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THE LIPID COMPOSITION OF MICROSOMAL
AND MYELIN FRACTIONS.

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II. A COMPARISON WITH CEREBRUM OF LIPID COMPOSITION
AND PROTEIN CONTENT. III. THE LIPID COMPOSITION
OF MICROSOMAL AND MYELIN FRACTIONS.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy in the Graduate School of
The Ohio State University

BY

BYRON NAGIB DE SOUSA, B.S., B.S., M.D.

* * * * *

The Ohio State University
1976

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Approved By
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Adviser
Department of Physiological Chemistry
I hear and I forget
I see and I remember
I do and I understand

Ancient Chinese Proverb
To my parents
ACKNOWLEDGMENT

This study was accomplished at the Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio, U.S.A.

I would like to express my sincere appreciation to my adviser, teacher and friend, Dr. Lloyd A. Horrocks, who has made it possible for me to carry out my scientific work through his encouragement, support and advice during my investigations. I would also like to thank the chairman of the Department, Dr. David Cornwell for his constructive criticism during our department seminars, which have been invaluable. I also wish to express my gratitude to Drs. Rao Panganamala, John Rieske, Howard Sprecher and Allan Yates for their discussions. I would also like to express my gratitude to Ms. Betty Harvey for the typing of this manuscript without whom this work would never have been accomplished.
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PUBLICATIONS

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ABSTRACTS


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A simple, fast, economic and damage-free procedure for rat spinal cord removal has been developed. It is hoped that this method will stimulate further, more systematic research on the biochemistry of rat spinal cord.

The increase in protein concentrations in cerebrum and spinal cord during maturation of the Sprague Dawley rat were rather similar, but at all ages the spinal cord contained a higher protein concentration than cerebrum. At all ages the concentrations of cholesterol, galactolipids and phospholipids were higher in spinal cord than in cerebrum. Marked changes in the phospholipid composition in both tissues were also observed during development. The proportion of ethanolamine plasmalogens increased from 9% to 19% in cerebrum and from 18% to 29% in spinal cord. Choline phsophoglyceride proportions decreased from 58% to 33% in cerebrum and from 54 to 25% in spinal cord, while the proportion of sphingomyelin increased moderately throughout maturation in both tissues. The marked increase in concentrations of protein,
cholesterol, galactolipids, and various phospholipids took place earlier in spinal cord than in cerebrum.

Substantial amounts of the microsomal fraction were obtained from spinal cord at all ages. Only trace amounts of the spinal cord myelin fraction were obtained from newborn and 1 day old rats. For isolated myelin during the first two weeks of life the mole ratio of cholesterol to lipid phosphorus gradually declined to the adult value of about 1.0. The mole ratio of lipid galactose to lipid phosphorus after 7 days of age gradually increased to a value of 0.5 at 30 days of age. Changes in the relative proportions of individual phospholipids were also detected in the myelin. Those changes are qualitatively similar to the ones found for the total tissue. In the microsomal fraction, the concentrations of cholesterol and lipid phosphorus were rather constant through 11 days of age. The microsomal lipid galactose concentration increased considerably from 5 to 30 days of age. The relative proportions of phospholipids also changed. The most pronounced changes included a decrease of the choline and an increase of the ethanolamine phosphoglyceride proportions. The changes in lipid content and composition are consistent with the continuing deposition of myelin, maturation of the myelin lipid composition and increasing proportions of oligodendroglial membranes in the microsomal fraction.
The Rat.

The Albino Norway Rat, *Mus norvegicus albinus*, has a life span of about three years and breeding begins at about three months in all seasons of the year, but most readily in the spring (Donaldson, 1924). The albino rat is born blind, hairless, with a short tail, closed ears and undeveloped limbs. It responds to contact, olfactory and taste stimuli, utters a squeaking sound and is capable of some locomotory movements which are a combination of wriggling and paddling.

Sexual maturity is reached in both males and females at the age of about two months or less. The estrous cycle of the albino rat is about five days. The gestation period in albino rats is about 21-22 days. However this period of gestation might be prolonged if the female is suckling more than five young rats.

During our investigation, litter sizes varied from four to seventeen pups. In large litters we observed a few underweight pups which also had low cerebrum and spinal cord weights.

When compared to man the albino rat has many similarities which are very significant for its use as an experimental animal. Like man the rat is omnivorous and capable of adapting itself to a large variety of diets. The albino rat doubles its birth weight in about six to seven days, which is one-thirtieth the time taken by man. For a ninety year man a three year old rat is taken as the equivalent, which again gives a 1:30 ratio for the equivalent ages. Therefore, we can assume
that the rat lives thirty times as fast as man, and that one day of rat
life is equivalent to one month of human life. The menopause occurs
at 15-18 months, which is equivalent to 38-45 years in man. In addition,
parameters such as the weight of the brain, and the percentage of water
in the brain change similarly at equivalent stages of growth in the rat
and man (Donaldson, 1918).

Neurogenesis.

The central nervous system of mammalian embryos can be recognized
as a long slipper-shaped area, known as the neural plate, shortly after
its appearance. The neural folds are formed by the elevation of the
lateral edges of this plate. The depression formed between the folds
forms the neural groove. As development proceeds the neural folds be­
come more elevated and approach each other in the midline and finally
fuse to form the neural tube. This process is simultaneous in the
cephalic and caudal directions and at the term of this fusion the
central nervous system is seen as a closed tubular structure with a
long caudal portion (the spinal cord) and a broader cephalic portion
characterized by a number of local swellings (the brain vesicles),
(Langman, 1968). While the central nervous system (CNS) derived from
the neural plate the peripheral nervous system (PNS) is partly derived
from the ectodermal epithelium of the neural plate and partly from
a region of the ectoderm called the neural crest.

Cells from the wall of the neural tube divide actively and are
organized in a pseudo stratified epithelium known as the primitive
ependyma. These matrix cells seem to be all of the same type (Herman &
Kauffman, 1966). Some escape from the division cycle and differentiate
into neurons, astrocytes and oligodendrocytes (de Vellis & Clemente,
(1970). Three major stages of cytological development are generally
described on the basis of microscopy, namely primordial neuroepithelial cells, early cellular differentiation (spongioblasts, neuroblasts, sympathoblasts) and final differentiation (neurons, oligodendrocytes, astrocytes). The main types of neurons derived from the neuroectoderm are interneurons, receptor neurons, adrenergic neurons, neurosecretory cells and motoneurons. The types derived from the neural crest are sympathetic neurons, chromaffine cells, and sensory ganglionic cells. Glial cells of the CNS are mainly of two kinds; astrocytes and oligodendrocytes. In the PNS the Schwann cell is the counterpart of the oligodendrocyte.

Myelin.Introduction

The orthodox idea that myelin behaves like an electrical insulator that wraps around the axon and resembles the insulation that surrounds an electrical cable is presumably in some degree correct. Nevertheless, the main role of this insulator seems to be the facilitation of neural impulse conduction, rather than the prevention of short-circuits between adjacent nerve fibers. In the central nervous system each oligodendrocyte provides myelin for only a segment of the axon. The myelin sheath does not furnish a continuous covering over the entire axon length. Between myelin segments, short portions of the axons are left uncovered. These breaks of continuity or interruptions, called nodes of Ranvier (Ranvier, 1871), play a crucial role in the transmission of neural impulses along the myelinated axons. The impedance of the myelin sheath is about 10 to 20 times higher than the extracellular salt solutions. The segments of myelin, which are periodically interrupted
by the nodes of Ranvier, increase the distance between active regions along the nerve fiber. The time between successive peaks of the action potential is little affected by the distance between active regions. Therefore, the conduction velocity increases as the distance between active regions increases. The myelin sheath prevents ions from moving across the membrane, and thus only the spaces between successive myelin sheaths (i.e., the nodes of Ranvier) are used for ionic movement. Hence the bioelectrical charge or the action potential generated at the low impedance region of the node by an impulse flows through the medium external to the sheath to activate the axonal membrane at the next node, rather than causing a continuous sequential depolarization of the membrane through the high impedance sheath. This type of conduction is known as saltatory conduction, because the action potential jumps from node to node. In unmyelinated fibers, increased conduction velocity is achieved by increasing the diameter of the axon. Conduction velocities in myelinated fibers are proportional to the diameter of the fiber and vary from 10 m/sec for a 2μm nerve to 100 m/sec for a 20μm nerve. On the other hand, the conduction velocity in unmyelinated fibers is roughly proportional to the square of the diameter and runs from 0.3 m/sec for a 0.7μm fiber to 25 m/sec for a 500μm squid giant axon.

