C	1.6 \pm 1.2	6.1 \pm 2.6	11.8 \pm 2.8	36.5 \pm 10.0
	U	0.3 \pm 0.5	3.7 \pm 2.3	11.8 \pm 0.7	21.5 \pm 1.2
	U as % of C	19%	61%	100%	59%
SPM Specific Activity (μ mole Pi/hr/mg prot.)	C	8.4 \pm 4.4	19.6 \pm 8.4	25.1 \pm 6.4	27.4 \pm 15.8
	U	2.8 \pm 3.1*	13.8 \pm 3.1	42.9 \pm 2.1*	53.2 \pm 8.4*
	U as % of C	33%	70%	171%	194%

(Na⁺-K⁺)-ATPase activity in developing control (C) and undernourished (U) rats. Each value represents the mean \pm the standard deviation of two separate experiments each done in triplicate (n=6). An * indicates that the values between the control and undernourished rats are different at p<0.05.

in rat brain synaptosomes as a result of neonatal undernutrition.

SPM PROTEINS

L-[4,5-³H]-Leucine (100 μ Ci in 10 μ l of saline) was injected intracerebrally into control and undernourished pups of selected ages: 9,13,19 and 33 day old. Pups were sacrificed 16 hours later by decapitation, and SPMs were prepared as described previously. There was an increased incorporation (total dpm and dpm/ μ g protein) of [³H]-leucine [Figure 7] into the brain and SPM protein of young (10- to 20-day-old) undernourished pups. This increase was statistically significant ($p < .05$) at several ages. The highest specific activity (dpm/ μ g protein) of [³H]-leucine in SPM and brain proteins was observed at the earliest age examined (10 days).

The SPM proteins were delipidated, solubilized and separated by electrophoresis on polyacrylamide gels as described in the methods section. There were two major protein bands in the SPMs and about 20 minor bands that stained with Fast-Green [Figure 8]. One of the major protein bands had an apparent molecular weight (96,000) similar to the subunit weight of ATPase (Morgan et al., 1973). The other band had an apparent molecular weight of 56,000, which is similar to that reported for tubulin (Kelly and Cotman, 1977; and Yen et al., 1977). The relative distribution of dye-binding capacity of the individual peaks

[³H] - LEUCINE INCORPORATION

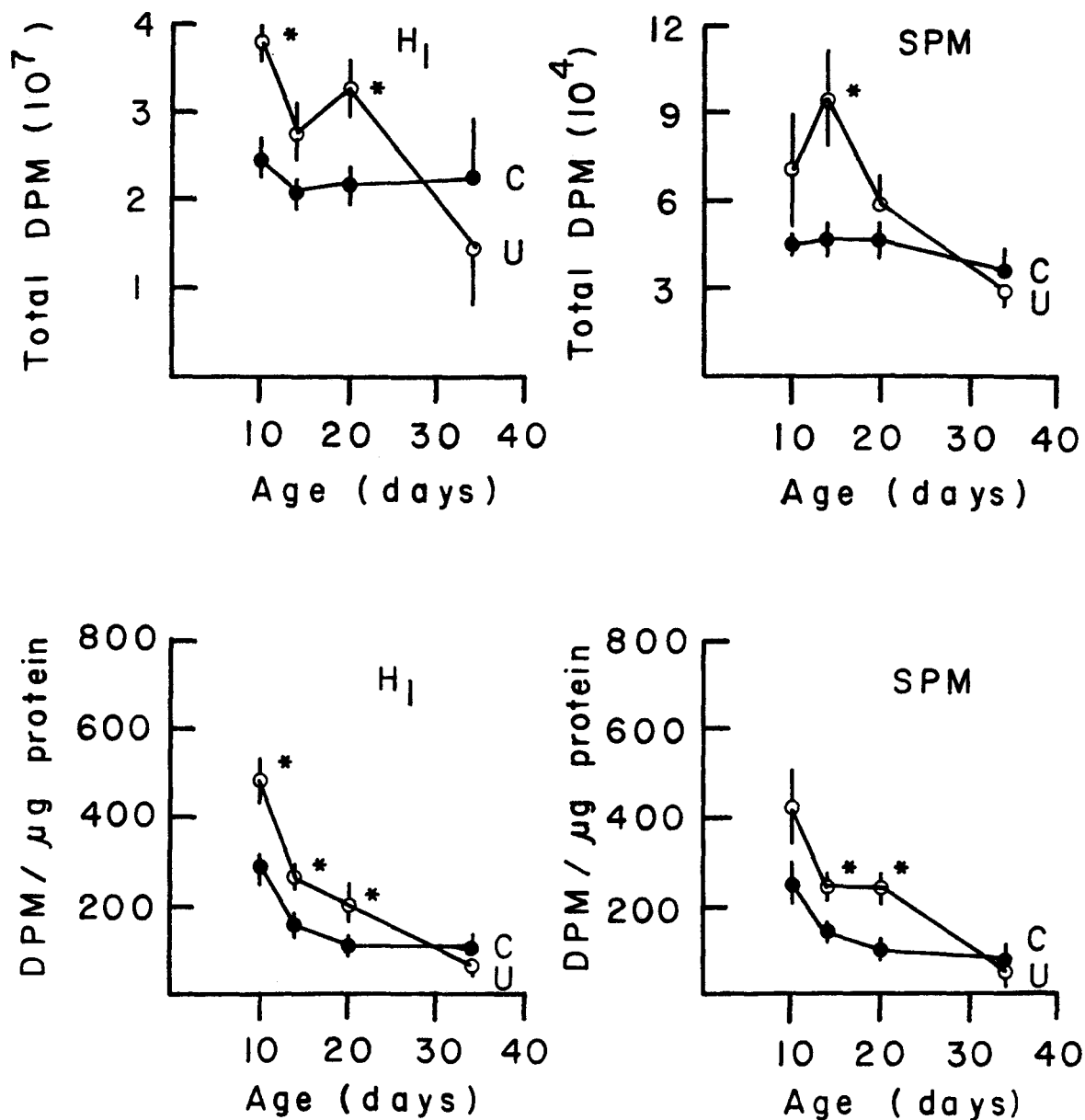


Figure 7. Incorporation of [³H]-leucine into whole brain (H₁) and SPMs of control (●) and undernourished (○) rat pups. Values represent the mean ± the standard deviation of six animals from two separate experiments. An * indicates that the difference between control and undernourished animals is significant at p < 0.05.

SPM PROTEIN PROFILE

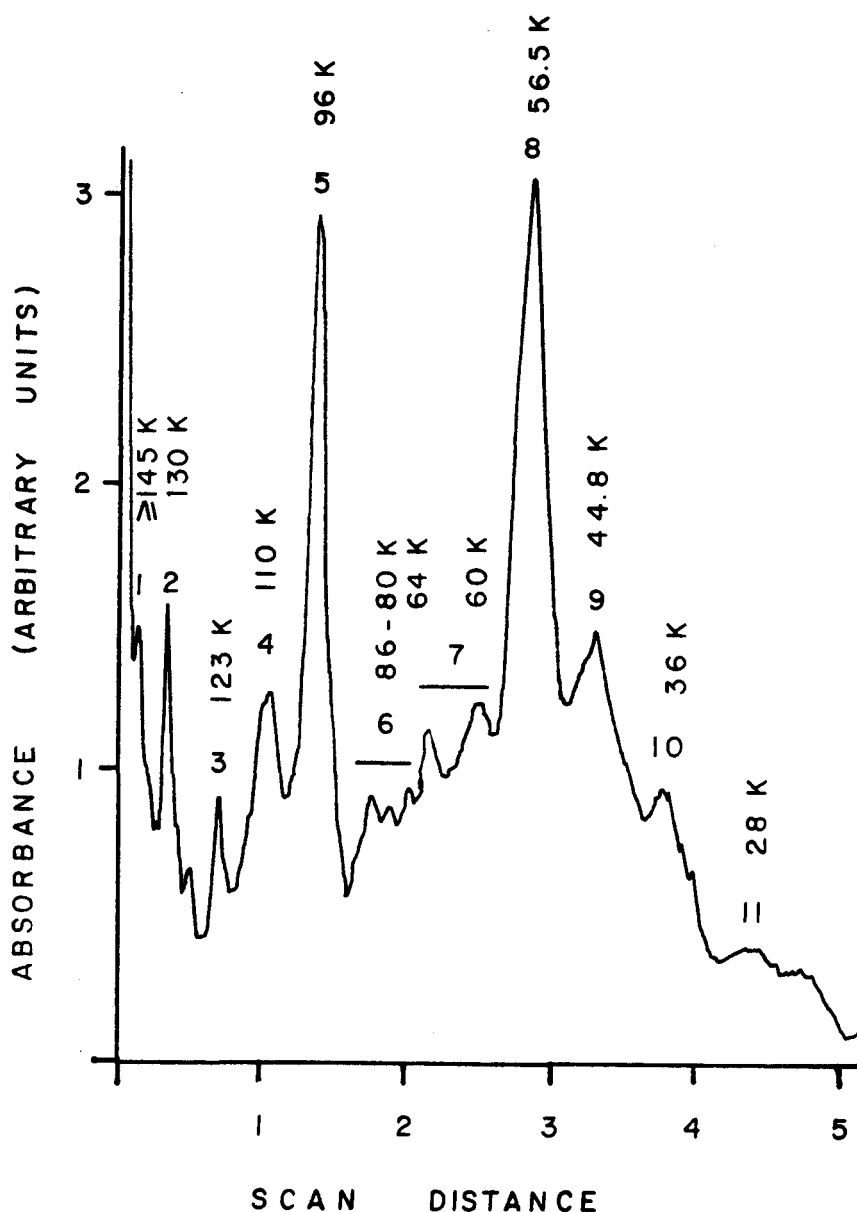


Figure 8. SPM protein densitometric tracing. SPM proteins (250 grams) from a 34-day-old control rat pup were delipidated with ether/ethanol, 3/2 (v/v), solubilized in SDS-urea and electrophoresed on SDS-7.5% polyacrylamide disc gels. The protein bands were stained with 1% Fast Green (in fixing solution: MeOH/HAc/H₂O, 45/10/45 (v/v) for two hours at 37°C and allowed to destain by diffusion in fixing solution. The gel was scanned densitometrically at 600 nm in a recording Guilford 2400 spectrophotometer with a scan rate of 1 cm/min and a chart speed of 1 in/min. Numbers 1-11 represent corresponding protein bands for which molecular weights and peak areas were determined.

per region of the gel in Figure 8 is presented in Table VII. The relative proportion of the SPM band with a molecular weight of $\sim 96,000$ (band 5) increased in normal animals from $\sim 18.2\%$ at 10 days to 27.5% at 34 days [Table VII]. Although the relative proportion of this band (ATPase) was comparable in 10- and 14-day-old control and undernourished pups, it was statistically decreased (20%) and increased (though not statistically) (15.6%) in 20- and 34-day-old undernourished pups, respectively. The undernourished animals had a statistically significant ($p < 0.05$) increase in the proportion of the band (8) with an apparent molecular weight of 56,000 at 20 days of age, in contrast to a statistically significant ($p < 0.05$) decreased proportion of this band at 34 days compared to controls.

