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Metabolism of Three Subfractions of Central Nervous System Myelin in Developing Rat Brain

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METABOLISM OF THREE SUBFRACTIONS OF CENTRAL NERVOUS SYSTEM MYELIN IN DEVELOPING RAT BRAIN

by

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VITA

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Druse-Manteuffel, M. J. and Hofteig, J. H. 1975. Development and Metabolism of Three Subfractions of Myelin in Rats. Neurosci. Abs. 5:792.

Hofteig, J. H. and Druse, M. J. 1976. Metabolism of Three Subfractions of Myelin in Developing Rats. Life Sci. 18:543-552.

Hofteig, J. H., Druse-Manteuffel, M. J., and Collins, M. A. 1976. The Effect of Maternal Alcohol Consumption on CNS Myelination in the Developing Rat. Neurosci. Abs. 6:604.

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Druse, M. J. and Hofteig, J. H. 1977. The Effect of Chronic Maternal Alcohol Consumption on the Development of Central Nervous System Myelin Subfractions in Rat Offspring. Drug. Alc. Depend. 2:421-429.

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LIST OF ABBREVIATIONS

Abbreviation	Identity
3'-AMP	3'-adenosine monophosphate
2',3'-cAMP	2',3'-cyclic adenosine monophosphate
CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
CPM	counts per minute
DPM	disintegrations per minute
EAE	experimental allergic encephalomyelitis
FAS	fetal alcohol syndrome
HSA	human serum albumin
PNS	peripheral nervous system
SDS	sodium dodecyl sulphate
TLC	thin layer chromatography

CHAPTER I

BACKGROUND AND LITERATURE REVIEW

INTRODUCTION

This dissertation reports neurochemical research designed to clarify the metabolism and ontogeny of three density subfractions of whole brain central nervous system (CNS) myelin, isolated and prepared from actively myelinating young rats. The metabolism and ontogeny of CNS brain myelin subfractions were studied by examining the protein and lipid metabolism of three myelin subfractions under a variety of experimental conditions designed to simulate either normal or abnormal development. Abnormal development was simulated by postnatal protein-calorie malnutrition and by two separate periods of maternal ethanol consumption (defined below as "acute" and "chronic" periods of maternal ethanol consumption).

This research is important for a number of reasons. These reasons include the unique, intrinsic properties of CNS myelin, the controversy currently surrounding the true significance of CNS myelin subfractions, and the need to determine the extent of stress-mediated perturbations in CNS myelin subfraction content and synthesis as a consequence of postnatal protein-calorie malnutrition and maternal ethanol consumption. Accordingly, each of these concerns will be discussed in light of what is currently known and the specific goals of the present study.

CNS MYELIN PROPERTIES

There are a number of unique and interesting porperties of myelin, especially CNS myelin, which account for the special attention given to myelination studies by basic scientists and clinicians. A number of significant biochemical, morphological, functional, and clinical aspects set myelin apart as an exceptional membrane system. Biochemically, myelin has a disproportionately high lipid content. Metabolically, it has relative stability which belies the feverish metabolic activity which characterizes the cell bodies, i.e., oligodendroglia in the CNS, Schwann cells in the peripheral nervous system (PNS), which synthesize and maintain the myelin sheath and with which continuity is maintained. Morphologically, it is extremely highly ordered while having complex spatial relationships with both the cell bodies which synthesize and maintain it and the nerve axon segments about which it is deposited. Functionally, it is responsible for saltatory nerve conduction. Clinically, it is the focus of serious de- and dysmyelinating diseases and is vulnerable to environmental, traumatic, and immunological insults, especially within the CNS where functional recovery from myelin lesions is less likely than in the PNS. This cursory review of important myelin properties will be documented in greater detail below.

CNS myelin has a very low water content, very high lipid content, and an interesting array of lipids and proteins. As will become clear, these properties of CNS myelin contribute to its membrane-molecular architecture, apparent stability, and functional role. With the exception of PNS myelin, which is somewhat similar, CNS myelin is unique.

By indirect measurements, the water content of myelin has been estimated at 40 percent by weight (Finean, 1960). This was determined from x-ray diffraction studies of drying nerve tissue. The low water content of white matter (72 percent by weight) compared to that of gray matter (82 percent) reflects the fact that by weight white matter is 50 percent myelin. Direct measurement of the water content of myelin is as yet impossible (Norton, 1976).

By dry weight, mammalian CNS myelin is 70 to 85 percent lipid and 15 to 30 percent protein. The three major lipids of CNS myelin by weight distribution are cholesterol (25 to 28 percent), galactosphingolipid (27 to 30 percent), and phospholipid (40 to 45 percent). The major two galactosphingolipids are cerebroside and sulfatide, comprising 23.7 and 7.1 percent, respectively, of total lipid in rat CNS myelin. The major phospholipids are the ethanolamine phosphatides, comprising 16.7 percent of total lipid, (ethanolamine phosphatides in the plasmalogen form comprise 14.1 percent of total lipid), and lecithin, comprising 11.3 percent of total lipid in rat CNS myelin. Minor but important phospholipids include the polyphosphoinositides which remain tightly bound to myelin proteins when lipids are not extracted with acid organic solvents. The brain triphosphoinositide stable to postmortem degradation is probably concentrated in CNS myelin and may be a myelin marker. This brain triphosphoinositide is of interest since in contrast to myelin's relative stability it has the highest turnover rate of any brain phospholipid. Triphosphoinositides represent 4 to 6 percent of total myelin phosphorus, while diphosphoinositides represent 1 to 1.5 percent. Sphingomyelin is a relatively minor constituent by weight, comprising 3.2 percent of total

lipid in rat CNS myelin. Minor galactolipids include mono- and digalactosyl diglycerides, comprising less than 1 percent by weight of total CNS myelin lipid. By molar ratios, the three major lipids, cholesterol, phospholipid, and galactolipid, are in the ratio 4:3:2, respectively, (Norton, 1976). CNS myelin lipids include limited amounts of ganglioside, as reflected by the yield of 40 to 50 µg sialic acid per 100 mg myelin. Although ganglioside is more abundant in other compartments, it is not considered a neuronal contaminant in myelin (Norton and Autilio, 1966; Suzuki, Poduslo, and Poduslo, 1968). The ganglioside, G_7, a unique sialosylgalactosylceramide derived from galactocerebroside, represents a major human CNS myelin ganglioside, and may serve as a myelin marker (Ledeen, Yu, and Eng, 1973; Norton, 1976).

With the possible exception of cardiolipin, which is present in non-myelin CNS compartments but not in myelin, there is no lipid of CNS non-myelin origin or myelin origin which is not also found in the other compartment. While there are no myelin specific lipids *per se*, cerebroside is chiefly associated with myelin. During brain development total cerebroside parallels CNS myelin content (Norton, 1976).

There are features which distinguish CNS myelin lipids from the corresponding lipids of the rest of the CNS. These differences concern primarily the nature of the associated fatty residues. A very high proportion of fatty aldehydes comprise myelin fatty residues. These fatty aldehydes derived from the plasmalogens, phosphatidalethanolamine, and less so phosphatidalserine, account for one-sixth of the total glycerylphosphatide fatty residues and on a mole percent basis for 12 percent of

the total hydrolyzable fatty residues of myelin lipids. The phospholipid fatty acids generally have a high oleic acid (18:1) acid content. and small amount of polyunsaturated fatty acids. Glycosphigolipids (cerebrosides and sulfatides) have unsubstituted and α-hydroxy fatty acids which may be saturated or monounsaturated. In contrast, sphigomyelin has only unsubstituted fatty acids. Sphingolipid acids are mainly long chain (i.e., 22 to 26 carbon atoms) with varying amounts of stearic acid (18:0)(Norton, 1976).

Differences are also apparent for the gangliosides. For example, mature rat and cow CNS myelin gangliosides have a different pattern than do those of human CNS gangliosides. In these non-human mammalian species the major monosialoganglioside, G_{M1}, accounts for 80 to 90 mole percent of total myelin ganglioside. Regional differences in lipid-protein ratios within the CNS for brain myelin are suspected but not well documented (Norton, 1976).

Non-lipid components associated with CNS myelin include a limited number of enzymes, some protein classes, glycoproteins, and insignificant amounts of mucopolysaccharides. There is some controversy regarding whether certain of the enzymes and protein classes associated with CNS myelin are in fact myelin specific.

The enzyme, 2',3'-cyclic nucleotide 3'-phosphohydrolase, (CNP), has enriched specific activity in CNS myelin, is absent from PNS myelin, and is generally accepted as a CNS myelin marker (Norton, 1976). Its activity in CNS (brain and spinal cord) parallels myelination during development (Kurihara and Tsukada, 1968; Olafson, Drummond, and Lee, 1969; Kurihara, Nussbaum, and Mandel, 1970; Braun and Barchi, 1972;

Toews, Horrocks, and King, 1976) and low levels of the enzyme are observed in two murine mutants (quaking and jimpy) which are deficient in myelin (Kurihara et al., 1970; Eto and Suzuki, 1973b; Matthieu, Quarles, Webster, Hogan, and Brady, 1974a; Matthieu, Brady, and Quarles, 1974b). Unfortunately, neither its physiological substrate nor function is known. Another enzyme, cholesterol ester hydrolase, pH optimum 7.2, is myelin associated and one of three such enzymes found in whole brain. It appears to be myelin specific (Eto and Suzuki, 1973a). Its physiological function also is unknown. Knowing that at least two enzymes may be myelin specific suggests that myelin, once thought to be enzymatically inert, may in fact have additional enzymatic activities. Recent evidence has been advanced for the presence of a protein kinase capable of phosphorylating myelin basic protein (Carnegie, Dunkley, Kemp, and Murray, 1974; Miyamoto and Kakiuchi, 1974). There are also reports suggesting the presence of the enzyme, UDP-galactose: ceramide galactosyl transferase, the enzyme catalyzing the final steps in cerebroside synthesis (Costantino-Ceccarini and Suzuki, 1975; Chou and Jungawala, 1976). In addition, peptidase activity has been reported, however, the evidence is equivocal whether this activity is truly undissociable from myelin (Norton, 1976).

Of the proteins associated with mammalian CNS myelin, the major three intrinsic proteins are proteolipid (30 to 50 percent of total myelin protein), basic protein (30 to 35 percent), and Wolfgram protein (a lower percentage). In addition, rodents have an additional smaller basic protein, and all mammals have a class of high molecular weight proteins, a

proteolipid-type protein doublet of lower molecular weight than proteolipid protein, and at least one glycoprotein. The relative amount of high molecular weight protein is species dependent, comprising a higher percentage of total myelin protein in mouse and rat myelin compared to bovine and human myelin. This species variance raises doubts concerning which of the high molecular weight proteins is (are) myelin specific (Norton, 1976).

The resolution of myelin proteins solubilized in sodium dodecyl sulphate (SDS) and electrophoresed in polyacrylamide gels depends upon the molecular sieving characteristics of the gel system employed, polyacrylamide concentration and the degree of polymer cross linking. It also depends upon whether one employs stacking or resolving gels. Depending upon the procedure employed, one may obtain greater resolution for the higher molecular weight or for the lower molecular weight proteins. The proteolipid-like protein called DM20 by its discoverer, Agrawal (Agrawal, Burton, Fishman, Mitchell and Prensky, 1972) may not be well resolved in certain gel systems. Regardless of the particular system employed, the myelin proteins migrate in order of decreasing molecular weight in the rat as follows: high molecular weight proteins including the Wolfgram doublet (Wolfgram, 1966) near the bottom of the high molecular weight protein bands, proteolipid protein, DM20 protein, large basic protein, and small basic protein. A few of the more interesting features of some of these proteins will be discussed below.

Proteolipid, as its name implies, has protein and lipid characteristics. Named after those who discovered and characterized it, the

Folch-Lees proteolipid protein is extractable from whole brain by chloroform-methanol, and once extracted, soluble in either chloroform or chloroform-methanol. Its amino acid content is 40 percent polar, 60 percent nonpolar. The extracted protein is heterogeneous, perhaps due to subunit aggregation. The major proteolipid species has a molecular weight of approximately 24,000 (Folch and Stoffyn, 1972). The so called DM20 proteolipid-like protein is extracted with proteolipid protein from whole brain, has an apparent molecular weight of approximately 20,000, and is enriched in tryptophan, cysteine, and methionine residues. PNS myelin, in contrast to CNS myelin, has little proteolipid protein (Norton, 1976).

CNS myelin glycoprotein may be labelled with fucose, glucosamine, or N-acetylmannosamine. It migrates with high molecular weight proteins during SDS polyacrylamide gel electrophoresis. Its molecular weight appears to decrease with development (Matthieu, Brady, and Quarles, 1975a). Numerous studies indicate that the major glycoprotein in isolated myelin is truly myelin-associated (Quarles, Everly, and Brady, 1972, 1973a, 1973b; Druse, Brady, and Quarles, 1974; Matthieu et al., 1974a; Matthieu et al., 1974b).

Basic protein, the most well characterized CNS myelin protein, is most widely known for its encephalitogenic activity (Eylar, 1972; Kies, Martenson, and Deibler, 1972a). When injected with Freund's adjuvant into an animal it evokes a cellular antibody response resulting in the autoimmune experimental allergic encephalomyelitis (EAE). EAE resembles multiple sclerosis in that it too presents with multifocal inflammatory and demyelinating CNS lesions. Basic protein cannot be ex-

tracted from whole brain by organic solvents but is soluble by action of chloroform-methanol (2:1) upon isolated myelin. It is easily extracted from either myelin or whole brain by dilute acid or salt solution, and upon extraction is water soluble. The complete primary structure of bovine (Eylar, 1972) and human (Carnegie, 1971; Eylar, 1972) basic protein is known. Bovine basic protein has an isoelectric point greater than pH 12, is highly unfolded, and devoid of tertiary structure. With a molecular weight of approximately 18,000, 54 percent of its residues are polar, 46 percent nonpolar. It is devoid of cysteine and has only one tryptophan residue. The additional smaller basic protein found in rodent CNS myelin has 40 less amino acid residues but shares the same N- and C-termini (Martenson, Deibler, Kies, McKneally, Shapira, and Kibler, 1972). P_1, the larger of two PNS myelin basic proteins, is identical to CNS myelin large basic protein. The smaller PNS myelin basic protein, P_2, is distinct from the smaller basic protein associated with CNS myelin of rodent origin (Brostoff, Karkhanis, Carlo, Reuter, and Eylar, 1975a).

Wolfgram protein, discovered and characterized by Wolfgram (1966), is a class of acid soluble proteolipid proteins appearing predominantly as a doublet or triplet separable by SDS polyacrylamide gel electrophoresis at the lower end of the high molecular weight proteins. Wolfgram protein is insoluble in water and neutral chloroform-methanol. Fifty-three percent of its residues are polar, 47 percent nonpolar (Norton, 1976). Waehneldt and Neuhoff (1974) noted that the appearance of Wolfgram protein in rat brain during postnatal development paral-

leled the deposition of basic protein and proteolipid protein, an indication of the close association of Wolfgram protein with myelinogenesis.

Current membrane-molecular models for the ultrastructural arrangement of lipid and protein in CNS myelin have evolved from fundamental studies employing polarized light, x-ray diffraction, and electron microscopy.

Birefringence under polarized light, an indication of long range order, had been observed in myelin as early as the last half of the nineteenth century. In 1913 Gothlin demonstrated lipid- and protein-dependent birefringence, the former being predominant (Schmitt, 1959). PNS polarization studies by Schmitt revealed the lamellar nature of myelin. In their model, similar to contemporary models, lipids are arranged radially and proteins tangentially with respect to the myelinated axon (Schmitt, 1959; Davison and Peters, 1970).

The lamellar character of myelin was further confirmed in x-ray diffraction studies by Schmitt and colleagues (Schmitt and Bear, 1939; Schmitt, Bear, and Palmer, 1941; Schmitt, 1959). They observed radial periodicity of 17 to 18 nm. This was significant because it was compatible with the dimensions requisite for two bimolecular lipid layers and associated protein coats in the simple Danielli and Davson (1935) membrane model. Further clarification of this model came from low angle x-ray diffraction studies by Finean (1953a, 1953b, 1960, 1961, 1965, 1969) and Finean, Hawthorne, and Patterson (1957). Electron density mappings from this work showed three peaks and two troughs with a repeat of 18 nm. Protein and lipid polar heads account for the electron dense

peaks while lipid hydrocarbon tails account for the troughs. The 8 nm repeat distance between peaks is compatible with a single bimolecular lipid layer with protein coating on either side. These data were interpreted to represent a repeating unit of protein-lipid-protein-lipid-protein. The model emerging so far is of two bimolecular lipid layers each with its own external (i.e., extracellular) protein coat and internal (i.e., intracellular) protein coats which have fused by close apposition of the internal (i.e., intracellular) protein coats of the two bimolecular lipid layers.

The early conclusions based on polarized light and x-ray diffraction studies of myelin, chiefly PNS myelin, have been confirmed by electron microscopy studies of PNS and CNS myelin (Sjostrand, 1950; Fernandez-Moran, 1950; Maturana, 1960; Peters, 1960a, 1960b, 1961a, 1961b, 1962, 1964a, 1964b, 1966). Myelin electron photomicrographs present alternating dark and less dark lines separated by unstained zones. The osmophilic lines represent protein layers, the unstained region represents the lipid hydrocarbon chains. The dark and less dark lines are referred to as the major dense line and the intraperiod line, respectively. The intraperiod line represents the protein closely apposed to the outer (i.e., extracellular) surface of the original (oligo or Schwann) cell plasma membrane. The major dense line represents the fusion of the inner (i.e., intracellular) protein layer from each of the two apposed (oligo or Schwann) cell plasma membranes. That the intraperiod line represents the outer surface of a single plasma cell membrane is confirmed from electron photomicrographs of PNS myelin swollen in hypotonic so-

lution; only at this line does myelin normally split, i.e., the extracellular space between adjacent myelin lamellae widens. The major dense line represents the fusion of protein coats within a single lamella (Robertson, 1966). More recent techniques now show the intraperiod line as two lines, indicating that extracellular outer sides of each unit membrane are not fused (Norton, 1976). Electron microscopy determined periodicity reflects the shrinkage occurring during tissue preparation. The current techniques for electron microscopy tissue preparation with in vivo fixation now show PNS myelin periodicity of 11.9 nm and CNS myelin periodicity of 10.7 nm (Finean, 1961; Karlsson, 1966; Norton, 1976). The smaller CNS myelin periodicity may reflect the fact that on the average, the extracellular space between lamellae is thinner in the CNS (Davison and Peters, 1970; Norton, 1976).

The most recent membrane-molecular models for CNS myelin have modified the classical picture described above to make it fit more consistently the fluid mosaic model of membrane structure. The details concerning this model refinement are discussed in the recent review by Braun (1977).

The complex morphology of myelin in situ is a consequence of the complicated manner in which myelin is deposited about axons in the CNS by oligodendrocytes and in the PNS by Schwann cells. The manner of deposition is more complex for CNS myelin.

A single oligo cell may send out as many as 30 to 50 tortuous pseudopod-like cytoplasmic processes, each of which is capable of investing a segment of a separate axon with 10 to 15 lamellae of compacted

myelin (Davison and Peters, 1970). Unlike the Schwann cell which may rotate about the single axon segment for which it provides a myelin sheath, it is impossible for a single oligo cell to rotate around multiple axon segments and effectively myelinate each axon segment. Accordingly, from all available electron photomicrographic evidence, it is surmised that each cytoplasmic projection of the oligo cell spirals internally about its respective axon segment independent of all other processes. While the exact mechanism eludes demonstration, there are cogent theories to explain the process subject to the three dimensional constaints present. Is is thought that a single oligo process grows around an axon segment in ameboid-like movement. Upon fully encircling the axon segment once, the opposing tongues of cytoplasm come into very close apposition to form an external and internal mesaxon. Myelin lamellae appear to form by inward spiralling of the internal mesaxon. During and/or following completion of several inward spiral rotations of the internal mesaxon, the cytoplasmic internal surfaces of the extended oligo plasma cell membrane within each lamella fuse as cytoplasm is excluded. The complete exclusion of cytoplasm and fusion of the apposed intracellular cytoplasmic surfaces of the extended oligo plasma cell membrane within each lamellae results in the structure which appears under electron microscopy as the major dense line. When the process is completed, cytoplasm has been effectively excluded from the entire extent of the myelin sheath except for three main regions, viz., the external mesaxon, the internal mesaxon, and pockets of cytoplasm at either longitudinal end of the sheath about a particular axon

segment, i.e., at the paranode. This is in contrast to the PNS in which considerable cytoplasm invests the outer lamella of myelin and is present in pockets throughout the sheath. In longitudinal section, CNS myelin shows a narrow ridge of cytoplasm on the outermost surface of the sheath. This represents the cytoplasmic process which still maintains continuity between the parent oligo cell body and the myelin sheath (Davison and Peters, 1970; Norton, 1976).

The actual process is considerably more complex. While the sheath may indeed grow by inward spiralling of the internal mesaxon, growth in other directions is requisite. The sheath must be capable of growth within each lamella along the longitudinal axis of the axon segment. Furthermore, as the axon diameter increases the myelin sheath must accommodate either by slippage of lamellae or by growth internally of each lamella. Like the PNS, cytoplasmic continuity between cytoplasm within the myelin sheath and the parent oligo cell body is postulated. However, unequivocal demonstration has not been possible, perhaps because of the tortuous growth of oligo processes following myelination in the developing CNS. Like the PNS, each axon is not ensheathed for its whole length, but rather in segments delineated by unmyelinated gaps, the nodes of Ranvier. At the paranodal region, outer concentric myelin lamellae extend out over each other approaching the center of the node. The paranodal cytoplasmic pocket of each lamella is in tight apposition to the surface of the axon, and it is here at the paranode that each lamella appears to open up at the major dense line. Unlike the PNS, at the center of the node of Ranvier there is a gap in which the axon is

bare, there being neither myelin lamellae nor oligo cell cytoplasmic processes abutting against the axon nor a basal lamina (Davison and Peters, 1970; Norton, 1976).

The high lipid content, ultrastructural arrangement of membrane constituents, and complex morphologic relations serve to account for the CNS myelin's chief function, to increase nerve conduction velocity by saltatory conduction. In an unmyelinated axon conduction velocity is limited by local ionic (Na^+, K^+, Ca^{++}) currents in and out of the axon plasma cell membrane as a consequence of alternating resting and active membrane potentials. The high lipid content of myelin confers upon it an electrical resistance 10 to 20 times higher than extracellular salt solutions. Those segments of axon invested by the myelin sheath are effectively "insulated" from local ionic currents. Were the entire length of the axon invested with a myelin sheath no nerve conduction would be possible. However, there are regular interruptions in the myelin sheath at the nodes of Ranvier. At the low resistance region of the node the bioelectrical gradient generates a current which acts through the extracellular salt solutions external to the myelin sheath to activate the axonal membrane distally at the next node of Ranvier. This saltatory (literally, dancing) current from node to node travels six times faster than local ionic current in an unmyelinated axon. Compared to unmyelinated axons of similar diameter, saltatory conduction per unit length of axon requires 1/300th the sodium ion flux and results in a similar or greater reduction in energy requirements. The myelin investment about an axon also lowers its capacitance per unit

length, thereby further increasing conduction velocity. Saltatory conduction results in retarded nerve conduction velocity if the myelinated axon diameter is less than 1 μ (Hodgkin, 1964; Norton, 1976).

The generally relative stability of CNS myelin membrane components belies the rapid synthetic activity during myelinogenesis. CNS myelin, once thought to be a relatively inert membrane system, is now generally regarded as a relatively stable membrane in which only a few enzymatic activities have been demonstrated with certainty. The exact reasons for myelin's relative stability are unknown. Generally its membrane components have a longer half-life than their counterparts in other CNS compartments. In contrast, rapid onset of synthetic activity characterizes the oligo cell during early myelinogenesis.

Work by Smith (1967) demonstrated that lipid precursors are incorporated into CNS myelin in young rats at rates comparable to incorporation into mitochondria. However, the varied half-lives of myelin lipid components are considerably greater than for their counterparts in mitochondria. CNS myelin lipid half-lives were reported as follows: phosphatidylinositol (5 weeks), lecithin (2 months), phosphatidylserine (4 months), and ethanolamine phospholipids, cholesterol, sphingomyelin, cerebrosides, and sulfatides (7 months to 1 year). This is in contrast to mitochondrial lipid half-lives ranging from 11 days (phosphatidylinositol) to 59 days (cerebroside). Half-life calculations are complicated by a certain amount of pool exchange and reutilization of certain metabolites (Norton, 1976). Half-lives of CNS myelin proteins are also greater than for CNS non-myelin proteins. CNS myelin basic

protein half-life in the rat is age dependent. Reported values include 14 to 21 days, 21 days, and 42 to 44 days. Proteolipid protein half-life is comparable to that of basic protein while that of Wolfgram protein is shorter. Proteolipid protein and basic protein are labelled more slowly than Wolfgram protein (Fischer and Morell, 1974; Norton, 1976).

Prior to myelination, the immature brain has relatively large amounts of cholesterol and phospholipids but little cerebroside and negligible enzymatic activity to synthesize cerebroside from UPD-galactose and ceramide. This enzymatic activity in the mouse peaks at 10 to 20 days, correlating well with the peak myelinogenetic activity. In the rat the high level of myelinogenetic activity by oligo cells at 20 days is suggested by rough calculations by Norton (1976). By his estimates the 3.5 mg per day production of myelin represents an output of myelin per oligo cell body equivalent in weight to three times the mass of the oligo cell.

In a more general sense, the time scale for CNS myelination may be related to chronological age, other neurodevelopmental events, and to systemic and regional time gradients of myelination. In the rat CNS, myelination is a postnatal event commencing at 10 to 12 days of age. Between 15 and 30 days of age there is a six-fold increase in myelin from approximately 4 mg at 15 days. Between 30 and 60 days there is a further 2.5-fold increase in myelin (Norton, 1976). Myelin deposition in the rat is believed to continue even beyond 425 days of age (Norton and Poduslo, 1973a). In the rat, the onset of CNS myelination follows closely the peak of cellular proliferation seen at 10 days postnatally

(Norton, 1976).

The biochemical content of CNS whole brain myelin changes with time (Davison and Peters, 1970; Norton, 1971; Norton and Poduslo, 1973). During maturation myelin galactolipids increase by about 50 percent while lecithin content decreases by approximately 50 percent. The limited amount of myelin desmosterol declines further. Polysialogangliosides wane while the monosialoganglioside, G_{M1}, becomes the predominant rat CNS myelin ganglioside. In rodents during the first two months the relative amount of basic protein and proteolipid protein increases while that of high molecular weight protein decreases (Morell, Greenfield, Costantino-Ceccarini, and Wisniewski, 1972). Generally, the PNS begins to myelinate first, followed by spinal cord, and finally the brain in a caudocranial temporal gradient. In general, myelination follows phylogenetic development (Yakovlev and Lecours, 1967; Rorke and Riggs, 1969).