In the peripheral nervous system, the myelin membrane is formed by the conjunction of Schwann cells to the axon of the neuron (Fig.1). The Schwann cells enclose almost completely the myelinated nerve fiber and the myelin lamellae form a spiral in continuity with the Schwann cell with respect to the axon. On the other hand, in the central nervous system, the myelin sheath arises from the extension and
FIGURE 1. Representation of myelin formation in the peripheral myelin sheath. (A) The plasma membrane of a Schwann cell partially surrounds the axon (B) to form the mesaxon. (C) The mesaxon elongates to form a spiral around the enclosed axon (Davison & Peters, 1970).
modification of the plasma membrane of oligodendroglial cells which form a spiral around the axon of nerve cells (Fig. 2). The myelin membranes are continuous with the oligodendrocyte plasma membranes (Bunge, 1968). Myelin is the paramount substance of white matter, including about 50% of its total dry weight. Myelin is responsible for the glistening pearly-white appearance of white matter.

Myelin has been one of the most studied nervous system components. In 1839, Schwann described the sheath and neurilemmal cells which are closely associated with the axon in the PNS and now bear his name. The name myelin was given by Virchow (1854) from the Greek word muelos (marrow, from mus, muscle) (German, mark) to describe the lipid-rich material that was then found in the medullar part of the brain. Since the myelin is wrapped around the axon in the form of a sheath, thus the inappropriate term myelin sheath (German, Markscheide).

In 1862, microscopic examinations under polarized light by Valentin gave evidence that myelin was a highly ordered structure. Later in 1889 Ranvier published his technique for osmium staining which advanced the histological study of myelin. The paracrystalline nature of myelin became apparent with the work of Lehman (1911, 1918), Friedel (1922), and Schmidt (1924, 1936). Ambronn (1890) had shown that alcohol reversed the sign of the uniaxial double refraction from positive to negative in myelinated nerve fibers, causing their optical properties to resemble those of unmyelinated fibers. Göthlin (1913) showed that myelin was not only found in vertebrate nerves but also in invertebrate nerves. He also observed that the birefringence of nerve fibers was mainly related to the lipid moiety of the myelin and secondarily to the protein moiety. Schmidt (1936) proposed that the myelin sheath was
FIGURE 2. Representation of myelin formation in the central nervous system. (A) The oligodendroglial plasma membrane process surrounds the axon partially. (B) The process completely envelops the axon, and retains cytoplasm throughout its entire extent (B') the cytoplasm is lost from the middle part of the process. (C) The process spirals around the axon with retained cytoplasm or (C') with it only at the outer and inner borders. (D) Finally, irrespective of whether or not cytoplasm is retained throughout the myelin-forming process, the structure of the resulting myelin sheath is the same in both cases. (Davison & Peters, 1970).
made of concentric cylindrical layers of protein sandwiched between double layers of lipids. Sjöstrand (1949) verified the laminar structure through electron micrographs of nerve tissue preparations. Fernández-Morán (1950a,b) and Sjöstrand (1950) confirmed such laminar structure. Geren (1954,1956) and Geren & Schmitt (1954) made the suggestion that myelin was laid down directly by the satellite cell in spirals and not in concentric layers as had been believed.

As early as 1854, von Bibra established that the lipid composition of the brain changes with age. Even though there was no idea of the relationship between brain lipids and myelin, a number of lipids were found in the brain in peculiar composition and such components varied with age, sex and pathological states. Thudichum (1884) conceived a kind of relation from his different studies on brain lipid composition. However, only with the work of Folch et al. (1951) was a quantitative method of extraction devised to examine brain lipids.

There were great controversies over which cell type in the central nervous system is responsible for myelination. Kolliker (1904) attributed this role to the axon and Hild (1957) reaffirmed this concept which he abandoned later (Hild, 1960). Rio-Hortega (1928) was the first to postulate the role of oligodendroglia in central myelination. This concept was modified by Alpers & Haymaker (1934) who suggested that in addition to oligodendrocytes, astrocytes are involved in myelination. Later, Luse (1956), from microscopic observations, proposed that the myelin sheath originated from the flattening of glial processes juxtaposed against the nerve axon. Those observations were reaffirmed by Ross et al. (1962) through studies of electron micrographs of myelinating cultures of rat brain.
De Robertis et al. (1958) postulated that the myelin sheath originates by fusion of membranes and vesicles inside the cytoplasm of glial cells located near the axon. Fernández-Morán & Finean (1957) proposed that myelin arises from a wrapping of the glial cell plasma membrane around the axon, which is consistent with a spiral arrangement of myelin sheaths similar to peripheral myelin sheaths. This was later supported by Maturana (1960) and Peters (1960) who showed that the number of lamellae in different parts of the same myelin sheath are in agreement with a spiral arrangement. The direct continuity between the myelin membranes and the oligodendroglial plasma membrane in the developing mammalian central nervous system (Bunge et al., 1962; Peters, 1964) is further evidence for the role of oligodendroglial processes (Bunge et al., 1961; Lampert, 1965).

During myelination glial cell cytoplasm can be visualized between loosely wrapped myelin lamellae (Karlsson, 1967). The cytoplasm disappears gradually as the number of lamellae increases, but at the loops nevertheless, cytoplasmic pockets have been found in pathological conditions.

Robertson (1959), from electron microscopic studies of peripheral myelin, proposed the "unit membrane" concept, which is meant to represent the minimum structure of a cell membrane and to be comprised of a bimolecular layer of lipid sandwiched between monolayers of protein. Gent et al. (1964), based on physicochemical studies, proposed that myelin is composed of a repeating unit containing lipids and proteins. Cuzner and her collaborators (1965), in support of the unit membrane concept, reported that the proteins and lipids of myelin have the same rate of turnover. Finean (1953) postulated the existence of a cholesterol-phospholipid complex that fits into the dimensions
obtained by X-ray diffraction techniques and which does not cause stress to the phospholipid molecule. Eng & Smith (1966), on the basis of turnover rates, divided the myelin lipids into "stable" and "labile". The "labile" myelin lipids include phosphatidyl inositol, phosphatidyl choline and phosphatidyl serine, which have a half-life of 5 weeks to 4 months. They also observed that the molar ratio of the sum of "stable" lipids closely approximates the molar concentration of cholesterol in myelin from various species. This remains close to one during maturation although the myelin composition changes (Eng et al., 1967). O'Brien (1967) postulated a model for the myelin membrane (Fig. 3) based on the drawings of Vandenheuvel (1963).

Before, myelin composition could not be determined by a direct approach since there were no methods to isolate myelin. Hence, myelin components were inferred by comparisons of white matter and gray matter composition, and by following the composition of the brain or its white matter as a function of the stage of myelination during brain development. This approach was used by Koch to attempt to define the myelin composition of rat, sheep, pig and human brains and spinal cords (Koch, 1904, 1905, 1907). According to his determinations, he defined three basic periods of development. During the first period of up to 10 days after birth, the rat brain shows a high water content, a low lipid content and a lack of histologically visible myelin. During the second period from 10 to 40 days of age, there is a decrease of the water content and deposition and accumulation of some lipids and proteins. The third and last period is when the myelin content becomes constant. MacArthur & Doisy (1919) did a more detailed work on the subject and Waelsh et al. (1940,1941), using radioisotopes, demonstrated that those lipids believed to participate
FIGURE 3. Molecular model of central nervous system myelin. The lipid acyl moieties are assembled in a bilayer having a distance of 55 to 60 Å between the polar headgroups (dark areas). In the center the lipid molecules of the two layers interdigitate. The smaller and asymmetrical blocks close to the outer edges of the bilayer represent cholesterol and the hook-like configuration represents a polyunsaturated fatty acid (O'Brien, 1967).
in the myelin composition were laid down during myelination.