The relative distribution of [^3H]-leucine derived radioactivity on SDS gels was calculated according to the following formula:

$$\% \text{ DPM/gel slice} = \frac{\text{DPM for each gel slice}}{\text{Total DPM in the whole gel}} \times 100\%$$

Approximately 70-80% of the total counts recovered from the gels were localized in the first half of the gels at all gels examined. There was no major incorporation into any particular protein with molecular weight below 40,000 [Figure 9]. Therefore, the first one half of each gel was cut into 1 mm sections. Results are shown for the first half of the [^3H]-leucine-labeled SPM protein samples from control and

Table VII
SPM PROTEIN PROFILE

Peak #	Apparent mol. wt. ($\times 10^{-3}$)	Age (Days)							
		10d		14d		20d		34d	
		C	U	C	U	C	U	C	U
1	145	2.2 \pm 1.6	2.9 \pm 1.6	3.4 \pm 1.5	2.4 \pm 1.5	1.8 \pm 1.5	0.6 \pm 0.2	0.4 \pm 0.1	0.4 \pm 0.0
2	130	2.8 \pm 2.0	3.3 \pm 1.5	1.6 \pm 0.4	2.4 \pm 1.1	2.8 \pm 1.0	2.3 \pm 1.0	2.7 \pm 0.1	2.4 \pm 0.2
3	123	1.3 \pm 1.5	2.1 \pm 1.5	1.2 \pm 0.6	1.4 \pm 0.9	1.7 \pm 0.5	1.7 \pm 0.5	1.4 \pm 0.4	1.3 \pm 0.2
4	110	6.6 \pm 2.1	7.2 \pm 2.6	8.2 \pm 1.7	7.0 \pm 1.2	9.2 \pm 0.8	6.4 \pm 2.4*	6.3 \pm 0.4	8.5 \pm 0.9
5	96	18.2 \pm 3.9	18.4 \pm 4.5	21.8 \pm 1.8	21.1 \pm 1.9	26.7 \pm 2.8	21.6 \pm 3.9*	27.5 \pm 2.3	31.8 \pm 2.7
6	80	6.4 \pm 0.9	5.4 \pm 2.4	6.2 \pm 0.4	5.6 \pm 1.3	5.9 \pm 1.5	4.5 \pm 1.3	5.0 \pm 0.4	5.4 \pm 0.5
7	64	10.0 \pm 5.2	6.7 \pm 1.3	9.6 \pm 1.7	8.0 \pm 1.4	9.7 \pm 4.4	10.2 \pm 4.2	13.8 \pm 0.4	14.5 \pm 0.4
8	56.5	33.2 \pm 8.3	37.3 \pm 3.6	29.7 \pm 6.6	36.4 \pm 6.7	23.9 \pm 2.9	31.3 \pm 3.4*	29.6 \pm 0.8	25.3 \pm 1.5*
9	44.8	8.0 \pm 2.7	6.6 \pm 2.5	7.8 \pm 2.7	6.0 \pm 1.4	9.4 \pm 3.8	9.5 \pm 3.8	6.6 \pm 2.0	5.3 \pm 2.0
10	36	6.1 \pm 1.6	5.8 \pm 1.4	6.4 \pm 2.4	6.8 \pm 1.3	5.3 \pm 1.4	8.6 \pm 5.0	3.8 \pm 0.6	3.8 \pm 0.7
11	28	5.1 \pm 3.4	5.4 \pm 2.1	3.8 \pm 2.9	3.5 \pm 2.9	2.2 \pm 1.2	4.1 \pm 2.6	3.0 \pm 0.1	1.2 \pm 0.4

SPM protein profile. SPM proteins from control (C) and undernourished (U) rat pups at 10, 14, 20 and 34 days of age were separated by electrophoresis on SDS-7.5% polyacrylamide disc gels. The protein peaks correspond to those indicated on Figure 8. The apparent molecular weights of the SPM proteins were determined by comparison to protein standards of known molecular weights on similar gels. The values represent the mean \pm the standard deviation of the relative peak areas obtained from densitometric tracings (n=6). An * represents that the difference between the control and undernourished animals is significant at $p < 0.05$.

[³H]-LEU INCORPORATION INTO SPM PROTEINS

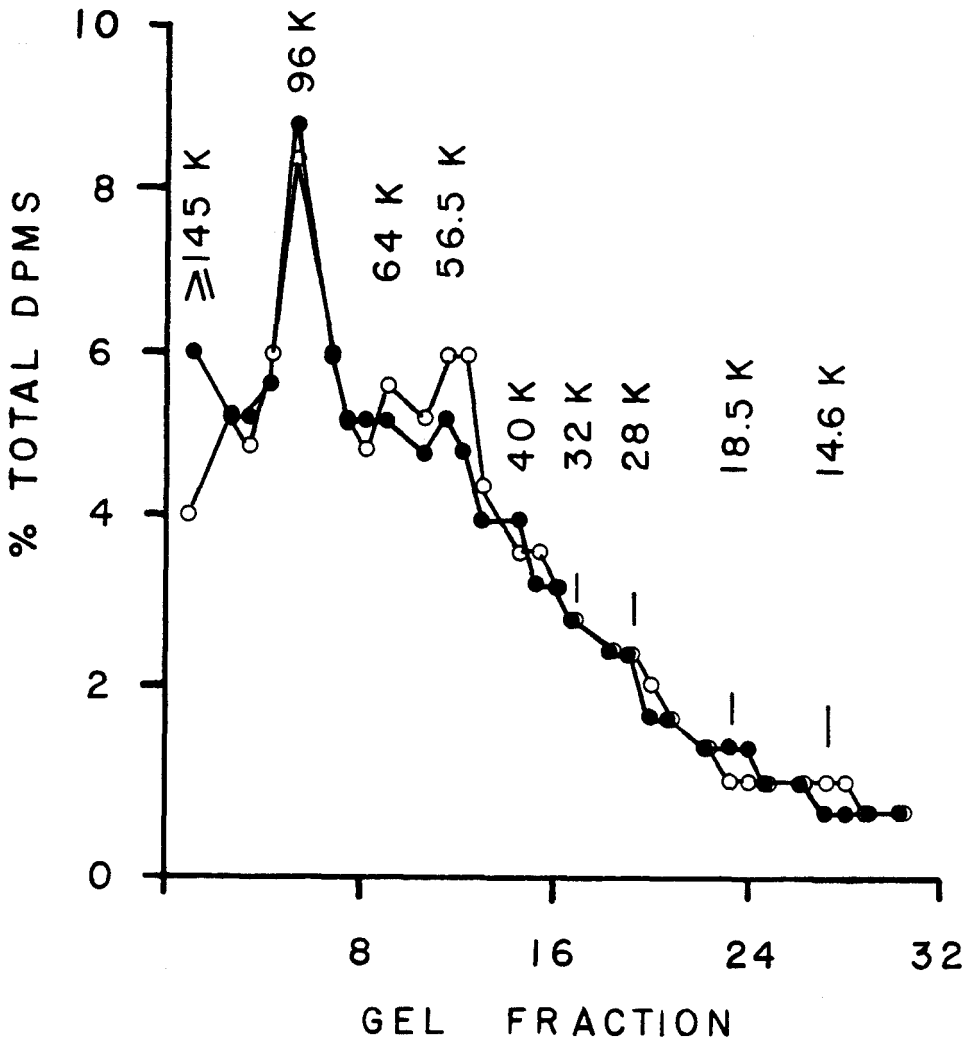


Figure 9. [³H]-Leucine (Leu) incorporation into SPM proteins. Control (●) and undernourished (○) rat pups at 10 days of age were injected intracranially with 100 μCi [³H]-leucine in 10 μl saline. Animals were sacrificed 16 hours later and SPMs were isolated. Individual SPM protein samples were electrophoresed on SDS-7.5% polyacrylamide disc gels. The gels were sliced into 2 mm slices and the radioactivity in each counted by liquid scintillation. Values are representative of two separate experiments each done in triplicates.

undernourished animals at each observed age (10, 14, 20 and 34 days) [Figure 10]. A large proportion of the [^3H]-radioactivity (DPM) was found associated with a protein with an apparent molecular weight of 96,000. There was a statistically significant increased relative incorporation in this 96,000 daltons protein band in the SPMs of the undernourished animals at 14, 20 and 34 days. There was a trend towards an increased incorporation of [^3H]-leucine into an SPM protein band with an apparent molecular weight of 64,000 daltons in the undernourished animals at 10 days of age. In contrast there was a trend towards a decreased relative incorporation into this 64,000 molecular weight protein in the undernourished animals at 34 days of age. There was also a statistically significant increased incorporation into a broad band region having an apparent molecular weight of approximately 56,500 in the undernourished pups at 10 and 14 days. This corresponds to the earlier finding that this band also represents a larger relative proportion of the SPM proteins (as determined by its dye binding capacity) in the undernourished pups than in the controls at 10 and 14 days of age.

SPM FUCOSYLATED GLYCOPROTEINS

Control and undernourished rat pups were injected intracranially with [^3H]-fucose (30 μCi) or [^{14}C]-fucose (9.6 μCi) at selected ages (13, 19 and 33 days). The animals were sacrificed 18 hours later, and the SPMs were isolated as