The research presented in this dissertation concerns CNS myelination in the rat. For purposes of reference, myelinogenesis commences in the human during the fifth month *in utero*, and peaks during the perinatal period. Myelinogenesis in the human continues throughout and possibly beyond the third decade of life, however, the bulk of CNS brain myelination is complete by the end of the second year (Norton, 1976).

CNS MYELIN SUBFRACTIONS

Concerning the controversy currently surrounding the true significance of CNS myelin subfractions, suffice it to say that there exists a plethora of procedures for isolating and preparing CNS myelin, myelin

subfractions, and myelin associated membrane fractions. There are divergent nomenclatures, criteria of purity, and experimental paradigms for metabolic studies. A variety of interpretations have been advanced concerning the origin and significance of myelin subfractions. Myelin subfractions may simply be operationally and arbitrarily defined. Alternatively, myelin subfractions could be merely artifacts of isolation and preparation procedures, e.g., owing to the complex spatial relations of the myelin sheath in situ, different portions of the sheath might be differentially vulnerable to shearing forces operative during subfractionation procedures. And finally, myelin subfractions may indeed be genuine correlates of anatomical structures and/or biochemical maturity having significance for the composition and ontogeny of myelin.

Another aspect of the current myelin subfraction controversy concerns the ontogenetic significance of myelin subfractions and myelin associated membrane fractions with respect to precursor-product relations, either between non-myelin and myelin compartments, or within myelin compartments. Precursor-product relationships are exceedingly difficult to demonstrate unequivocably. The indirect, suggestive evidences thus far presented are compatible with certain precursor-product relations; however, additional data are needed to more adequately demonstrate the reality of these and other similar relationships. These matters will be documented and discussed below with respect to the

previously published findings of workers in the field.

Several laboratories have described procedures for isolating CNS myelin, myelin subfractions, and myelin associated membrane fractions. (Autilio, Norton, and Terry, 1964; Cuzner and Davison, 1968; Adams and Fox, 1969; Shapira, Binkley, Kibler and Wundram, 1970; McMillan, Williams, Kaufman, and Day, 1972; Morell et al., 1972; Benjamins, Miller, and McKhann, 1973; Matthieu, Quarles, Brady, and Webster, 1973; Norton and Poduslo, 1973b; Agrawal, Trotter, Burton, and Mitchell, 1974; Waehneldt and Neuhoff, 1974). CNS myelin is generally prepared by ultracentrifugation techniques following homogenization of tissue in low ionic strength sucrose solutions. During homogenization myelin lamellae are stripped away from the axon and reform as large vesicles of high lipid content. These vesicles have the lowest density of any CNS membrane. In the presence of high ionic strength sucrose solutions myelin adheres to axon fragments and can not be isolated free of contaminants. Ultracentrifugation of sucrose homogenates upon appropriate discontinuous or continuous sucrose, CsCl, or sucrose-Ficoll gradients takes advantage of the great buoyant density of the crude myelin vesicles (Norton, 1976).

Two main methods for CNS myelin preparation employ the principles stated above. In the first, the tissue is differentially centrifuged to yield a crude mitochondrial fraction containing mitochondria, synaptosomes, and myelin, and a nuclear fraction. Each of these two fractions is resuspended in isotonic sucrose (0.3 M) and layered above or below 0.8 M sucrose. Upon centrifugation crude

myelin is recovered as interfacial material. Alternatively, whole tissue may be homogenized directly in isotonic sucrose and the homogenate layered above or below 0.85 M sucrose. Centrifugation again yields crude myelin as interfacial material. The purity of crude myelin varies with the source of the tissue and the age of the brain. Younger animals yield crude myelin with greater impurity, the chief impurities being axoplasmic and microsomal contaminants. These are removed by osmotic shock in distilled water by two methods. The crude myelin suspended in distilled water is slowly centrifuged, sedimenting myelin and leaving axoplasmic and microsomal material in the supernatant. Alternatively, crude myelin may be resuspended in isotonic sucrose solutions reapplied to gradients and centrifuged repeatedly. Depending upon the gradient medium employed, myelin will band out at different densities. On a sucrose gradient myelin bands at a density of 1.08 g/ml, the density of 0.65 M sucrose. On CsCl gradients banding occurs at greater density, on sucrose-Ficoll gradients at lower density (Norton, 1976). Myelin represents a continuum of densities within a defined range (Detering and Wells, 1976). Accordingly, one can operationally select specific gradient densities in order to isolate whole myelin or myelin subfractions of specific density range.

Since procedures for isolation and subfractionation of CNS myelin are of necessity somewhat arbitrary, it becomes necessary to select appropriate criteria of purity. Criteria advanced have included morphology as revealed by electron microscopy, the presence of chemical and enzymatic markers specific to myelin, the absence of

markers specific to non-myelin structures, and the appearance of SDS protein gel electrophoregrams. Isolated CNS myelin does retain its lamellar appearance with its fundamental protein-lipid-protein-lipid-protein structure and a radial periodicity of approximately 12 nm when examined under electron microscopy. The electron photomicrographic fields may also be examined for obvious contamination by nuclear, mitochondrial, and axonal structures. Unfortunately, beyond a certain level of purity it is impossible to exclude contamination by small membrane vesicles of microsomal origin. The presence of enriched specific activity of CNP, a CNS myelin marker, is a powerful criterion of purity. Enrichment in galactosphingolipid, especially cerebroside, is another useful criterion of purity. The absence of succinic dehydrogenase activity excludes mitochondrial contamination. The absence of Na^+, K^+-activated ATPase and 5'nucleotidease activity and nucleic acid are criteria for the exclusion of contamination by plasma cell membrane, ribosomes, microsomes, and nuclei. Enrichment in myelin specific proteins, basic protein, proteolipid protein, and Wolfgram protein, is a further demonstration of CNS myelin purity (Norton, 1976).

The research reported in this dissertation was prompted in large measure by the interesting observations made by Matthieu et al., (1973) and Zimmerman, Quarles, Webster, Matthieu, and Brady (1975) concerning the correlation between density and maturity of three density subfractions (Matthieu et al., 1973) of whole brain CNS myelin (Norton and Poduslo, 1973b). The CNS whole brain myelin was recovered as

interfacial material following ultracentrifugation (75,000 g avg., 30 minutes) of an 0.32 M sucrose homogenate of brain tissue layered over 0.85 M sucrose. The CNS whole brain myelin was subsequently further purified by osmotic shocking (Norton and Poduslo, 1973b). Purified whole brain CNS myelin was subfractionated into light, medium, and heavy myelin (Matthieu et al., 1973). These subfractions were recovered as two interfacial bands and a pellet following ultracentrifugation (75,000 g avg., 30 minutes) of a gradient containing myelin suspended in 0.32 M sucrose layered over a discontinuous gradient of 0.62 M and 0.70 M sucrose. It is significant that most of the subfractionated myelin resedimented to the same position in the 0.32/0.62/0.70 M sucrose discontinuous gradient. Matthieu's procedure separates myelin subfractions on the basis of actual differences in physical properties and not artifactually by aggregation phenomena (Matthieu et al., 1973 ; Zimmerman et al., 1975).

The observations by Matthieu et al. (1973) and Zimmerman et al. (1975) concerning the correlation between myelin density and maturity are very provocative. On morphologic and biochemical grounds both Matthieu and Zimmerman observed that light myelin is most like classical mature myelin, heavy most like immature myelin, and medium myelin intermediate in character. These conclusions were based on preparations from whole brain of 16-day-old rat and from the cortical region and brain stem of 40 day-old rat brain (Zimmerman et al., 1975) and from whole brain of 60-day old rat (Matthieu et al., 1973). Morphologically CNS myelin subfractions from all regions and at all ages examined showed greater maturity in lighter subfractions.

Light myelin appeared compact and multilamellar. Heavy myelin appeared vesicular and was enriched in single membranous structures. Medium myelin was intermediate in appearance. At all ages and in all regions examined light myelin was enriched in basic protein. Heavy myelin, by contrast, was enriched in high molecular weight protein. Medium myelin had a protein composition intermediate between light and heavy myelin. In 16-day-old whole brain heavy myelin had less than half the specific activity of CNP and less than half the total content of the major myelin associated glycoprotein than did either light or medium myelin. In preparations from 40- and 60-day-old rat brain tissue there was an enrichment in both CNP and the major myelin associated glycoprotein from light to heavy myelin. Heavy myelin from 16-day-old rat brain had less proteolipid protein than did light myelin while in 60-day-old adult whole brain Matthieu observed comparable amounts of proteolipid protein in all three subfractions. According to Matthieu the good correlation between enrichment of CNP and the major myelin associated glycoprotein in adult heavy myelin suggests a similar locus for these components either in loose myelin or oligo plasma cell membrane. Both agree that heavy myelin is enriched in loose uncompacted lamellae, oligo plasma cell membrane, and oligo plasma cell membrane. These membranes are thought to be in transition towards myelin of greater morphological and biochemical maturity.

It is significant that at the ages examined, i.e. 16-, 40-, and 60-days-old, a given Matthieu CNS myelin subfraction retains similar morphological and biochemical properties. As any other subfractionation procedure, the Matthieu procedure is arbitrary. However,

it is a reproducible technique and appears to be valuable in examining selected density ranges of CNS brain myelin over a wide range of ages.

The morphological and biochemical properties of the Matthieu CNS light, medium, and heavy myelin subfractions as observed by Matthieu et al. (1973) and Zimmerman et al. (1975) are in general accord with the properties of "lighter" and "heavier" CNS myelin subfractions as prepared and investigated by other workers. The lighter subfractions of myelin typically have a high specific activity of CNP (Benjamins et al., 1973; Matthieu et al., 1973; Agrawal et al., 1974; Zimmerman et al., 1975), low activity of acetylcholinesterase (Matthieu et al., 1973; Agrawal et al., 1974), a multilamellar morphology as revealed by electron microscopy (Benjamins et al., 1973; Matthieu et al., 1973; Agrawal et al., 1974; Zimmerman et al., 1975), significant amounts of basic protein (Matthieu et al., 1973; Agrawal et al., 1974; Waehneldt and Neuhoff, 1974; Zimmerman et al., 1975), and significant amounts of galactolipid (Cuzner and Davison, 1968; McMillan et al., 1972; Benjamins et al., 1973; Agrawal et al., 1974). The heavier subfractions characteristically are composed of many single vesicular structures (Morell et al., 1972; Benjamins et al., 1973; Matthieu et al., 1973; Agrawal et al., 1974), enriched in high molecular weight proteins (Adams and Fox, 1969; Eng and Bignami, 1972; Morell et al., 1972; Benjamins et al., 1973; Matthieu et al., 1973; Agrawal et al., 1974; Zimmerman et al., 1975; Benjamins, Gray, and Morell, 1976a; Fujimoto, Roots, Burton, and

Agrawal, 1976), and enriched in phospholipid content (Cuzner and Davison, 1968; McMillan et al., 1972; Benjamins et al., 1973; Agrawal et al., 1974). The increase in the protein to lipid ratio observed by Matthieu et al. (1973) and Zimmerman et al. (1975) from light to heavy myelin, accounting in large measure for the differing sucrose densities at which the three Matthieu subfractions are isolated, is similar to that reported by Autilio et al. (1964) for their light and heavy bovine myelin.

As Quarles (1977) indicates, there is good reason to believe that the Matthieu heavy myelin subfraction is enriched in myelin related membranes rather than deriving its biochemical properties by admixture of unrelated, non-myelin membranes. Heavy myelin, compared to whole myelin from which it was subfractionated and compared to light and medium myelin, has higher levels of CNP, the major Wolfgram protein, and the major myelin associated glycoprotein. The significance of high CNP activity, Wolfgram protein content, and content of the major myelin associated glycoprotein as markers of myelin was alluded to previously.

The current controversy concerning the true significance of myelin subfractions arises in large measure from the uncertainty regarding the ultrastructural origins of the myelin subfraction and related membrane fractions. It is at this point that one must appreciate the incredibly complex morphological relations of the myelin sheath in situ vis-a-vis the axon and the oligo extended plasma membrane. In a contemporary review of this problem, Quarles (1977)

discusses the technical problems inherent in the task of labelling, isolating, and characterizing discrete microenvironments within the oligodendrocyte-extended oligo plasma cell membrane-myelin sheath-axon complex. Until techniques of sufficient sophistication are developed, ultimate solution is not available. Pending the solution of this mystery, Quarles (1977) discusses the prevailing theories concerning the origin of myelin subfractions and related membrane fractions and provides cogent reasons for accepting the viewpoint that myelin subfractions are indeed morphological and biochemical correlates of the tissue complex in situ.

The suggestion that myelin subfractions are simply operationally and arbitrarily defined may be discounted easily. While differences in lipid to protein ratio obviously contribute to the "arbitrary" nature of myelin subfraction selection by density gradient ultracentrifugation, the arbitrary nature of such a buoyant density selection criterion does not obscure the fact that once selected, the various density subfractions have well documented morphological and biochemical differences beyond the density factors operative in their selection.

Another prevailing explanation is that myelin subfractions represent regional differences per se within the CNS. Regional differences within the CNS are generally known and acknowledged. For example, the ratio of basic protein to proteolipid in myelin varies within the brain and is higher in spinal cord (Morell, Lipkind, and Greenfield, 1973; Zgorzalewicz, Neuhoff, and Waehneldt, 1974; Smith

and Sedgewick, 1975). Zimmerman et al. (1975), examining myelin from 40-day-old rat brain, observed a four-fold greater yield of myelin per g fresh weight tissue and a higher proportion of light myelin from brain stem compared to the cortical region. Similarly, Williams, Hogan and Brostoff (1976a), isolating myelin by means of the isosmotic procedure of McMillan et al. (1972) observed a greater proportion of their lightest myelin fraction regionally: cerebrum < brain stem < spinal cord. However, Quarles (1977) proposes that regional differences are of only apparent significance. The morphology of the myelinated axon population is probably more significant. Brain stem and spinal cord have a higher content of large myelinated axons, which also are myelinated earlier than those in other regions of the CNS and at any given age would be expected to be more mature.

Since isolated light and medium myelin subfractions are enriched in multilamellar material, they obviously must be derived from classical compact myelin. The ultrastructural origin of heavy myelin is more obscure since the identification of isolated single membranes and vesicles does not necessarily resolve the question of whether they represent heavier myelin per se or simply artifactual fragments of lighter myelin subfractions.

A further explanation which has been advanced implies a certain degree of artifactual origin behind the distinction between the lighter subfractions and the heavy subfractions. If one assumes that all subfractionated myelin is derived from compact classical

multilamellar myelin, then one must also assume that heavy myelin subfractions represent small fragments of mature myelin which constitute a select population of myelin membranes with unique biochemical properties. Such a select population of membranes could include the inner or outer portion of the myelin sheath, lamellae which are particularly vulnerable to the shearing forces operative during myelin isolation and subfractionation procedures. However, Quarles (1977) and Matthieu et al. (1973) suggest that heavy myelin subfractions are also enriched in membranes transitional between the extended plasma membrane of oligodendroglia and multilamellar myelin.

The explanation of Quarles (1977) and Matthieu et al. (1973) that heavy myelin subfractions are enriched in transitional membranes is supported by other investigators (Agrawal, Banik, Bone, Davison, Mitchell, and Spohn, 1970; Morell et al., 1972; Waehneldt and Mandel, 1975). If this hypothesis concerning the origin of the heavy myelin subfractions is correct, such myelin fractions should have properties somewhat similar to those of oligo plasma membrane. Calf oligo plasma membrane has been purified and biochemically characterized by Poduslo (1975). Bovine oligo plasma membranes have a protein to lipid ratio greater than that of myelin, a large proportion of high molecular weight protein, a protein of the same electrophoretic mobility as proteolipid protein, relatively high CNP activity, and glycoproteins.

The precise locus of each of the membranes within the continuum between oligo plasma cell membrane and multilamellar myelin is unknown.

It is reasonable, however, to assume that they would be found in the following loci: the inner and outer loops (i.e., the inner and outer mesaxon), lateral loops (i.e., paranodal membranes), and the process connecting oligo to the myelin sheath (Quarles, 1977). Common morphological characteristics of these loci include the presence of oligodendroglial cytoplasmic pockets and an uncompacted extended oligo plasma membrane presumably at some stage in the continuum. Such oligodendrocyte derived membranes may be represented not only by heavy myelin subfractions but also by the so-called "myelin-like" fraction (Banik and Davison, 1969; Agrawal et al., 1970; Agrawal, Trotter, Mitchell, and Burton, 1973) and the so-called "floating fraction" or "dissociated myelin" (Norton, Poduslo, and Suzuki, 1966; Smith,1973; Matthieu, Zimmerman, Webster, Ulsamer, Brady, and Quarles, 1974c; Cammer, Rose, and Norton, 1975).

The "myelin-like" fraction is derived from material which does not sediment at low g force following osmotic shock of crude myelin. The purity of the "myelin-like" fraction has not been established and its predominantly vesicular morphology is dissimilar to myelin. However, it does have some biochemical properties in common with myelin, e.g., relatively high CNP activity though lower than purified myelin, presence of high molecular weight protein, and low levels of basic protein, proteolipid protein, and galactocerebroside. According to Quarles (1977) it is probably enriched in small membranes of myelin membrane or other oligo plasma cell derived membranes. Similarly isolated material derived from supernatant material following osmotic

shock of crude myelin has been analyzed, e.g., the myelin related membrane fraction designed "SN-4" (Waehneldt and Mandel, 1972) and the CsCl gradient derived fractions designated as "lower layer" material (Morell et al., 1972). Waehneldt's (1975) SN-4 fraction of rat origin had an enrichment in CNP activity increasing with age, approximately 1.5 to 2.5 fold higher than myelin. Its protein composition included Wolfgram protein as the major protein, proteolipid, and DM20 protein, as well as periodic acid-Schiff positive protein (the myelin associated glycoprotein?).

Quarles (1977) suggests that the so-called "floating fraction" represents a form of degrading myelin since it is primarily observed in association with specific neuropathologies, e.g., subacute sclerosing leukoencephalitis (Norton et al., 1966), triethyl tin-induced demyelination (Smith, 1973), and hexachlorophene intoxication (Matthieu et al., 1974c; Cammer et al., 1975). Smith (1973) suggested that it is enriched in more recently synthesized myelin composed of lamellae splitting off near the external mesaxon and paranodal cytoplasmic pockets.

The CNS brain myelin subfraction metabolic studies to be reported herein will only concern the light, medium, and heavy myelin subfractions as defined by the procedure of Matthieu et al. (1973) and characterized in non-metabolic studies by Matthieu et al. (1973) and Zimmerman et al. (1975). The foregoing extended discussion of what Quarles (1977) prefers to call oligodendroglial derived membrane fractions is significant, nonetheless, for two reasons. First,

it provides a background against which results reported herein may be discussed and evaluated. Secondly, the oligo derived membrane fractions as a whole, of which Matthieu's heavy myelin subfraction is only a part, are of considerable interest because of reports presented consistent with the association of oligo derived membrane fractions with myelin precursor-product or apparent myelin precursor-product roles. Precursor roles are exceedingly difficult to document; however, the overall picture is supportive of and consistent with important precursor-product roles for membranes in this population, especially the heavier myelin subfractions and the myelin-like membrane fraction. The preliminary data obtained thus far are very suggestive. Continued effort is warranted to confirm and clarify the extent and significance of such precursor roles in order to obtain a more complete biochemical understanding of CNS brain myelinogenesis.

That the heavier myelin subfractions and certain myelin associated membranes are involved in significant myelinogenetic precursor-product relationships is suggested by a number of metabolic studies (Benjamins et al., 1973; Agrawal et al., 1974; Sabri, Tremblay, Banik, Scott, Gohil, and Davison, 1975; Figlewicz and Druse, 1976a, 1976b; Benjamins et al., 1976a, Benjamins, Miller, and Morell, 1976b). The details of these metabolic studies will be considered during the discussion of the data presented in this dissertation. Only a summary of the conclusions of these studies is presented here.

Benjamins et al. (1973) concluded that an early, very active

microsomal pool of newly synthesized sulfatide was accepted by their rapidly turning over heaviest myelin subfraction and subsequently accepted and accumulated in lighter myelin subfractions. Agrawal et al. (1974) concluded that their myelin-like and membrane fractions more rapidly incorporated [2,3-^{3}H] tryptophan early on, followed by equilibration and incorporation of newly labelled protein into lighter myelin subractions. Sabri et al. (1975) concluded that [^{3}H] lysine was more rapidly incorporated in myelin-like material and subsequently more rapidly lost from myelin-like material than in purified whole myelin. Furthermore, at very young ages, 0-20 days, what little CNP and basic protein there was, appeared to be enriched in microsomal and myelin-like fractions compared to purified whole myelin. Benjamins et al. (1976a, 1976b) concluded that selected protein and lipid precursors were incorporated into myelin density subfractions in a complex manner. Phosphatidylethanolamine and its plasmalogen analogue and proteolipid protein appeared to enter more rapidly into heavier myelin subfractions, suggestive of precursor-product relationships between myelin associated fractions and heavier myelin subfractions on the one hand, and lighter myelin subfractions on the other hand. However, the entry of phosphatidylcholine, its plasmalogen analogue, cerebroside, sulfatide, galactosyl diglyceride, basic protein, and Wolfgram protein appeared to enter the heavy and lighter myelin subfractions in an independent and simultaneous manner.

Figlewicz and Druse (1976a, 1976b) studied abnormal myelin-

ogenesis. Figlewicz and Druse (1976a) concluded that quaking mouse mutants could incorporate L-[4,5-^{3}H]leucine into their heavy myelin subfraction more easily than into their lighter myelin subfractions when compared to normal litter mate controls. They suggested that the apparent ability to synthesize heavy myelin contrasted against the apparent difficulty in making lighter myelin reflects a blockage in the normal conversion or modification of heavy myelin requisite for its maturation into the lighter subfractions. Figlewicz and Druse (1976b) concluded from a study of abnormal myelinogenesis caused by pre- and postnatal protein deficiency that disproportionate amounts of heavy myelin were synthesized during periods of nutritional rehabilitation. It was this disproportionate amount of the morphologically and biochemically immature heavy myelin which accounted for the observed final recovery of near normal amounts of total myelin protein in malnourished pups after rehabilitation. The work by Figlewicz and Druse (1976a, 1976b) is particularly pertinent to the present study because their metabolic studies also employed the myelin subfractionation procedure described by Matthieu et al. (1973).

Further interest in the possible importance of the role of the heavier myelin subfractions in early myelinogenetic events comes from developmental studies of the major myelin associated glycoprotein. Quarles et al. (1973b) reported higher apparent molecular weight of this glycoprotein in immature rat whole brain myelin. The apparent molecular weight of this glycoprotein may be a sensitive marker of myelin maturity as further evidenced by the observation of a slightly

larger glycoprotein isolated from whole brain myelin in the quaking murine mutant (Matthieu et al., 1974b), hypothyroid rats (Matthieu, Reier, and Sawchak, 1975a), and copper deficient rats (Zimmerman, Matthieu, Quarles, Brady, and Hsu, 1976). More pertinent to the developmental distinctions between the Matthieu light, medium, and heavy myelin subfractions was the observation by Zimmerman et al. (1975) in 16-day-old rat brain of higher apparent glycoprotein molecular weight in medium and heavy subfractions.

Although Matthieu et al. (1973) and Zimmerman et al. (1975) have described the Matthieu myelin subfractions in considerable detail, their published work did not include any reports concerning the metabolism of these subfractions. The protein and lipid metabolism of Matthieu myelin subfractions reported in this dissertation have been presented previously (Druse and Hofteig, 1975; Hofteig and Druse, 1976). Additional reports of normal metabolism of the Matthieu myelin subfractions have not appeared in the literature.

POSTNATAL PROTEIN-CALORIE MALNUTRITION

The determination of the extent of stress-mediated perturbations in CNS brain myelin subfractions as a consequence of postnatal protein-calorie malnutrition and maternal ethanol consumption is important because serious neurological functional deficits are observed in similarly stressed human neonates. Such determinations should be able to quantitate at a more basic level the neuropathological consequences of maternal malnutrition and ethanol consump-

tion upon the neonate. Further, to the extent that these animal models for similar kinds of stress perturb CNS myelinogenesis, it may be possible to gain a greater appreciation of normal myelinogenetic mechanisms. Numerous studies have appeared (see below) concerning the effects of various kinds of malnutritional stress upon CNS myelinogenesis. However, only a limited number of such reports have actually considered the metabolism of myelin subfractions.

To date, the CNS myelin subfractionation procedure of Matthieu et al. (1973) is the only subfractionation procedure employed in metabolic studies of abnormal CNS myelinogenesis (Figlewicz and Druse, 1976a, 1976b; Hofteig, Druse, and Collins, 1976; Druse and Hofteig, 1977; Figlewicz, Hofteig, and Druse, 1977). It was also employed by Trapp and Bernsohn (1977) in a non-metabolic study of the effects of an experimentally induced essential fatty acid deficiency in developing rat brain.

It had been appreciated for some time that growth retardation due to malnutrition during critical periods of brain development in the human is frequently associated with irreversible deficit in higher mental function (Dobbing, 1971a). It was also appreciated that the neurological effects of malnutrition are subject to reversal upon dietary supplementation provided the malnutrition stress was sufficiently mild or of short duration.

A fundamental concept which has shaped neurochemical research with stress during periods of CNS brain development is the vulnerable period hypothesis (Dobbing, 1966; Davison and Dobbing, 1966; Dobbing, 1968; Dobbing, 1970, 1971; Adlard and Dobbing, 1971). The vulnerable

period hypothesis states that each particular cell line is most vulnerable to stress or insult during the relatively brief period of growth hyperplasia. The mammalian brain growth spurt during brain development is associated with the most rapid and profound increase in brain weight. Generally, it is defined by two overlapping periods, first, neuronal cellular proliferation, and secondly, glial cellular proliferation. Myelination is associated with the latter half of the brain growth spurt. The vulnerable period also coincides with the period of rapid increases in enzymatic activity. Accordingly, the clinical manifestations of stress during this period may reflect perturbation in neuronal population size and differentiation, glial population size and differentiation, enzymatic activity, relative timing of key events, and formation of synaptic connections, etc.

The failure to myelinate properly may or may not be the more fundamental etiologic factor in the neonatal malnutrition-growth retardation associated neurological dysfunction observed in malnourished humans. However, deficits in CNS brain whole myelin have been observed in experimental animal models of malnutrition.