The most systematic study on brain and spinal cord composition is still that of Branté (1949). He made inferences about myelin composition, and identified cholesterol, cerebrosides and sphingomyelin as typical myelin lipids. Johnson and his collaborators (1950) named them "myelin lipids". Later McColl & Rossiter (1952a&amp;b) reported a similar type of study.

When Polch (1951) and Polch-Pi (1955) characterized phosphatidyl inositol and proteolipids as myelin constituents further advances in myelin chemistry were achieved.

**Myelin: Isolation and Purification.**

The isolation of different classes of subcellular particles from tissue homogenates can be accomplished basically by two different centrifugation procedures, namely isopycnic centrifugation when significant differences exist in their buoyant densities, and differential centrifugation if significant differences exist in their sedimentation rates. The sedimentation rate is dependent on the size, density and shape of the particles. The buoyant densities of biological membranes are mainly determined by the relative amounts of protein and lipid forming the membranes. The buoyant density is directly proportional to the ratio of protein to lipid. Therefore, if the ratio of protein to lipid decreases the buoyant density decreases, and vice-versa.

As sub-cellular fractionation methods were improved for the isolation and purification of myelin a great advance in the study of the composition of isolated and purified myelin was made. Different groups, using variations of the subcellular fractionation procedure
of Schneider & Hogeboom (1952), obtained small amounts of relatively pure myelin from brains of different animals (August et al., 1961; Mandel et al., 1961 and Patterson & Finean, 1961). Laatsch et al. (1962) obtained myelin from a dispersion medium containing 0.88M sucrose. Horrocks (1967) used 1.0M sucrose, then 0.80M sucrose for purification.

According to Cotman (1972), during dispersion of brain membranes shear into vesicles of a size determined by the "shear conditions" which include such things as the clearance between the pestle and the homogenizer tube, the rotation speed and the number of up and down strokes of the pestle, and the duration of the process. Since the main variable in the separation by rate sedimentation is particle size, the dispersion technique will certainly affect the resulting separation. This is of special importance when subcellular particles from different tissues are being compared.

When sucrose solutions are used to disperse nervous tissue the myelin lamellae bulge out, peel off the axons, and form spherical vesicles of about the size of nuclei and mitochondria, and hence sediment with these fractions during differential centrifugation (Norton, 1971). Since among the subcellular fractions myelin has the lowest protein to lipid ratio and is less dense than 0.8M sucrose, it floats on it whereas denser particles as nuclei, mitochondria and synaptoosomes sediment at the bottom of the centrifuge tube.

In order to remove fragments of axoplasm trapped within myelin vesicles during dispersion, it is essential to shock the vesicles osmotically. In a hypotonic medium, these myelin vesicles will swell and break open, releasing the contaminating axoplasm into the surrounding medium.
Adams et al. (1963) and Cuzner et al. (1965) suspended brain crude mitochondrial fractions on 0.8M sucrose solutions. After centrifugation and osmotic shock they obtained myelin relatively free of axoplasm and mitochondria and which had low succinate dehydrogenase and ATPase activity and low levels of RNA.

Horrocks (1967) purified myelin from squirrel monkey spinal cord by three different methods. In the first, tissue was dispersed in 1.0M sucrose and the floating layer was then purified by reflation on 0.8M sucrose, osmotically shocked and repurified on a final flotation on 0.8M sucrose. In the second, tissues were dispersed in 0.32M sucrose and myelin was isolated from the crude mitochondrial fraction by flotation on 0.8M sucrose. After an osmotic shock, the myelin was purified by sedimentation through a sucrose density gradient. Two bands, corresponding to the "light" and "heavy" myelin fractions described by Autillo et al. (1964) were recovered. The third method was similar to the second, except that the osmotically shocked crude myelin was purified by flotation on 0.8M sucrose. Myelin isolated by all three methods was very pure as analysed under the electron microscope, by chemical composition and through solubility in chloroform/methanol (2:1, by vol.).

Norton & Poduslo (1973a) have described a method for myelin isolation modified from Autillo et al. (1964). This procedure yields myelin of low and equal levels of contamination during all phases of maturation. The myelin isolated by this procedure was very pure as judged by chemical composition. Myelin is the only subcellular fraction obtained by this method.

Spohn & Davison (1972) have described a procedure that allows the isolation of microsomes, mitochondria, synaptosomes as well as myelin.
In this method, myelin is isolated from the crude nuclear and crude mitochondrial fractions by flotation on 0.8M sucrose followed by osmotic shock and reflotation on 0.85M sucrose.

Many authors have suggested that myelin is not homogeneous and can be separated into sub-fractions of different densities. Myelin isolated from animals in early stages of development has been fractionated into a compact myelin and a "myelin-like" fraction by means of discontinuous sucrose gradients (Banik & Davison, 1969; Agrawal et al. 1970, 1973). The myelin-like fraction was found to have a higher density (greater than 1.0M sucrose) than the compact myelin (0.65M sucrose). The myelin-like fraction resembles myelin in its enzyme activity, but no cerebroside was found (Agrawal et al., 1970). According to Agrawal and collaborators, the myelin-like fraction represents a transition between the oligodendroglial plasma membrane and compact myelin.

There are evidences that myelin from brain and spinal cord may not be similar in all aspects. Wolfram & Kotorii (1968) have reported such dissimilarities for protein content. Morell et al. (1973) have found differences in myelin proteins. Evidence for differences in the stability of the myelin sheath in spinal cord and brain has also been presented (Smith, 1973b; Smith & Tagg, 1973). The problem of regional differences in the central nervous system has not yet been completely resolved and deserves additional study.

Toews et al. (1976a) described a method for the simultaneous isolation of purified microsomal and myelin fractions from rat spinal cord. The purified microsomes contained insignificant amounts of myelin as analyzed by electron microscopy and polyacrylamide gel electrophoresis. The myelin fraction was very pure as analyzed by electron microscopy,
enzyme activities and polyacrylamide gel electrophoresis. In contrast with brain, crude microsomal fractions from spinal cord contain a large part of the myelin. These methods developed for the isolation of brain myelin cannot be applied directly to spinal cord.
CHAPTER II

MATURATION OF RAT SPINAL CORD.

A RAPID METHOD FOR EJECTION FROM THE VERTEBRAL COLUMN.
The mammalian spinal cord has been a difficult tissue to study because it is enclosed within a large number of vertebrae and it is extremely sensitive to trauma. Dissection of the spinal cord from the vertebral column requires at least 20 minutes for the adult rat. This dissection is inevitably accompanied by multiple traumatic injuries to the cord plus the insult of ischemia for a 20 minute period. Ischemia can be avoided by maintaining the animal with a respirator during removal of spinal cord tissue. This method is time-consuming, difficult, and introduces the variable of anesthetic agents. Another possibility is freezing of the whole animal or vertebral column in liquid nitrogen, but freezing of the spinal cord is not immediate and removal of the frozen spinal cord is quite difficult. In the present paper we describe a method for removal of the intact spinal cord in less than one minute with minimal trauma.