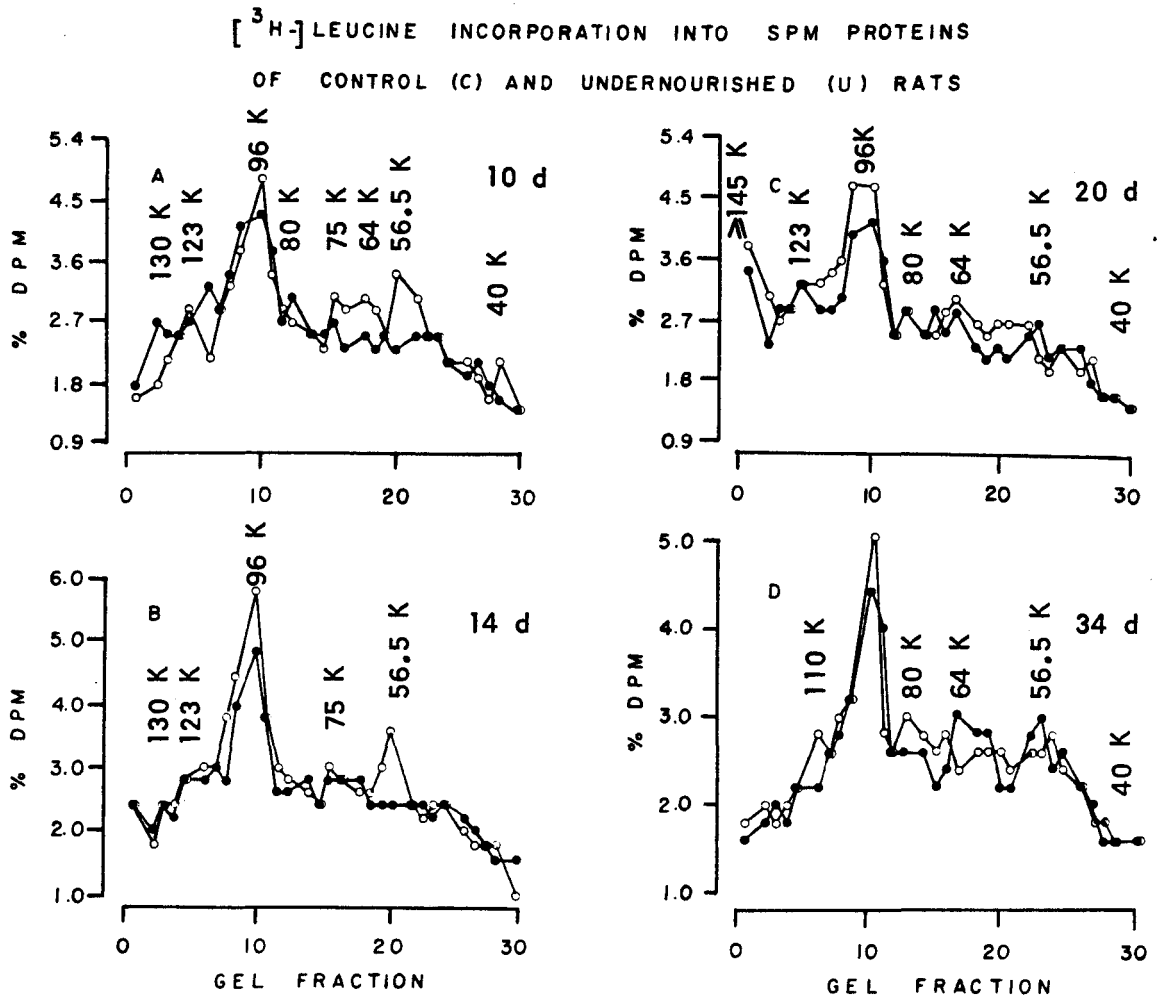


Figure 10. [³H]-Leucine incorporation into SPM proteins. Control (●) and undernourished (○) rat pups at A) 10, B) 14, C) 20 and D) 34 days of age were injected intracranially with 100 μCi [³H]-leucine in 10 μl saline. Animals were sacrificed 16 hours later and the SPMs were isolated. Individual SPM protein samples were electrophoresed on SDS-7.5% polyacrylamide disc gels. The top half of the gels were sliced in 1 mm slices and the bottom half of the gels were sliced in 2 mm slices. The radioactivity in each slice was determined by liquid scintillation. Each graph represents only the top half of the gels containing proteins with molecular weights ≥ 40,000. Values are representative of two separate experiments each done in triplicate.

described previously. The SPM proteins were electrophoresed on 7.5% polyacrylamide disc gels and stained with Fast-Green, as also described previously. Individual protein bands were excised, and radioactivity was determined by liquid scintillation counting. The major fucosylated SPM glycoproteins had apparent molecular weights of 110,000, 96,000, 86,000, 80,000, 64,000, 56,500, 36,000 and 28,000 and corresponded to gel slice numbers 4, 6, 7, 8, 10, 12, 15 and 17, respectively [Figure 11].

Several developmentally-related trends in the relative distribution of fucose-associated radioactivity were noted in control rats, i.e., 1) a developmentally related increase (8.6 to 9.8%) in the band with apparent molecular weight of 96,000, 2) a decrease (between 14 to 20 days) in the band with an apparent molecular weight of 86,000, and 3) an increase (between 14 and 20 days) in the band that corresponds to tubulin (apparent molecular weight of 56,500).

An [³H]-labeled control sample was combined with a [¹⁴C]-labeled sample from an undernourished animal prior to electrophoresis. The order of the assigned isotopes was reversed in approximately half of the samples. The dual-label studies demonstrated several significant differences between the relative distribution of radioactivity among fucosylated SPM glycoproteins from control and undernourished pups. The SPMs from the 14-day-old undernourished pups [Figure 12] have a significantly higher proportion of radioactivity in the SPM

FUCOSYLATED SPM GLYCOPROTEINS

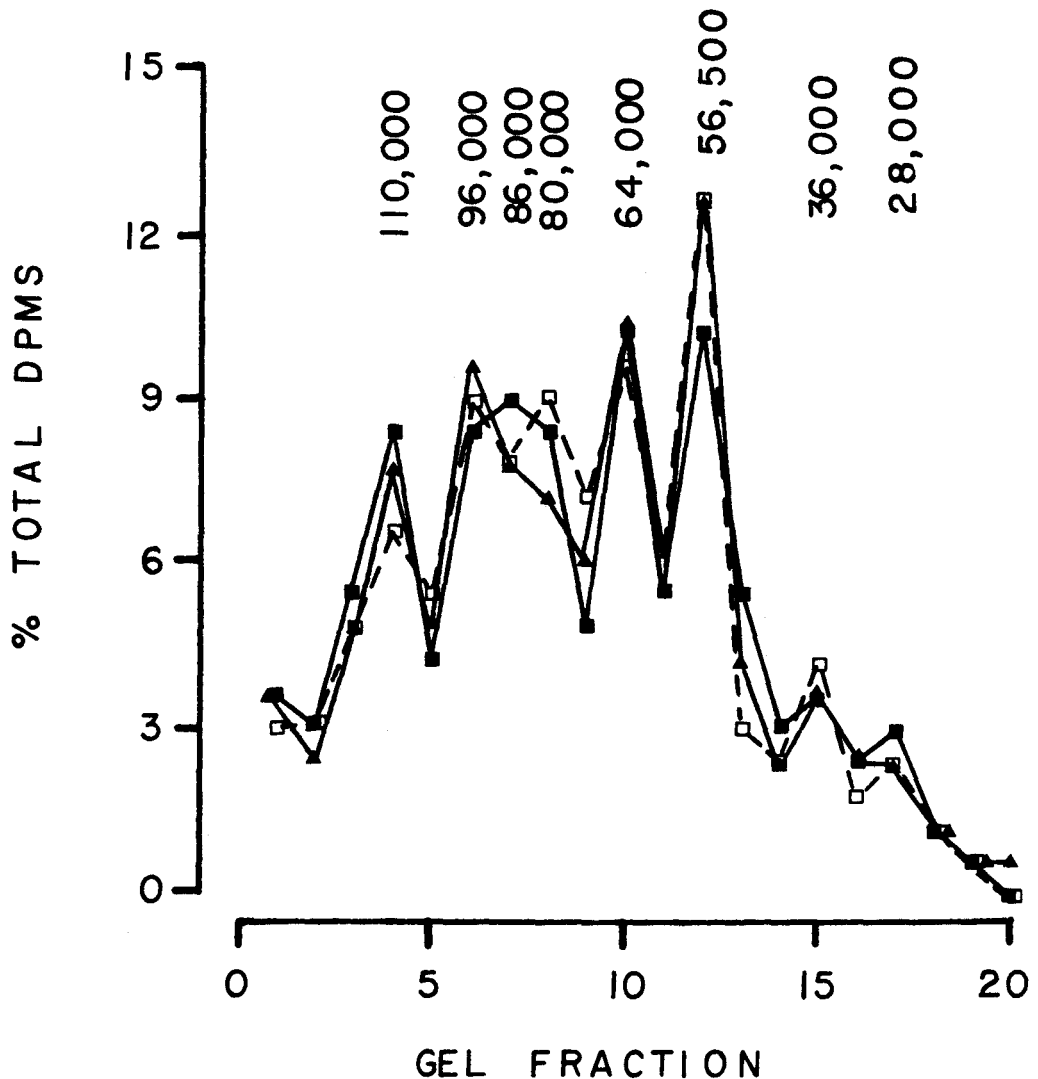


Figure 11. Developmental alteration of fucosylated SPM glycoproteins. Control animals at 14 (■), 20 (□) and 34 (▲) days of age were injected intracranially with 30 μ Ci [3 H]-fucose. Animals were sacrificed 18 hours later and SPMs were isolated. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide disc gels. The gels were sliced according to particular protein bands and the radioactivity in each counted by liquid scintillation. The apparent molecular weight for each major fucosylated glycoprotein is indicated above each peak. Values represent the mean of 2-3 samples each.

FUCOSYLATED GLYCOPROTEINS IN 14-DAY-OLD PUPS

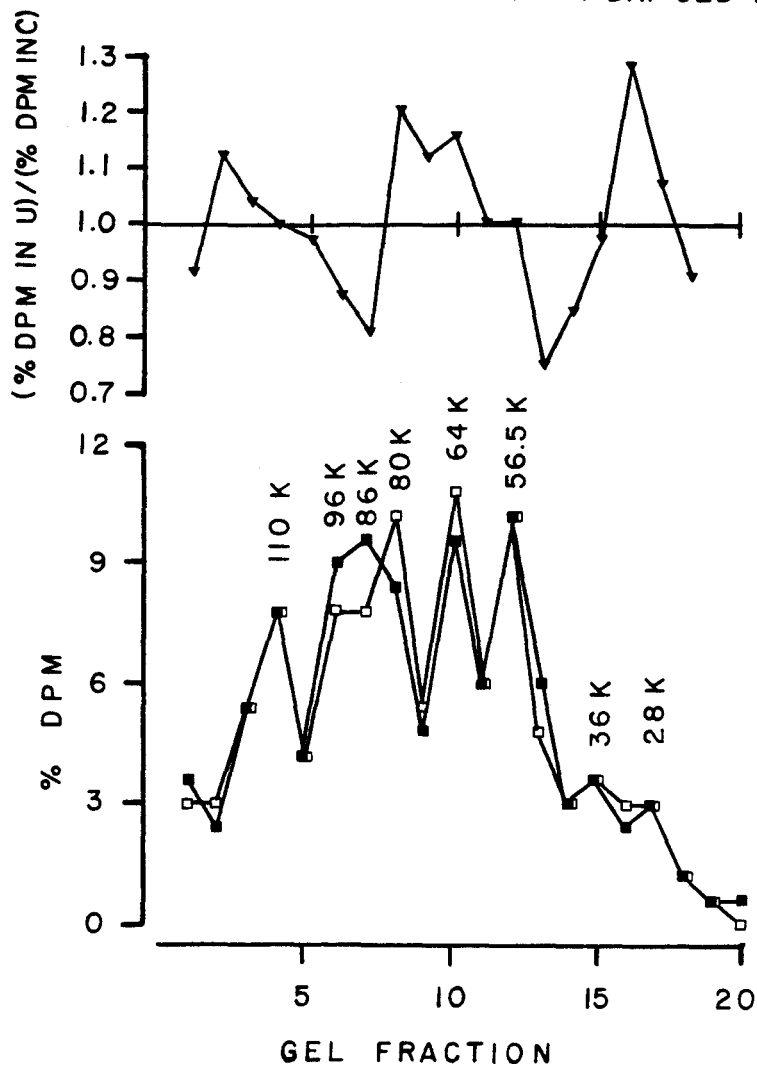


Figure 12. Fucosylated SPM glycoproteins in 14-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 12,400 dpm [³H]-fucose in the control and 4,250 dpm [¹⁴C]-fucose in the undernourished.

band with molecular weight of 64,000 and a significantly decreased proportion in the protein band with molecular weights of 96,000. At 20 days of age [Figure 13], undernourished rats had a decreased proportion of radioactivity in SPM glycoproteins with molecular weights of 80,000, 56,000. At 34 days of age [Figure 14], no consistent differences in incorporation of fucose into SPM glycoproteins were detected between the undernourished and the control pups.