Malnutrition during the vulnerable period of brain development can result in at least a temporary deficit of CNS whole myelin during or following malnutrition (Benton, Moser, Dodge, and Carr, 1966; Chase, Dorsey, and McKhann, 1967; Fishman, Prensky, and Dodge, 1969; Geison and Waisman, 1969; Bass, Netsky, and Young, 1970; Fishman, Madyastha, and Prensky, 1971; Wood, 1973; Fox, Fishman, Dodge, and Prensky, 1972; Krigman and Hogan, 1976; Wiggins, Miller, Benjamins, Krigman, and

Morell, 1976; Simons and Johnston, 1976). Severe and prolonged malnutrition results in a persisting deficit in CNS whole myelin (Chase et al., 1967; Bass et al., 1970; Fishman et al., 1971; and Simons and Johnston, 1976). However, near normal levels of CNS whole brain myelin may be recovered if nutritional rehabilitation is begun early enough (Benton et al., 1966; Geison and Waisman, 1969).

Interpretation of previous studies of CNS brain myelination and malnutrition necessitates some appreciation of the variety of types of nutritional stress and periods of stress employed. Early postnatal starvation was employed by Benton et al. (1966), Chase et al. (1967), Geison and Waisman (1969), Bass et al. (1970), Fishman et al. (1971), Wood (1973), Wiggins, Benjamins, Krigman, and Morell (1974), Krigman and Hogan (1976), and Wiggins et al. (1976). Protein deficiency during lactation was studied by Nakhasi, Toews, and Horrocks (1975), and during both gestation and lactation by Stewart, Merat, and Dickerson (1974) and Simons and Johnston (1976).

Among workers in the field there is controversy concerning whether CNS brain whole myelin formed during nutritional stress or during post-stress periods of nutritional rehabilitation efforts is normal in composition. Fox et al. (1972) and Fishman et al. (1971) reported that malnourished children had decreased amounts of total CNS brain whole myelin, with normal lipid composition. However, Wiggins et al. (1974), Nakhasi et al. (1975), Wiggins et al. (1976), and Simons and Johnston (1976) report abnormal biochemical composition of CNS brain whole myelin isolated from malnourished and pre-

viously malnourished rats after periods of attempted nutritional rehabilitation.

The research reported herein concerning the effects of postnatal protein-calorie malnutrition upon CNS brain myelin subfraction protein and lipid metabolism was prompted in part by this controversy. Knowing that the heavier CNS brain myelin subfractions are less mature than lighter subfractions, one can assess the maturity of myelin synthesized during and following periods of malnutrition. The use of approrpiate isotopically labelled protein and lipid precursors permits assessment of synthetic rates of the various subfractions.

CHRONIC AND ACUTE MATERNAL ALCOHOL CONSUMPTION

The fetal alcohol syndrome (FAS) is now recognized as a well documented serious public health problem. It was clearly characterized as a distinct multiple pathology syndrome in neonates directly associated with serious chronic maternal alcohol consumption by Lemoine, Harousseau, Borteyru, and Menuet (1968) in a very large retrospective case history analysis in France. Since that first clinical account, numerous clinical reports have appeared (Ulleland, 1972; Jones and Smith, 1973; Jones, Smith, Ulleland, and Streissguth, 1973a; Jones, Smith, Streissguth, and Myrianthopoulos, 1974a, 1974b; Palmer, Ouellette, Warner, and Leichtman, 1974; Root, Reiter, Andriola, and Duckett, 1975; Mulvihill and Yeager, 1976; Mulvihill, Klimas, Stokes, and Risemberg, 1976; Ouellette, Rosett, Rosman, and Weiner, 1977).

The frequency of severe chronic alcoholism in the United States is conservatively estimated at 1 in 1000 to 2000 pregnancies (Jones et al., 1974b; Hanson et al., 1976). It is estimated that 30 to 50 percent of such pregnancies will present with FAS signs (Hanson et al., 1976). Other complicating factors may include socio-economic status, inadequate access to medical care, poor nutrition, hereditary factors, etc. These may be excluded as major factors in as much as carefully controlled prospective studies have demonstrated FAS signs independent of socio-economic status, and even when vitamin supplementation, adequate nutrition, and optimal access to medical care were provided (Jones et al., 1974b; Hanson et al., 1976). In a typical retrospective review of 41 pregnancies in a population of well documented chronic abusers of ethanol, Hanson et al. (1976) found a very high incidence of growth and performance abnormalities (i.e., prenatal growth deficiency, postnatal growth deficiency, microcephaly, developmental delay or mental deficiency, and fine motor dysfunction), a high incidence of craniofacial abnormalities (i.e., short palpebral fissures, midfacial hypoplasia, and epicanthic folds), moderate frequency of limb abnormalities (i.e., abnormal palmar creases and joint anomalies), less frequent miscellaneous abnormalities (i.e., cardiac defects, external genital anomalies, hemiangiomas, and ear anomalies), and a number of infrequent abnormalities of minor to major seriousness.

The seriousness of the fetal alcohol syndrome is revealed by the following facts. There is a 17 percent perinatal mortality asso-

ciated with the FAS (Jones et al., 1974b; Hanson et al., 1976). Most FAS offspring are classified as small for date (Ulleland, 1972; Jones and Smith, 1973; Jones et al., 1973, 1974b; Palmer et al., 1974; Root et al., 1975; Hanson et al., 1976). Nearly half of FAS neonates are mentally deficient (Jones et al., 1974b; Palmer et al., 1974; Hanson et al., 1976; Mulvihill et al., 1976; Mulvihill and Yeager, 1976) and have altered EEG patterns (Root et al., 1975). The most serious aspect of the FAS is the observation that if the FAS presentations are serious at birth, growth and mental retardation is refractory to all rehabilitation efforts including "enriched" social environment and nutritional supplementation, even through the age of 5 or 6 years of age, and presumably beyond (Jones et al., 1973; Hanson, 1976).

Except for the observations cited concerning mental retardation and altered EEG patterns, little is known about the effects of maternal alcohol consumption upon brain development. The mechanism of the neurological damage is unknown. Furthermore, it is still unknown whether ethanol itself, its major metabolite, acetaldehyde, or some other ethanol derived agent is responsible for the FAS. It is generally accepted that placental transfer of ethanol from mother to fetus occurs throughout the term of the pregnancy with rapid equilibration of maternal and fetal blood values (Dilts, 1970; Idanpaan-Heikkila, Fritchie, Ho, and McIsaac, 1972; Akesson, 1974; Randall, 1977). However, acetaldehyde, does not cross the placenta in appreciable amounts in the near term rat apparently because of placental metabolism of acetaldehyde (Sippel and Kesaniemi, 1975; Kesaniemi and

Sippel, 1975; Randall, 1977).

The research to be reported herein concerning the effect of selected periods of maternal alcohol consumption upon brain myelin subfraction protein and lipid metabolism is prompted by the suggestion that abnormalities in myelinogenesis may be either causative, or contributory, or might be present as a reflection of more underlying neuronal or glial abnormalities.

The association of mental deficiency caused by maternal ethanol consumption with myelination abnormalities is an attractive hypothesis. Mental retardation and CNS myelin abnormalities are both seen in phenylketonuria (Alvord, Stevenson, Vogel, and Engle, 1950; Menkes, 1967; Clark and Lowden, 1969) and in some children malnourished early in life (Fishman et al., 1969; Osofskey, 1969; Chase, Welch, Dabiere, Vasan, and Butterfield, 1972; Fox et al., 1972).

SUMMARY

The unique biochemical, morphological, and functional properties of CNS brain myelin have been presented, The current theories concerning the mechanisms of myelinogenesis by oligodendroglial cells have been reviewed. The general techniques and theory of CNS myelin isolation and subfractionation have been discussed with particular reference to the techniques in current usage. The biochemical properties, including putative precursor-product relationships as they are currently understood, have been reviewed in detail for myelin, myelin subfractions, and myelin related membrane fractions. The

various appraisals of the significance of CNS myelin subfractions have been documented as has been the complexity of the task inherent in correlating precisely the biochemical properties of a particular myelin related membrane fraction or myelin subfraction with its specific original *in situ* locus. The need to clarify the significance of CNS brain myelin subfractions has been made manifest. The interesting and useful properties of the Matthieu CNS myelin subfractions have been reviewed in detail, especially their potential for further clarifying the biochemical mechanisms operative in normal myelinogenesis as well as the mechanisms operative during stress and post-stress efforts at rehabilitation. The association of myelination abnormalities with malnutrition associated mental dysfunction has been reviewed. The lack of knowledge concerning the mechanisms operative in the pathogenesis of FAS associated neurological dysfunction has been reviewed, and the suggestion has been made that myelination abnormalities at the very least may be associated with the FAS.

The research to be presented herein begins with several hypotheses which are worthy of consideration and which need to be confirmed, clarified, tested, revised, or discarded as the case may be after having completed the research. The major assumption is that myelin subfractions are actual correlates of morphological and biochemical maturity. The Matthieu subfractions are considered to be particularly valuable and useful models especially because of the observed correlation between density and maturity. It is assumed that elucidation of CNS myelin subfraction protein and lipid metabolism under a variety of normal and

stress conditions is a necessary task with promise of further clarifying the ontogenetic significance of CNS myelin subfractions, further defining at a more basic level the pathogenetic mechanisms operative in malnutrition and maternal alcohol consumption mediated stress, and further assessing the quantitative and qualitative effectiveness of post-stress rehabilitation efforts following stressed myelinogenesis.

CHAPTER II

MATERIALS AND METHODS

ANIMALS

Sprague-Dawley albino rats were purchased from Holtzman (Madison, Wi.) and Locke-Erikson Laboratories (Melrose Park, Il.).

DIET

Different dietary protocols were employed, i.e., normal ad libitum laboratory chow, and defined periods of protein-calorie malnutrition and maternal ethanol consumption. Except during defined periods of dietary stress, all animals were given free access to water and standard laboratory rat chow (Purina Laboratory Chow, Purina, St. Louis, Mo.) containing casein (27%), starch (59%), vegetable oil (10%), and salt mixture and vitamin fortification (4%).

Protein-calorie malnutrition from parturition to 18 days postpartum was accomplished by total daily chow allotment of 14 to 16 grams chow per dam. The daily allotment of 14 to 16 grams chow per dam was selected empirically. This level of dietary restriction resulted in significant decrements in offspring body and brain weights. More severe dietary restriction resulted in nearly complete offspring mortality by the age of 12 to 18 days. Litters whose dams were restricted in protein-calorie intake were weaned at 29 days. Ad libitum chow fed control litters were weaned at 24 to 25 days of age. At all times both experimental and control animals had free access to water.

Maternal ethanol consumption was controlled in a pair feeding paradigm employing the isocaloric control and alcohol diets described by Freund (1969) and by Lieber and DeCarli (1974). Daily and cumulative average daily volume, calorie, and alcohol consumption were recorded. The isocaloric control and alcohol diets were identical in vitamin, mineral, lipid, protein, and calorie content. The only difference between the control and alcohol diets was the isocaloric substitution of ethanol in the alcohol diets for calories in the control diets otherwise supplied by either sucrose in the case of the diets described by Freund (1969) or maltose-dextrins in the case of the diets described by Lieber and DeCarli (1974). The Freund and Lieber DeCarli diets are characterized in greater detail below. Basically, the Freund diet is based on Metrecal (Mead Johnson, Cincinnati, Oh.) with variable supplementation by sucrose and ethanol. The Lieber DeCarli diet, commercially available (Bio-Serv, Frenchtown, N.J.), has a fixed alcohol content of 6.7% (v/v) in the alcohol diet. The composition of the control and alcohol Lieber DeCarli liquid diets is described in Table I.

While the Lieber DeCarli diet was used at a fixed percent alcohol content, the Freund diet was used with variable concentrations of alcohol in the alcohol diet, i.e., 5.1, 7.0, 9.0, and 11.0% (v/v). Furthermore, by using liquid Metrecal Shape (Mead Johnson), addition of isocaloric solutions of either 63.33% (v/v) stock solution of ethanol or 87.0% (v/v) stock solution of sucrose, resulted in variable enrichment in carbohydrate as a percent of total calories while dilut-

TABLE I

Lieber DeCarli Liquid Diet

Substitutent	Control Gram/Liter	Control Percent Calories	Ethanol Grams/Liter	Ethanol Percent Calories
Protein:		16.9%		16.9%
Casein, vitamin free:	41.4		41.4	
L-Cystine:	.5		.5	
DL-Methionine:	.3		.3	
Carbohydrates (Non-Alcoholic):		47.5%		11.7%
Maltose-Dextrins:	114.0		24.4	
Dextrose Vitamin Mixture:	5.0		5.0	
Carbohydrates (Alcoholic)		0.0%		35.8%
Ethanol:			50.0	
Fats:		35.6%		35.6%
Corn Oil:	8.5		8.5	
Olive Oil:	28.4		28.4	
Ethyl Linoleate:	2.7		2.7	
Salt Mixture:	10.0		10.0	

ing protein and fat as a percent of total calories. The composition of control and alcohol Freund liquid diets is described in Table II.

Administration of these liquid diets was scheduled over two separate periods defined as "chronic" and "acute". During the chronic paradigm, female rats were maintained on either the isocaloric control or alcoholic diet for approximately 2 months prior to conception through the third day postpartum. In contrast, the acute period extended from the fifth day of gestation through the third day postpartum. During the entire acute period, the 6.7% (v/v) alcoholic Lieber DeCarli diet or its isocaloric control was used. In the chronic paradigm the Metrecal based Freund diet was administered until midway through gestation, followed by the Lieber DeCarli diet through the third postpartum day. The specific ethanol percent (v/v) content in the diet during that period in which the Freund diet was used was, in chronological order: one week at 5.1%, two weeks at 7.0%, one week at 9.0%, one week at 11.0%, and four weeks at 7%. In both the chronic and acute study dams fed the alcohol diets were withdrawn from ethanol over a three day period during which withdrawal was moderated by half strength (3.3%, v/v) alcohol diet and free access to chow. During the same three day withdrawal period control dams were given free access to chow in addition to control liquid diet. Actual consumption of ethanol in the chronic and acute studies was approximately 10 grams ethanol per kilogram body weight per day.

ISOTOPE

L- [4,5-^{3}H] leucine (47.6 Ci/mmole) was purchased from Inter-

TABLE II

Freund Liquid Diet

Diet:	Alcohol Based Diet	Control Diets Isocaloric to Metrecal With Ethanol Concentration (v/v) Of:			
	0.0%	5.1%	7.0%	9.0%	11.0%
Substitutent:					
Protein As Percent Of Total Calories:	26.3%	19.9%	18.1%	16.4%	14.9%
Fat As Percent Of Total Calories:	12.4%	9.4%	8.5%	7.8%	7.0%
Metrecal Derived Carbohydrate As Percent Of Total Calories:	61.3%	46.5%	42.3%	38.3%	34.8%
Added Ethanol Or Sucrose As Percent Of Total Calories:	0.0%	24.2%	31.1%	37.5%	43.3%
Total Carbohydrate As Percent Of Total Calories:	61.3%	70.7%	73.4%	75.8%	78.1%

national Chemical and Nuclear Corporation (Irvine, Ca.). D-[U-^{14}C]-glucose (210 mCi/mmole) was purchased from Amersham/Searle (Arlington Heights, Il.).

Leucine was selected as a protein precursor and glucose as a lipid precursor. The metabolic lability of each of these compounds necessitated a determination of the extent to which the leucine ^{3}H-label and the glucose ^{14}C-label actually were partitioned respectively between protein and lipid compartments in subsequently recovered myelin subfraction material.

ADMINISTRATION OF ISOTOPE

An isotope solution containing either 150 µCi L-[4,5-^{3}H]-leucine (100 mCi/mmole) or both 150 µCi L-[4-5-^{3}H] leucine (100 mCi/mmole) and 20 µCi D-[U-^{14}C] glucose (10 mCi/mmole) in 10 µl of 0.85% (w/v) NaCl was prepared. The isotopically labelled solution was administered intracerebrally in young rats either by means of a single 10 µl injection along the midline of the skull or in two divided injections, each 5 µl, on either side of the midline of the skull. Similarly injected dye solutions appeared to be concentrated intraventricularly shortly after injection. Inpenetrability of the skull in pups greater than 50 days in age necessitated a single 10 µl injection intraoccipitally. Injections were made without benefit of stereotaxic technique.

SCHEDULE OF ISOTOPIC INJECTIONS AND SUBSEQUENT MYELIN SUBFRACTIONATION

Pups were injected with either the radioisotopically labelled protein precursor or both the radioisotopically labelled protein and radioisotopically labelled lipid precursors and subsequently sacrificed for preparation of CNS brain myelin subfractions according to a schedule appropriate to the goals of each particular experimental sequence.

Normal myelin subfraction protein and lipid metabolism was examined in young rats in a long term study extending from 18 hours to 85 days following administration of both the protein and lipid radioisotopically labelled precursors to 12-day-old pups. During the interim, myelin was recovered at six to seven selected ages. The long term study was repeated at similar ages and time intervals and with both labelled precursors. Normal myelin subfraction protein metabolism was also examined in young rats in a short term study at intervals of 1, 12, and 24 hours following administration of only the radioisotopically labelled protein precursor to 12-day-old pups.

During each of the above normal series litter size was adjusted to 12 pups per litter at birth, and all litters were weaned at approximately 21 days. However, litter size was not maintained by replacement of sacrificed pups by cold pups prior to weaning.

Myelin subfraction protein and lipid metabolism as perturbed by protein-calorie malnutrition was examined 18 hours following administration of both the radioisotopically labelled protein and lipid

precursors to pups at 17, 24, and 52 days of age. Normal myelin subfraction protein and lipid metabolism was similarly studied in normal, ad libitum chow fed animals. Normal litter size was maintained at 10 pups. The protein-calorie malnourished litters were adjusted to 12 pups per litter at three days of age.

Myelin subfraction protein and lipid metabolism as perturbed by maternal alcohol consumption of either chronic or acute duration was examined in 18-, 25-, and 53-day-old pups following 18-hour pulse labelling by both the protein and lipid radioisotopically labelled precursors. Control, isocalorically fed pups were similarly studied. In the chronic study alcohol and control litters were adjusted to 11 to 12 pups per litter at birth and weaned at 26 days of age. In the acute study, alcohol and control litters were adjusted to 10 to 11 pups per litter at birth.

ISOLATION OF PURIFIED CNS BRAIN WHOLE MYELIN

Animals were sacrificed by decapitation. Whole brain was rapidly removed. Brains were kept at 0^{o} C and myelin preparation followed immediately. Purified whole brain myelin was prepared according to the method of Norton and Poduslo (1973b) employing ultracentrifugation equipment available in this laboratory. Throughout the procedure tissue was kept between 0 and 4^{o} C.

A 5% (w/v) homogenate of whole brain was prepared in 0.32 M sucrose in a powered tissue homogenizer (Kontes Glass Co., Vinland, N.J.) equipped with a tight fitting conical teflon-coated pestle until

the tissue was well homogenized and dispersed. One ml of this homogenate was reserved for subsequent analysis. The remaining 0.32 M sucrose homogenate was layered over 12 to 17 ml of 0.85 M sucrose. The 0.32 M/0.85 M sucrose gradient was centrifuged according to one of the following procedures similar to the procedure originally described by Norton and Poduslo (1973b).

1. SW-25.1 rotor, 23,500 RPM, 40 min, (56,200 $g_{avg.}$ X 40 min = 2,248,000 $g_{avg.}$·min);
2. SW-27 rotor, 22,000 RPM, 40 min, (64,100 $g_{avg.}$ X 40 = 2,564,000 $g_{avg.}$·min);
3. SW-27 rotor, 25,000 RPM, 30 min, (82,500 $g_{avg.}$ X 30 min = 2,475,000 $g_{avg.}$·min).

Interfacial material was recovered by Pasteur pipet and suspended in 38 ml of ice cold distilled water by hand homogenization in a Dounce homogenizer (Kontes Glass Co., Vinland, N.J.) and pelleted by centrifuging for at least 30 minutes at the highest possible speed in either an SS-34 fixed angle rotor, 17,500 RPM (28,200 $g_{avg.}$), or in an SW-27 rotor, 25,000 RPM (82,500 $g_{avg.}$). Pelleted material was osmotically shocked in 38 ml of ice cold distilled water and homogenized by hand in a Dounce homogenizer. Following osmotic shock, material was centrifuged for at least 20 minutes at a $g_{avg.}$ force ranging from 13,000 to 14,000 in an SW-27 or SS-34 rotor.

Pelleted osmotically shocked myelin was resuspended in a total volume of 20 ml of 0.32 M sucrose by hand homogenization in a Dounce homogenizer and the 0.32 M sucrose homogenate layered over 12 to 17 ml

of 0.85 M sucrose. The 0.32 M/0.85 M sucrose gradient was centrifuged as described above. Interfacial material was recovered by Pasteur pipet , suspended in ice cold distilled water and pelleted as described previously.

MYELIN SUBFRACTIONATION

Purified CNS brain whole myelin obtained according to the procedure of Norton and Poduslo (1973b) was immediately subfractionated according to the procedure of Matthieu et al. (1973). Whole myelin was resuspended in 13 ml of 0.32 M sucrose by hand homogenization in a Dounce homogenizer and the 0.32 M sucrose homogenate layered over 12 ml of 0.62 M sucrose layered over 12 ml of 0.70 M sucrose. This 0.32 M/0.62 M/0.70 M sucrose gradient was centrifuged as described previously for the 0.32 M/0.85 M sucrose gradients. Light and medium myelin were recovered at the 0.32 M/0.62 M sucrose and 0.62 M/0.70 M sucrose interfaces, respectively, while heavy myelin was removed as the pellet. The heavy myelin which pelleted through the 0.70 M sucrose layer was a component of material which had previously floated above the 0.85 M sucrose layer. Recovered myelin subfractions were washed and pelleted as described above.

Washed, pelleted, purified myelin subfraction material was taken up by Pasteur pipet in a final volume of one to two ml ice-cold distilled water and kept frozen at or below -20^{o} C until subsequent assays and analytic procedures were performed.

In the above procedures for myelin isolation, purification, and subfractionation stated volumes were adjusted in accordance with

varied rotor capacity. All gradient centrifugations were done with the highest acceleration possible initially, with no braking. Pelleting procedures were done with maximum initial acceleration possible and with modest braking as available on a specific centrifuge.

For all myelin isolation, purification, and subfractionation procedures three brains from a given dietary regime at a given age were processed separately. The only exception was in the case of 18-day-old protein-calorie malnourished pups for which the initial interfacial material from independent preparations was combined in pairs during subsequent steps in the procedures.

Live body weights and fresh wet weights of brains were recorded for all stressed pups and their corresponding controls.

ASSAYS AND ANALYTICAL PROCEDURES

Aliquots of each brain homogenate and water suspension of myelin subfraction were reserved for liquid scintillation counting and protein assay. Additional aliquots were reserved for assay of CNP activity in both long term normal studies. Remaining aqueous suspensions of brain homogenate and myelin subfraction were lyophilized. Lyophilized material in whole or in part was taken from selected representative light, medium, and heavy subfractions from each dietary protocol and at each age and committed for delipidation of protein in preparation for subsequent SDS polyacrylamide gel electrophoretic characterization of myelin subfraction proteins. Similarly, lyophilized material from representative myelin subfractions labelled with D-[U-^{14}C] glucose was committed for extraction of lipids and subsequent lipid analysis follow-

ing thin layer chromatography (tlc) procedures. The specific assays and analytical procedures are described in detail below.

LOWRY PROTEIN ASSAY

Protein content of aliquots of brain homogenate, myelin subfractions, and myelin subfraction protein at various stages in preparation of SDS polyacrylamide gel electrophoresis was determined by the protein assay of Lowry, Rosebrough, Farr, and Randall (1951).

Preparation of Solutions

1. 1 N Sodium Hydroxide

   40 grams of NaOH was dissolved in distilled water to a volume of one liter.

2. 2% (w/v) Sodium Potassium Tartarate

   10 grams of $NaKC_4H_4O_6.4H_2O$ were dissolved in distilled water to a volume of 500 ml.

3. 1% (w/v) Cupric Sulphate

   10 grams of $CuSO_4$ were dissolved in distilled water to a volume of 1 liter.

4. 2% (w/v) Sodium Carbonate

   10 grams of Na_2CO_3 anhydrous were dissolved in distilled water to a volume of 500 ml.

5. 1 N Phenol Reagent

   100 ml of commercially available 2 N preparation of Folin and Ciocalteu Phenol Reagent (Harleco, Philadelphia, Pa.) was dissolved in distilled water to a volume of 200 ml and refrigerated.

6. 0.1% (w/v) Human Serum Albumin

3 mg of Human Serum Albumin (HSA) were dissolved in distilled water to a volume of 3 ml and refrigerated.

Aqueous samples of material to be assayed in less than 60 µl were added to small test tubes. To each sample was added 100 µl of 1 N NaOH. Each test tube was vortexed. Following a 30 minute incubation at room temperature, 1 ml of a mixture of NaKTartarate-$CuSO_4$-Na_2CO_3 (0.1:0.1:10, v/v/v) prepared by addition of reagents in this order was added to each sample. Test tubes were vortexed and incubated at room temperature for 10 minutes. To each test tube was added 100 µl of 1 N Phenol Reagent, followed by immediate vortexing of each test tube, and a 30 minute incubation at room temperature. In the presence of protein, a blue color developed. Before spectrophotometric assay, the blue color was clarified by extraction of any remaining lipid material by addition of 1 ml of $CHCl_3$, vortexing, and low speed centrifugation in a desk top centrifuge. Any remaining lipid material was thereby partitioned into the lower phase material; only the upper phase material was assayed spectrophotometrically. The absorbance of the clarified bluish upper phase was measured spectrophotometrically against a reagent blank at 700 nm. A standard curve was prepared for each assay using 5 to 60 µl of a 1 µg/ul HSA standard solution.

LIQUID SCINTILLATION COUNTING TECHNIQUES

The radioactivity of ^3H or of ^3H and ^{14}C in a number of different kinds of preparations was measured. Regardless of the techniques

employed to extract and solubilize the radioactivity present in such samples, once solubilized, all radioactivity was counted using a toluene soluble POPOP/POP liquid scintillation fluor system.

Preparation of Solutions

1. Liquid Scintillation Fluor

   To one gallon scintillation grade Toluene (ScintillAR, Malinckrodt, Inc., St. Louis, Mo.), was added 200 ml Toluene, 16 grams PPO, (2,5-Diphenyloxazole), scintillation grade (Amersham/Searle, Arlington Heights, Il.), and 0.4 gram POPOP, (p-Bis[2-(5-Phenyloxazolyl)]-benzene), scintillation grade (New England Nuclear, Boston, Ma.).

Most samples were solubilized by NCS Tissue Solubilizer (Amersham/Searle, Arlington Heights, Il.) prior to addition of fluor, except for thin layer chromatography plate scrapings of separated lipid classes and electrophoresed sodium dodecyl sulphate polyacrylamide gel protein bands. The radioactivity in separated lipid bands from thin layer chromatograph plate scrapings was eluted with 0.5 ml benzene according to the procedure of Druse and Hogan (1972). Radioactivity in separated electrophoresed SDS polyacrylamide gel protein bands was eluted by digestion of gel slices in Protosol Tissue Solubilizer (New England Nuclear, Boston, Ma.), Protosol:water (9:1, v/v), at room temperature. To samples suspended in a maximum aqueous volume of 100 µl in a liquid scintillation counting vial was added one ml of NCS, 100 µl of 0.1 N acetic acid, and 10 ml of fluor, in this order.