MATERIALS AND METHODS

Sprague-Dawley (Madison, Wisc.) rats were decapitated at 0, 1, 2, 3, 5, 7, 9, 11, 12, 13, 14, 15, 17, 20, 25, 30, 40, 58, 127, 190 and 300 days of age. Two parallel longitudinal incisions were made on each side of the vertebral column at the sacral level. After insertion of forceps to hold the vertebral column, it was transected at the most distal position. For older animals, longer incisions along the vertebral column are necessary in order to sever more of the spinal roots. Selection of a syringe and needle depends on the age of the rat (Fig.4). For rats older than 30 days, a 20cc syringe is used. Syringes of 5 or 10cc capacity are used with smaller
FIGURE 4 Material used for rat spinal cord removal.
rats. The needle should approximate the inside diameter of the vertebral canal. Up to 7 days of age, I use 20 gauge needles, then 18 or 19 gauge needles for rats of 7 to 21 days of age. For adult rats a 16 gauge needle is necessary. For younger rats I have also been able to use plastic tips for micropipettes after removing a portion of the large end so that the remainder fits inside a Luer-Lok hub.

A gauze pad is placed immediately in front of the rat. The syringe is filled with cold water, the vertebral column is held with forceps and the syringe needle is introduced about 5 mm into the caudal end of the vertebral canal. Pressure is then applied to the syringe plunger with the thumb until the spinal cord is ejected from the vertebral canal (Fig. 5). The cord is blotted on the gauze and immediately placed in a Petri dish. Graph paper under the dish was used to measure the length of the cord. The adhering meninges were dissected off, any remaining spinal nerves were trimmed, then the spinal cord was placed in a freezer (-20°) until required for chemical determinations. Cerebrums were removed by dissection. Weights of the tissues were determined after freezing. With appropriate modifications of the syringe needle, this method may also be used with mice and cats.

RESULTS

The rat spinal cord increased from 34 mg and 25 mm at birth to 743 mg and 120 mm in the adult (127 days of age). The weight of the spinal cord at birth is only 5 percent of the adult weight (Fig. 6). A marked increase in weight began during the third week of life. From 14 to 21 days, the weight nearly doubled. The spinal cord length at birth is 21 percent of the adult length (Fig. 7a8). A gradual increase during development was observed. Since changes were taking place in both the
FIGURE 5 The carcass of an adult rat just after the ejection of the spinal cord by hydraulic pressure from a syringe.
FIGURE 6  The weight ± s.e.m. of rat spinal cords during maturation. Note that the age scale is logarithmic.
FIGURE 6
FIGURE 7  The length ± s.e.m. of rat spinal cords during maturation.
FIGURE 8 Spinal cords from 3 & 7 days of age and adult rat.
weight and length, we also plotted the ratio of weight to length as a measure of "thickness" of the spinal cord (Fig. 9). This parameter more than doubled between 7 and 20 days of age, then doubled again between 20 days and adulthood. Between 11 and 120 days of age, the increase of "thickness" was proportional to the logarithm of the age. Part of this increase is due to the deposition of myelin (Chapter IV). Partly because of the pronounced change in length of the spinal cord during development, the change in weight is much greater for the spinal cord than for the brain. However, even the "thickness" parameter, mg/cm of spinal cord, increased several-fold during the course of development. Increases in the "thickness" began shortly after birth, were of greatest magnitude during the third week of life, then gradually diminished but with detectable increases beyond 100 days of age.

DISCUSSION

Removal of the spinal cord by pressurized ejection is much faster than previous methods which require dissection of the spinal cord from the vertebral column. By analogy with brain, energy supplies are depleted (Lowry et al., 1964), free fatty acids are released (Bazan, 1971; Cenedella et al., 1975), mitochondria are uncoupled (Ozawa et al., 1967), and many other metabolic changes take place in the tissue during the time required for dissection. The present method may be used to study the effects of stagnant anoxia on spinal cord.

The spinal cord is extremely sensitive to impact trauma and compression (Dohrmann, 1973; Osterholm, 1974). Among the sequelae are hemorrhagic necrosis of the gray matter (White et al., 1969; Ducker & Perot, 1971), demyelination of the white matter (Dohrmann et al., 1972 Horrocks et al., 1973) and a permanent loss of function in areas below the site of
FIGURE 9 The "thickness" (ratio of weight to length) of rat spinal cords during maturation.
FIGURE 9
injury (Albin et al., 1968). Compression and trauma of the cord are unavoidable during dissection of the vertebral column. With removal of the spinal cord by pressurized ejection, any injury to the cord occurs immediately before the intact cord is obtained and the cord can be frozen or otherwise processed within seconds.
CHAPTER III

MATURATION OF RAT SPINAL CORD.

A COMPARISON WITH CEREBRUM OF LIPID CONCENTRATION

AND COMPOSITION AND PROTEIN CONCENTRATION.
INTRODUCTION

During the maturation of rodent brains there is a progressive increase in the content of lipids, particularly cholesterol, ethanolamine phosphoglycerides, galactolipids and sphingolipids (Branté, 1949; McColl & Rossiter, 1952a; Wells & Dittmer, 1967; Dickerson & Walmsley, 1967; Dickerson, 1968; Geison & Waismam, 1970; Ansell, 1973; Norton & Poduslo, 1973b; Odutuga et al., 1973; Rajalakshmi & Nakashi, 1974). Alling & Karlsson (1973) also studied the lipid composition of rat cerebrum. The highest rate of lipid deposition coincides with the highest rate of accumulation of myelin lipids. Most of these studies on the lipid composition during maturation have been done with whole brain. The two most systematic studies on spinal cord lipids are those of Branté (1949) and McColl & Rossiter (1952b). Since then, new classes of lipids have been characterized and new methods have been developed. Previous studies on the spinal cord have been reviewed by Levi (1969). In this study, the protein content, lipid content, and the lipid composition of rat spinal cord have been investigated more thoroughly and have been compared with cerebrum from the same animals.

MATERIAL AND METHODS

Sprague-Dawley (Madison, Wisc.) pregnant rats were maintained on a standard pellet diet (see appendix) and observed every morning until delivery. Litter sizes varied from four to seventeen. Rats were
decapitated without anesthesia at 0, 1, 2, 3, 5, 7, 9, 11, 13, 14, 15, 17, 20, 21, 25, 30, 40, 58, 90, 127, 190 and 300 days of age in a cold room and the cerebrums were removed rapidly. The spinal cords were removed by ejection in less than one minute after death (Chapter II). The tissues were stored at -20° until required and pooled as necessary. After weighing, the cerebrums were dispersed in 10 volumes of ice-cold 0.01M Tris-HCl buffer pH 7.4 and spinal cords were dispersed in 0.85M sucrose, 0.01M Tris-HCl buffer pH 7.4. The dispersions were carried out in a Potter-Elvejhem tissue grinder with a motor-driven Teflon pestle (Tri-R model S-63 tissue homogenizer, variable speed setting, 7.5) for about 30 seconds using 6-8 up and down strokes of the pestle.

A portion of each spinal cord or cerebrum dispersion was assayed for protein (Lowry et al., 1951) with bovine serum albumin as the standard. Lipid extraction and the separation into neutral lipid, galactolipid and phospholipid fractions by chromatography on a small column of silicic acid have been described (Horrocks & Sun, 1972). The appropriate fractions were assayed for cholesterol (Bowman & Wolf, 1962), galactose (Carroll, 1960; Neskovic et al., 1972) and phosphorus (Gottfried, 1967; Bartlett, 1959). The phospholipid composition was determined by a two-dimensional TLC procedure (Horrocks & Sun, 1972) with modified solvents, namely chloroform-methanol-acetone-acetic acid-0.1M ammonium acetate (120:50:60:2.5:15, by vol.) for the second dimension (G.Y. Sun, personal communication, 1973).

RESULTS

Animal and Tissue Weight. The rat body weight (Fig.10) increased rapidly from birth up to the age of 127 days, after which the growth
FIGURE 10  The rat body weight during maturation.
FIGURE 10
curve leveled off. There was some variance in the rat body weight probably due to differences in litter size. The interval of most rapid growth was between 30 and 58 days of age.