SPM SIALYLATED GLYCOPROTEINS

Sialic acid has been shown to be a constituent of both glycoproteins and glycolipid (ganglioside) components. N-Acetylmannosamine has been shown to be a specific in vivo precursor for sialic acid (Yohe et al., 1980). The incorporation of radiolabeled N-acetylmannosamine into SPMs (combined glycoproteins and gangliosides) of undernourished pups was increased, over that in controls at all ages examined although only the difference at 14 days was statistically significant [Figure 15]. Protein bands corresponding to those taken in the fucosylated glycoprotein study were also cut and counted for radioactivity by liquid scintillation. It appears that N-acetylmannosamine was incorporated into the sialic acid moiety of several SPM glycoproteins that previously were also shown to be fucosylated. However, there was an absence of sialic acid labeling of a chloroform:methanol insoluble fucosylated glycoprotein with a molecular weight of 80,000.

FUCOSYLATED GLYCOPROTEINS IN 20-DAY-OLD PUPS

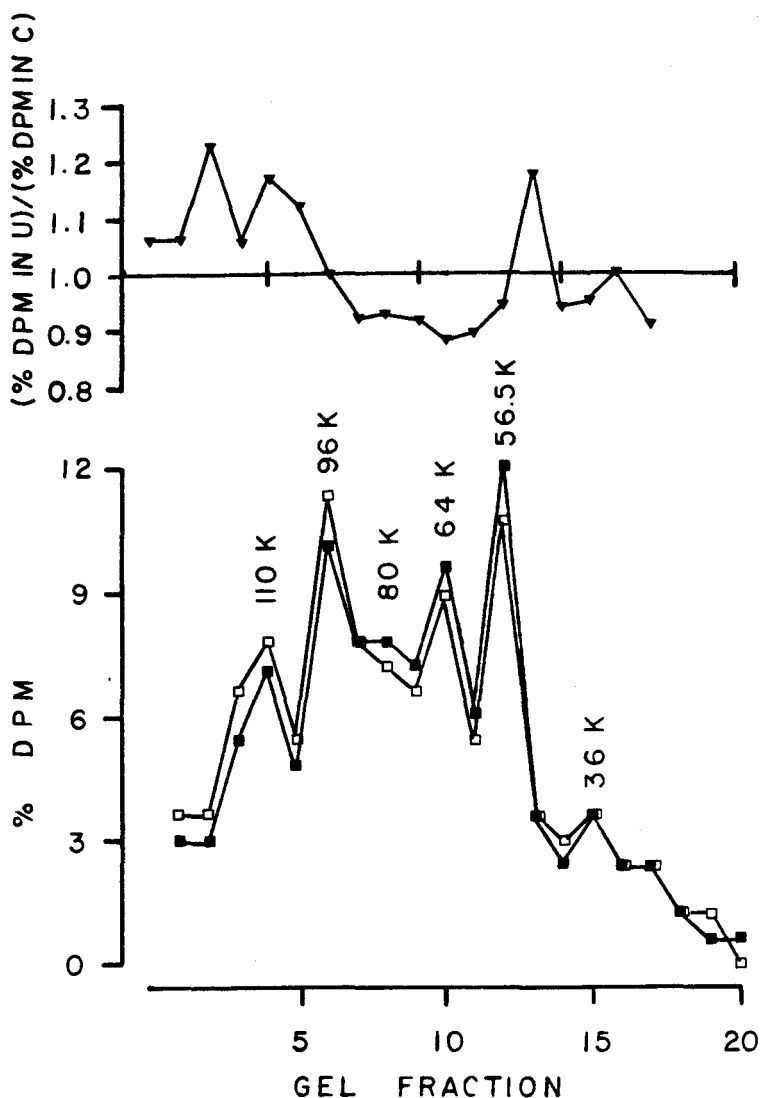


Figure 13. Fucosylated SPM glycoproteins in 20-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 29,350 dpm [³H]-fucose in the control and 3,300 dpm [¹⁴C]-fucose in the undernourished.

FUCOSYLATED GLYCOPROTEINS IN 34-DAY-OLD PUPS

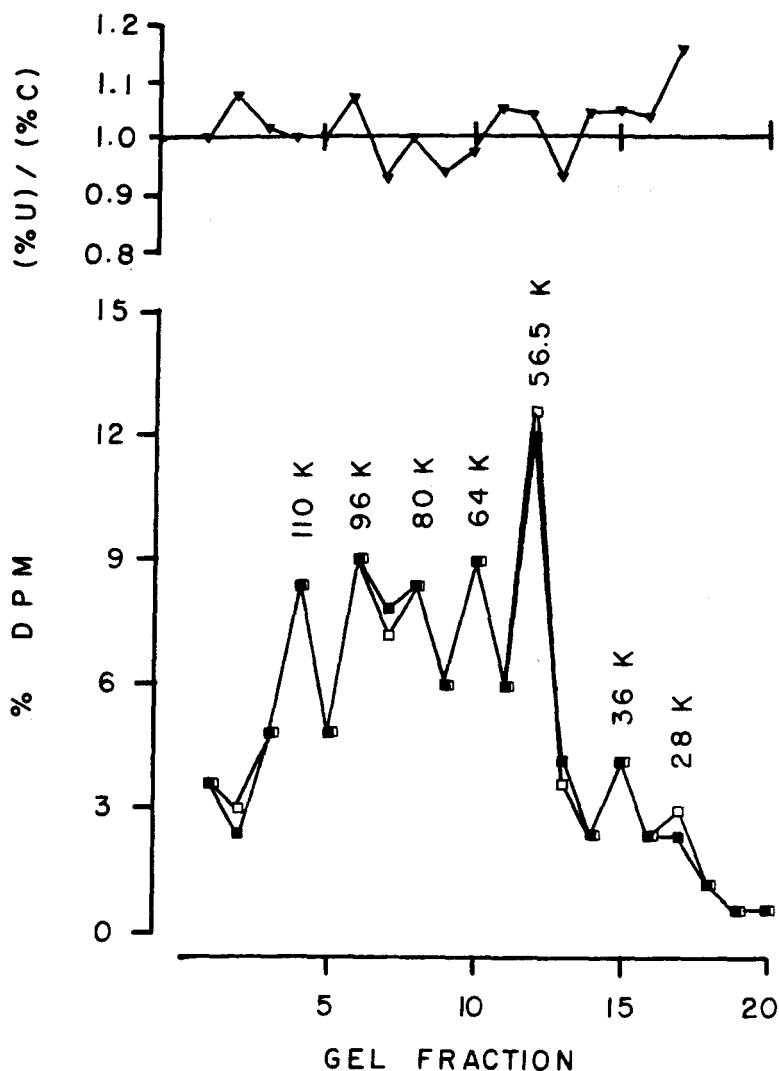


Figure 14. Fucosylated SPM glycoproteins in 34-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 2,500 dpm [^{14}C]-fucose in the control and 13,500 dpm [^3H]-fucose in the undernourished.

INCORPORATION OF N-ACETYLMANNOSAMINE

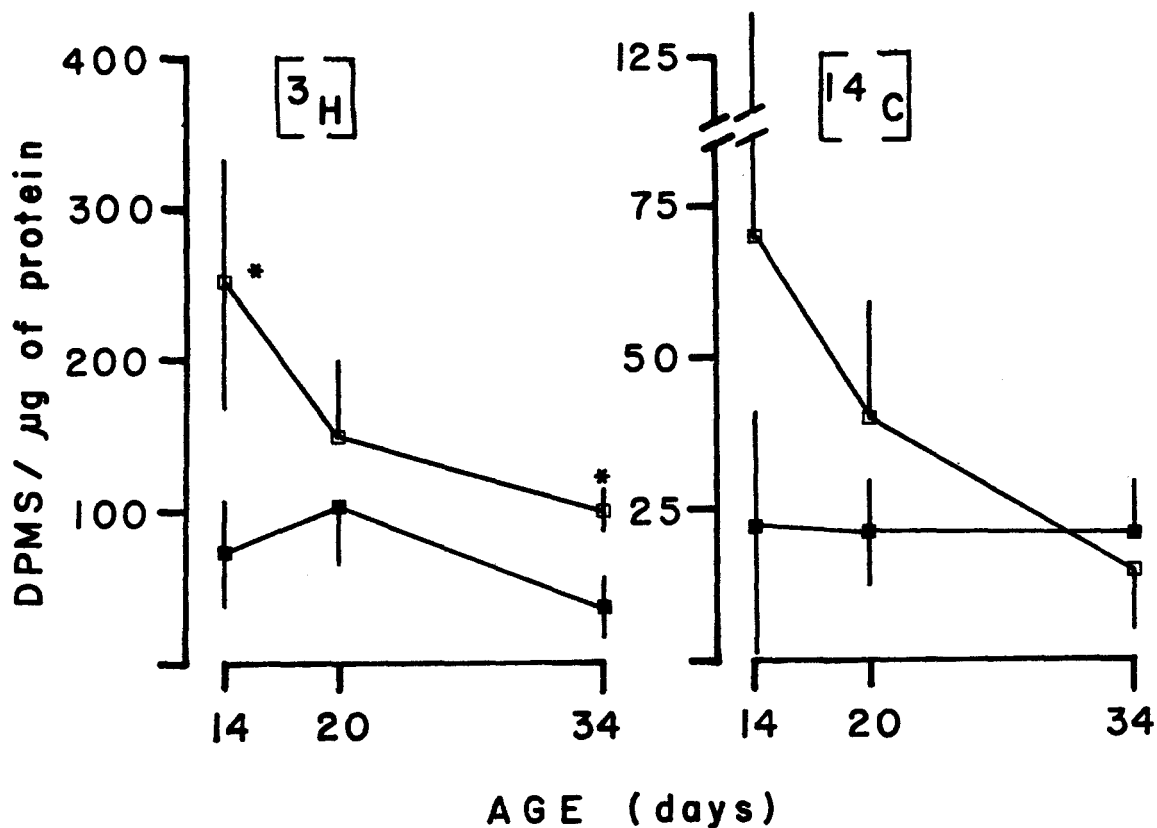


Figure 15. Specific incorporation of radiolabeled N-acetylmannosamine into SPMs of developing control (■) and undernourished (□) rat pups. Values represent the mean \pm standard deviation from two separate experiments (n=8). An * represents the difference to be significant at $p < 0.05$.