All samples were counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Ca.) in accordance with a counting protocol in use in this laboratory and previously developed before any of the within described research was begun. The predetermined counting protocol permitted counting of ^{3}H and dual labelled ^{3}H and ^{14}C samples. The following instrument settings were used for all counting runs:

Liquid Scintillation Counting Settings

1. Counting Mode: Auto + Std, (automatically counts sample and determines external standard ratio).
2. Gain Setting: 550 divisions.
3. External Standard Ratio Setting: 0.784.
4. Automatic Quench Control (AQC) Setting: 800 divisions.
5. Fixed Discriminator Channel Isosets: ^{3}H below ^{14}C, and ^{14}C above ^{3}H.

A quench correction curve, previously determined before any of the within described research was begun, was used to assign counting efficiencies to observed external standard ratios. At the settings used approximately 5.0% of actual ^{3}H activity spilled into the ^{14}C counting channel while approximately 14.7% of the actual ^{14}C activity spilled into the ^{3}H counting channel. Observed cpm (counts per minute) data were transformed to dpm (disintegrations per minute) accordingly.

PARTITIONING OF L-[4,5-^{3}H]LEUCINE AND D-[U-^{14}C]GLUCOSE LABEL BETWEEN PROTEIN AND LIPID COMPARTMENTS

The use of dual labels, L-[4,5-^{3}H]leucine and D-[U-^{14}C]glucose, as precursors, respectively, for protein and lipid, necessitated a determination of the extent to which each label was partitioned between the protein and lipid compartments. Partitioning of the ^{3}H label was determined by determining the percent of recovered ^{3}H activity distributed between ether-ethanol (3:2, v/v) insoluble material (i.e., delipidated protein) and soluble material (i.e., lipid). Partitioning of the ^{14}C label was determined by determining the percent of recovered ^{14}C activity distributed between chloroform-methanol (2:1, v/v) soluble material (i.e., lipid) and insoluble material (i.e., protein). The two separate procedures for isolating material from one or the other compartment was necessitated by the different solubility of proteolipid in the two solvent systems. Ether-ethanol (3:2, v/v) extraction leaves all protein in the insoluble material. Chloroform-methanol (2:1, v/v) extracion solubilizes all lipid as well as proteolipid bound protein. It was so determined that 70 percent of the total myelin recovered ^{3}H activity was incorporated into the protein compartment while 30 percent of this activity was incorporated into the lipid compartment. (The incorporation of the leucine ^{3}H label into myelin lipids has been reported by Smith, 1974.) The total recovered myelin ^{14}C activity was partitioned 90 percent into lipid incorporation and 10 percent into protein incorporation. Accordingly, in analysing subsequent data, determination of corrected radioactivity expressed in terms of dpm necessitated transformation of cpm data to reflect label partitioning as well as spill be-

tween the ^{3}H and ^{14}C channels and counting efficiencies. The observed label partitioning necessitated transformation of cpm into dpm on the basis of spillover and counting efficiencies because all samples to be counted contained significant proportions of both ^{3}H and ^{14}C in dual labelled experiments with the exception of delipidated myelin protein prepared for electrophoresis which contained negligible ^{14}C. Accordingly, the distribution of ^{3}H amongst electrophoretically separated myelin proteins was nearly equivalent expressed either in terms of cpm or dpm.

CNP ASSAY

The activity of 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNP) was determined by the assay of Kurihara and Tsukada (1967). The assay was applied to aliquots of material taken from aliquots of homogenates and myelin subfractions taken from the two long term normal studies. The enrichment of CNP in myelin subfraction material compared to brain homogenate as a measure of myelin purity was of greater interest than the distribution of the enzymatic activity between the light, medium, and heavy myelin subfractions during development. Since this enzyme activity is membrane bound, activity was uniformly assayed after only one freezing and thawing of the material to be assayed. Repeated freezing and thawing may artifactually increase enzymatic assay levels by release of bound enzyme.

Preparation of Solutions

1. 0.03 M Sodium Adenosine 2',3'-Cyclic Phosphate

   11.62 mg Na Adenosine 2',3'-Cyclic Phosphate (2',3'-cAMP)·$2H_2O$ per ml water, MW = 387.4 (Sigma Chemical Co., St. Louis, Mo.)

2. 0.2 M Sodium Dibasic Phosphate - 0.1 M Citric Acid Buffer, pH 6.2

   16.9 ml of 0.1 M Citric Acid ($C_6H_7O_8$), (1.92 g/100 ml), was added to 33.1 ml of 0.2 M NaDibasicPO_4, (5.38 g/100 ml) and the mixture diluted with distilled water to a volume of 1 liter.

3. 1% (v/v) Triton X-100

   1 ml of Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was diluted with distilled water to a volume of 100 ml and the mixture refrigerated.

4. Glacial Acetic Acid

5. Isopropanol:Concentrated NH_4OH:H_2O (7:1:2, v/v/v)

6. 0.01 N Hydrochloric Acid

   Concentrated HCl, 11.6 N, was diluted with distilled water 1160-fold.

Prior to incubation the following was added to test tubes at room temperature: 50 µl phosphate-citric acid buffer (pH 6.2), and 80 µl distilled water. Samples whose CNP enzymatic activity was to be assayed were dissolved in 100 µl total volume of 1% (v/v) Triton X-100 such that 20 µl of this stock solution of solubilized assay material contained approximately 5 µg homogenate protein or 2 to 4 µg myelin protein based on previous protein assay (Lowry et al., 1951) determinations. Test tubes containing pre-dispensed substrate, buffer, and water were placed in a 37° C shaking water bath. At regular intervals, 20 µl of stock solution of solubilized enzymatic activity to be assayed were added to each incubated test tube. The incubation reaction was

timed and stopped at 20 minutes by the addition of 20 µl glacial acetic acid and removal of incubated test tubes to a bed of crushed ice. Unreacted substrate (i.e., 2',3'-cAMP) and product (i.e., 3'-adenosine monophosphate) (3'-AMP) were separated in a subsequent paper chromatographic procedure. Measurement of quantity of product formed per unit weight of protein per unit time was determined thereafter by a spectrophotometric assay.

Paper chromatographic separation of substrate and product was effected by spotting Whatman paper (Whatman Ltd., England) with 20 µl of the incubated reaction mixture and subsequently chromatographing in an equilibrated tank containing isopropanol:concentrated NH_4OH:water (7:1:2, v/v/v) for approximately three-and-a-half hours. Following the chromatographic run and drying of paper, spots indicating product (nearest the origin) and substrate (nearest the solvent front) were identified by visualization under uv light and circled. Areas of the paper containing product, substrate, and any comparably sized region of the paper below the solvent front and above the substrate (designated as the paper blank) were cut out, shredded, and absorbed material was eluted from the paper by incubation at room temperature with 4 ml of 0.01 N HCl for 2 hours in test tubes placed in a shaker.

The optical densities of substrate and product eluates were read against the paper blank at 260 nm in a spectrophotometer. Total activity of CNP expressed as µmole product formed per hour per mg protein was determined according to the following calculations:

Calculation of Total and Specific Activity of CNP

1. S = (Optical density of substrate) - (Optical density of paper blank).
2. P = (Optical density of product) - (Optical density of paper blank).
3. X = $\frac{S}{1.5}$ = Optical density per µmole product formed per 20 µl aliquot.
4. Y = (3) · (P) ÷ (X) = µmole product formed per 20 µl of assay per hour.
5. Z = (Y) ÷ (mg protein present in 20 µl of assay) = µmole product formed per hour per mg protein = specific activity.
6. Total activity = (Z) · (total mg protein) = µmole product formed per hour.

CHARACTERIZATION OF MYELIN SUBFRACTION PROTEINS BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Characterization of myelin subfraction proteins by SDS polyacrylamide gel electrophoresis involved a number of steps, i.e., delipidation of myelin subfraction protein, solubilization of protein, gel preparation, actual electrophoresis, fixation, staining, and destaining of gels, desitometric analysis of gel scans, and recovery of radioactivity in separated protein.

Lyophilized myelin subfraction material was delipidated with diethyl ether:ethanol (3:2, v/v) according to the procedure of Greenfield, Norton, and Morell (1971). One ml of diethyl ether:ethanol (3:2, v/v) was added to a sample of lyophilized myelin subfraction

containing 1 to 3 mg myelin protein in a test tube and vortexed. After centrifugation for 10 minutes at 2500 to 2000 RPM in a table top centrifuge the ether:ethanol supernatant was drawn off by Pasteur pipet and discarded. Delipidation was similarly repeated once or twice. Samples were dried carefully under a stream of nitrogen, and immediately solubilized.

Delipidated lyophilized myelin subfraction protein was solubilized according to the procedure of Quarles et al. (1973b). Protein to be solubilized was vortexed and macerated as necessary in solubilizing solution for a minimum of 4 to 8 hours in tightly capped test tubes. Solubilizing solution was used in the proportions of 1 µl solubilizing solution to 1 to 2 µg protein. Solubilizing solution was prepared fresh monthly or more frequently. Solubilizing solution was 2.5% (w/v) in sodium dodecyl sulphate (SDS), 1.0% (w/v) in sodium carbonate, and 10.0% (v/v) in β-mercaptoethanol.

Preparation of Solubilizing Solution

1. To 100 ml of a stock solution of 5.0% (w/v) SDS (50 grams SDS per liter) was added 20 ml of a stock solution of 10.0% (w/v) Na_2CO_3 (50 grams Na_2CO_3 per 500 ml) and 20 ml of β-mercaptoethanol with dilution by distilled water to a volume of 200 ml.

Upon completion of solubilization, solubilized protein was dialyzed overnight against a dialyzing solution prepared fresh. The dialyzing solution was 0.1% (w/v) in SDS, 0.01 M in sodium phosphate buffer (pH 7.2), 1.6 M in urea, and 0.05% (w/v) in dithiothreitol.

Sufficient dialyzing solution was prepared to provide 100 to 200 ml dialyzing solution per ml of solubilized protein solution.

Preparation of Solutions

1. 1 M Phosphate Buffer, pH 7.2 (pH range 7.1 to 7.2 acceptable)

   39.0 grams Sodium Monobasic Phosphate·H_2O ($NaH_2PO_4 \cdot H_2O$) and 192.0 grams Sodium Dibasic Phosphate·$7H_2O$ ($Na_2HPO_4 \cdot 7H_2O$) were dissolved in distilled water to a volume of 1 liter.

2. Dialyzing Solution

   To 20 ml of a stock solution of 5.0% (w/v) SDS was added 10 ml of 1 M Sodium Phosphate Buffer, 200 ml of a stock solution of 8 M Urea (CH_4N_2O) (480.5 grams Urea per liter), and 500 mg Dithiothreitol ($C_4H_{10}O_2S_2$) with dilution by distilled water to a volume of 1 liter. Proportions were adjusted to make the minimum volume necessary for total volume of solubilized protein solution currently being dialyzed.

The optimal amount of protein to be electrophoresed on a given gel for visualization of the stained protein and resolution of bands was 150 to 200 µg. Larger amounts were electrophoresed to recover greater radioactivity from electrophoresed gels. SDS polyacrylamide gels were prepared and myelin subfraction protein electrophoresed according to the procedure of Druse et al. (1974).

Preparation of Solutions

1. Gel Solution A1

   To 8 ml of a stock solution of 5.0% (w/v) SDS was added 40 ml of

1 M sodium Phosphate Buffer (pH 7.2) with dilution by distilled water to a volume of 180 ml. Solution A1 was made fresh monthly.

2. Gel Solution A2

   40 grams Acrylamide (Eastman Kodak Co., Rochester, N.Y.) and 1.040 grams N,N'-methylenebisacrylamide (Eastman Kodak Co., Rochester, N.Y.) were dissolved in distilled water to a volume of 180 ml. Solution A2 was made fresh monthly.

3. Gel Solution B

   90 mg Ammonium Persulfate and 50 ml of N,N,N',N'-Tetramethylethylenediamine (TEMED), (Eastman Kodak Co., Rochester, N.Y.) were dissolved in distilled water to a volume of 10 ml. This solution was made fresh immediately before making gels.

4. Electrophoresis Chamber Buffer Solution

   Buffer Solution 0.1% (w/v) in SDS and 0.1 M in Phosphate Buffer (pH 7.2) was made by adding 20 ml of a stock solution of 5.0% (w/v) SDS to 100 ml of 1.0 M Phosphate Buffer (pH 7.2) with dilution by distilled water to a volume of 1 liter. This solution was made fresh in sufficient volume for adequate electrophoresis buffering capacity.

Gels were prepared by quickly mixing 22.5 ml each of gel solutions A1 and A2 with 5 ml of gel solution B. The final mixture was deaerated for 30 to 60 seconds. Approximately 2.5 ml of this mixture was quickly and carefully pipetted into previously cleaned glass gel tubes (internal diameter 6 mm). Gels were immediately overlayered with a few drops of water. Optimal polymerization was indicated

by appearance, disappearance, and reappearance of a sharp interfacial difference in refractive index between the gel solution and the overlayered water, visible within 30 minutes. Polymerized gels of approximately 5 to 6 cm in length were used within a day of preparation. Dialyzed, solubilized protein in a load of 150 to 200 µg protein, as determined by protein assay (Lowry et al., 1951), was electrophoresed in electrophoresis chambers with sufficient buffer present. Gels were electrophoresed usually for approximately 24 hours. Maximum voltage never exceeded 50 volts, and maximum amperage per gel never exceeded 6 to 7 ma. Bromophenol Blue was used as a tracking dye migrating ahead of any protein. Gel electrophoresis was terminated when the dye front was within 5 mm of the gel tube end. Gel material below the lower half of the dye was discarded after completion of electrophoresis.

Gels were rapidly removed from gel tubes after incubation in ice-cold water for a maximum of one hour. Gels were fixed, stained, and destained according to the procedure of Greenfield et al. (1971).

Preparation of Solutions

1. Fixing Solution

   Methanol:Glacial Acetic Acid:Distilled Deionized Water (45:10:45, v/v/v)

2. 1.0% (w/v) Fast Green Staining Solution

   10 grams Fast Green Dye dissolved in Fixing Solution, Methanol: Acetic Acid:Water (45:10:45, v/v/v) to a volume of 1 liter. Staining solution was mixed and filtered prior to use.

3. Destaining Solution

Identical to Fixing Solution

Electrophoresed gels were fixed in capped test tubes and gently shaken for variable periods ranging from 3 to 7 days minimum with frequent changes of fixing solution. Fixation was continued until optimum clarity of gels were observed and the appearance of white flocculent material was minimal. Gels were stained with 1% (w/v) Fast Green for 1.5 to 2.0 hours. At the end of the staining period gels were immediately rinsed with tap water, distilled water, and fixing solution. They were kept in capped test tubes and gently shaken in a shaker with frequent changes of fixing solutions until destaining was optimal. Gels were kept in fixing solution as long as gels were kept intact. Alternatively, gels were destained in a diffusion destainer.

Relative distribution of dye binding amongst the various myelin subfraction proteins was quantitated by densitometric scanning in a densitometer reading absorbance at 560 nm followed by quantification of the weight under peaks in a copy of the desitometric scan tracing according to the procedure of Greenfield et al. (1971). No correction was made for difference in dye binding between the major subfraction proteins.

As described earlier, the radioactivity in separated myelin subfraction myelin protein bands from electrophoresed SDS polyacrylamide gels was eluted by digestion of gel slices in Protosol:water (9:1, w/v). Digestion at room temperature was continued for approx-

imately three to six days with periodic vortexing of minced gel slices before fluor was added to scintillation vials.

CHARACTERIZATION OF MYELIN SUBFRACTION LIPIDS BY THIN LAYER CHROMATOGRAPHY

Characterization of myelin subfraction lipids by thin layer chromatography (tlc) involved a number of steps, i.e., extraction of lipid, the thin layer chromatographic separation itself, visualizing chromatographically separated components, and recovery of radioactivity from separated lipids. These procedures were only applied to representative myelin subfractions labelled with D[U-^{14}C]glucose.

Lipid was extracted from lyophilized myelin subfraction material according to the method of Folch-Pi, Lees, and Sloane-Stanley (1957). One ml of chloroform:methanol (2:1, v/v) was added to a sample of lyophilized myelin subfraction containing 1 to 3 mg protein in a test tube and vortexed. Extraction was continued for at least 30 minutes followed by centrifugation of test tubes at 2500 to 3000 RPM for at least 10 minutes in a table top centrifuge. Extracted lipid was drawn off by Pasteur pipet . Lipid extraction was similarly repeated 2 to 3 times, and lipid extracts were pooled. One ml ether was added to the remaining insoluble material. Following similar extraction, vortexing, and centrifuging the ether soluble material was pooled with chloroform:methanol (2:1 v/v) soluble material. The insoluble material was discarded. The soluble material was carefully dried under a stream of nitrogen. Lipid was resolubilized in a known volume of chloroform:methanol (2:1, v/v) and kept in tightly capped

test tubes.

Aliquots of extracted lipid were separated into major lipid classes, i.e., in order of decreasing mobility during tlc separation, cholesterol, cerebroside, sulfatide, and phospholipid, according to the procedure of O'Brien, Fillerup, and Mead (1964). Extracted lipid, solubilized in chloroform:methanol (2:1, v/v) was spotted on Silica-G, 250 μ thick tlc plates (New England Nuclear, Boston, MA.) and chromatographed in an equilibrated tank containing chloroform:methanol:concentrated NH_4OH (80:20:0.4, v/v/v). Either a single lane was spotted with each of the cerebroside and sulfatide standards (Supelco, Bellefonte, Pa.) or separate standard lanes were spotted with one or the other standard, respectively.

Chromatographically separated lipid classes were visualized and tentatively identified by spraying dried plates with 2',7'-dichlorofuorescein (Supelco, Bellefonte, Pa.). Separated lipid classes were visualized under uv light. Plates were marked to indicate the location of separated lipids. Individually separated lipid classes were scraped from the plate into scintillation counting vials. Confirmation of the identity of separated lipid classes was effected by spraying the standard lanes with Gal's spray, Ammonium Bisulfate, 30% (w/v) according to the procedure of Gal (1968). The plate was charred by incubation in an oven at 140^{o} C for at least 30 minutes.

The radioactivity in plate scraping was eluted with 0.5 ml of benzene according to the procedure of Druse and Hogan (1972) as described previously. Following vortexing, 10 ml of fluor was added, and

samples were counted by liquid scintillation counting as previously described.

CHAPTER III

EXPERIMENTAL RESULTS

NORMAL LONG TERM METABOLISM

Whole brain homogenate and myelin subfraction protein and lipid metabolism were studied in normal Holtzman albino rats maintained on a normal ad libitum feeding regimen. Various parameters of protein and lipid metabolism were examined over the interval ranging from approximately 18 hours to approximately 90 days following intracerebral injection of both L- [4,5-^{3}H] leucine and D- [U-^{14}C] glucose at 12 days of age.

Over the range of age from 13 to 97 days of age while brain homogenate protein increased 1.5 fold (Table III), dramatic increases were observed in each of the myelin subfractions (Table IV). Over this age range myelin accretion was approximately 18-fold for light myelin, 44-fold for medium myelin, and 9-fold for heavy myelin. Concomitant to dramatic myelin subfraction protein accretion there was also observed a shift in distribution of total myelin protein amongst the three subfractions. At the earliest age examined, 13 days, over half (53 percent) of total myelin protein was recovered in heavy myelin (Table IV). With increasing age the predominant proportion of total myelin protein was recoverable in light myelin.

Incorporation of the ^{3}H label, primarily into protein, and the ^{14}C label, almost exclusively into lipid, over the period of approximately 18 hours to 90 days following intracerebral injection of 12-day-old pups

TABLE III

PROTEIN CONTENT OF BRAIN HOMOGENATES OF DEVELOPING RATS

AGE IN DAYS:	13	16	22	29	43	64	97
HOMOGENATE PROTEIN MG:	107 ± 8	106 ± 12	129 ± 1	188 ± 20		183 ± 13	164 ± 27
NUMBER OF SAMPLES:	6	6	3	3	0	2	2

Each value represents the mean ± the standard deviation.

TABLE IV

PROTEIN CONTENT AND DISTRIBUTION IN MYELIN SUBFRACTIONS OF DEVELOPING RATS

AGE IN DAYS	MG PROTEIN/BRAIN			PERCENT OF TOTAL MYELIN PROTEIN IN INDIVIDUAL SUBFRACTIONS			NUMBER OF SAMPLES
	LIGHT	MEDIUM	HEAVY	LIGHT	MEDIUM	HEAVY	
13	0.27 ± 0.06	0.10 ± 0.04	0.41 ± 0.23	34	13	53	6
16	0.49 ± 0.08	0.19 ± 0.08	0.22 ± 0.07	54	21	24	3
22	1.48 ± 0.27	0.47 ± 0.07	0.08 ± 0.05	69	22	9	3
29	2.97 ± 0.42	0.98 ± 0.11	0.90 ± 0.52	61	20	19	6
43	6.36 ± 0.66	2.30 ± 0.08	2.20 ± 0.33	59	21	20	3
64	6.01 ± 1.03	3.66 ± 1.35	2.97 ± 0.71	48	29	23	5
97	5.04 ± 0.96	4.40 ± 1.11	3.77 ± 1.37	38	33	28	3

Each value represents the mean ± the standard deviation.

resulted in patterns of incorporation different for myelin subfractions compared to whole brain homogenate. Over the interval 18 hours to 85 days post injection the recovery of ^{3}H DPM in myelin subfractions increased approximately 2-fold for light myelin, 8-fold for medium myelin, and 5-fold for heavy myelin, whereas during the same interval the recovery of ^{3}H DPM in whole brain homogenate decreased 94 percent (Table V). A somewhat similar pattern obtained when measuring ^{14}C DPM radioactivity. Over the same interval ^{14}C DPM radioactivity increased approximately 2-fold for light myelin, 3- to 4-fold for medium myelin, and 2-fold for heavy myelin, whereas a 91 percent decrement was observed for whole brain homogenate (Table VI).

Specific radioactivity is tabulated in Tables VII and VIII for two separate but similar long term series and also graphically presented for Series I in Figure 1. Table VII was prepared by computing the quotient of total ^{3}H DPM per µg protein for each fraction. Table VIII was prepared by computing the quotient of total ^{14}C DPM per µg protein for each fraction. Because approximately 70 percent of total recoverable myelin ^{3}H radioactivity is found in the protein compartment, Table VII is an approximate measure of specific radioactivity for the protein compartment. Because approximately 90 percent of total recoverable myelin ^{14}C activity is found in the lipid compartment, Table VIII is an indirect measure of specific radioactivity for the lipid compartment. Since by dry weight myelin is composed of approximately 70 percent lipid and 30 percent protein the foregoing approximate measures need to be transformed into specific radioactivities per µg protein or lipid. Figure 1 represents this transformation for data from Tables

TABLE V

METABOLISM OF [^{3}H] LEUCINE IN MYELIN SUBFRACTIONS OF DEVELOPING RATS

TIME AFTER INJECTION	^{3}H X 10^{-3} (DPM) LIGHT MYELIN	MEDIUM MYELIN	HEAVY MYELIN	^{3}H X 10^{-6} (DPM) BRAIN HOMOGENATE
18 HOURS	84 ± 36	13 ± 11	13 ± 7	16.2 ± 1.8
4 DAYS	108 ± 44	39 ± 12	27 ± 11	13.5 ± 2.3
10 DAYS	119 ± 29	22 ± 4	9 ± 1	3.0 ± 0.6
17 DAYS	160 ± 5	31 ± 6	19 ± 4	3.4 ± 0.1
31 DAYS	183 ± 56	42 ± 4	41 ± 11	*
52 DAYS †	183 ± 7	53 ± 6	49 ± 6	1.4 ± 0.2
85 DAYS †	175 ± 11	109 ± 13	68 ± 3	0.9 ± 0.1

Twelve-day-old rats were injected with [^{3}H] leucine and sacrificed at intervals from 18 hours to 85 days later. Myelin was subfractionated as described in the text. Each value represents the mean of three values ± the standard deviation. *Indicates that no sample was available for analysis. †Indicates that only two rats were available for analysis at ages 52 and 85 days.

TABLE VI

METABOLISM OF [^{14}C] GLUCOSE IN MYELIN SUBFRACTIONS OF DEVELOPING RATS

TIME AFTER INJECTION	^{14}C X 10^{-3} (DPM) LIGHT MYELIN	MEDIUM MYELIN	HEAVY MYELIN	^{14}C X 10^{-6} (DPM) BRAIN HOMOGENATE
18 HOURS	13 ± 5	2 ± 1	2 ± 0.6	0.97 ± 0.25
4 DAYS	19 ± 10	6 ± 1	4 ± 0.7	0.77 ± 0.18
10 DAYS	18 ± 4	5 ± 4	4 ± 3	0.21 ± 0.04
17 DAYS	24 ± 0.5	8 ± 6	3 ± 0.5	0.36 ± 0.07
31 DAYS	29 ± 8	5 ± 1	4 ± 1.2	*
52 DAYS †	25 ± 2	6 ± 0.1	4 ± 0.1	0.18 ± 0.01
85 DAYS †	18 ± 2	7 ± 0.8	3 ± 0.1	0.09 ± 0.001

Twelve-day-old rats were injected with [^{14}C] glucose and sacrificed at intervals from 18 hours to 85 days later. Myelin was subfractionated as described in the text. Each value represents the mean of three values ± the standard deviation. *Indicates that no sample was available for analysis. †Indicates that only two rats were available for analysis at ages 52 and 85 days.

TABLE VII

^{3}H SPECIFIC RADIOACTIVITY (DPM/μ GRAM PROTEIN)

Series	Age In Days	Days After Injection	Light Myelin	Medium Myelin	Heavy Myelin	Brain Homogenate
I	13	1	283.7 ± 82.5	69.1 †	66.1 ± 29.6	148.3 ± 19.5
I	16	4	210.0 ± 60.9	212.3 ± 32.5	146.1 ± 94.7	121.3 ± 15.6
I	22	10	78.7 ± 8.2	47.6 ± 8.0	51.0 ± 9.7	23.6 ± 5.7
I	29	17	61.7 ± 6.3	32.0 ± 3.6	28.1 ± 2.5	18.4 ± 2.7
I	43	31	28.0 ± 8.1	18.5 ± 2.3	18.2 ± 3.9	*
I	64	52	32.6 †	24.1 †	19.8 †	7.9 †
I	97	85	32.3 †	26.7 †	23.4 †	5.9 †
II	13	1	193.1 ± 26.5	200.2 ± 32.9	127.3 ± 6.9	134.2 ± 19.7
II	15	3	170.1 ± 32.3	1917. ± 176.4	1327.4 ± 742.8	*
II	21	9	80.4 ± 31.0	68.9 ± 28.5	68.0 ± 24.5	*
II	52	40	37.5 ± 14.4	22.7 ± 3.6	20.8 ± 4.7	10.4 ± 2.4
II	68	56	38.4 ± 20.0	19.4 ± 7.3	14.5 ± 4.8	6.3 †
II	104	92	15.3 ± 2.8	13.8 ± 1.0	13.4 ± 2.1	3.8 ± 0.8

Values represent the mean of three samples ± the standard deviation. †Indicates mean values only in cases for which only two samples were available. *Indicates that no sample was available for analysis. Animals in both Series I and II received similar injections of isotopically labelled precursors at 12 days of age. Series II animals represent a separate population of subjects begun on a similar experimental protocol after Series I was in progress. That is, all animals within a given series received identical injections from a common isotopic preparation prepared fresh for each series. Additional details of isotopic administration are provided in text. Data for each fraction represent the quotient of total recovered ^{3}H DPM divided by total protein. It is not corrected for spill of the leucine ^{3}H label into lipid material. As stated, the data are relative measures of protein metabolism.