The weight of the cerebrum (Fig.11) increased rapidly from birth up to 20 days of age. At 20 days the weight of the cerebrum was 71% of the weight in adult rats. The increase in weight after 20 days was very gradual.

As reported in Chapter II, the spinal cord weight (Fig.6) increased from 34 mg up to 743 mg at 127 days. The rate of weight increase was most accelerated between 1 and 5 days. From 14 to 21 days of age, the spinal cord weight nearly doubled. The spinal cord length (Fig.7) increased from 25 mm at birth to 120 mm in the adult. At 17 days of age, the length of the spinal cord nearly doubled. The "thickness" of the spinal cord (Fig.8) more than doubled between 7 and 20 days of age.

**Protein Concentration.** The increases in protein concentration (Fig.12) in both tissues during maturation were rather similar, but at all ages the spinal cord contained a higher protein concentration than cerebrum. In cerebrum the protein concentration increased slowly up to 15 days of age and very rapidly between 17 and 40 days. In spinal cord the most rapid increase in concentration was found between 11 and 21 days. The protein concentration in cerebrum at 1 day was only 53 per cent and in spinal cord was only 48 per cent of the adult concentration.

**Lipid Concentration.** At all ages the concentrations of cholesterol, galactolipid and phospholipids were higher in spinal cord than in cerebrum (Tables 1 & 2). Increases in the concentration of lipids were most rapid during the second and third weeks of life.
FIGURE 11 The weight of rat cerebrum during maturation.
FIGURE 11
FIGURE 12  Protein concentration of rat spinal cord and cerebrum during maturation.
FIGURE 12
TABLE 1  Lipid Composition of Rat Cerebrum During Maturation

<table>
<thead>
<tr>
<th>Lipid</th>
<th>birth</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>11</th>
<th>13 day</th>
<th>14</th>
<th>15</th>
<th>20</th>
<th>21</th>
<th>25</th>
<th>40</th>
<th>90</th>
<th>127</th>
<th>190</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chol</td>
<td>7.96</td>
<td>7.97</td>
<td>11.30</td>
<td>11.50</td>
<td>9.20</td>
<td>13.52</td>
<td>12.90</td>
<td>18.80</td>
<td>19.90</td>
<td>22.20</td>
<td>26.31</td>
<td>27.50</td>
<td>33.93</td>
<td>50.30</td>
<td>51.74</td>
<td>50.36</td>
<td>47.66</td>
</tr>
<tr>
<td>PL</td>
<td>7.61</td>
<td>7.31</td>
<td>14.71</td>
<td>15.10</td>
<td>10.96</td>
<td>15.05</td>
<td>23.36</td>
<td>27.38</td>
<td>27.03</td>
<td>27.87</td>
<td>30.40</td>
<td>30.20</td>
<td>34.52</td>
<td>60.17</td>
<td>60.15</td>
<td>52.04</td>
<td>46.94</td>
</tr>
<tr>
<td>GAL</td>
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<td>0.10</td>
<td>0.18</td>
<td>0.32</td>
<td>0.44</td>
<td>0.99</td>
<td>1.58</td>
<td>1.78</td>
<td>2.01</td>
<td>2.38</td>
<td>2.95</td>
<td>3.02</td>
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<td>26.17</td>
<td>27.20</td>
<td>23.1</td>
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<tr>
<td>C/P</td>
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<td>1.09</td>
<td>0.77</td>
<td>0.76</td>
<td>0.84</td>
<td>0.90</td>
<td>0.55</td>
<td>0.69</td>
<td>0.74</td>
<td>0.80</td>
<td>0.86</td>
<td>0.91</td>
<td>0.97</td>
<td>0.83</td>
<td>0.85</td>
<td>0.97</td>
<td>1.01</td>
</tr>
<tr>
<td>G/P</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.07</td>
<td>0.08</td>
<td>0.10</td>
<td>0.11</td>
<td>0.12</td>
<td>0.52</td>
<td>0.52</td>
<td>0.95</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Results are expressed in μmoles/g tissue.

The abbreviations are Chol, cholesterol; PL, phospholipid; GAL, galactolipid; C/P, cholesterol to phospholipid molar ratio; G/P, galactolipid to phospholipid molar ratio.
### TABLE 2 Lipid Composition of Rat Spinal Cord During Maturation

<table>
<thead>
<tr>
<th>Lipid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>17</th>
<th>20</th>
<th>21</th>
<th>25</th>
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<th>40</th>
<th>90</th>
<th>127</th>
<th>190</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chol</td>
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<td>15.31</td>
<td>16.92</td>
<td>19.60</td>
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<td>22.73</td>
<td>26.14</td>
<td>31.10</td>
<td>34.49</td>
<td>35.81</td>
<td>39.20</td>
<td>60.40</td>
<td>61.20</td>
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<td>70.35</td>
<td>69.22</td>
<td>99.00</td>
<td>95.32</td>
<td>97.80</td>
</tr>
<tr>
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<td>18.21</td>
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<td>17.70</td>
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<td>25.60</td>
<td>34.61</td>
<td>34.40</td>
<td>36.10</td>
<td>37.73</td>
<td>56.30</td>
<td>55.91</td>
<td>58.19</td>
<td>67.89</td>
<td>62.77</td>
<td>91.11</td>
<td>85.26</td>
<td>88.63</td>
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<td>Gal</td>
<td>3.21</td>
<td>4.11</td>
<td>6.57</td>
<td>9.61</td>
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<tr>
<td>C/P</td>
<td>0.89</td>
<td>0.83</td>
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<td>1.07</td>
<td>1.02</td>
<td>0.90</td>
<td>1.00</td>
<td>0.99</td>
<td>1.04</td>
<td>1.07</td>
<td>1.09</td>
<td>1.09</td>
<td>1.04</td>
<td>1.10</td>
<td>1.09</td>
<td>1.12</td>
<td>1.10</td>
</tr>
<tr>
<td>G/P</td>
<td>0.18</td>
<td>0.19</td>
<td>0.25</td>
<td>0.28</td>
<td>0.29</td>
<td>0.34</td>
<td>0.41</td>
<td>0.48</td>
<td>0.52</td>
<td>0.52</td>
<td>0.53</td>
<td>0.65</td>
<td>0.60</td>
<td>0.62</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Results are expressed in μmoles/g tissue.

The abbreviations are Chol, cholesterol; PL, phospholipid; Gal, galactolipid; C/P, cholesterol to phospholipid molar ratio; G/P, galactolipid to phospholipid molar ratio.
The concentration of cholesterol in rat cerebrum increased from 8.0 μmoles/g tissue at birth to 50.4 μmoles/g tissue in the adult (127 days of age). In rat spinal cord it was 16.0 μmoles/g tissue at 1 day of age and 95.3 μmoles/g tissue at 127 days.

Galactolipids were not detected in the newborn rat cerebrum. In spinal cord the galactolipid concentration was too low for an accurate determination between the ages of 1 and 5 days. The concentration of this lipid was found to be more than six-fold greater in spinal cord than in cerebrum between 7 and 25 days. This ratio also increased during that period up to 21 days of age then decreased to the adult ratio of two-fold greater in spinal cord than in cerebrum.

Changes in the concentration of total phospholipids in cerebrum and spinal cord coincide qualitatively with those in cholesterol concentration. In cerebrum the cholesterol to phospholipid molar ratio is slightly above one at birth, 0.77 at two days of age and then increased moderately throughout maturation until it reaches one in the adult rat. In spinal cord this ratio was 0.89 at 1 day, became greater than one at 5 days, and remained close to one throughout maturation.