In this study, [^3H]- and [^{14}C]-sialic acid labeled samples from control and undernourished animals were combined as described for the fucose study. Several important differences were observed in the relative distribution of radioactivity among sialylated SPM glycoproteins from control and undernourished rats at 14 days of age [Figure 16]. The undernourished rats consistently had an increase in the proportion of radioactivity in sialylated SPM glycoproteins of molecular weights: 110,000, 86,000, 64,000, 40,000 and 36,000, and a decreased proportion in the sialylated SPM glycoproteins with molecular weight of 56,500. At 20 days of age, there was a consistent increase in the proportion of sialylated radioactivity in SPM glycoproteins with apparent molecular weights of > 140,000, 110,000, 86,000, 64,000, 36,000 and 28,000 in the undernourished pups compared to controls [Figure 17]. At 34 days of age, there were very few significant differences between the sialylated SPM glycoproteins of the control and undernourished pups. However, there was a consistent increase in the proportion of radioactivity among sialylated glycoproteins with molecular weight of > 140,000 in the undernourished rats and a consistent decrease in the proportion of radioactivity associated with molecular weight of 96,000 compared to controls [Figure 18].

SIALYLATED GLYCOPROTEINS IN 14-DAY-OLD PUPS

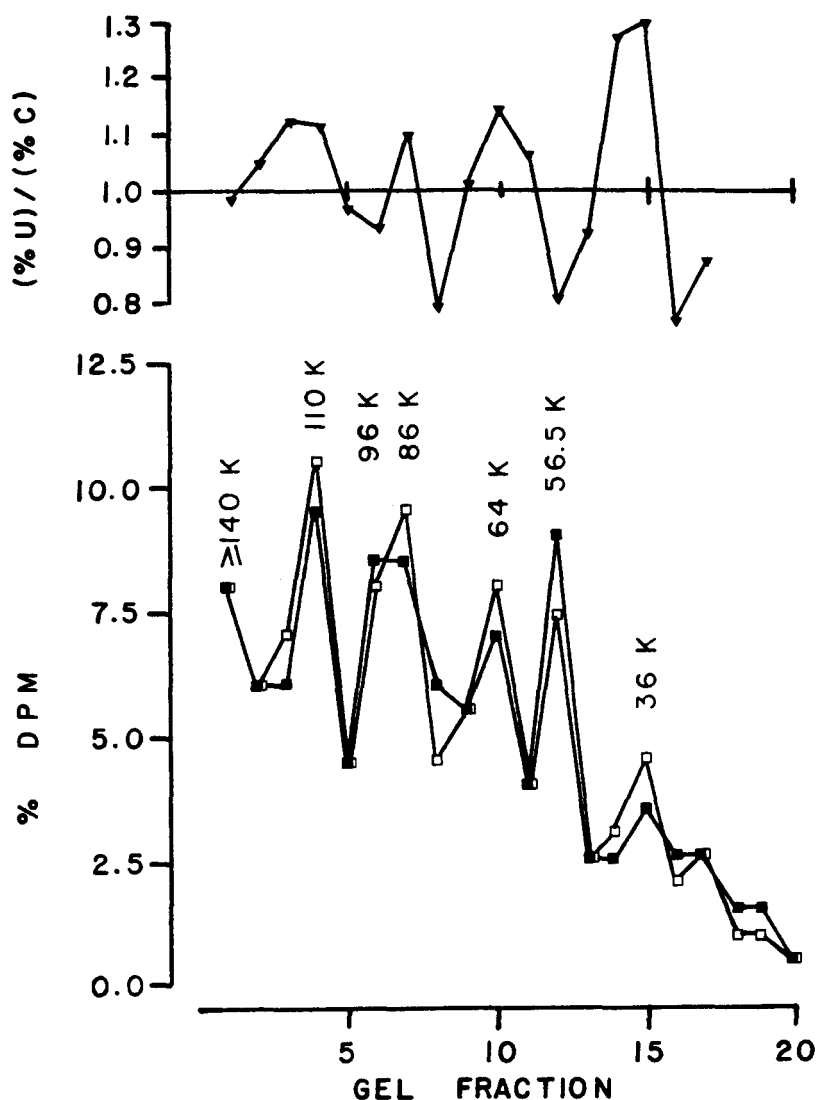


Figure 16. Sialylated SPM glycoproteins in 14-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 1600 dpm [^3H]-N-acetylmannosamine in the undernourished and 700 dpm [^{14}C]-N-acetylmannosamine in the control.

SIALYLATED GLYCOPROTEINS IN 20-DAY-OLD PUPS

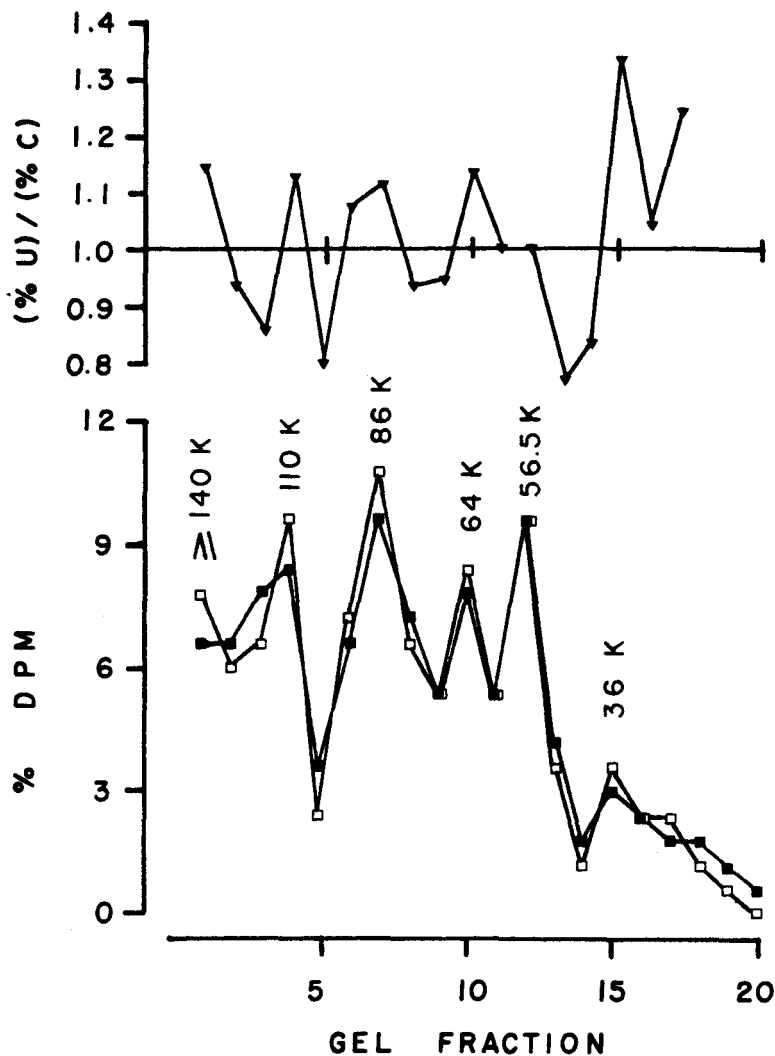


Figure 17. Sialylated SPM glycoproteins in 20-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 2200 dpm [3 H]-N-acetylmannosamine in the control and 700 dpm [14 C]-N-acetylmannosamine in the undernourished.

SIALYLATED GLYCOPROTEINS IN 34-DAY-OLD PUPS

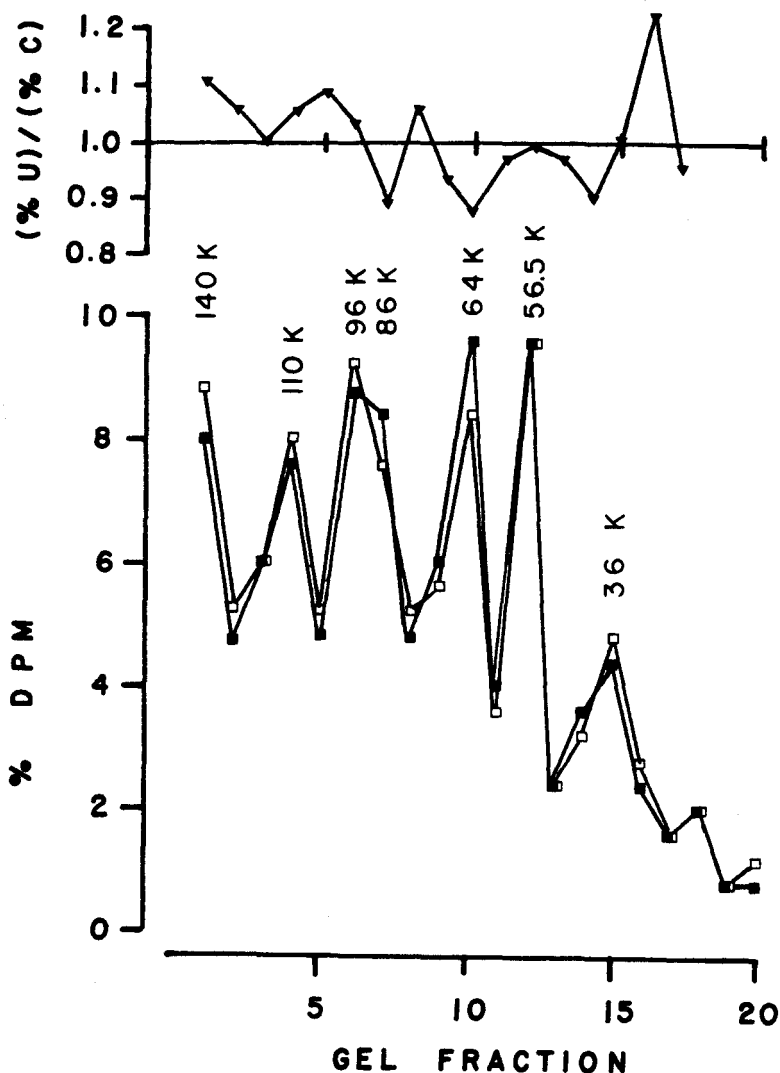


Figure 18. Sialylated SPM glycoproteins in 34-day-old control (■) and undernourished (□) rats. The lower graph shows the results of a single control-undernourished pair that is representative of four such pairs. SPM protein/glycoprotein samples were electrophoresed on SDS-7.5% polyacrylamide cylindrical disc gels. The gels were sliced according to particular protein bands (as shown on Figure 11) and the radioactivity in each counted by liquid scintillation. The top graph shows these same results expressed as the ratio: (%dpm in the undernourished)/(%dpm in the control) for each gel slice. The recovery of isotopes in this pair was 6200 dpm [^3H]-N-acetylmannosamine in the undernourished and 1400 dpm [^{14}C]-N-acetylmannosamine in the control.