TABLE VIII

^{14}C SPECIFIC RADIOACTIVITY (DPM/μ GRAM PROTEIN)

Series	Age in Days	Days After Injection	Light Myelin	Medium Myelin	Heavy Myelin	Brain Homogenate
I	13	1	45.9 ± 10.0	19.0 ± 14.3	13.3 ± 1.4	8.8 ± 1.0
I	16	4	37.8 ± 8.8	30.3 ± 5.6	18.5 ± 8.7	6.9 ± 1.5
I	22	10	11.9 ± 0.8	5.6 ± 1.6	6.0 ± 0.7	1.6 ± 0.4
I	29	17	9.3 ± 1.3	7.8 ± 5.8	4.1 ± 1.2	2.0 ± 0.6
I	43	31	4.4 ± 1.0	2.0 ± 0.6	1.6 ± 4	*
I	64	52	4.6 †	2.7 †	1.8 †	0.8 †
I	97	85	3.3 †	1.7 †	0.9 †	0.6 †
II	13	1	23.2 ± 4.7	22.3 ± 5.0	10.1 ± 0.7	6.6 ± 1.3
II	15	3	9.5 ± 6.2	364.7 ± 167.3	107.1 ± 64.5	*
II	21	9	8.8 ± 3.2	5.8 ± 2.5	2.9 ± 0.7	*
II	52	40	4.2 ± 1.6	2.2 ± 0.5	1.3 ± 0.3	0.8 ± 0.1
II	68	56	4.3 ± 2.8	1.9 ± 1.0	1.2 ± 0.7	0.4 †
II	104	92	1.9 ± 0.5	1.5 ± 0.1	1.4 ± 0.1	0.3 ± 0.03

Values represent the mean of the samples ± the standard deviation. †Indicates mean values only in cases for which only two samples were available. *Indicates that no sample was available for analysis. Animals in both Series I and II received similar injections of isotopically labelled precursors at 12 days of age. Series II animals represent a separate population of subjects begun on a similar experimental protocol after Series I was in progress. That is, all animals within a given series received identical injections from a common isotopic preparation prepared fresh for each series. Additional details of isotopic administration are provided in text. Data for each fraction represent the quotient of total recovered ^{14}C DPM divided by total protein. It is not corrected for entry of the glucose ^{14}C label into lipid primarily nor for the high lipid content of myelin. As stated, data are relative measures of lipid metabolism.

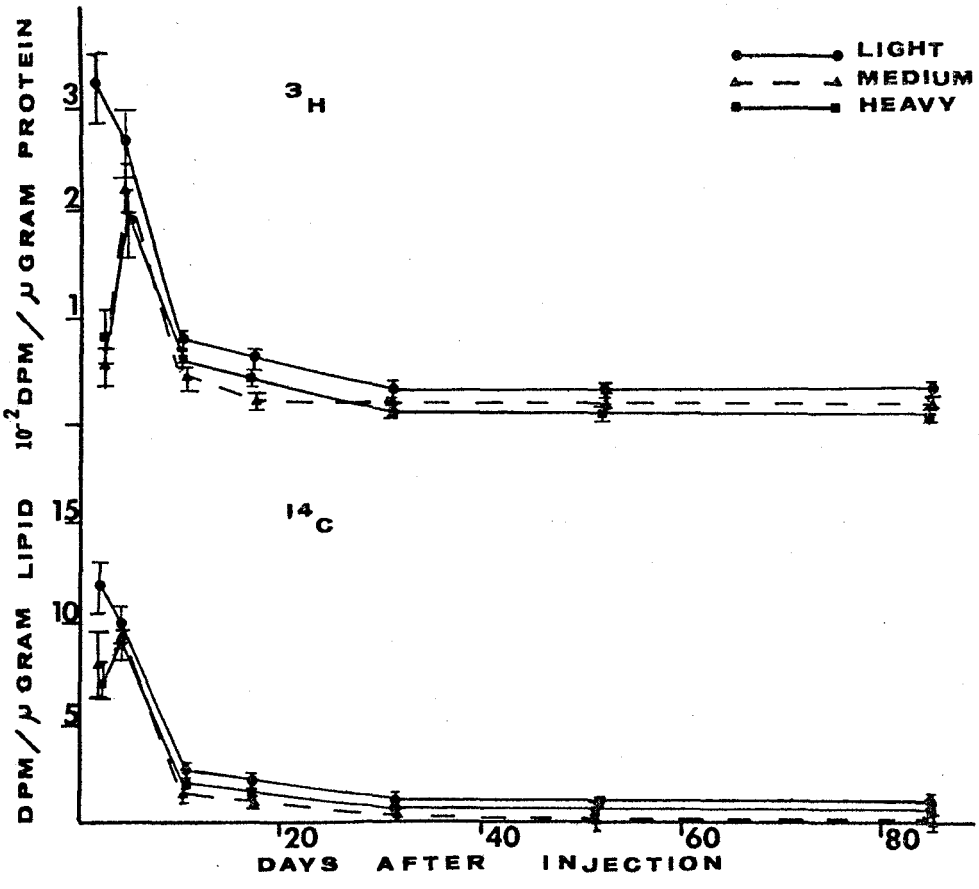

FIGURE 1. Graphical representation of specific radioactivity (^{3}H DPM/μ gram protein and ^{14}C DPM/μ gram lipid) changes in myelin subfractions following administration of [^{3}H]leucine and [^{14}C]glucose at 12 days of age. Data represent information, corrected accordingly, from Tables VII and VIII for animals in Series I. Graphic representation is corrected to reflect actual partitioning of either label into protein and lipid compartments and to reflect the proportion of protein and lipid present in myelin. A similar graphical representation would obtain were information from Tables VII and VIII corrected accordingly for Series II animals. Standard deviations are indicated by vertical bars.

VII and VIII corrected accordingly to more accurately reflect protein and lipid specific radioactivities.

Tables VII and VIII and Figure 1 demonstrate similar trends for specific radioactivities of protein and lipid in either Series I or II. Over the two approximately 90-day intervals following administration of isotopically labelled protein and lipid precursors the following trends were observed. Specific radioactivities declined in time for all three subfractions of myelin and for whole brain homogenate except for an early peak in specific activities in medium and heavy myelin seen at 4 and 3 days following injection in Series I and II, respectively. A similar "delayed" peak in specific radioactivity was observed in somewhat similarly prepared "heavy myelin" and "membrane fraction" 2 days following administration of [2,3-^{3}H] tryptophan (Agrawal et al, 1974a).

In Series I over the interval 18 hours to 85 days post injection protein specific radioactivity declined 89, 61, 65, and 96 percent, respectively, in light, medium, and heavy myelin and whole brain homogenate. Similarly, in Series II over the interval 18 hours to 92 days post injection corresponding declines in protein specific radioactivity were 92, 93, 89, and 97 percent, respectively. The early peak in protein specific radioactivity in medium and heavy myelin, respectively, was 3 times greater and 2.2 times greater after 4 days than at the original 18 hour time point in Series I, contrasted with corresponding peaks in protein specific radioactivity 9.5 times greater and 10.4 times greater, respectively, after 3 days than at the original 18 hour time point in Series II. In Series I lipid specific radioactivity declined 93, 91, 93, and 93 percent, respectively, in light, medium, and heavy myelin subfractions

and in whole brain homogenate. Similarly, in Series II corresponding percentage declines in lipid specific radioactivity were 92, 93, 86, and 95 percent, respectively. The early peak in lipid specific radioactivity in medium and heavy myelin, respectively, was 1.5 times greater and 1.4 times greater after 4 days than at the original 18 hour time point in Series I, contrasted with corresponding peaks in lipid specific radioactivity 16 times greater and 10.6 times greater, respectively, after 3 days than at the original 18 hour time point in Series II.

Representative Fast Green stained electrophoresed SDS gels of myelin protein are illustrated in Figure 2. The electrophoretic pattern illustrated for each myelin subfraction was similar at each age point.

An analysis of Fast Green stained electrophoresed SDS gels of myelin subfractions over the age range 13 to 97 days of age revealed a common pattern. Large basic protein in the system employed here appeared as a split band as reported previously (Allison, Agrawal, and Moore, 1974). Basic protein accounted for approximately 53, 45, and 30 percent, respectively, of total dye binding in light, medium, and heavy myelin subfractions. Proteolipid protein accounted for approximately 25, 28, and 31 percent of total dye binding in light, medium, and heavy myelin subfractions. HMW accounted for approximately 22, 26, and 38 percent of total dye binding in light, medium, and heavy myelin subfractions. In agreement with Matthieu, et al (1973) basic protein was enriched in light myelin and high molecular weight proteins in heavy myelin. In contrast to Matthieu, proteolipid protein was somewhat more enriched in heavy myelin than lighter subfractions.

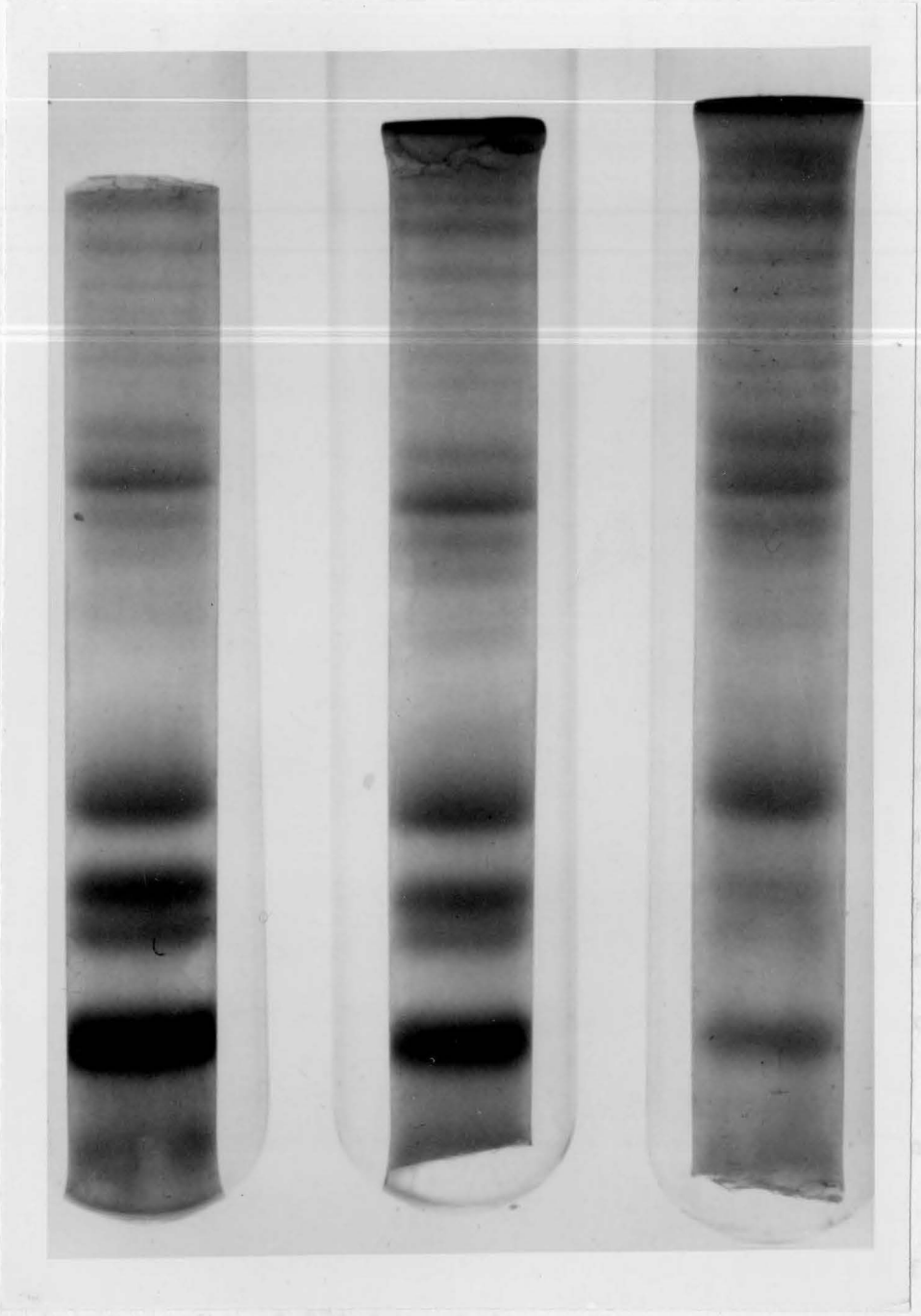

FIGURE 2. SDS-polyacrylamide gel electrophoresis of myelin proteins from the (A) light, (B) medium, and (C) heavy subfractions of 64-day-old rats. Each gel was loaded with 200 µg of myelin protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

The percentage distribution of ^{3}H incorporation into major myelin proteins separated by electrophoresis on SDS gels was determined over the interval 18 hours to 85 days post injection. Approximately 60-70 percent of solubilized ^{3}H radioactivity was associated with basic protein and proteolipid proteins in the light and medium myelin subfraction whereas in the heavy myelin subfraction over 50 percent of the solubilized ^{3}H radioactivity recovered from electrophoresed gels was found associated with high molecular weight proteins.

Also over the time interval 18 hours to 85 days post injection the distribution of solubilized, recovered ^{14}C DPM radioactivity was determined amongst the major lipid classes separated by thin layer chromatography. The share of recovered ^{14}C DPM radioactivity found associated with galactosphingolipids (cerebroside and sulfatide) was approximately 53, 46 and 43 percent respectively in light, medium, and heavy myelin subfractions, respectively. The percent of ^{14}C DPM radioactivity associated with phospholipids was approximately 17-18 percent in the light and medium myelin subfractions and 25 percent in the heavy myelin subfraction. While there was a modest enhanced relative incorporation of ^{14}C into galactosphingolipids of lighter subfractions and into phospholipids of heavier subfractions, the pattern observed for relative incorporation of ^{14}C into cholesterol was equivocal. The proportion of recovered ^{14}C DPM radioactivity found associated with cholesterol varied from approximately 25 to 31 to 28 percent, respectively for light, medium, and heavy subfractions. Proteolipid which comigrates with phospholipids in the tlc system employed contributed

negligibly to the radioactivity recovered in the phospholipid band. This conclusion was drawn from the observation of negligible ^{14}C radioactivity associated with separated proteolipid protein on SDS gels.

The specific activity of the enzyme CNP was determined for myelin subfractions and whole brain homogenate over the age range 13 to 97 days of age (Table IX). The specific activity declined with age, except for an increase in specific activity in medium and heavy subfractions between 13 and 16 dyas of age. There were no other developmental or subfraction differences. The specific activity of CNP was higher in each of the myelin subfractions than in whole brain homogenate at all the ages examined.

TABLE IX

SPECIFIC ACTIVITY† OF CNP

(2',3'-cyclic NUCLEOTIDE 3'-PHOSPHOHYDROLASE)

Age in Days	Light Myelin	Medium Myelin	Heavy Myelin	Brain Homogenate
13	1054 ± 298 (5)	1255 ± 395 (6)	636 ± 394 (6)	498 ± 30 (3)
16	894 ± 472 (5)	2049 ± 596 (4)	1368 ± 197 (5)	*
22	569 ± 154 (6)	602 ± 149 (6)	690 ± 468 (6)	*
29	765 ± 299 (5)	837 ± 383 (6)	845 ± 252 (4)	324 ± 43 (3)
43	493 ± 83 (3)	525 ± 55 (3)	521 ± 108 (3)	*
64	493 ± 161 (5)	546 ± 166 (5)	510 ± 187 (5)	333 ± 93 (5)
97	484 ± 76 (5)	570 ± 41 (5)	535 ± 160 (5)	270 ± 67 (5)

CNP activity assayed according to the procedure of Kurihara and Tsukada (1967a). †Specific activity expressed as μmoles 3'-AMP formed/hour/mg protein as the mean ± the standard deviation. Number of samples assayed in parentheses. *Indicates that no sample was available for analysis.

SHORT TERM METABOLISM

Whole brain homogenate and myelin subfraction protein metabolism was studied in normal Holtzman albino rats maintained on a normal, ad libitum feeding regimen. Parameters of protein metabolism were examined at intervals, 1, 12, and 24 hours following intracerebral injection of L- [4,5-^{3}H] leucine at 12 days of age.

Over the 24-hour period examined while brain homogenate protein increased 7 percent (Table X), myelin subfraction protein accretion was very rapid. During that interval protein accretion was 8-fold for light myelin, 14-fold for medium myelin, and 3-fold for heavy myelin (Table XI). Concurrent to this period of rapid protein accretion, the proportion of total myelin protein recovered in the heavy subfraction decreased from 77 to 52 percent while the proportionate share represented by light myelin increased from 17 to 28 percent.

During this early 24-hour period of rapid myelin protein accretion, while recoverable ^{3}H radioactivity declined in brain homogenate by 61 percent (Table XI) the amount of recoverable ^{3}H radioactivity in heavy myelin declined by only 6 percent whereas recoverable radioactivity in each of the light and medium subfractions increased 12-fold (Table XI). It is of interest that immediately after injection heavy myelin accounted for 90 percent of the recoverable myelin protein radioactivity whereas 12 and 24 hours following injection this proportion declined to 45 and 42 percent. During the same interval the proportion of recoverable myelin subfraction protein radioactivity increased from 6 to 29 percent in light myelin and from 4 to 20 percent in medium. One would expect

TABLE X

BRAIN HOMOGENATE PROTEIN CONTENT, ^{3}H DPM, AND SPECIFIC RADIOACTIVITY IN 12- TO 13-DAY-OLD RATS

HOURS AFTER INJECTION	MG PROTEIN / BRAIN HOMOGENATE	^{3}H / BRAIN HOMOGENATE	
		X 10^{-6} (DPM)	DPM/μ GRAM PROTEIN
1	143 ± 17	17.1 ± 5.9	119 ± 31
12	151 ± 24	11.1 ± 3.6	74 ± 26
24	153 ± 4	6.7 ± 7.3	45 ± 49

Each value represents the mean of three samples ± the standard deviation. Specific radioactivity expressed as dpm/μgram protein.

TABLE XI

MYELIN SUBFRACTION PROTEIN CONTENT, ^{3}H DPM, AND SPECIFIC RADIOACTIVITY IN 12- TO 13-DAY OLD RATS

HOURS AFTER INJECTION	MG PROTEIN/BRAIN			^{3}H X 10^{-3} DPM (DPM/μGRAM PROTEIN)		
	LIGHT MYELIN	MEDIUM MYELIN	HEAVY MYELIN	LIGHT MYELIN	MEDIUM MYELIN	HEAVY MYELIN
1	0.05 ± .01	0.02 ± .002†	0.23 ± .20†	3 ± 0.7 (60)	2 ± 0.5 (100)†	47 ± 18 (204)†
12	0.18 ± .13	0.15 ± .08	0.33 ± .14	33 ± 9 (183)	22 ± 3 (147)	45 ± 30 (136)
24	0.42 ± .09	0.29 ± .10	0.77 ± .26	36 ± 11 (86)	25 ±10 (86)	44 ± 5 (57)

Each value represents the mean of three samples ± the standard deviation. Specific radioactivity expressed as dpm/μgram protein is presented in parentheses. †Indicates that only two samples were available for analysis.

newly synthesized myelin protein to be radioactively labelled. It is to be noted that while light and medium myelin subfractions continued to accumulate both newly synthesized protein and incorporated radioactivity, the heavy myelin subfraction accumulated newly synthesized protein without accumulating additional radioactive labelling.

Specific radioactivity for whole brain homogenate and myelin subfraction protein was determined over the 24-hour period following isotope administration (Tables X and XI). Protein specific radioactivity declined 62 percent in brain homogenate and 72 percent in heavy myelin. Light myelin protein specific activity had a 3-fold peak observed at 12 hours while medium myelin had a more modest 1.5-fold peak in protein specific radioactivity at 12 hours.

After allocating appropriate aliquots of myelin subfractions for determinations of protein and ^{3}H radioactivity remaining material was committed to preparation of Fast Green stained electrophoresed SDS gels. One such gel was prepared for each subfraction at each age except for medium myelin at the 1-hour time point which was not available. Sufficient radioactivity could be solubilized from cut gels to determine the percent distribution of the incorporated ^{3}H label into major myelin proteins.

The proportion of solubilized ^{3}H radioactivity from electrophoresed gels was determined for gels in the series. The proportion associated with high molecular weight protein was approximately 63, 72, and 81 percent, respectively, for light, medium, and heavy myelin. The proportion associated with proteolipid was approximately 24, 15, and 11

percent, respectively, for light, medium, and heavy myelin. The proportion associated with basic protein was approximately 13, 13, and 8 percent, respectively, for light, medium, and heavy myelin. Compared to the pattern observed earlier in the long term study, the present study reflects an enrichment in the proportion of radioactivity associated with high molecular weight protein and a deficiency of radioactivity associated with myelin basic protein and proteolipid protein at 12 to 13 days of age.

CHRONIC MATERNAL ETHANOL CONSUMPTION:

EFFECTS ON OFFSPRING CNS MYELIN METABOLISM

Whole brain homogenate and myelin subfraction protein and lipid metabolism was studied in the offspring of Sprague-Dawley female rats whose consumption of a liquid diet supplemented with ethanol was for a chronic period extending 2 months prior to conception through the third day postpartum. Controls were the offspring of females pair fed an isocaloric diet containing additional non-alcoholic carbohydrate in lieu of any ethanol during an identical period. The period of the special dietary regime will be referred to as chronic. Offspring in the two sets are hereinafter referred to as chronic ethanol pups and control pups, respectively. A variety of parameters of myelin subfraction protein and lipid metabolism was examined at 18, 25, and 53 days of age following an 18-hour pulse labelling with intracerebrally injected L-[4,5-^{3}H] leucine and D- [U-^{14}C] glucose.

Throughout the age range examined, chronic ethanol pups had modest but consistent and sustained decrements in live body weight and fresh wet weight (Table XII and Figure 3). Chronic ethanol pups had decrements in body weight of approximately 12, 16, and 9 percent, respectively, at 18, 25, and 53 days of age. Corresponding decrements in brain fresh wet weight were approximately 7, 4, and 16 percent, respectively, at 18, 25, and 53 days of age.

In contrast to observed decrements in body and brain weights, chronic ethanol pups had higher total myelin protein at 18 and 25 days

TABLE XII

BODY AND BRAIN WEIGHTS

Age	Animal	Body Wt. Grams (n)	Brain Wt. Grams (n)
18	Control	27.4 (3)	1.30 (3)
18	Chronic-Ethanol	24.2 (3)	1.21 (3)
25	Control	50.9 (3)	1.41 (3)
25	Chronic-Ethanol	43.0 (3)	1.35 (3)
53	Control	189 (3)	1.73 (3)
53	Chronic-Ethanol	172 (3)	1.45 (3)

Control and ethanol pups exposed to effects of chronic maternal consumption of pair-fed isocaloric liquid diets for a chronic period as described in text. Body weight represents mean of live body weights only for those number (n) of animals sacrificed, and is comparable to mean of live body weights for all animals of a given age as graphed in accompanying figure.

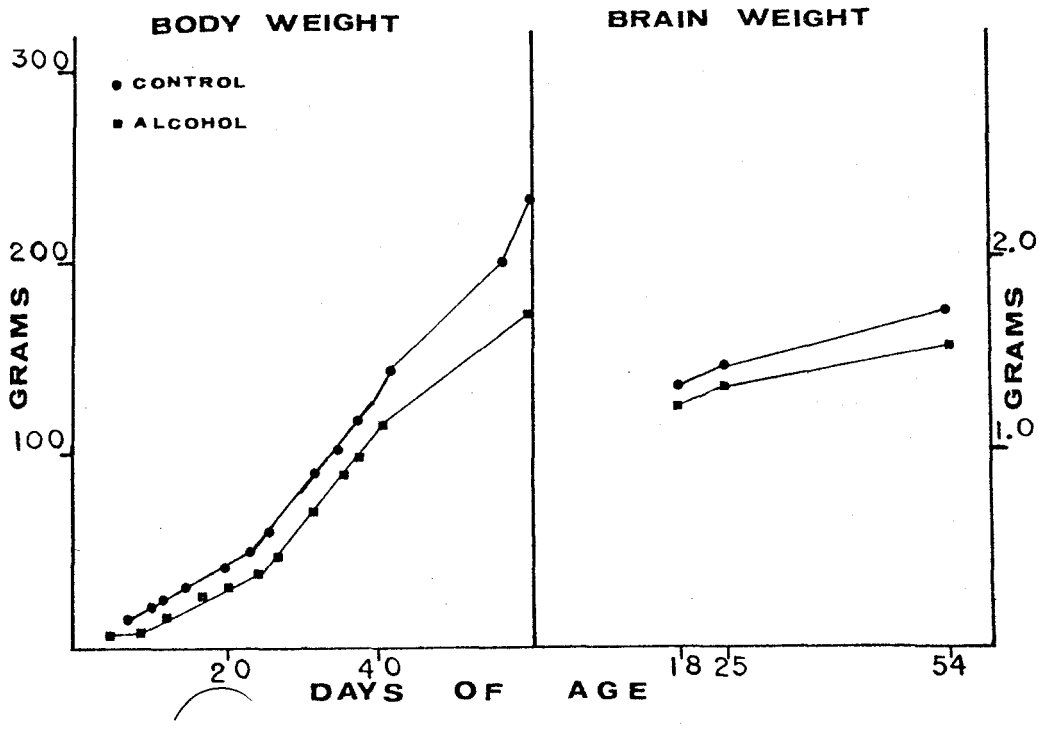

FIGURE 3. Brain and body weights of chronic ethanol and control rats. The body weights represent the mean of 3-12 rats. Each brain weight represents the mean of three brains. Ethanol pups are offspring of females exposed to ethanol for a chronic period as described in text.

of age (Table XIII). The statistically significant ($p < 0.05$) increase in total myelin protein in 18- and 25-day-old chronic ethanol pups was due exclusively to a statistically significant ($p < 0.05$) increase in the chemically and morphologically immature heavy myelin subfraction. At the age of 18 and 25 days the amount of protein in whole brain homogenate and in the light and medium subfractions in chronic ethanol pups was essentially the same as respective fractions in control pups. At 53 days of age both chronic ethanol pups and control pups had essentially the same amount of protein, respectively, in whole brain homogenate and heavy myelin. At 53 days of age the chronic ethanol pups had statistically significant ($p < 0.05$) reduced light and medium myelin protein. Fifty-three-day-old chronic ethanol pups also had reduced total myelin protein, however, the total reduction in myelin protein was not statistically significant.