Phospholipid Composition. Marked changes in the phospholipid composition in both tissues (Tables 3 & 4) were also observed during development. The proportion of ethanolamine plasmalogens increased from 9% to 19% in cerebrum and from 18% to 29% in spinal cord. In neither tissue were changes in proportion of acid-stable ethanolamine phosphoglycerides very large. Choline phosphoglyceride proportions decreased from 58% to 33% in cerebrum and from 54% to 25% in spinal cord, while the proportions of sphingomyelins increased moderately throughout maturation.
### TABLE 3 Percent Distribution of Phospholipids from Rat Cerebrum During Maturation

<table>
<thead>
<tr>
<th>Lipid</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>20</th>
<th>21</th>
<th>25</th>
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<th>40</th>
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<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPG</td>
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<td>20.0</td>
<td>20.5</td>
<td>21.2</td>
<td>21.0</td>
<td>21.6</td>
<td>22.1</td>
<td>23.3</td>
<td>23.7</td>
<td>23.5</td>
<td>23.9</td>
<td>23.7</td>
<td>23.7</td>
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The abbreviations are EPG, acid-stable ethanolamine phosphoglyceride; EPla, ethanolamine plasmalogens; CFG, choline phosphoglycerides; Sph, sphingomyelin; SPG, serine phosphoglycerides; IPG, inositol phosphoglycerides.
### TABLE 4 Percent Distribution of Phospholipids from Rat Spinal Cord During Maturation

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</table>

The abbreviations are EPG, acid-stable ethanolamine phosphoglyceride; EPla, ethanolamine plasmalogens; CPG, choline phosphoglycerides; Sph, sphingomyelin; SPG, serine phosphoglycerides; IPG, inositol phosphoglycerides.
in both tissues. Neither serine phosphoglyceride nor inositol phosphoglyceride proportions changed markedly during development.

DISCUSSION

The protein concentrations presented in this paper for rat cerebrum are nearly identical to those of Alling & Karlsson (1973) for the period of 0 to 45 days of age. After 45 days, the protein concentration declined slightly. Human cerebral protein concentrations from 25 weeks of gestation to adulthood (Toews & Horrocks, 1975b) are similar to those for rat cerebrum except that adult values were reached at 2 years in the human cerebrum and at 40 days in the rat cerebrum. The increase in protein concentration took place at an earlier age in the spinal cord than in cerebrum in both the rat and the human (Toews & Horrocks, 1976b). The adult values are higher for rat spinal cord than for rat cerebrum and human spinal cord.

Large changes in the lipid content and concentration of rat cerebrum and spinal cord were observed. For cerebrum, these changes are rather similar to those reported by Wells & Dittmer (1967), Rouser et al. (1971), Ansell (1973) and Norton & Poduslo (1973b) for developing rat brain and by Alling & Karlsson (1973) for developing rat cerebrum except that the latter authors reported somewhat higher phospholipid concentrations throughout development. The concentrations of cholesterol, galactolipid and phospholipid increased throughout maturation. This is in agreement with a high rate of lipid biosynthesis during myelination and maturation of the CNS (Rouser & Yamamoto, 1969; Paoletti, 1971; Ansell, 1971; Rouser et al., 1971; Mokrasch, 1972; Horrocks, 1973; and Norton & Poduslo, 1973b). The pattern of glycolipid deposition in cerebrum and spinal cord is qualitatively similar to those found by
El-Eishi (1967) for chick embryo spinal cord, Odutuga et al. (1973) for rabbit brain, Yates & Wherrett (1974) for rabbit sciatic nerve and Sweasey & Patterson (1975) for pig spinal cord. With the exception of ethanolamine plasmalogen and sphingomyelin there is a slow rate of accumulation of phospholipids. This is in agreement with the observations of Odutuga et al. (1973) for the developing rabbit brain.

Increases of cholesterol, ethanolamine plasmalogens, galactolipids and sphingomyelin signal the maturation of the central nervous system and particularly its myelination. Adult concentrations of these myelin components occur earlier in spinal cord than in either cerebrum or brain as expected because the spinal cord is phylogenetically older.

The highest rate of cholesterol, galactolipid and phospholipid accumulation coincides with the highest rate of increase of spinal cord "thickness". Similarly, the highest rate of increase in body weight occurs around the same time.

Rat cerebrum, but not spinal cord is richer in phospholipid than in cholesterol during all phases of maturation except in the mature animal. Galactolipid, phospholipid and cholesterol concentrations are much higher in spinal cord than in cerebrum at all ages.

The concentration of ethanolamine plasmalogen in spinal cord was found to be higher than that of acid-stable ethanolamine phosphoglycodies. This observation was reversed in cerebrum. This is in agreement with the studies of Webster (1960). Seige (1960) reported the concentrations of plasmalogen and other lipids during maturation of human spinal cord. Other studies on spinal cord lipids have been reviewed by Levi (1969) and Rouser et al. (1972). Life span changes in brain lipids were reviewed by Rouser et al. (1972) & Horrocks et al. (1975).
CHAPTER IV

MATURATION OF RAT SPINAL CORD.

THE LIPID COMPOSITION OF MICROSOMAL AND MYELIN FRACTIONS.
INTRODUCTION

The composition of myelin fractions from brain has been studied extensively during maturation (Norton, 1971; Horrocks, 1973). Rather marked changes in lipid composition are found which may be due to the presence of membranes that are transitional between the oligodendroglial plasma membranes and mature myelin (Agrawal et al., 1970; Poduslo & Norton, 1972). The composition of rat spinal cord myelin has not been studied during the first two weeks of life when pronounced changes in spinal cord lipid concentrations take place (Chapter III). The myelins from brain and spinal cord do not have identical compositions (Smith, 1973; Toews et al., 1976a). Some changes in the lipid composition of mouse brain microsomes were found during maturation (Horrocks, 1968b). Procedures for the isolation of a purified microsomal fraction from rat spinal cords (Toews et al., 1976a) and for the rapid removal of rat spinal cords (Chapter II) have made possible the present study of microsomal and myelin fractions from rat spinal cords during maturation.

MATERIAL AND METHODS

Sprague-Dawley (Madison, Wisc.) pregnant rats were maintained on a standard pellet diet (see appendix) and observed every morning until delivery. Litter sizes varied from four to seventeen pups. Rats were decapitated without anesthesia at 0, 1, 3, 5, 7, 9, 11, 13, 17, 20, 21, 25, 30, 40 and 127 days of age in a cold room and spinal cords were removed by
ejection as described in Chapter II and frozen at -20° until required. They were weighed and dispersed in 0.85M sucrose, 0.01M Tris-HCl buffer pH 7.4 in a Potter-Elvehjem tissue homogenizer with a motor-driven Teflon pestle (Tri-R model S-73 tissue homogenizer, variable speed setting, 7.5). The dispersions were carried out for about 30 seconds using 6-8 up and down strokes of the pestle.

Subcellular Fractions. Spinal cord dispersions were subjected to subcellular fractionation using a procedure (Fig. 13) which was modified from Toews, Horrocks & King (1976).

A Beckman Spinco model L5-65 ultracentrifuge was used to prepare the tissue fractions. An SW 40 swinging bucket rotor was used for all steps except steps 6 and 7 for which a 60 Ti rotor was used. Dilutions were made with 0.01M Tris-HCl buffer, pH 7.4.