SPM GANGLIOSIDES

To assess any qualitative alteration in particular ganglioside species in undernourished offspring, whole brain and SPM gangliosides were separated by thin-layer chromatography as described previously. Ganglioside positions were compared with those of ganglioside standards (GT_1 , GD_{1a} , GM_1 and GM_2). The positions of additional gangliosides were tentatively identified by their relative positions in comparison with those previously published. The following additional ganglioside species were tentatively identified: GQ_{1b} , GT_{1b} , GD_{1b} , GD_3 , and GM_3 . Since gangliosides GD_2 and GT_{1a} did not separate from each other, the data from them were combined. GD_3 (which migrates between GD_{1a} and GM_1 in the solvent system used) appeared as an easily-separated doublet. A slower migrating of the doublet was designated "carbohydrate(CHO)- GD_3 " as such because it gave a slight yellow or brown color when visualized with the resorcinol-HCL spray.

There were no major differences in the proportion of individual ganglioside species between control and undernourished rat pups in either brain or SPM gangliosides at all ages examined (14, 20 and 34 days). There were several small differences. However, they were consistently observed between control and undernourished animals. There was a consistent decrease in GD_{1b} in the whole brain of the undernourished animals at 14 days [Figure 19]. There were

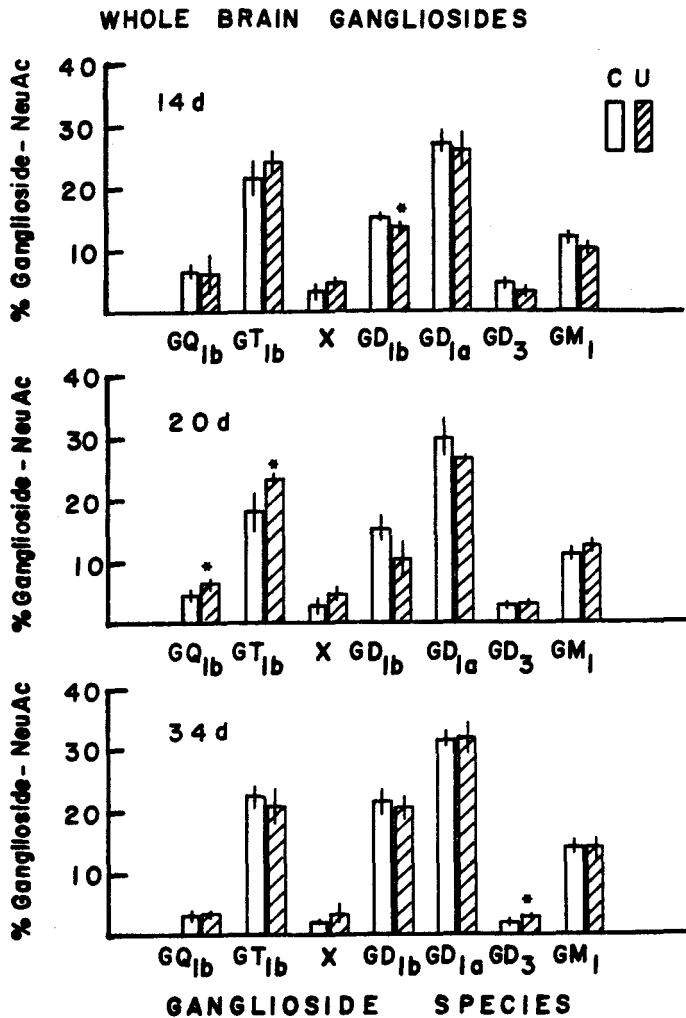


Figure 19. Whole brain ganglioside distribution in control (C) and undernourished (U) rats at 14, 20 and 34 days of age. Aliquots of whole brain gangliosides were spotted on 20cm x 20 cm silica gel G-60 thin layer chromatography (tlc) plates. The plates were developed in a saturated tank containing CHCl_3 :MeOH: 0.25% (w/v) CaCl_2 , 60:35:8 (v/v), Solvent System I. After chromatography, the ganglioside spots were visualized with resorcinol-HCl reagent. The tlc plate was densitometrically scanned at 580 nm using a Kratos Schoeffel SD 3000 densitometer. Individual ganglioside species were quantitated by measuring the peak areas from the densitometric scans. Values represent the mean \pm the standard deviation of the relative distribution (% ganglioside-NeuAc) for each species. An * indicates that the difference between the control and undernourished animals is significant at $p < 0.05$. (The ganglioside species indicated by "X" is unidentified and may be due to lactone formation.) Abbreviations used are tlc and NeuAc for thin layer chromatography and N-acetyl neuraminic acid, respectively.

also consistent increases in GQ_{1b} and GT_{1b} in the whole brain of the undernourished animals at 20 days and of GD_3 in the undernourished at 34 days of age. There was a consistent decrease in GT_{1b} in the SPMs of the undernourished animals at 14 days of age and a consistent increase in GD_{1a} in the SPMs of the undernourished at 34 days [Figure 20]. The relative distribution of the ganglioside species was comparable between whole brain and SPMs with the notable exception of a possible enrichment of GD_{1b} in the SPMs of both control and undernourished rats over whole brain gangliosides.

The effects of the maternal protein deficiency on the incorporation of radiolabeled N-acetylmannosamine into brain and SPM gangliosides was also examined using the dual-label, [3H]-/[^{14}C]-isotopic precursor (N-acetylmannosamine) technique described previously. The SPM gangliosides were separated by thin layer chromatography as in the quantitative ganglioside study. The ganglioside species were visualized using iodine (I_2) vapors and identified according to co-chromatographed ganglioside standards. The ganglioside spots were scraped from the plates, and radioactivity was determined by liquid scintillation counting. There were no statistically significant abnormalities in the relative distribution of radioactivity among SPM gangliosides from 14-, 20- and 34-day-old undernourished rats [Figure 21].

There were several developmentally related trends in the distribution of the radiolabel among SPM gangliosides.

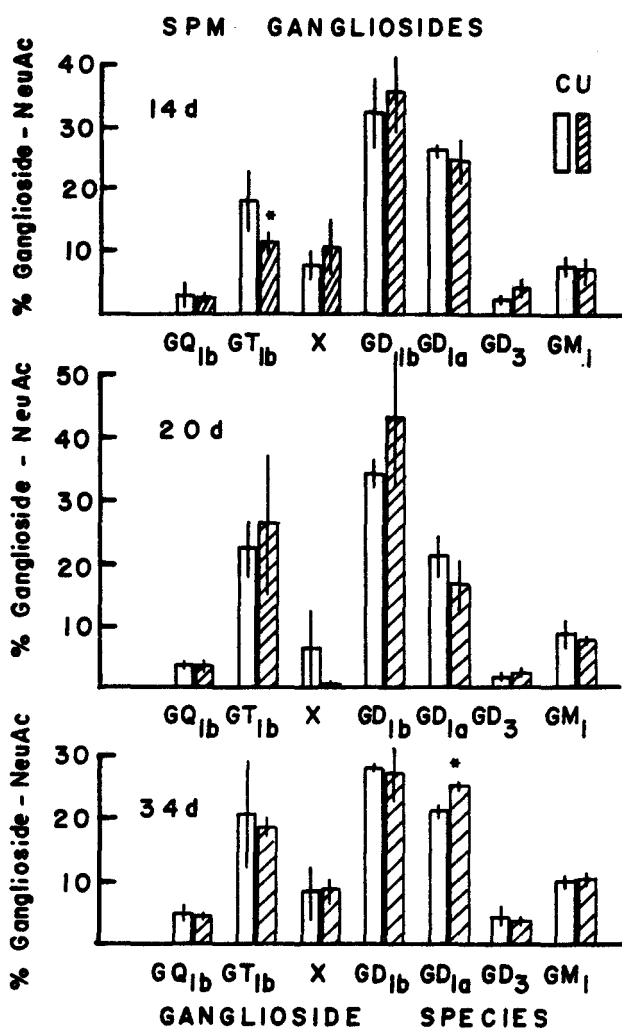


Figure 20. SPM ganglioside distribution in control (C) and undernourished (U) rats at 14, 20 and 34 days of age.² Aliquots of SPM gangliosides were spotted on 20x20 cm² silica gel G-60 thin layer chromatography (tlc) plates. The plates were developed in a saturated tank containing CHCl₃: MeOH: 0.25% (w/v) CaCl₂, 60:35:8 (v/v/v), Solvent System I. After chromatography, the ganglioside spots were visualized with resorcinol-HCl reagent. The tlc plate was densitometrically scanned at 580 nm using a Kratos Schoeffel SD 3000 densitometer. Individual ganglioside species were quantitated by measuring the peak areas from the densitometric scans. Values represent the mean \pm the standard deviation of the relative distribution (% ganglioside-NeuAc) for each species. An * represents that the difference between the control and undernourished animals is significant at $p < 0.05$. (The ganglioside species indicated by "X" is not identified and may be due to lactone formation.) Abbreviations used are tlc and NeuAc for thin layer chromatography and N-acetylneuraminic acid, respectively.

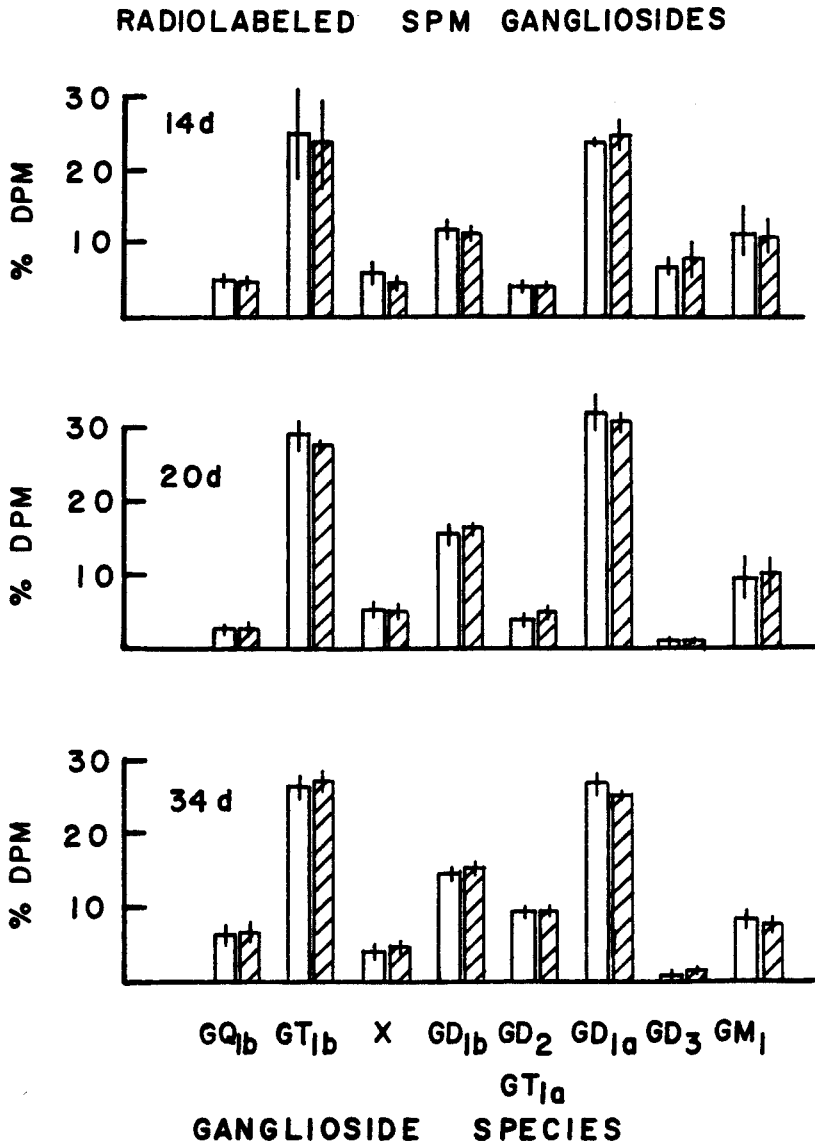


Figure 21. Distribution of radiolabeled N-acetylmannosamine among SPM gangliosides of control (C) and undernourished (U) rats at 14, 20 and 34 days of age. The combined dual-labeled samples were simultaneously extracted, purified and chromatographed (using System I). The gangliosides were visualized using iodine (I₂) vapors. The separated ganglioside species were counted by liquid scintillation. The values represent the mean \pm the standard deviation of the relative percent distribution of the radiolabel in four control-undernourished pairs at each of the ages indicated.