There were also different patterns of radioactive incorporation of ^{3}H- and ^{14}C-labelled precursors for chronic ethanol pups and control pups (Table XIV and XV). At 18 days of age chronic ethanol pups had statistically significant ($p < 0.05$) increases in total myelin incorporation of both ^{3}H and ^{14}C which was due to a statistically significant ($p < 0.05$) increase of incorporation into the chemically and morphologically immature heavy myelin subfraction. As well, there were increases in incorporation of both labels in light and medium myelin; however, these increments were not statistically significant. Incorporation of both labels into whole brain homogenate at 18 days of age was essentially similar for both chronic ethanol pups and control pups. At

TABLE XIII

BRAIN AND MYELIN SUBFRACTION PROTEIN

Animal (age in days)	Brain Homogenate	Total Myelin	Light Myelin	Medium Myelin	Heavy Myelin
Control (18)	132 ± 19	1.42 ± 0.25	0.88 ± 0.24	0.35 ± 0.18	0.19 ± 0.07
Chronic Ethanol (18)	134 ± 10	2.05 ± 0.10*	0.98 ± 0.02	0.37 ± 0.11	0.71 ± 0.10*
Control (25)	164 ± 12	3.04 ± 0.73	2.38 ± 0.45	0.47 ± 0.23	0.19 ± 0.12
Chronic Ethanol (25)	165 ± 8	4.78 ± 0.34*	2.42 ± 0.28	0.75 ± 0.16	1.60 ± 0.28*
Control (53)	209 ± 9	10.96 ± 0.83	5.61 ± 0.58	2.14 ± 0.15	3.21 ± 0.37
Chronic Ethanol (53)	208 ± 21	8.99 ± 1.67	4.39 ± 0.64*	1.44 ± 0.25*	3.15 ± 0.71

Each value represents the mean of 3 samples ± the standard deviation. *Value obtained for the ethanol rats statistically different from the control value at $p < 0.05$. Ethanol rats offspring of females exposed to ethanol chronically as described in text.

TABLE XIV

^{3}H RADIOACTIVITY IN BRAIN AND MYELIN SUBFRACTIONS

Animal (age in days)	10^{-6} DPM/brain	10^{-3} DPM/brain			
	Brain Homogenate	Total Myelin	Light Myelin	Medium Myelin	Heavy Myelin
Control (18)	19.9 ± 2.0	251.0 ± 35.5	165.6 ± 56.3	48.1 ± 25.8	38.3 ± 1.9
Chronic Ethanol (18)	19.3 ± 4.1	363.5 ± 78.6*	206.5 ± 42.9	64.9 ± 20.2	92.1 ± 20.0*
Control (25)	7.1 ± 0.5	96.1 ± 37.0	75.6 ± 25.4	17.2 ± 9.2	3.3 ± 2.5
Chronic Ethanol (25)	10.3 ± 0.2*	365.5 ± 63.4*	188.8 ± 38.1*	58.6 ± 17.1*	118.1 ± 25.8*
Control (53)	5.2 ± 0.3	135.0 ± 24.7	65.1 ± 17.5	24.4 ± 7.1	45.4 ± 0.5
Chronic Ethanol (53)	1.9 ± 1.8*	53.0 ± 14.6*	23.4 ± 6.2*	10.0 ± 3.3*	10.6 ± 2.6*

Each value represents the mean of three samples ± the standard deviation. *Value obtained for the ethanol rats statistically different from the control value at $p < 0.05$. DPM = disintegrations/min. Ethanol rats offspring of females exposed to ethanol chronically as described in text.

TABLE XV

^{14}C RADIOACTIVITY IN BRAIN AND MYELIN SUBFRACTIONS

Animal (age in days)	10^{-6} DPM/brain	10^{-3} DPM/brain			
	Brain Homogenate	Total Myelin	Light Myelin	Medium Myelin	Heavy Myelin
Control (18)	1.28 ± 0.22	33.4 ± 7.3	21.9 ± 9.1	6.7 ± 4.8	4.8 ± 0.4
Chronic Ethanol (18)	1.14 ± 0.17	51.2 ± 5.8*	31.4 ± 4.2	8.5 ± 1.7	11.3 ± 0.8*
Control (25)	0.47 ± 0.03	15.6 ± 5.2	13.1 ± 3.7	2.1 ± 1.3	0.4 ± 0.3
Chronic Ethanol (25)	0.68 ± 0.05*	39.9 ± 2.2*	23.5 ± 0.6*	5.9 ± 0.8*	10.6 ± 0.3*
Control (53)	0.24 ± 0.06	10.1 ± 2.2	5.1 ± 1.4	2.0 ± 0.5	3.0 ± 0.4
Chronic Ethanol (53)	0.13 ± 0.05*	5.1 ± 0.6*	2.5 ± 0.3*	0.9 ± 0.2*	1.7 ± 0.2*

Each value represents the mean of three samples ± the standard deviation. *Value obtained for the ethanol rats statistically different from the control value at $p < 0.05$. DPM = disintegrations/min. Ethanol rats offspring of females exposed to ethanol chronically as described in text.

25 days of age both ^{3}H and ^{14}C incorporation was statistically significantly ($p < 0.05$) elevated in chronic ethanol pups in all myelin subfractions and in whole brain homogenate. At 53 days of age chronic ethanol pups had statistically significantly ($p < 0.05$) reduced ^{3}H and ^{14}C incorporation in all myelin subfractions and in whole brain homogenate when compared to control pups.

Thus, chronic ethanol pups, compared to controls, demonstrated greater myelin synthesis at younger ages and reduced synthesis at the oldest age examined.

Figure 4 illustrates the appearance of gels prepared from 25-day-old light, medium, and heavy subfraction delipidated protein from chronic ethanol pups. As documented in Table XVI similar and normal electrophoretic patterns were observed in terms of the percent distribution of dye-binding between major myelin proteins. That is, light myelin was enriched in basic protein and deficient in high molecular weight protein while the pattern was reversed for heavy myelin and intermediate for medium myelin. All three myelin subfractions had nearly equivalent proportions of proteolipid protein.

^{3}H radioactivity was solubilized and eluted from gels and analyzed for the relative distribution of the incorporation of the label between the major myelin protein classes. As indicated in Table XVII experimental and control pups had very similar patterns within each subfraction. The proportion of ^{3}H incorporation into high molecular weight proteins was slightly less in light myelin than in either medium or heavy myelin, while light myelin had some enrichment in the proportion of label

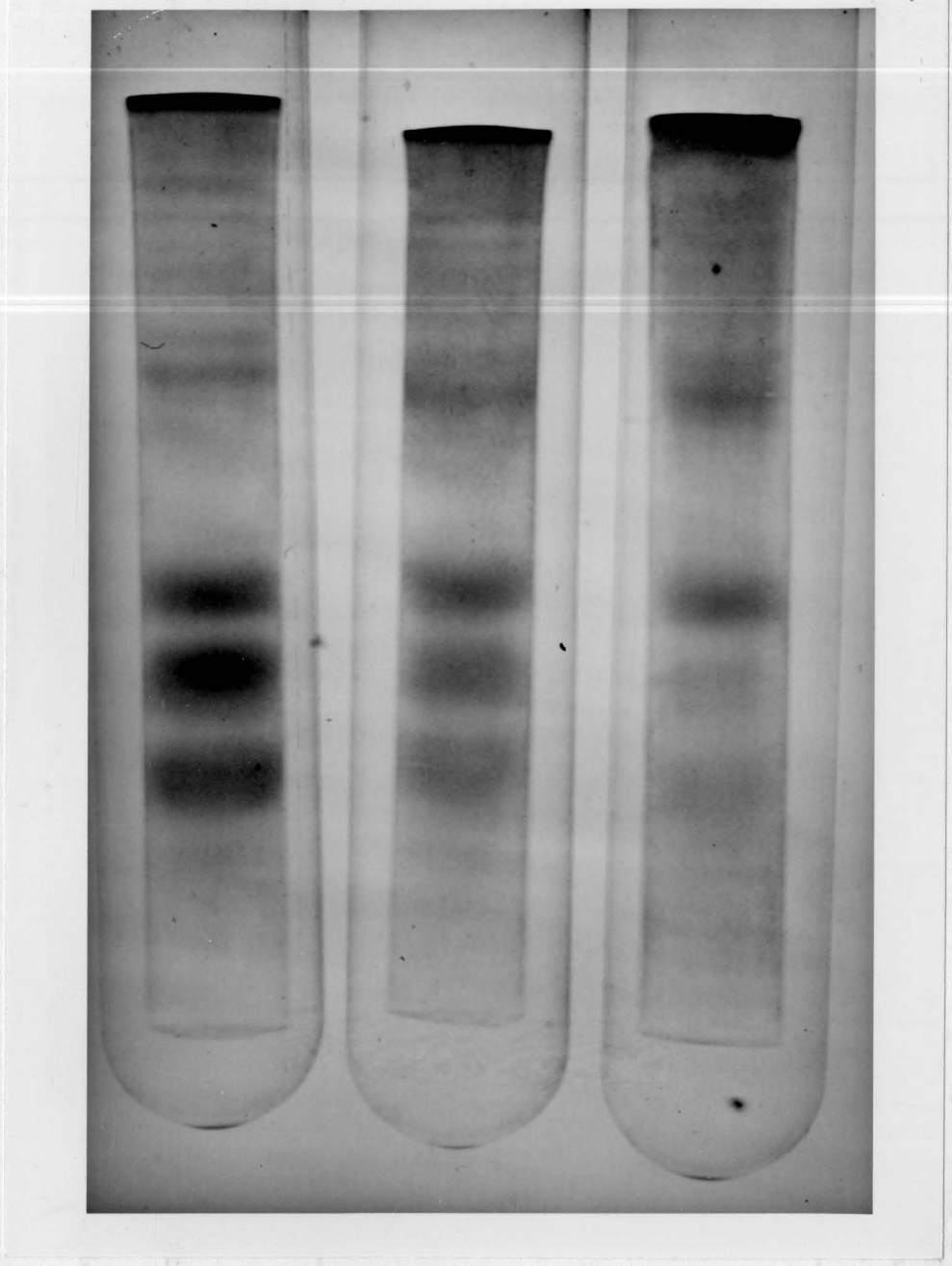

FIGURE 4. SDS polyacrylamide gels of myelin protein from the (A) light, (B) medium, and (C) heavy subfractions isolated from 25-day-old chronic ethanol pups. Each gel was loaded with 200 µg of protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

TABLE XVI

PROTEIN DISTRIBUTION OF MAJOR PROTEIN BANDS ON SDS GELS

Myelin Fraction-Animal	Percent Dye Binding			
	HMW	PLP	LBP	SBP
Light-Control	46 ± 6	15 ± 3	21 ± 2	18 ± 5
Light-Chronic Ethanol	38 ± 5	18 ± 2	26 ± 3	18 ± 2
Medium-Control	55 ± 4	12 ± 2	17 ± 2	16 ± 1
Medium-Chronic Ethanol	64 ± 10	11 ± 4	14 ± 4	13 ± 8
Heavy-Control	74 ± 12	11 ± 6	8 ± 5	6 ± 2
Heavy-Chronic Ethanol	61 ± 12	15 ± 9	11 ± 2	13 ± 6

Each value represents the mean ± the standard deviation of values obtained from SDS gels at 18, 25, and 53 days of age. Values were calculated by dividing the area under densitometric peaks of specific major proteins by total gel protein densitometric area. Each value was multiplied by 100 to determine the percent distribution. Values reflect uncorrected dye binding only. Ethanol pups are offspring of female rats exposed to chronic alcohol consumption as described in text. HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic proteins, SBP = small basic proteins.

TABLE XVII

^{3}H DISTRIBUTION IN MYELIN PROTEINS

Fraction-animals	HMW	PLP	BP
Light-Control	67 ± 7	14 ± 2	20 ± 5
Light-Chronic Ethanol	68 ± 2	15 ± 1	16 ± 3
Medium-Control	76 ± 10	12 ± 5	12 ± 4
Medium-Chronic Ethanol	72 ± 1	16 ± 5	12 ± 4
Heavy-Control	71 ± 6	13 ± 1	16 ± 6
Heavy Chronic Ethanol	75 ± 1	14 ± 1	11 ± 0

Values were calculated by dividing the ^{3}H radioactivity associated with a specific category of proteins by the total ^{3}H radioactivity solubilized from an entire gel. Each value was multiplied by 100 to determine the percent distribution of radioactivity. Each value represents the mean ± the standard deviation of values obtained at 18 and 25 days of age. Values from 54-day-old rats were not included because the ^{3}H solubilized from each gel slice was too low to be meaningful. HMW = high molecular weight proteins; PLP = proteolipid protein; BP = sum of large and small basic proteins. Ethanol rats offspring of females exposed to ethanol chronically as described in text.

incorporated in basic proteins. All myelin subfractions had essentially the identical proportion of ^{3}H radioactivity incorporated in proteo-lipid protein.

Thus, myelin protein from chronic ethanol pups in terms of electrophoretic patterns and proportion of ^{3}H incorporation into major myelin proteins appeared to be quite normal.

The distribution of ^{14}C incorporation into major myelin lipids separated by thin layer chromatography was determined for each sub-fraction from experimental and control pups. Similar distributions of ^{14}C incorporation into major myelin lipids was observed for each myelin subfraction from either the experimental or control pups. Approximately 48, 25, and 25 percent of the recovered ^{14}C radioactivity was found associated with phospholipids, galactosphingolipids (cerebrosides and sulfatides) and cholestrol, respectively.

Thus, in terms of the proportionate ^{14}C incorporation into major myelin lipids, the myelin lipid from chronic ethanol pups appeared normal.

The chief overall effect of chronic maternal ethanol consumption upon offspring myelination appeared to be an aberrant pattern of myelin synthesis. Compared to controls, at various ages chronic ethanol pups had statistically significant alterations in the total amount, maturity, and synthesis of CNS myelin. However, having isolated myelin protein and lipid from such animals, no quantitative or qualitative aberrations were evident by the electrophoretic and thin layer chromatography methods herein employed.

ACUTE PERIOD OF MATERNAL ETHANOL CONSUMPTION: IN UTERO EFFECTS ON CNS MYELIN METABOLISM

Whole brain homogenate and myelin subfraction protein and lipid metabolism were studied in the offspring of Sprague-Dawley female rats whose consumption of a liquid diet supplemented with ethanol was for an acute period extending from the fifth day of gestation through the third day postpartum. Controls were the offspring of females pair-fed an isocaloric diet containing additional non-alcoholic carbohydrate in lieu of any ethanol during an identical period. Offspring in the two sets are hereinafter referred to as acute ethanol pups and control pups. The period of the special dietary regime will be referred to as either acute or *in utero*. A variety of parameters of myelin subfraction protein and lipid metabolism was examined at 18, 25, and 53 days of age following an 18-hour pulse labelling with intracerebrally injected L- [4,5-^{3}H] leucine and D- [U-^{14}C] glucose.

In contrast to the chronic ethanol study, the effects of acute *in utero* exposure to ethanol were, with few exceptions, minimal.

The body and brain weights of acute ethanol pups were very close to those of control pups (Table XVIII and Figure 5). The difference in body weight between experimental and control pups at 53 days is explained in Table XVIII. When the body weights of all animals at 53 days of age, including animals not sacrificed, are considered (Figure 5) no apparent differences were present.

The yield of whole brain homogenate protein and myelin sub-

TABLE XVIII

BODY AND BRAIN WEIGHTS

Age	Animal	Body Wt. Grams (n)	Brain Wt. Grams (n)
18	Control	29.7 (3)	1.34 (3)
18	Acute-Ethanol	29.8 (3)	1.32 (3)
25	Control	48.0 (3)	1.44 (3)
25	Acute-Ethanol	55.9 (3)	1.42 (3)
53	Control	222 (3)	1.85 (3)
53	Acute-Ethanol	121* (3)	1.81 (3)

*Discrepancy between mean live body weight between 53-day-old control and experimental pups reflects sex differences. All three control pups were male; one ethanol pup was male (229 grams) while two ethanol pups were female (53.7 and 80.1 grams). Control and ethanol pups exposed to in utero effects of maternal consumption of pair-fed isocaloric liquid diets for an acute period of time as described in text. Body weight represents mean of live body weights only for those number (n) of animals sacrificed, and is comparable to mean of live body weights for all animals of a given age as graphed in accompanying figure.

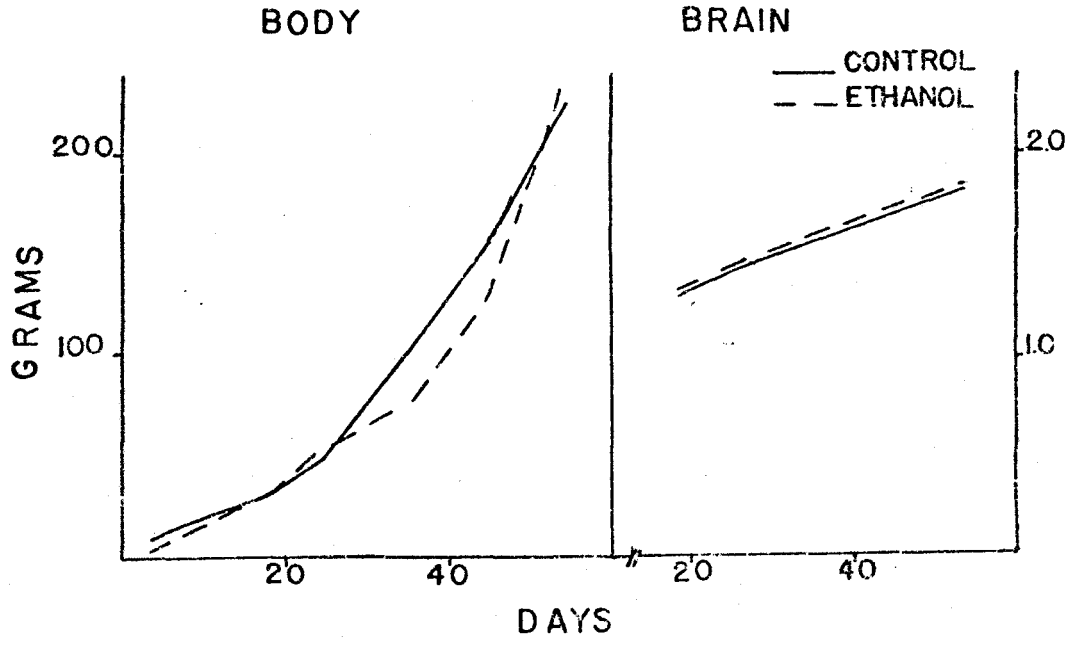

FIGURE 5. Brain and body weights of acute ethanol and control rats. The body weights represent the mean of 6-12 rats. Each brain weight represents the mean of three brains. Ethanol pups are offspring of females exposed to ethanol for an acute period as described in text.

fraction protein is tabulated in Table XIX. Generally, acute ethanol pups had amounts of protein comparable to those present in control pups. However, at 53 days of age acute ethanol pups had a statistically significant ($p < 0.05$) increase in the chemically and morphologically immature heavy myelin subfraction. At 53 days of age acute ethanol pups also had reductions in the more mature light and medium myelin subfractions and in total myelin protein. However, these reductions were not statistically significant.

^{3}H and ^{14}C radioactivity recovered from myelin subfractions was normalized with respect to whole brain homogenate radioactivity to correct for variability in injection (Tables XX and XXI). At 18 days of age acute ethanol pups had statistically significant ($p < 0.05$) increments in normalized ^{3}H incorporation into light and medium myelin subfractions and total myelin. Normalized incorporation into heavy myelin was decreased in acute ethanol pups, but not statistically significantly. A similar trend was observed for ^{14}C normalized incorporation at 18 days of age. Normalized incorporation of ^{14}C in 18-day acute ethanol pups was elevated in light and medium myelin subfractions and whole brain homogenate but decreased in heavy myelin. However, only the increment in the medium subfraction was statistically significant. At 25 and 53 days of age there were no statistically significant differences in normalized ^{3}H and ^{14}C incorporation in any fraction from control or acute ethanol pups.

^{3}H radioactivity was solubilized and eluted from Fast Green stained electrophoresed SDS gels. The percent distribution of the

TABLE XIX

MYELIN SUBFRACTION PROTEIN

Animal-Age in Days	Homogenate	Mg. Protein/Brain			
		Total	Light	Medium	Heavy
Control-18	151 ± 1	2.11 ± .29	0.79 ± .18	0.66 ± .14	0.66 ± .17
Acute Ethanol-18	161 ± 3	1.99 ± .21	0.79 ± .05	0.74 ± .12	0.47 ± .15
Control-25	187 ± 9	5.44 ± .40	2.90 ± .37	1.26 ± .21	1.28 ± .17
Acute Ethanol - 25	168 ± 10	4.30 ± .08	2.80 ± .21	1.14 ± .15	1.36 ± .03
Control-53	235 ± 9	13.70 ± 1.36	6.62 ± .06	4.90 ± 1.48	2.19 ± .28
Acute Ethanol-53	219 ± 6	12.01 ± 1.84	5.65 ± 1.84	3.30 ± .18	3.05 ± .30*

Each value represents the mean of 3 samples ± the standard deviation. A * indicates that the experimental value is different than the control at $p < .05$. Ethanol pups exposed to ethanol for an acute period *in utero* as described in text.

TABLE XX

^{3}H RADIOACTIVITY IN MYELIN SUBFRACTIONS

Animal-Age in Days	^{3}H DPM—Myelin Fraction/Homogenate x 100%			
	Total	Light	Medium	Heavy
Control-18	2.20 ± .24	.90 ± .10	.68 ± .13	.61 ± .19
Acute-Ethanol-18	2.53 ± .09*	1.18 ± .12*	.95 ± .07*	.40 ± .13
Control-25	5.95 ± .97	3.77 ± .99	1.16 ± .11	1.02 ± .22
Acute Ethanol-25	4.91 ± .69	2.88 ± .44	1.09 ± .22	.94 ± .05
Control 53	4.61 ± .89	2.04 ± .95	1.64 ± .40	.93 ± .59
Acute-Ethanol-53	4.51 ±1.35	2.05 ± .70	1.28 ± .41	1.17 ± .25

Each value represents the mean of 3 samples ± the standard deviation. A * indicates that the experimental value is different than the control value of $p < .05$. The ethanol pups exposed to ethanol for an acute period *in utero* as described in text.

TABLE XXI

^{14}C RADIOACTIVITY IN MYELIN SUBFRACTIONS

Animal-Age in Days	^{14}C DPM——Myelin Fraction/Homogenate x 100%			
	Total	Light	Medium	Heavy
Control-18	4.41 ± .50	2.05 ± .29	1.35 ± .14	1.01 ± .34
Acute Ethanol-18	4.90 ± .33	2.47 ± .38	1.78 ± .02*	.65 ± .22
Control-25	8.74 ± 1.52	5.90 ± 1.61	1.58 ± .21	1.26 ± .11
Acute Ethanol-25	7.57 ± 1.13	4.87 ± .68	1.52 ± .38	1.18 ± .11
Control-53	5.82 ± 2.25	2.90 ± 1.35	1.92 ± .69	1.00 ± .47
Acute Ethanol-53	5.20 ± 1.93	2.60 ± 1.15	1.41 ± .47	1.19 ± .31

Each sample represents the mean of 3 samples ± the standard deviation. A * indicates that the experimental value is different than the control at $p < .05$. Ethanol pups exposed to ethanol for an acute *in utero* period as described in text.

^{3}H incorporated into the major myelin proteins was determined (Table XXII). Close agreement between experimental and control subfractions was observed except for a statistically significant ($p < 0.05$) increase in the proportion of ^{3}H radioactivity associated with proteolipid protein in acute ethanol pups. Compared to the light myelin subfraction, heavier myelin subfractions from both experimental and control pups demonstrated a modest increase in the proportion of ^{3}H radioactivity associated with high molecular weight protein. Compared to heavier myelin subfractions, the light myelin subfraction from both experimental and control pups demonstrated a modest increase in the proportion of ^{3}H radioactivity associated with proteolipid protein.

The percentage distribution of ^{14}C incorporation into major myelin lipids separated by thin layer chromatography was determined (Table XXIII). There was very close agreement between experimental and control animals for each subfraction. The proportion of ^{14}C radioactivity associated with cholesterol was 26-27 percent for all subfractions. The proportion of ^{14}C radioactivity associated with galactosphingolipids (cerebrosides and sulfatides) was 35-43 percent with increased proportions in the lighter subfractions. The proportion of ^{14}C radioactivity associated with phospholipids ranged from 27 to 35 percent, respectively, for light to heavy myelin subfractions.

Compared to effects of chronic maternal alcohol consumption the effects of <u>in utero</u> exposure to alcohol were rather modest.

TABLE XXII

^{3}H DISTRIBUTION IN MYELIN SUBFRACTION PROTEINS

Myelin Fraction-Animal	Percent Total ^{3}H DPM/GEL		
	High Molecular Weight	Proteolipid	Basic Proteins
Light-Control	70.0 ± 2.8	18.7 ± 2.0	11.3 ± 1.0
Light-Acute Ethanol	67.1 ± 3.4	21.7 ± 2.8	11.2 ± 2.3
Medium-Control	78.2 ± 7.2	8.2 ± 2.7	13.6 ± 4.7
Medium-Acute Ethanol	73.4 ± 4.9	18.0 ± 2.3*	8.7 ± 2.9
Heavy-Control	80.3 ± 4.7	11.8 ± 3.5	7.8 ± 2.1
Heavy-Acute Ethanol	78.5 ± 3.2	12.5 ± 1.3	9.0 ± 2.3

Each value represents the mean ± the standard deviation of values obtained at 18, 25 and 53 days of age. Values were calculated by dividing the ^{3}H radioactivity associated with a specific category of proteins by the total ^{3}H radioactivity solubilized from a gel. Each value was multiplied by 100 to determine the percent distribution of radioactivity. A * indicates that the experimental value is different than the control value at $p < .05$. Ethanol pups exposed to ethanol for an acute period in utero as described in text.