Subcellular fractionation steps
1. Approximately nine-tenths of each spinal cord fraction was placed in centrifuge tubes and spun at 40,000g for 60 minutes.
2. The tubes were then sliced, the floating layer was removed by syringe, diluted to 0.32M sucrose with buffer and recentrifuged at 20,000g for 20 minutes.
3. The pellet from step 2 was suspended in buffer and kept at 4° for 30 minutes. It was then centrifuged at 20,000g for 20 minutes and the supernate was discarded.
4. The pellet from step 3 was suspended in 0.80M sucrose and spun at 40,000g for 60 minutes.
5. Step 2 was repeated. This pellet was the purified myelin fraction.
6. The combined infranate and pellet from step 2 was diluted to 0.32M sucrose with buffer and centrifuged at 13,500g for 15 minutes.
FIGURE 13 Subcellular fractionation procedure. A Beckman Spinco model L5-65 was used to prepare the tissue fractions. An SW 40 swinging bucket rotor was used for all steps, except for the purification of microsomes, in which a 60 Ti rotor was used.
SPINAL CORD

DISPERSED IN 10 VOLUMES ICE-COLD 0.85M SUCROSE

60 min at 40,000g

FLOATING LAYER

DILUTE SUCROSE TO 0.32M
WITH 0.01M TRIS-HCL, pH 7.4

20,000 g/20 min

PELLET

SUSPEND IN 0.01 M TRIS-HCL
BUFFER, pH 7.4 KEEP AT 4°C
FOR 30 min

20,000 g/20 min

PELLET

SUSPEND IN 0.80 M SUCROSE
SPIN 40,000g/60 min

FLOATING LAYER

SUSPEND IN 0.01 M TRIS-HCL
BUFFER, pH 7.4

20,000 g/20 min

PELLET

SUSPEND IN 0.01 M TRIS-HCL
BUFFER, pH 7.4

20,000 g/20 min

FIGURE 13
The pellet was discarded.

7. The supernate from step 6 was centrifuged at 100,000g for 60 minutes. The pellet was the purified microsomal fraction. The supernate was discarded.

The purified myelin and microsomal fractions were diluted to 10 ml with buffer and frozen at -20°C until required for lipid studies.

RESULTS

The weight, length and thickness of the spinal cords are described in Chapter II. Substantial amounts of the microsomal fraction were obtained from spinal cords at all ages. Only trace amounts of a spinal cord myelin fraction were obtained from 1 day old rats. The concentration of lipids recovered in the myelin fraction increased gradually during the first two weeks of life, then increased very rapidly during the third week (Table 5).

For isolated myelin during the first two weeks of life, the mole ratio of cholesterol to lipid phosphorus gradually declined to the adult value of about 1.0 (Table 6). Lipid galactose was not detectable until 7 days of age. The mole ratio of lipid galactose to lipid phosphorus then gradually increased to a value of 0.5 at 30 days of age. Changes in the relative proportions of individual phospholipids were also detected in the myelin (Table 7). The proportion of phosphatidyl cholines in the isolated myelin fraction decreased from one-half of the total phospholipid to less than one-third in the spinal cord of the adult rat. This decrease was offset primarily by an increase in the proportion of ethanolamine plasmalogens from 17 to 33% of the total phospholipid. At the same time there was an increase in the proportion
TABLE 5 Lipid Composition of the Myelin and Microsomal Fractions Purified from Rat Spinal Cord During Maturation

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<th>age, days</th>
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<th></th>
<th>Microsomes</th>
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<td>PL</td>
<td>Gal</td>
<td>Chol</td>
<td>PL</td>
<td>Gal</td>
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</table>

Results are expressed in \( \mu \)moles/g tissue.

The abbreviations are Chol, cholesterol; PL, phospholipid; Gal, galactolipid.
TABLE 6 Relative Proportions of Cholesterol, Galactolipids and Phospholipids in Myelin and Microsomal Fractions from Rat Spinal Cord During Maturation

<table>
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<tr>
<th>age, days</th>
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<td>0.40</td>
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<td>0.98</td>
<td>0.49</td>
<td>0.76</td>
<td>0.22</td>
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</table>

The values are mole ratios of cholesterol (Chol) or lipid galactose (Gal) to lipid phosphorus (LP).
TABLE 7 Percent Distribution of Phospholipids in the Myelin Fraction from Rat Spinal Cord During Maturation

<table>
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<th>Lipid</th>
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<th>13</th>
<th>20</th>
<th>25</th>
<th>30</th>
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<th>127</th>
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<tbody>
<tr>
<td></td>
<td>age, days</td>
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<td></td>
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<td>39.2</td>
<td>34.5</td>
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<tr>
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<td>7.0</td>
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<td>1.9</td>
<td>1.6</td>
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<td>3.4</td>
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The abbreviations are EPG, ethanolamine phosphoglycerides; EPla, ethanolamine plasmalogens; CPG, choline phosphoglycerides; Sph, sphingomyelin; SPG, serine phosphoglycerides; IPG, inositol phosphoglycerides.
of sphingomyelins.

In the microsomal fraction, the concentrations of cholesterol and lipid phosphorus were rather constant through 11 days of life (Table 5). From 13 to 40 days of age, these concentrations were considerably higher. The mole ratio of cholesterol to lipid phosphorus of about 0.73 was rather constant throughout maturation (Table 6). The microsomal lipid galactose concentration increased considerably from 5 to 30 days of age. During this same period, the mole ratio of lipid galactose to lipid phosphorus increased to 0.22. The relative proportions of phospholipids also changed (Table 8). The proportion of phosphatidyl choline in the microsomal fraction decreased from 60 to 33% between 7 and 40 days of age. Substantial increases in the proportions of ethanolamine phosphoglycerides were found for the same time period. The proportion of phosphatidyl ethanolamine increased from 10 to 26% and the proportion of ethanolamine plasmalogens increased from 8 to 17%. Smaller increases were found for sphingomyelins and phosphatidyl serines.

DISCUSSION

When brain tissue is dispersed in 0.32M sucrose solutions, the myelin forms large vesicles which are almost completely sedimented by 2 x 10^5 g-min. This supernate, when centrifuged for 6 x 10^6 g-min, gives a sedimented microsomal fraction of small vesicles. Application of the same procedure to a spinal cord dispersion produces a microsomal fraction which contains more myelin than microsomes (Toews et al., 1976a). The myelin could be separated from the microsomes by flotation on 0.85M sucrose. In order to isolate all of the myelin in one fraction, regardless of particle size, the spinal cord tissue was dispersed in 0.85M
### TABLE 8 Percent Distribution of Phospholipids in the Microsomal Fraction from Rat Spinal Cord During Maturation

<table>
<thead>
<tr>
<th>Lipid</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>13</th>
<th>17</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>age, days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPG</td>
<td>10.4</td>
<td>13.7</td>
<td>21.3</td>
<td>17.4</td>
<td>16.5</td>
<td>19.8</td>
<td>25.4</td>
<td>25.8</td>
</tr>
<tr>
<td>EPla</td>
<td>7.9</td>
<td>6.9</td>
<td>7.9</td>
<td>9.2</td>
<td>13.0</td>
<td>7.8</td>
<td>11.3</td>
<td>17.4</td>
</tr>
<tr>
<td>CFG</td>
<td>60.5</td>
<td>59.6</td>
<td>51.7</td>
<td>57.5</td>
<td>48.9</td>
<td>44.3</td>
<td>37.5</td>
<td>33.5</td>
</tr>
<tr>
<td>Sph</td>
<td>6.0</td>
<td>4.3</td>
<td>4.8</td>
<td>4.8</td>
<td>6.7</td>
<td>8.4</td>
<td>11.3</td>
<td>9.7</td>
</tr>
<tr>
<td>SPG</td>
<td>8.0</td>
<td>9.7</td>
<td>8.1</td>
<td>8.7</td>
<td>10.1</td>
<td>13.3</td>
<td>9.7</td>
<td>12.1</td>
</tr>
</tbody>
</table>

The abbreviations are EPG, ethanolamine phosphoglycerides; EPla, ethanolamine plasmalogens; CFG, choline phosphoglycerides; Sph, sphingomyelin; SPG, serine phosphoglycerides.

Inositol phosphoglycerides were not detected at any of the studied ages, except at 30 days of age, where the value was 2.2% of the total phospholipid.
sucrose and centrifuged to produce a floating layer of crude myelin. The myelin was purified by osmotic shock and refloation on 0.8M sucrose. This procedure is similar to Method A (Horrocks, 1967) which gave a myelin preparation from squirrel monkey spinal cord which had a low protein content, was soluble in chloroform-methanol (2:1,v/v), had the typical appearance of myelin by electron microscopy, and had a lipid composition typical of myelin. In the present study, the myelin fraction is that material which floats on 0.8M sucrose after osmotic shock. The microsomal fraction is the material that does not float on 0.85M sucrose and sediments between 2 x 10^6 g-min and 6 x 10^6 g-min.