There was a developmentally related increase in the proportion of radiolabel incorporation associated with GD_2 and GT_{1a} in both the control and undernourished animals (from 4% of the total dpms at 14 days to less than 9% at 34 days).

CHAPTER V

DISCUSSION

In agreement with a number of previous reports (Guthrie and Brown, 1968; Adlard et al., 1970; Dobbing and Sands, 1971; Chase, 1972; Balazs and Patel, 1973; Dyson and Jones, 1976; West and Kemper, 1976), neonatal undernutrition caused a decrease in body and brain weights in rat pups. Undernutrition also caused a significant decrease in the brain protein content. This decrease in brain protein content is directly correlated with a decreased brain weight since the brain protein per gram wet weight of tissue in the undernourished pups was equal to that in the age-matched controls.

Undernutrition also caused a decrease in the SPM protein content. The decreased brain weight could not fully account for the deficit in SPM protein, as evidenced by the fact that the SPM protein concentration (μg protein per gram wet weight of brain tissue) was also decreased in the undernourished animals. There are several possible explanations for this decreased amount of SPM protein in the undernourished animals: 1) decreased number of synapses per brain; 2) a decreased size of the synapse although normal number of synapses; or 3) a decreased quantitative isolation of the SPMs from the undernourished animals. These possibilities are discussed in greater detail below.

Undernutrition has been reported to cause a decrease in dendritic arborization. This has been demonstrated with morphological evidence of decreased synaptic density (Dyson and Jones, 1976) and a decreased synapse-to-neuron ratio (Cragg, 1972; Bedi et al., 1980; Thomas et al., 1980). There is also substantial biochemical evidence of decreased synaptosomal protein in undernourished rats (Rabie and LeGrand, 1973; Smart et al., 1974).

In addition to affecting the number of synapses, undernutrition affects the structure of the synapse. Yu and Yu (1977) found abnormalities within the presynaptic terminal of young undernourished animals. Dyson and Jones (1976) found a decrease in the length of the synaptic cleft and also a decreased thickening in both the pre- and postsynaptic membranes in the offspring of dams that were fed a protein-calorie restricted diet during gestation and lactation.

Although there is evidence from the cited studies that neonatal undernutrition can affect both the number and size of synapses, one must also consider that the deficiency of SPM protein may have also resulted from an increased difficulty in the isolation of synaptic membranes from the undernourished animals. A delay in synaptic maturation could possibly affect the density of the synaptosomes and SPMs, since the buoyant density of both have been shown to increase during normal development (Gonatas et al., 1971; Oberjat and Howard, 1977). The synaptosomes were separated from myelin

fragments and mitochondria using Ficoll-sucrose gradients. The gradient density separation was considered to be large enough to allow isolation of synaptosomes with a broad density with minimal contamination, although the exact densities necessary to achieve this in the event of undernutrition was not checked.

The yield of isolated SPM protein reported here is somewhat less than the 1.5-2.0 mg of SPM protein from 1-2 grams of tissue weight reported by other investigators (Cotman and Matthews, 1971; Gurd et al., 1974). There are several possible reasons for this; i.e., 1) whole brain was used in this study versus cerebral cortices which may contain enrichment of synaptic connections; 2) the extent of homogenization has been shown to cause an effect in the yield of SPMs (Cotman, 1974); and 3) Gurd et al. (1974) also stated that the method of removing the supernatants from synaptosomal pellets may cause a loss in synaptosomal material (also Smith, Tonetti and Druse, unpublished observations). However, since this was a comparative study of the effects of undernutrition on the development of the SPMs, and since samples were processed in similar manner, one would expect the trends of the present observations to be reliable.

There was a developmental increase in tissue protein concentration in each of the brain regions studied - prefrontal lobes, hippocampus, cerebral cortex and cerebellum.

The most dramatic rate of increase was observed in the hippocampus from 69 mg of protein/gm wet weight tissue at 14 days of age to 105 mg protein/gm wet weight at 34 days. This may be due to the rapid migration of neurons into the hippocampus between day 14 and 15 in the rat pup (Altman and Das, 1966). Undernutrition could have caused a decrease in the migration of neurons into the hippocampus, thus resulting in fewer synaptic connections. It was demonstrated in these studies that neonatal undernutrition resulted in a significant deficit in SPM protein concentration which was observed only within the hippocampus at 14 and 20 days of age. However, the SPM protein concentration in the hippocampus in the undernourished animals at 34 days of age had returned to normal. At present it is not known if this recovery would have occurred by itself or if it was due directly to the effects of nutritional rehabilitation in the previously undernourished pups.

The purity of the SPMs from both control and undernourished pups at 20 days of age was measured by assaying for marker enzymes specific for desired membranes and possible contaminating membranes. There was a significant enrichment in the specific activity for plasma membrane specific markers in the SPM fraction; $(\text{Na}^+ - \text{K}^+) - \text{ATPase}$ and alkaline phosphatase specific activity were increased in the SPMs from 4.8- and 5.7-fold, respectively, in control animals and 8- and 13-fold, respectively, in undernourished animals.

Although the enrichment of (Na^+-K^+) -ATPase in these SPM preparations over that in whole brain may be somewhat lower than the 5- to 10-fold enrichment reported by several other workers (Cotman and Matthews, 1971; Morgan et al., 1971; Gurd et al., 1974), it should be noted that the animals used in this study were only 20 days of age versus considerably more mature animals in the other studies. Also, the SPM fraction isolated from whole brain may be less enriched in membranes that contain a higher specific activity of (Na^+-K^+) -ATPase than from a particular brain region (i.e., cerebral cortices).

There was also an enrichment (1.5-3 fold) of 5'-nucleotidase in the SPM fraction in both control and undernourished pups over that in whole brain. However, this marker is not as specific for synaptic membranes since it is more enriched within the membrane fraction that migrates above that of the SPMs on the sucrose gradient (Cotman and Matthews, 1971). This marker enzyme may be more specific for vesicular and endoplasmic reticulum membranes. Therefore, its presence within the SPM fraction may be indicative of some degree of contamination by these membranes or indicate a nonspecific distribution. Gurd et al. (1974) advocated the use of several washes of the synaptosomal fraction in 0.32 M sucrose before hypotonic lysis in order to reduce the amount of contamination by microsomal membranes.

There was approximately a 2-fold enrichment of acid phosphatase within the SPM fractions from both control and undernourished pups. This may be an indication of contamination of the SPMs by lysosomal membranes (Cotman and Matthews, 1971). However, it has also been proposed that acid phosphatase may indeed be a component of the SPMs (Verity et al., 1973; Gurd et al., 1974).

Assays of other marker enzymes suggest relatively little or no contamination of the SPMs by either inner (less than 0.2%) or outer mitochondrial membrane. There may, however, be some contamination by myelin membranes as determined by the activity of CNPase in the SPM fraction from control animals (about 0.2%) and undernourished animals (0.4%). This might present a potential problem when doing the comparative studies between control and undernourished animals; however, the degree of contamination was considered to be minor. Also, when the SPM proteins were separated by SDS-polyacrylamide gel electrophoresis, there were no observed protein bands characteristic of myelin membranes. Myelin contamination of the SPM preparations could have become a problem as the quantity of myelin increased at 34 days of age in both the control and undernourished animals. This could possibly be reduced by increasing the volume separating the 10/25% sucrose interface from the 25/32.5% sucrose interface (Smith, Tonetti and Druse, personal observations).

Since (Na^+-K^+) -ATPase is highly enriched in the SPM preparations, it was decided to examine the effects of neonatal undernutrition on the development of ouabain-sensitive (Na^+-K^+) -ATPase in both whole brain and the SPMs. Before measuring the ATPase activity in these membranes, it was necessary to examine several parameters in order to achieve optimal enzyme activity. The effects of several membranes solubilizing detergents were tested on the SPM ATPase activity. It was discovered that concentrations of 0.02% to 0.04% (w/v) SDS would maximally stimulate the ATPase activity. Concentrations of SDS greater than 0.04% caused a decrease in the ATPase activity below that of control (no SDS) levels. This decrease in activity may in part be due to an alteration of the lipid environment of the enzyme or perhaps denaturation of the enzyme. Triton X-100 and deoxycholate were also tested and were able to stimulate ATPase activity above control levels, although the levels of activation at any of the examined concentrations of Triton X-100 or DOC did not stimulate as much as that of 0.02% SDS. It was not determined in these studies if SDS activation was comparable for ATPase in the SPMs from both control and undernourished pups.

There was a large (10-fold) increase in total whole brain (Na^+-K^+) -activated, ouabain-sensitive ATPase during normal neonatal development from 10 to 34 days of age (350 $\mu\text{mole Pi/hr}$ to 3000 $\mu\text{mole Pi/hr}$, respectively). This is in

general agreement with previous reports (Samson and Quinn, 1967; Abdel-Latif et al., 1967). There is, however, considerable controversy concerning the effects of malnutrition/undernutrition on brain (Na^+-K^+) -ATPase activity. Hernandez (1979) demonstrated that both gestational malnourished and large-litter-induced malnutrition caused a significant increase in the specific activity of (Na^+-K^+) -ATPase in the brain cortex of the rat while Reddy and Sastry (1978) demonstrated that protein malnutrition in 3-week-old rats did not affect the specific activity of ouabain-sensitive ATPase. As found in this study, undernutrition caused a significant reduction in (Na^+-K^+) -ATPase content at 14, 20 and 34 days of age. Adlard et al. (1973) reported that 21-day-old undernourished rats had a decreased ability of maintaining Na^+-K^+ gradients in the whole brain.