TABLE XXIII

^{14}C INCORPORATION INTO MYELIN SUBFRACTION LIPIDS

Myelin Fraction-Animal	Percent Total ^{14}C DPM		
	Cholesterol	Galacto-sphingolipids	Phospho-lipids
Light-Control	26.5 ± .9	42.9 ± 3.4	27.8 ± 4.2
Light-Acute Ethanol	28.3 ± 2.7	41.7 ± 2.0	27.2 ± 4.8
Medium-Control	25.9 ± 1.5	41.9 ± 5.0	29.6 ± 5.5
Medium-Acute Ethanol	26.1 ± 1.9	41.0 ± 3.0	30.1 ± 5.2
Heavy-Control	27.4 ± 2.4	35.2 ± 5.8	34.9 ± 7.2
Heavy-Acute Ethanol	25.9 ± 2.3	36.6 ± 3.6	34.9 ± 6.3

Each value represents the mean ± the standard deviation of values obtained at 18, 25 and 53 days of age. Values were calculated by dividing the ^{14}C radioactivity associated with a specific category of lipids by the total ^{14}C radioactivity solubilized from a thin layer chromatography lane. Each value was multiplied by 100 to determine the percent distribution of radioactivity. Ethanol pups exposed to ethanol for an acute period *in utero* as described in text.

POSTNATAL MATERNAL PROTEIN-CALORIE MALNUTRITION EFFECTS ON OFFSPRING CNS MYELIN METABOLISM

Whole brain homogenate and myelin subfraction protein and lipid metabolism was studied in the offspring of Sprague-Dawley female rats protein-calorie malnourished by dietary restriction during the first 18 days postpartum, after which nutritional remediation was begun with ad libitum access to normal laboratory rat chow. Controls were the offspring of females fed normal laboratory rat chow ad libitum at all times. A variety of parameters of myelin subfraction protein and lipid metabolism was examined at 18, 25, and 53 days of age following an 18-hour pulse labelling with intracerebrally injected L-[4,5-^{3}H] leucine and D-[U-^{14}C] glucose.

Body and brain weights of postnatally protein-calorie malnourished pups were decreased throughout the period examined (Table XXIV and Figure 6). The decrement in body and brain weights was greatest before nutritional remediation was begun. The body weight of protein-calorie malnourished pups was 49, 62, and 81 percent of control body weight, respectively, at 18, 25 and 53 days of age. Brain weights of protein-calorie malnourished pups followed a similar pattern. Experimental brain weights were 77, 88 and 92 percent of control brain weight, respectively, at 18, 25 and 53 days of age.

A similar pattern was also observed for whole brain homogenate and myelin subfraction protein content in experimental and control pups (Table XXV). In protein-calorie malnourished pups the content of

TABLE XXIV

BODY AND BRAIN WEIGHTS

Age	Animal	Body Wt. Grams (n)	Brain Wt. Grams (n)
18	Control	28.8 (3)	1.24 (3)
18	Postnatal-Protein-Calorie Malnutrition	14.2 (6)	0.95 (6)
25	Control	51.4 (3)	1.46 (3)
25	Postnatal-Protein-Calorie Malnutrition	32.0 (3)	1.28 (3)
53	Control	182.0 (3)	1.67 (3)
53	Postnatal-Protein-Calorie Malnutrition	148.0 (3)	1.53 (3)

Control animals fed ad libitum normal laboratory rat chow at all times. Staging and severity of postnatal protein-calorie malnutrition is described in text. Body weights represent mean of live body weights only for those number (n) of animals sacrificed, and is comparable to mean of live body weights of all animals of a given age as graphed in accompanying figure.

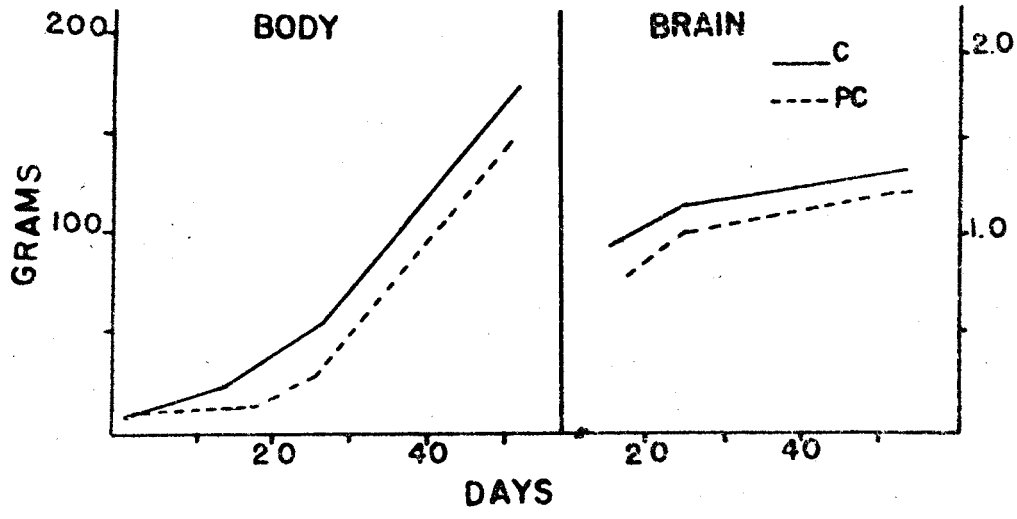

FIGURE 6. Brain and body weights of postnatally protein-calorie malnourished and control pups. The body weights represent the mean of 3-12 rats. Each brain weight represents the mean of three brains except the value for 18-day-old brain weight of protein-calorie malnourished pups which represents the mean of six brains. C = control, PC = protein-calorie malnourished.

TABLE XXV

BRAIN AND MYELIN PROTEIN

Age-Animal	Mg. Protein/Brain		% of Total Myelin Protein	
	Brain Homogenate	Total Myelin	Light & Medium	Heavy
18—Control	163 ± 6	1.45 ± .52	63.4 ± 5.9	36.4 ± 5.9
18—Postnatal-Protein-Calorie	109 ± 10*	.63 ± .22*	56.0 ± 7.3	44.0 ± 7.3
25—Control	187 ± 9	5.44 ± .40	76.4 ± 1.4	23.6 ± 1.4
25—Postnatal-Protein-Calorie	144 ± 15*	3.09 ± .58*	60.9 ± 2.6*	39.1 ± 2.6*
53—Control	229 ± 11	10.72 ± .80	65.9 ± 2.0	34.1 ± 2.0
53—Postnatal-Protein-Calorie	193 ± 21*	9.91 ± 1.04	78.8 ± .72*	21.2 ± .72*

Each value represents the mean of 3 values ± the standard deviation. A * indicates that the experimental value is different than the control value at $p < .05$. Details of the staging and severity of dietary stress are provided in text.

homogenate protein was statistically significantly ($p < 0.05$) reduced at all ages, especially at younger ages during which nutritional remediation was incomplete. The experimental pups had brain homogenate protein content 67, 77, and 84 percent of control, respectively, at 18, 25, and 53 days of age. Similarly, total myelin protein of experimental pups was 43, 57, and 92 percent of control total myelin protein, respectively, at 18, 25, and 53 days of age. The reduction in total myelin protein at 18 and 25 days of age was statistically significant ($p < 0.05$).

The proportion of total myelin either in light and medium myelin subfractions combined or in heavy myelin alone was also determined (Table XXV). At 18 days of age total myelin protein was apportioned between the lighter subfractions and heavy myelin in a comparable manner in both experimental and control pups. However, at 25 and 53 days of age there was statistically significant differences ($p < 0.05$) in the apportionment of total myelin protein between the lighter subfractions and heavy myelin subfractions. At 25 days of age protein-calorie malnourished pups had a greater proportion of the chemically and morphologically immature heavy myelin subfraction. But at 53 days of age protein-calorie malnourished pups had a greater proportion of the chemically and morphologically more mature lighter myelin subfractions.

With longer periods of nutritional remediation the deficit in body and brain weight and in brain homogenate and total myelin protein became less pronounced. A further measure of this improvement is afforded by calculating the yield of mg total myelin protein per gram wet weight of brain. This ratio increased over the age range 18 to 53

days of age for experimental pups from 0.66 to 2.41 to 6.48 compared with controls for which the corresponding ratios were 1.17, 3.73, and 6.42. This measure of myelin protein concentration converged towards normality at 53 days of age, 35 days following initiation of nutritional rehabilitation.

^{3}H and ^{14}C radioactivity incorporation data were normalized with respect to homogenate radioactivity to correct for injection variability (Tables XXVI and XXVII). Radioactivity in the lighter myelin subfractions was combined. A similar pattern of radioactive incorporation was observed for both isotopes.

At 18 days of age there were no statistically significant differences in normalized radioactive incorporation of either ^{3}H or ^{14}C in lighter myelin subfractions and heavy myelin between experimental and control animals. At 25 days of age protein-calorie malnourished pups had statistically significantly ($p < 0.05$) greater normalized ^{3}H radioactivity incorporation in heavy myelin and total myelin. At 25 days of age protein-calorie malnourished pups had statistically significantly ($p < 0.05$) greater normalized ^{14}C radioactivity incorporation in lighter myelin subfractions, heavy myelin, and total myelin. The greatest increases in normalized ^{3}H and ^{14}C radioactivity incorporation were seen in 53-day-old protein-calorie malnourished offspring lighter myelin subfractions and total myelin. The approximately 2-fold increase in normalized radioactivity in the lighter subfractions and the approximately 1.7-fold increase in normalized radioactivity in total myelin were both statistically significant ($p < 0.05$). Normalized radio-

TABLE XXVI

^{3}H DPM-MYELIN FRACTION/HOMOGENATE X 10^{3}

Age-Animal	Light & Medium	Heavy	Total
18—Control	3.01 ± 1.30	3.60 ± .43	6.61 ± 1.42
18—Postnatal-Protein-Calorie	6.83 ± 2.97	3.62 ± .96	10.46 ± 3.73
25—Control	12.74 ± 3.00	4.33 ± .88	17.08 ± 2.37
25—postnatal-Protein-Calorie	17.28 ± 1.82	9.67 ± 2.09*	26.95 ± 1.81*
53—Control	17.78 ± 8.80	9.06 ± 4.30	26.85 ±12.92
53—Postnatal-Protein-Calorie	38.87 ± 4.86*	9.67 ± .08	48.54 ± 4.82*

Each value represents the mean of values obtained from 3 animals ± the standard deviation. A * indicates that the experimental value is different from the control value at $p < 0.05$. The timing and severity of the postnatal stress is described in the text.

TABLE XXVII

^{14}C DPM-MYELIN FRACTION/HOMOGENATE X 10^3

Age-Animal	Light & Medium	Heavy	Total
18—Control	8.27 ± 3.77	7.64 ± 1.32	15.92 ± 4.37
18—Postnatal-Protein-Calorie	10.61 ± 4.77	4.88 ± 1.32	15.49 ± 5.93
25—Control	20.28 ± 3.38	5.75 ± 1.29	26.03 ± 2.11
25—Postnatal-Protein-Calorie	30.15 ± 1.56*	12.78 ± 2.19*	42.93 ± 1.47*
53—Control	24.62 ± 6.41	12.49 ± 2.00	37.11 ± 8.41
53—Postnatal-Protein-Calorie	46.85 ± 1.23*	11.08 ± 1.85	57.92 ± 2.05*

Each value represents the mean of values obtained from 3 animals ± the standard deviation. A * indicates that the experimental value is different from the control value at $p < 0.05$. The timing and severity of the postnatal stress is described in the text.

activity in 53-day-old experimental and control pups was essentially the same in heavy myelin for each of the isotopes.

Hence, during the period of nutritional remediation following 18 days of age, synthesis as measured by normalized radioactivity incorporation was sustained and elevated in protein-calorie malnourished pups. After the first 7 days of nutritional remediation there was increased synthesis in all myelin fractions. At 53 days of age after an additional 28 days of nutritional remediation in protein-calorie malnourished pups there was elevated synthesis only in the lighter, more mature myelin subfractions.

Densitometric scans of Fast Green stained electrophoresed SDS gels were analyzed to determine the percent dye binding by the major myelin proteins (Table XXVIII). At all ages the densitometric pattern for control medium and control heavy myelin subfraction proetins was age-independent. However, there were interesting age-dependent changes in the densitometric patterns of gels obtained from protein-calorie malnourished medium and heavy myelin subfraction proteins. The enrichment in high molecular weight proteins and the relative deficiency in basic proteins which characterizes heavier myelin subfractions when compared to light myelin was further accentuated in 18- and 25-day-old protein-calorie malnourished pups. At 53 days of age the additional enrichment in high molecular weight protein and relative additional deficiency in basic protein in heavier myelin subfractions was abolished. That is, only at 53 days of age did the heavier myelin subfraction protein electrophoregrams from protein-calorie malnourished pups assume

TABLE XXVIII

RELATIVE DISTRIBUTION OF MYELIN PROTEINS IN MYELIN SUBFRACTIONS

Subfraction	Age - Animal	% Of Total Dye Binding Capacity On Fast Green Stained Gels			
		HMW	PLP	LBP	SBP
Light	Avg-Control	46.5 ± 2.7	18.6 ± 1.3	18.6 ± 0.6	16.1 ± 2.0
Light	Avg - Postnatal-Protein-Calorie	43.9 ± 2.6	18.6 ± 1.9	20.1 ± 1.1	17.4 ± 1.2
Medium	Avg-Control	56.9 ± 5.7	17.8 ± 2.0	14.5 ± 0.9	10.8 ± 3.0
Medium	18-Postnatal-Protein-Calorie	63.6	13.1	12.7	10.6
Medium	25-Postnatal-Protein-Calorie	67.8	14.5	10.3	7.4
Medium	53-Postnatal-Protein-Calorie	47.1	19.9	18.1	15.0
Heavy	Avg-Control	64.7 ± 4.6	17.1 ± 0.5	10.3 ± 1.3	7.3 ± 1.8
Heavy	18-Postnatal-Protein-Calorie	79.2	16.1	3.4	1.3
Heavy	25-Postnatal-Protein-Calorie	76.1	19.4	4.8	1.8
Heavy	53-Postnatal-Protein-Calorie	66.0	17.9	9.6	6.6

Avg = Average. Malnutrition as described in text. Average values are averages of values obtained at 18, 25, and 53 days of age ± the standard deviation. Averaged values stated for those subfractions for which there was no significant variation in gel protein patterns as a function of age. HMW = High Molecular Weight Proteins, PLP = Proteolipid Protein, LBP = Large Basic Protein, SBP = Small Basic Protein. Values were calculated by dividing the area under densitometric peaks of specific major proteins by total gel protein densitometric area. Each value was multiplied by 100 to determine the percent distribution. Values reflect uncorrected dye binding only.

a normal appearance. Rather than labelling this phenomenon "age-dependent," it would be more correct to label it "remediation-dependent." With longer periods of nutritional remediation the electrophoretic protein pattern became more normal in appearance in the heavier myelin subfractions of postnatally protein-calorie malnourished pups. Such an "age-dependent" or remediation-dependent" effect was not observed for proteolipid protein.

The appearance of selected control and experimental myelin subfraction protein Fast Green stained electrophoresed SDS gels is illustrated in Figures 7, 8, 9, and 10.

^{3}H radioactivity was solubilized and eluted from gels and analyzed for the relative distribution of the incorporation of the label between the major myelin protein classes (Table XXIX). As indicated in Table XXIX for each subfraction there was close agreement between experimental and control pups. The greatest proportion of recovered ^{3}H radioactivity was associated with high molecular weight protein, with increased association in the heavier myelin subfractions. There was a modest enrichment in the proportion of ^{3}H radioactivity associated with proteolipid protein and the basic proteins in the light myelin subfraction.

The distribution of ^{14}C incorporation into major myelin lipids separated by thin layer chromatography was determined for each subfraction from experimental and control pups (Table XXX). The proportion of ^{14}C radioactivity associated with cholesterol followed no particular pattern, accounting for approximately 27, 31, and 24 percent of recovered

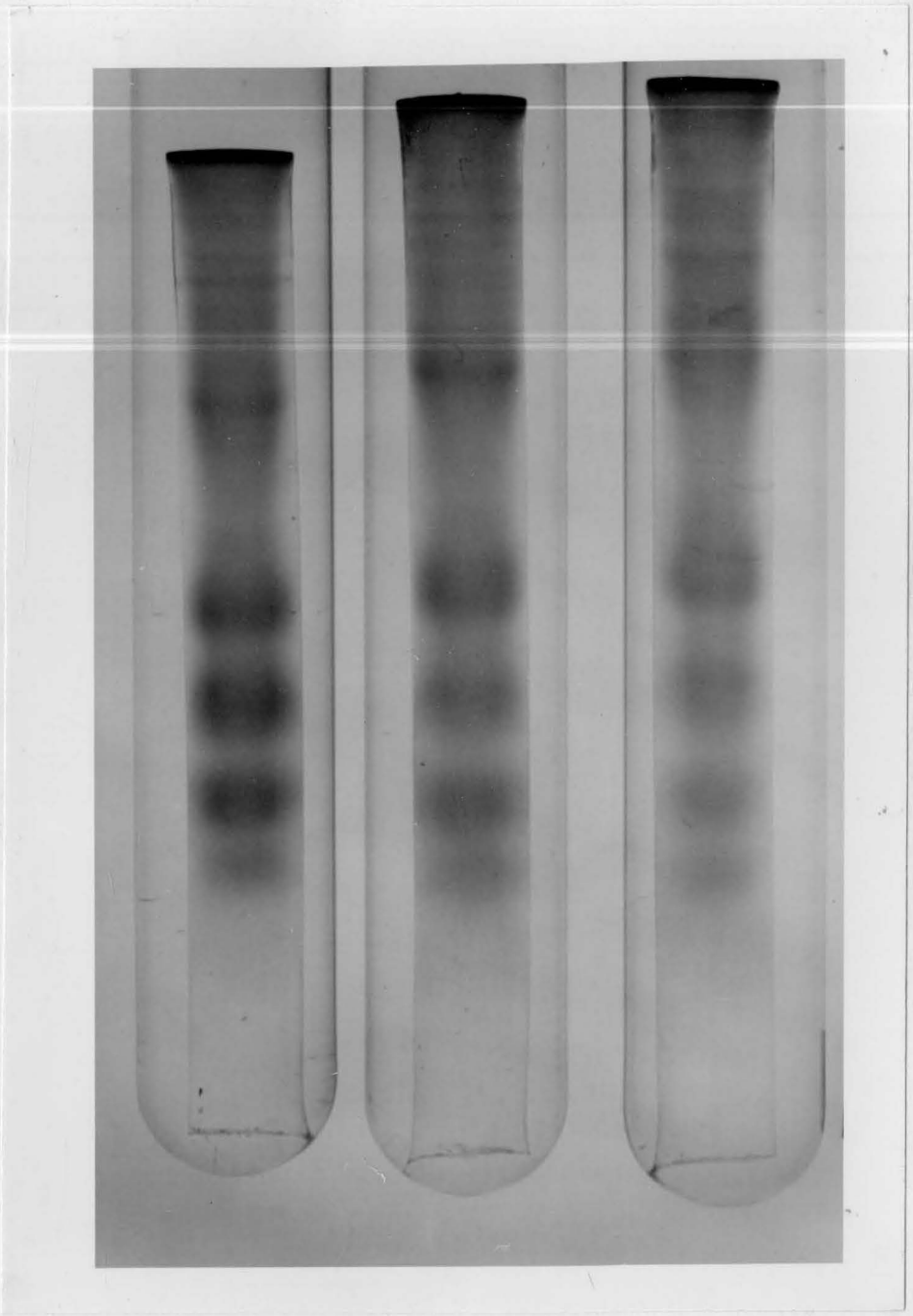

FIGURE 7. SDS polyacrylamide gels of myelin protein from the (A) light, (B) medium, and (C) heavy subfractions isolated from 25-day-old control rats. Each gel was loaded with 200 µg of protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

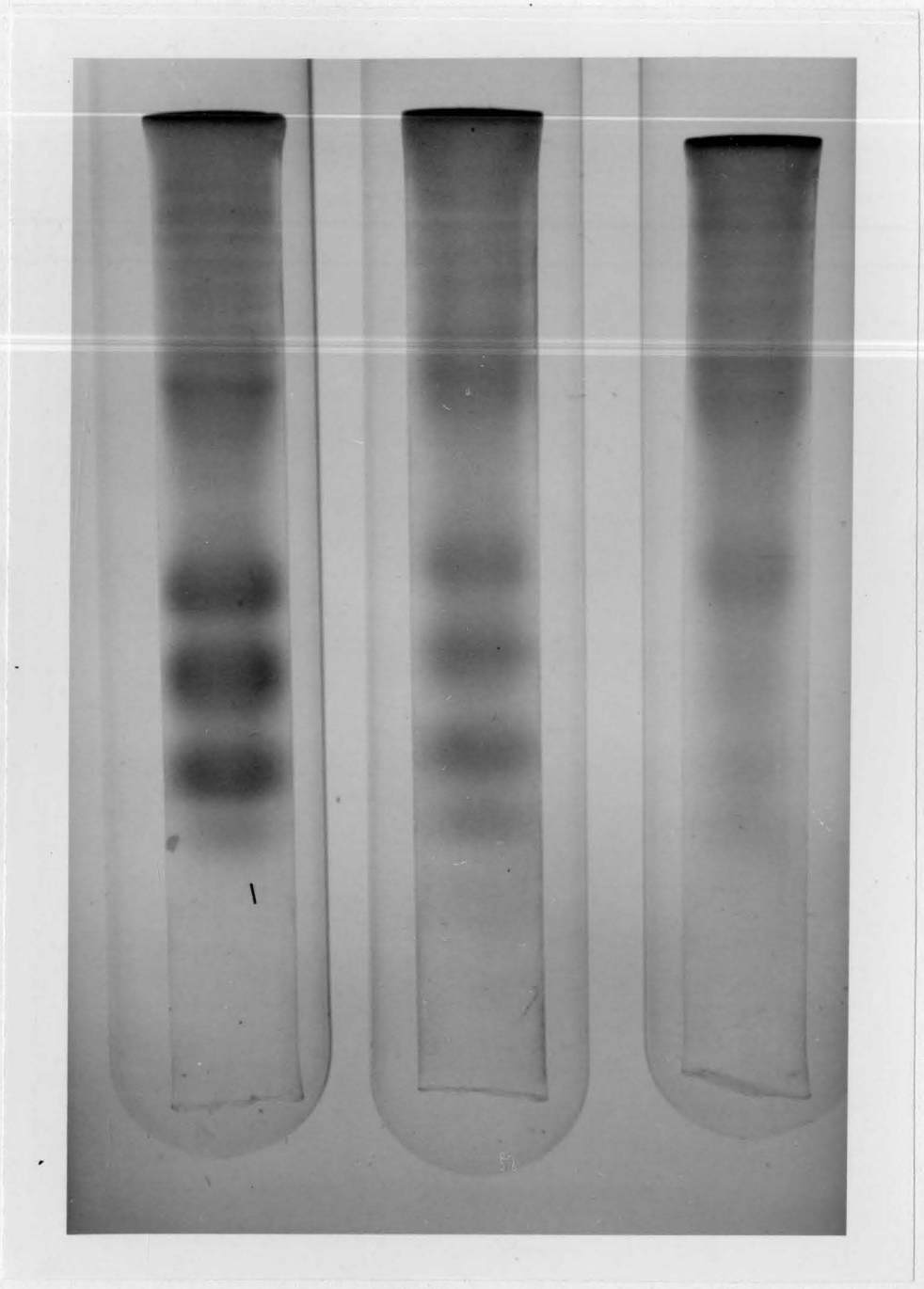

FIGURE 8. SDS polyacrylamide gels of myelin protein from the (A) light, (B) medium, and (C) heavy subfractions isolated from 18-day-old postnatally protein-calorie malnourished pups. Each gel was loaded with 200 µg protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

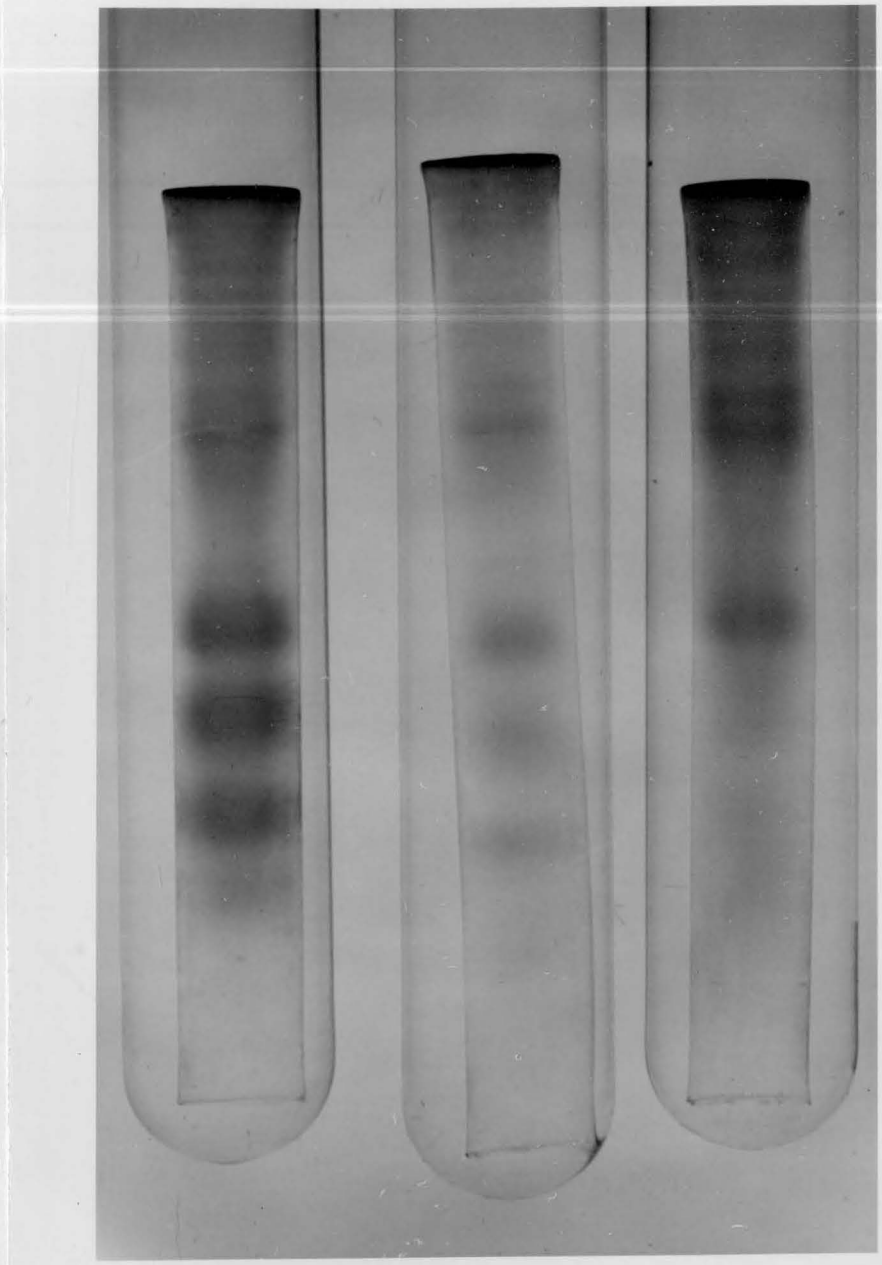

FIGURE 9. SDS polyacrylamide gels of myelin protein from the (A) light, (B) medium, and (C) heavy subfractions isolated from 25-day-old postnatally protein-calorie malnourished pups. Each gel was loaded with 200 µg protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