Subcellular fractions containing nerve endings isolated from whole brain homogenates show the highest specific activity of (Na^+-K^+) -ATPase (Abdel-Latif and Abood, 1964; Hosie, 1965; Albers et al., 1965; DeRobertis, 1967; Morgan et al., 1971). (Na^+-K^+) -ATPase is considered to be an important metabolic pump in maintaining proper Na^+-K^+ ionic concentrations across the synaptic terminal membrane and may be involved in neurotransmitter release.

Kissane and Hawrylewicz have studied the effects of protein malnutrition on the ATPase activity in cerebellar and

cerebral synaptosomes during the perinatal period (Kissane and Hawrylewicz, 1975) and postweaning period (Kissane and Hawrylewicz, 1978) in rat pups. They found that ATPase specific activity in isolated cerebral synaptosomes was significantly reduced in pups protein malnourished either during gestation or lactation. The decrease in ATPase specific activity in cerebral and cerebellar synaptosomes was most pronounced when the dams were subjected to protein malnutrition during both gestation and lactation. The deficit in cerebral synaptosomal ATPase reached normal levels after weaning in both malnourished groups. This is in contrast with the observation that protein malnutrition during lactation alone does not decrease the ATPase specific activity in cerebellar synaptosomes (Kissane and Hawrylewicz, 1975).

The enrichment of (Na^+-K^+) -ATPase within the SPMs suggests a particular localization of this enzyme within the synaptic membranes. It was demonstrated here that there was a significant increase in the specific activity of ATPase in the SPMs from undernourished animals at 20 and 34 days of age. This is in contrast with observations by Kissane and Hawrylewicz (1975, 1978) who showed that neonatal under-nutrition caused a decrease in (Na^+-K^+) -ATPase specific activity in rat brain synaptosomes. The differences between the studies reported here and those of Kissane and Hawrylewicz (1975, 1978) could be due to the fact that

synaptosomes also contain synaptic vesicles and mitochondria in addition to the synaptic plasma membranes. Further evidence which supports an increase in the ATPase in SPMs comes from the observation of an increased incorporation of [³H]-leucine into this SPM protein band [Figure 10]. There are several possible explanations for this higher specific activity of ouabain-sensitive (Na⁺-K⁺)-ATPase in the SPMs of the undernourished animals at 20 and 34 days of age. 1) There could be a conservation of this protein within the synapse, at the expense of proteins that are less important in terms of their involvement in synaptic transmission. 2) Alternatively, there could be increased synthesis of this enzyme to maintain proper intraterminal ionic concentrations because of an increased leakiness in the synaptic membranes as a result of the undernutrition. 3) The isolation of a particular population of synaptosomes/SPMs more enriched in ATPase concentration as a result of undernutrition.

Despite the decreased content of brain and SPM proteins, the incorporation of [³H]-leucine into the proteins of both of these fractions were increased in the 10- to 20-day-old undernourished pups. The higher specific activities in the undernourished pups may reflect an attempt to "catch-up" to the control levels of brain and SPM proteins. An increased incorporation of [³H]-leucine into CNS myelin subfractions has been observed during an attempted "catch-up" period in undernourished rats (Figlewicz et al., 1978).

Synaptic plasma membranes were isolated from rat brain and the proteins separated by SDS-polyacrylamide gel electrophoresis. The protein/polypeptide profile obtained from synaptic plasma membranes differs significantly from that of whole brain, mitochondria, myelin and soluble cytoplasmic proteins. However, the protein/polypeptide profiles from synaptic vesicles and SPM contain many similar bands (Morgan et al., 1973 a,b). This is not surprising since the synaptic vesicle fraction also contains synaptic membrane fragments and the synaptic membrane fraction often contains synaptic vesicles attached to the synaptic membrane.

The SPM protein/polypeptide profile, obtained from adult rat SPMs, contains several major bands (apparent molecular weights 95,000, 52-56,000, and 45,000 daltons) and at least twenty minor bands that are visible after staining with coomassie blue. Although the postsynaptic density protein pattern was not examined in this study, the band with an apparent molecular weight of 52,000 daltons is reportedly the major protein in the post synaptic density (Yen et al., 1977; Kelly and Cotman, 1978). Other proteins have been tentatively identified in the SPM fractions separated by SDS-PAGE: the two subunits of tubulin at 54,000 and 56,000 daltons; actin at 45,000 (Blitz and Fine, 1974); troponin at 37,000 and 24,000 (Mahendran and Berl, 1977); and $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ at 95,000 (Morgan et al., 1973; Mahler, 1977). Protein kinase activity has been detected in

the SPMs and has been identified with the SDS-PAGE protein molecular weights of 40,000 for the catalytic subunit and 48,000 for the regulatory subunit (Miyamoto et al., 1973). Great care should be taken, however, in the identification of particular SPM proteins (at least those separated by SDS-PAGE in one dimension). Each protein band may contain heterogeneous populations of proteins and also certain proteins can be broken down into several subunits by the SDS treatment.

There was a developmental alteration (from 10 to 34 days) in the profile of SPM protein/polypeptides separated by SDS-PAGE on 7.5% polyacrylamide gels [Table VI] that has also been shown by others (Kelly and Cotman, 1981). Although the SPM protein/polypeptide pattern as observed by using Fast-Green protein dye and that obtained by Kelly and Cotman (1981) using coomassie blue are quite similar. Any differences between these two studies may be due to: 1) differences in the dye-binding capacity for the protein/polypeptides between the two stains; 2) differences in the concentrations of polyacrylamide gels leading to better resolution of certain bands; or 3) the method used in determining the developmental alterations. In the study reported here, the relative proportion of the total dye-binding capacity of all SPM proteins was measured, whereas Kelly and Cotman (1981) measured increases or decreases in the staining intensity for particular proteins.

In this study it was reported that a major protein (as determined by its proportion to the total dye-binding capacity) had an apparent molecular weight of 56,500 [Table VI]. In the SPMs from the undernourished animals at 10-20 days of age, this protein band was greater in proportion than in age-matched controls. This may indicate a conservation of the basal cytoskeletal structure of the synapse in the event of undernutrition.

The higher specific activity of ouabain-sensitive ($\text{Na}^+ - \text{K}^+$)-ATPase in the SPMs of the undernourished pups at 20 and 34 days was consistent with the higher proportion of this band in the SPM protein profile of 34-day-old undernourished animals and with the increased incorporation of [^3H]-leucine into this SPM band [Figure 9c, 9d]. There was very little incorporation (less than 20% of the total dpms) into any SPM proteins with apparent molecular weights below 40,000. This is consistent with observations that most of the SPM proteins stained with Fast-Green had molecular weights at or above 40,000. There was very little significant difference in the incorporation of [^3H]-leucine into any particular SPM protein between the control and undernourished pups at any of the observed ages. The only significant difference was an increased incorporation of [^3H]-leucine by the undernourished animals into SPM protein with an apparent molecular weight of 96,000.

Using a dual-label isotope technique as previously described, a [^{14}C]-fucose labeled SPM sample from an undernourished animal was combined with a [^3H]-fucose labeled SPM sample from an age-matched control (or vice versa). The dual-label isotope technique was chosen in order to eliminate any variances between separation techniques; it was hoped that even minor abnormalities in the glycoprotein composition between undernourished and controls would be detected. It was shown in these studies that there were only transient abnormalities in the fucosylation of the SPM glycoproteins in the offspring of the protein deficient rats. Abnormalities in the relative distribution of radioactivity among fucosylated SPM glycoproteins were only seen at 14 and 20 days. By 34 days of age, the distribution in the undernourished animals had returned to a near-normal pattern.

In dual-label [^3H]/[^{14}C]-sialylated SPM glycoprotein study, it was noticed that the distribution of radiolabeled N-acetylmannosamine into SPM glycoproteins was very similar to the distribution of the labeled fucose. The exception to this statement is that there was no incorporation of radiolabeled N-acetylmannosamine into an SPM protein/glycoprotein band with an apparent molecular weight of 80,000. Any observed abnormalities in the relative distribution of sialylated glycoproteins (significant only at 14 days of age), were transient. The distribution was generally normal at later ages. Since this return to normal

distribution of SPM glycoproteins occurs after weaning, when the undernourished pups were given a chance for nutritional rehabilitation, it is not known if the abnormalities were due to a delayed maturation process (which would eventually reach normal values) or was totally dependent upon the period of nutritional rehabilitation. Since only a few of the many SPM proteins and glycoproteins have been identified, it is difficult to state how the functional significance of these minor alterations due to undernutrition might affect proper neural connectivity.

The effects of neonatal undernutrition on brain and SPM gangliosides were examined both quantitatively and qualitatively. The reported findings indicate that there is an enrichment in the proportion of GD_{1b} in the SPMs from both control and undernourished animals over that in whole brain gangliosides. There were no major developmental alterations in the distribution in either whole brain or SPM gangliosides in the controls. There were only minor abnormalities in the distribution in certain gangliosides between the control and undernourished in either whole brain or SPMs. This is in general agreement with Merat and Dickerson (1974) who showed that malnutrition did not seem to cause any change in the normal ganglioside distribution patterns among the several brain regions examined. It may be that whole brain SPMs mask any abnormalities in the NANA content or ganglioside

patterns that may be present in specialized brain regions where particular groups of synapses may be more affected.

SPM gangliosides were studied metabolically by the incorporation of radiolabeled N-acetylmannosamine. There were only minor abnormalities within the distribution of the radiolabels in the SPM gangliosides between the control and undernourished pups at the ages examined (14, 20 and 34 days). Therefore, it may be concluded that neonatal undernutrition in the rats does not cause any significant abnormalities in either the content or composition of whole brain or SPM gangliosides and that any abnormalities seen may only be transient at most.

It has been suggested here from studies on the ouabain-sensitive (Na^+ - K^+)-ATPase content and concentration in SPMs isolated from undernourished rat pups that neonatal undernutrition may affect an altered synaptic function. Indeed, it was demonstrated by Stoch and Smythe (1967) that children who had been previously malnourished exhibited several E.E.G. abnormalities.

In addition to finding a reduced amount of SPM proteins and abnormalities within certain SPM proteins, the studies presented here demonstrate small and transient abnormalities within SPM gangliosides and glycoproteins in the offspring of protein deficient rats. Although several SPM enzymes have been reported to be glycoprotein in character (Salvaterra et al., 1977), it is presently not

known if or how these small abnormalities might affect neurological function. It has generally been considered that SPM glycoproteins are important in interneuronal recognition, adhesion and synaptic connectivity (Brunngraber, 1968; Barondes, 1970; Mahler, 1979).

One aim of this dissertation was to examine how maternal protein deficiency might affect the SPM gangliosides and glycoproteins in the developing offspring with the possibility of altering normal neuronal synaptic connectivity. Although the observed SPM ganglioside and glycoprotein abnormalities were small and transient, it is currently not known which of these are most crucial during the critical period of synaptogenesis. These results do not preclude the possibility that maternal protein deficiency can result in larger abnormalities of specialized synapses in the offspring. Since the SPMs isolated here presumably came from a heterogeneous population of synapses, differences within a small population of specialized synapses might not be detected.

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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.

September 28, 1982
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