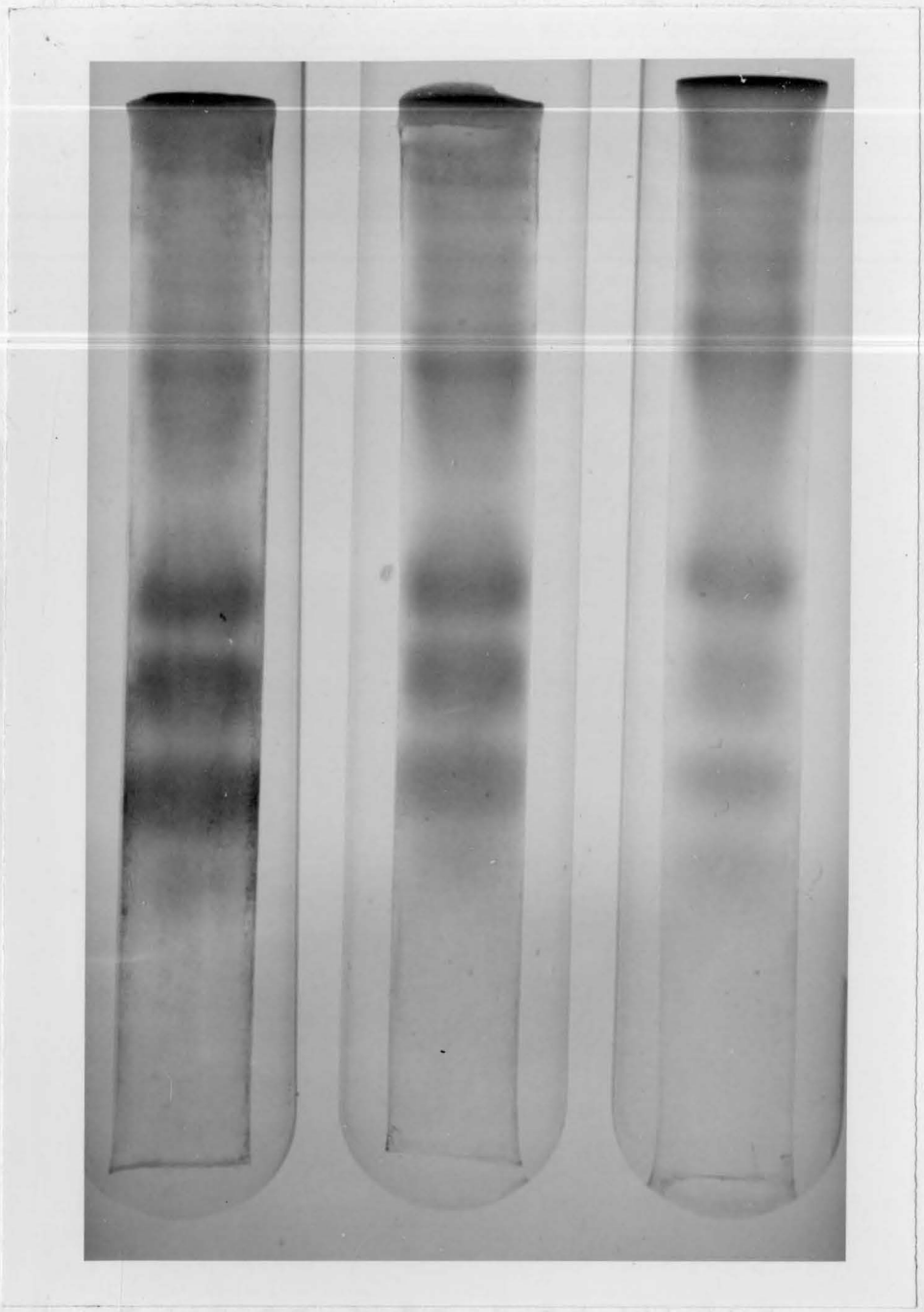

FIGURE 10. SDS polyacrylamide gels of myelin protein from the (A) light, (B) medium, and (C) heavy subfractions isolated from 53-day-old postnatally protein-calorie malnourished pups. Each gel was loaded with 200 µg protein prior to electrophoresis. Labels: HMW = high molecular weight proteins, PLP = proteolipid protein, LBP = large basic protein, and SBP = small basic protein.

TABLE XXIX

RELATIVE DISTRIBUTION OF ^{3}H MYELIN PROTEINS

Subfraction	Animal	% of Total ^{3}H Recovered From SDS-Gels			
		HMW	PLP	LBP	SBP
Light	Control	64.1 ± 1.6	19.9 ± 0.7	9.6 ± 1.0	6.4 ± 1.7
Light	Postnatal-Protein-Calorie Malnutrition	63.8 ± 1.4	21.4 ± 1.9	9.9 ± 1.8	4.8 ± 0.6
Medium	Control	71.7 ± 0.3	15.6 ± 1.3	8.5 ± 0.7	4.2 ± 0.6
Medium	Postnatal-Protein-Calorie Malnutrition	77.1 ± 2.3	12.1 ± 0.6	6.8 ± 1.5	4.1 ± 1.2
Heavy	Control	74.9 ± 5.0	15.6 ± 4.0	6.1 ± 0.9	3.4 ± 1.1
Heavy	Postnatal-Protein-Calorie Malnutrition	75.0 ± 9.1	17.2 ± 7.6	5.3 ± 1.0	2.4 ± 1.1

Each value represents the mean of samples obtained at 18, 25, and 53 days of age ± the standard deviation. HMW = High Molecular Weight Proteins, PLP = Proteolipid Protein, LBP = Large Basic Protein, SBP = Small Basic Protein. Protein-calorie malnutrition is described in text. Values were calculated by dividing the ^{3}H radioactivity solubilized from an entire gel. Each value was multiplied by 100 to determine the percent distribution of radioactivity.

TABLE XXX

RELATIVE DISTRIBUTION OF ^{14}C IN MYELIN LIPIDS

Subfraction	Animal	Cholesterol	Cerebrosides & Sulfatides	Phospholipids
Light	Control	24.9 ± 3.0	38.4 ± 6.0	35.9 ± 9.3
Light	Postnatal-Protein-Calorie Malnutrition	29.8 ± 5.4	24.9 ± 1.7*	42.4 ± 5.9
Medium	Control	33.5 ± 1.5	24.6 ± 3.4	40.1 ± 4.4
Medium	Postnatal-Protein-Calorie Malnutrition	28.7 ± 5.3	25.3 ± 3.7	44.0 ± 4.1
Heavy	Control	20.4 ± 3.8	31.0 ± 4.8	49.1 ± 8.4
Heavy	Postnatal-Protein-Calorie Malnutrition	26.6 ± 8.1	24.9 ± 3.2	46.1 ± 4.7

Each value represents the mean of samples obtained at 18, 25, and 53 days of age ± the standard deviation. Protein-Calorie malnutrition is described in text. Values were calculated by dividing the ^{14}C radioactivity associated with a specific category of lipids by the total ^{14}C radioactivity solubilized from a thin layer chromatography lane. Each value was multiplied by 100 to determine the percent distribution of radioactivity.

^{14}C radioactivity, respectively, in light, medium, and heavy myelin subfraction lipids. The proportion of ^{14}C radioactivity associated with phospholipids followed an expected pattern of enrichment in heavier subfractions. The proportion of ^{14}C radioactivity associated with phospholipids was approximately 39, 42,and 48 percent, respectively, in light, medium, and heavy myelin subfraction lipids. There was a statistically significant ($p < 0.05$) one-third decline in the proportion of ^{14}C radioactivity associated with galactosphingolipids (cerebrosides and sulfatides) in light myelin from malnourished pups compared to light myelin from control pups. The proportion of ^{14}C radioactivity associated with galactosphingolipids in medium and heavy myelin subfractions was 25 and 28 percent, respectively.

CHAPTER IV

DISCUSSION

NORMAL LONG-AND SHORT-TERM METABOLISM OF MYELIN SUBFRACTIONS

Matthieu et al. (1973) have suggested that their heavy myelin subfraction is enriched in membranes in transition from oligodendroglial membranes to mature myelin. Accordingly, one would expect heavy myelin to account for a greater proportion of total myelin during early myelinogenesis than late myelinogenesis. This expectation was borne out by the present research. During the long-term study 53 percent of total recoverable myelin protein was in the heavy myelin subfraction in 13-day-old pups. At subsequent times, the light myelin accounted for either a majority or a plurality of total myelin protein. Similarly, in the short-term study approximately 50 percent of myelin protein recovered 12 and 24 hours following injection of 12-day-old pups was from the heavy myelin subfraction. The proportion of heavy myelin protein was even greater in the more immature 12-day-old brains examined one hour following injection in the short-term study. These observations are in accord with those of Morell et al. (1972) who observed a greater proportion of their lower layer material in early murine myelinogenesis.

The ^{3}H radioactivity associated with myelin proteins and the ^{14}C radioactivity associated with myelin lipids increased throughout most of the 85-day period following administration of the leucine and glucose

derived ^{3}H and ^{14}C labels in the long-term study. The increase in recoverable protein-associated ^{3}H radioactivity was greater than that observed for lipid-associated ^{14}C radioactivity. The observed protein and lipid radioactivity increases are in accord with several reports of the incorporation of a variety of isotopically labelled precursors (Davison and Gregson, 1966; Suzuki, 1970; Banik and Davison, 1971; Jungawala and Dawson, 1971; Benjamins et al., 1973; Druse et al. 1974). However, with the exception of Druse et al. (1974) who observed an 80-day increase in protein-associated ^{3}H radioactivity following [^{3}H] fucose labelling of glycoprotein, and the present study, sustained increases in radioactivity have not been reported for periods longer than 21 days.

The observed increase in total radioactivity associated with myelin subfraction protein and lipid may be due to various factors which Druse et al. (1974) consider in detail. Basically, there are two major possibilities to consider: either labelled compounds in whole brain pools are reutilized or radiolabelled material from a specific myelin precursor pool is subsequently incorporated in myelin. The present study supports the latter explanation.

It is unlikely that the brain would reutilize compounds such as leucine and glucose. These compounds are metabolically labile, easily catabolized, easily incorporated into a variety of whole brain protein and lipid products, and rapidly equilibrated with total body pools. Furthermore, during the 18-hour to 85-day period examined, total recoverable whole brain homogenate ^{3}H and ^{14}C radioactivity decreased by ap-

proximately 90 percent. Reutilization of ^{3}H and ^{14}C labels from a shrinking whole brain pool of radioactivity becomes a less likely explanation for recovery of increasing radioactivity in myelin subfractions.

It is more probable that a large proportion of the initial increase in myelin radioactivity is derived from a membranous precursor of very high specific radioactivity. The appearance of peaks of radioactivity in medium and heavy myelin at 4 days following injection in Series I and 3 days in Series II is consistent with the incorporation of highly labelled precursor pool materials. Agrawal et al. (1974), Sabri et al. (1975), and Benjamins et al. (1976a, 1976b) have suggested that the myelin-like fraction is the precursor membrane. Benjamins et al. (1973, 1976a, 1976b) also report data consistent with a myelin membrane precursor role for the microsomal fraction. The peak in ^{3}H specific radioactivity in the heavy myelin subfraction observed at one hour after injection in the short-term study may be due to the rapid incorporation of highly labelled proteins into heavy myelin.

The present study is compatible with - but does not unequivocably prove - the attractive hypothesis that a membranous myelin precursor is first converted to the heaviest isolatable myelin fractions, which subsequently are converted to lighter myelin, presumably by insertion of myelin specific proteins and lipids. This hypothesis was supported by Benjamins et al. (1973); however, in more recent studies Benjamins et al. (1976a, 1976b) reported a more complex model. As was reviewed previously, in these studies they observed certain protein and lipid myelin components entering myelin density subfractions sequen-

tially from heavier to lighter subfractions (i.e., in support of the hypothesis) and others entering subfractions simultaneously (i.e. not in support of the hypothesis). The report of Figlewicz and Druse (1976a) concerning CNS myelin dysgenesis in the quaking mutant mouse is consistent with a blockage in the subsequent maturation of heavy myelin into lighter myelin subfractions, in support of the hypothesis. One cannot rule out the possibility that various myelin density subfractions are under independent genetic control.

CHRONIC- AND ACUTE- MATERNAL CONSUMPTION OF ETHANOL: EFFECTS ON OFFSPRING MYELIN SUBFRACTION METABOLISM

Two technically similar studies were designed as possible animal models for the fetal alcohol syndrome (FAS). The effects of chronic maternal ethanol consumption were significantly more profound than those seen following acute (in utero) ethanol consumption. The different effects observed in the two model systems may be pertinent to a consideration of the phenomena operative in the etiology of FAS associated neurological dysfunction.

Chronic exposure to ethanol prior to and including gestation through the third day postpartum resulted in dramatic alterations in the patterns of incorporation of ^{3}H and ^{14}C labelled precursors and accretion of myelin subfraction protein. However, once having separated myelin subfractions, no apparent significant alterations were seen in either the electrophoretic profile of myelin subfraction proteins, or the pattern of radioactivity incorporation into electrophoretically separated proteins and thin layer chromatographically separated major lipid classes from myelin subfractions. The normal electrophoretic pattern of myelin subfraction protein from chronic ethanol pups is in accord with the only other study of the effects of maternal ethanol consumption specifically upon CNS myelinogenesis (Szoke, Malone, and Rosman, 1977). Szoke et al. (1977) observed normal electrophoretic profiles of whole brain CNS myelin protein from pups exposed to ethanol early during gestation. The only abnormality which they observed was a

significant decrease in lipid soluble whole brain protein from 32- to 40-day-old ethanol pups.

The conclusion to be drawn from the altered patterns of protein precursor incorporation is that chronic maternal ethanol consumption causes premature onset and cessation of active myelination in the offspring.

At 18 and 25 days of age there was statistically significantly elevated total myelin protein recovered from chronic ethanol pups due to a statistically significantly elevated amount of the immature heavy myelin subfraction. Conversely, at 53 days of age there was slightly less total myelin protein recoverable from chronic ethanol pups and statistically significant declines in the amount of the more mature lighter myelin subfractions. The increment in recovered heavy myelin protein from chronic ethanol pups at 18 and 25 days of age is not due to an artifactual increase in contaminating membranes. Heavier myelin subfractions are more likely to be contaminated with other myelin-associated membranes than are the lighter subfractions. However, in the present study identical procedures were employed for myelin isolation and subfractionation throughout the study. Furthermore, the electrophoretic patterns of myelin subfraction proteins were normal.

At 18 days of age both ^{3}H and ^{14}C incorporation were statistically significantly elevated in total myelin and heavy myelin in chronic ethanol pups. At 25 days of age there were statistically significant elevations in incorporation of both labels in all brain homogenate and myelin subfraction preparations from chronic ethanol pups.

Conversely, at 53 days of age chronic ethanol pups had statistically significant decrements in incorporation of both labels in each myelin subfraction as well as in brain homogenate.

In many ways the observed effects of chronic maternal ethanol consumption are completely dissimilar to the typical patterns seen in pre- and postnatally malnourished offspring. Chronic ethanol pups had comparable whole brain homogenate protein, whereas malnourished offspring typically have decreased brain protein (Nakhasi et al., 1975; Krigman and Hogan, 1976). In contrast to accelerated or premature CNS myelination observed in the chronic ethanol pups, malnourished offspring typically have delayed CNS myelination (Geison and Waisman, 1969; Fishman et al., 1971; Nakhasi et al., 1975; Krigman and Hogan, 1976). Furthermore, in contrast to the observed normal electrophoretic patterns observed in chronic ethanol pups, malnourished offspring typically have abnormal myelin protein electrophoretic patterns (Wiggins, et al., 1976).

The only reported causes of accelerated CNS myelinogenesis have been exercise stimulation (Sammeck, 1975) and early postnatal hyperthyroidism (Hamburgh and Bunge, 1964; Hamburgh,1968). The first cause seems unlikely here. The second cause was not investigated but merits further inquiry. Perhaps chronic maternal ethanol consumption and/or withdrawal therefrom may result in maternal or fetal/neonatal abnormalities in endocrine balance or general metabolism.

Because CNS myelination is a postnatal event in the rat, any proposed mechanism must consider the fact that the alcoholic diet was removed at least a week or more before the usual onset of myelination in the rat. Therefore, the effect(s) which ethanol exerts must be med-

iated by either pre- or perinatal action, presumably, on neurons, axons, and/or oligodendroglia. Since the presence of healthy, functional neurons, axons, and oligodendroglia, each in appropriate numbers, is requisite for proper myelination there exist multiple loci which might be involved. Observed CNS myelination abnormalities may be contributory to FAS associated neurological dysfunction. More likely, the observed CNS myelination abnormalities reflect more underlying neural abnormalities.

Other neural effects of in utero exposure to ethanol have been reported. Alterations in certain neurotransmitter substances have been reported. Rawat (1977) reported increased levels of γ-aminobutyric acid (GABA) and glutamate and decreased acetylcholine and acetyl CoA. Elis, Drisiak, Paschlova, and Masek (1976) reported alterations in serotonin levels. Branchey and Friedhoff (1976) reported slight elevation in the activity of the biosynthetic enzyme, tyrosine hydroxylase.

In contrast to the chronic study, maternal ethanol consumption for an acute (in utero) period resulted in near normal myelination synthetic patterns. The major effects observed in the acute ethanol pups were increased incorporation of [^{3}H]leucine into light and medium myelin subfractions and total myelin and increased incorporation of [^{14}C]glucose into the medium myelin subfraction from 18-day-old acute ethanol pups.

In contrast to chronic ethanol pups, the acute ethanol pups had near normal patterns of CNS myelin subfraction protein accertion except for a statistically significant increase in heavy myelin protein from 53-day-old acute ethanol pups. However, 53-day-old acute ethanol pups

had near normal amounts of light and medium myelin; a small increment in the immature heavy myelin subfraction is, accordingly, of questionable physiological significance.

In accord with the observations of Szoke *et al.* (1977) myelin subfraction protein from acute ethanol pups had normal electrophoretic profiles. Also, no differences were observed in the pattern of ^{14}C incorporation into major myelin subfraction lipid classes separated by thin layer chromatography.

Since *in utero* ethanol exposure resulted in nominal effects while chronic ethanol exposure prior to and including the *in utero* period did cause profound effects, ethanol probably is not exerting a teratogenic effect *per se* with respect to CNS myelination. More probably, it is exerting its chronic effect by perturbation in maternal or offspring endocrine or metabolic balance. The present study, however, cannot prove or disprove that interesting possibility. While no teratogenic effects were observed in this particular *in utero*, acute paradigm, negative results from this study do not preclude the possibility of *in utero* teratogenic effects of ethanol if dosage of ethanol were sufficiently high.

It is interesting that the typical mother reported in human FAS case histories has been a chronic abuser of ethanol for several years. Jones *et al.* (1973) report an average of 9.4 years (ranging 2-23 years) chronic ethanol abuse in their case history population.

POSTNATAL PROTEIN-CALORIE MALNUTRITION:
EFFECTS ON OFFSPRING CNS MYELIN
SUBFRACTION METABOLISM

Postnatally protein-calorie malnourished pups had rather profound decrements in body and brain weights, total myelin protein, alterations in the pattern of incorporation of isotopically labelled protein and lipid precursors, and abnormal distribution of incorporated ^{3}H and ^{14}C radioactivity in electrophoretically separated myelin proteins and thin layer chromatographically separated myelin lipids. The severity of many of these effects was mitigated during increasing periods of nutritional remediation.

The present study is in accord with previous studies which examined postnatal malnutritional effects on CNS whole myelin. In agreement with Nakhasi et al. (1975) and Krigman and Hogan (1976), malnourished offspring had deficits in whole brain homogenate protein throughout the study. In agreement with Geison and Waisman (1969), Fishman et al. (1971), Nakhasi et al. (1975), and Krigman and Hogan (1976), the stress employed here also resulted in a temporary deficit in total CNS myelin which was restored upon nutritional remediation. In agreement with Wiggins et al. (1976) malnourished pups at early ages had abnormal electrophoretic profiles of myelin protein from medium and heavy myelin subfractions. In the present study the increase in the proportion of high molecular weight proteins and the decrease in the proportion of myelin basic proteins were more significant than the decrement in proteolipid at early ages in medium and heavy myelin from post-

natally protein-calorie malnourished pups.

Because the heavy myelin subfraction described by Matthieu et al. (1973) and Zimmerman et al. (1975) has a composition similar to CNS myelin isolated from immature brain, an excess or disproportionate share of heavy myelin could be a useful measure of CNS immaturity. The observed relative excess of heavy myelin at 25 days of age might be explained by a significant increase in the number or proportion of smaller diameter axons invested with fewer myelin lamellae. Heavy myelin, thought to be enriched in transitional membranes, presumably forms the myelin in closest contact with the axon. Histological studies were not done in the present study. However, Krigman and Hogan (1976) observed smaller axons and a decreased number of myelin lamellae per axon diameter in postnatally starved rat pups.

Compared to controls, the ^{3}H radioactivity data demonstrated statistically significantly increased synthesis of heavy myelin and total myelin in 25-day-old stressed pups and increased synthesis of lighter myelin subfractions and total myelin in 53-day-old stressed pups. The ^{14}C radioactivity data demonstrated statistically significantly increased synthesis of lighter and heavy myelin subfractions in 25-day-old stressed pups and increased synthesis of lighter myelin subfractions and total myelin in 53-day-old stressed pups.

The protein accretion and radioactivity incorporation data are consistent with greater synthesis of heavy myelin at 25 days and greater synthesis of lighter myelin at 53 days of age. This altered pattern of CNS myelinogenesis is compatable with a temporary delay in

the conversion of heavy to lighter myelin.

If normal myelinogenesis involves the conversion of heavier myelin subfractions into lighter myelin subfractions, the process of maturation presumably involves the synthesis and insertion of myelin specific proteins and lipids into the immature membranes. Either an inability to synthesize or to insert would be manifested in delayed or defective myelination. The protein electrophoretic studies indicated a temporary deficit of myelin basic protein. The present study is unable to determine whether this defect reflects delayed synthesis or delayed insertion of myelin basic protein. However, it is interesting to note that in stressed pups there were decreased proportions of ^{14}C in galactosphingolipids in light and heavy myelin, the decrease in light being statistically significant. Relative changes in the composition of myelin lipids have been reported in protein deprived pups by Nakhasi et al (1975) and Simons and Johnston (1976). Membrane lipid defects might result in inability to make normal insertions.

As discussed before, normal myelinogenesis requires healthy, functional neurons, axons, and oligodendroglia, each in adequate numbers and proportions. Any alterations or defects at these loci may affect myelinogenesis. Since myelinogenesis is a postnatal event in the rat, it is reasonable that the abnormalities observed here reflect abnormalities in oligodendroglia which proliferate and myelinate axons postnatally. This assumption is borne out by the report of Krigman and Hogan (1976). They observed a deficit in the number of oligodendroglia in severe postnatal malnutrition. It is also possible that substrate

availability limitations were also contributory to the observed delayed and abnormal pattern of myelination in the present study.

CHAPTER V

SUMMARY

Purified central nervous system (CNS) whole brain myelin (Norton and Poduslo, 1973b) was subfractionated into myelin of light, medium, and heavy density (Matthieu et al., 1973). Protein and lipid metabolism of these myelin subfractions was investigated in developing rat brain using radioisotopically labelled precursors injected intracerebrally. L-[4,5-^{3}H]leucine and D-[U-^{14}C]glucose were chosen as labelled precursors, respectively, of protein and lipid.

Myelin subfraction metabolism was investigated in normal developing rat brain during a long term period extending 18 hours to approximately 90 days following administration of both the ^{3}H and ^{14}C labelled precursors to 12-day-old pups, and at intervals of 1, 12, and 24 hours following administration of the ^{3}H labelled precursor to 12-day-old pups. Myelin subfraction metabolism was also investigated in developing rat brain in three experimental paradigms: chronic maternal ethanol consumption, acute (in utero) ethanol consumption, and postnatal protein-calorie malnutrition. The stress paradigms were designed to investigate the extent and possible significance of abnormalities in myelinogenesis in animal model systems of the human fetal alcohol syndrome (FAS) (Lemoine et al., 1968) and malnutrition, each of which is associated with neurological dysfunction in affected neonates.

Stress paradigms were different in each case. In the chronic ethanol study females were maintained on either control or ethanol liquid isocaloric diets (Freund, 1969; Lieber and DeCarli, 1974) for a pe-

riod beginning two months before gestation and ending on the third postpartum day. In the acute ethanol study the diets were employed from approximately the fifth day of gestation through the third postpartum day. In the malnutrition study, lactating females were restricted in total quantity of an otherwise normal diet during the first 18 postpartum days. In each stress paradigm, myelin subfractions were investigated at 18, 25, and 53 days of age following an 18 hour pulse labelling with both radioisotopically labelled precursors.

Normal long and short term studies revealed sustained accretion of myelin protein and incorporation of both ^{3}H and ^{14}C throughout the period examined. Evidence was obtained consistent with the hypothesis (Matthieu et al., 1973) that the chemically and morphologically immature heavy myelin is converted to the mature lighter myelin subfractions. The existence of a myelin precursor membrane was also supported by total and specific radioactivity data.

The fetal alcohol syndrome is a multiple pathology syndrome of unknown etiology associated with chronic maternal ethanol abuse. Evidence was obtained that acute (in utero) exposure to ethanol causes only minimal and transient perturbations in CNS myelinogenesis, whereas chronic ethanol exposure results in premature onset and cessation of myelination, abnormal proportions of myelin subfraction protein, and apparently normal myelin protein electrophoretic patterns from separated myelin subfractions. The observed aberrant myelinogenesis probably reflects more underlying neural abnormalities of unknown nature. The effect of ethanol in the present study does not appear to be teratogenic; it may reflect abnormalities in maternal or fetal/neonatal endocrine or metabolic

status as a consequence of chronic ethanol exposure or withdrawal therefrom.

In agreement with several investigators, severe deficits in body and brain weights, deficits in brain and myelin protein, and abnormal electrophoretic profiles of myelin protein were observed following protein-calorie malnutrition. With increasing periods of nutritional remediation these effects were minimized. The pattern of myelinogenesis was delayed and altered. The association of neonatal malnutrition with neurological dysfunction and with dysmyelination was reviewed.

The research reported represents the first account of the metabolism of the Matthieu myelin subfractions, the first report of the effect of maternal ethanol consumption upon myelinogenesis, and the first report of the effect of postnatal protein-calorie malnutrition on the metabolism of separated myelin subfractions.

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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.

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