Toews et al. (1976a) prepared a microsomal fraction from adult rat spinal cord with the same steps but in a different order. Their microsomal fraction was substantially free of myelin proteins.

The lipid compositions of rat (Cuzner et al., 1965; Herschkowitz et al., 1968; Toews et al., 1976a) and mouse (Horrocks, 1968b; Sun & Horrocks, 1970) brain microsomes and adult rat spinal cord microsomes (Toews et al., 1976a) have been reported. Lower proportions of cholesterol, galactolipids and ethanolamine plasmalogens were found in rat spinal cord microsomes during development then in the adult microsomes isolated by Toews et al. (1976a). Except for the proportion of cholesterol, these differences were also found for different ages during development (Table 5 & 8). Throughout maturation, with regard to lipid composition, the microsomes from developing spinal cord changed from a close resemblance to brain microsomes to a greater similarity to myelin. This was accomplished by increases in the proportions of galactolipid, acid-stable ethanolamine phosphoglycerides and ethanolamine plasmalogens and by decrease in the proportion of choline phosphoglycerides. Some of these changes were also evident for brain
microsomes (Horrocks, 1968b). These changes coincide with the proliferation of oligodendrocytes and thus may reflect an increasing proportion of membrane fragments of oligodendroglial origin in the microsomal fraction.

The lipid composition of rodent brain myelin fractions changes during development by increases in the proportions of cholesterol, galactolipids and ethanolamine plasmalogen and by a decrease in the proportion of choline phosphoglycerides (Horrocks, 1968b; Norton, 1971; Horrocks, 1973; Norton & Poduslo, 1973a). Changes in the composition of spinal cord myelin fractions during maturation were found in this study. With the exception of cholesterol, the changes in lipid composition of brain myelin were also seen in spinal cord myelin. These changes in composition may be due to decreasing proportions of a "myelin-like" fraction (Agrawal et al., 1974), an increase in the lighter myelin sub-fractions (Benjamins et al., 1973), or a relative decrease in the proportion of oligodendroglial plasma membranes Zanetta et al. (1972). Changes in the lipid composition of myelin fractions from rabbit spinal cords have also been reported (Dalal & Einstein, 1969).
SUMMARY

1. A simple, fast, economic and damage-free procedure for rat spinal cord removal has been developed. It is hoped that this method will stimulate further, more systematic research on the biochemistry of rat spinal cord.

2. The increases in protein concentration in cerebrum and spinal cord during maturation were rather similar, but at all ages the spinal cord contained a higher protein concentration than cerebrum.

3. At all ages the concentrations of cholesterol, galactolipids and phospholipids were higher in spinal cord than in cerebrum.

4. Marked changes in the phospholipid composition in both tissues were also observed during development. The proportion of ethanolamine plasmalogen increased from 9% to 19% in cerebrum and from 18% to 29% in spinal cord. Choline phosphoglyceride proportions decreased from 58% to 33% in cerebrum and from 54% to 25% in spinal cord, while the proportion of sphingomyelin increased moderately throughout maturation in both tissues.

5. The marked increase in concentrations of protein, cholesterol, galactolipids, and various phospholipids took place earlier in spinal cord than in cerebrum.

6. Substantial amounts of the microsomal fraction were obtained from spinal cord at all ages. Only trace amounts of a spinal cord myelin fraction were obtained from newborn and 1 day old rats.
7. For isolated myelin during the first two weeks of life the mole ratio of cholesterol to lipid phosphorus gradually declined to the adult value of about 1.0. The mole ratio of lipid galactose to lipid phosphorus after 7 days of age gradually increased to a value of 0.5 at 30 days of age. Changes in the relative proportions of individual phospholipids were also detected in the myelin. Those changes are qualitatively similar to those found for the total tissue.

8. In microsomal fraction, the concentrations of cholesterol and lipid phosphorus were rather constant through 11 days of age. The microsomal lipid galactose concentration increased considerably from 5 to 30 days of age. The relative proportions of phospholipids also changed. The most pronounced changes included a decrease of the choline and an increase of the ethanolamine phosphoglyceride proportions.

9. The changes in lipid content and composition are consistent with the continuing deposition of myelin, maturation of the myelin lipid composition and increasing proportions of oligodendroglial membranes in the microsomal fraction.
APPENDIX
To myself without whose tireless, and irrefragable efforts, this work would never have been accomplished.

Mus norvegicus albinus
MEMOIRS OF A RAT RUNNER

My Dearest Rat,

It's been long ago since we first met. It's been very nice
since then though. I am very sorry for the sacrifices that I carry
on with you, but there is no other way of getting to know you better.
Therefore, forgive me for those unfortunate moments and please, try
to understand that this is a way of life! You have always been stoic-
ally understanding, and considering the rat runner that I am, it makes
me very pleased to have you as my friend. Perhaps, excluding the
insane life of 24 hours everyday, you know I am O.K. You're O.K. too,
even when you ramble around your "castle" complaining about almost
everything. But, I myself understand that you're just trying to break
the monotony of a "castle" life. We know what it's all about. Neverthe-
less, your philosophy has been most outstanding and you have been able
to hold the title of Mus norvegicus albinus for almost one century now.
Ever since you conquered the scientist you have been the most success-
ful of all beings. You have developed quite well and become the tutor
of so many science "bugs". Yes, you've been very bright even when
you're young and hot tempered. You've been just marvelous! I know
the blue ribbon you won was the thing you most hoped for in life and
as your friend, I must say that nobody but you deserved it. I truly
recognize your efforts to cooperate and run all the experiments in the
best way you can, even in the most dramatic moments of your existence.
Your spinal cord, I know sometimes it bothers, but imagine what a beautiful cord you have. This is not nonsense! What do you mean? Is it absurd to talk about the contributions it has given to medicine? You know very well my friend, that without your tireless efforts, this work would never have been accomplished. Who but you could ever subject yourself to the most different series of experiments with the suasion never found in anybody else?

They say that you're feeble and ill developed, that you cannot withstand the arduous environment of a peaceful life in a laboratory. However, as you well know, this is just a vulgar way of expression of the instinctive verbalizers of the unlettered mass. I hope it won't cause you any grief and I'm sure you know that all of us are liable to this sort of ludicrous nonsense. We are all tired of this lack of recognition, but since you're self sufficient and ..... never mind! Put on your white lab coat and let's work.
RAT DIET COMPOSITION

The composition of the standard pellet diet described in the anterior chapters was: protein 23%, fat 4.5%, carbohydrate 69% and minerals 2.5%. The ingredients used for preparation of this diet were: ground extruded corn oil, soybean meal, ground oat groats, dried beet pulp, wheat germ, fish meal, dehydrated alfalfa meal, dried milk, wheat middlings, brewers' dried yeast, animal fat (preserved with BHA), animal liver meal, salt, calcium carbonate, dicalcium phosphate, calcium iodate, vitamin B₁₂, methionine hydroxy analogue calcium, calcium pantothenate, choline chloride, folic acid, riboflavin, thiamin, niacin, pyridoxine hydrochloride, ferrous sulfate, vitamin A, vitamin D, vitamin E, iron sulfate, iron oxide, manganous oxide, copper oxide, zinc oxide and cobalt carbonate.
TABLE 9  Rat Body, Cerebrum and Spinal Cord Weights (grams) and Spinal Cord Length (cm) During Maturation.

<table>
<thead>
<tr>
<th>age, days</th>
<th>n</th>
<th>body weight (g)</th>
<th>cerebrum weight (g)</th>
<th>spinal cord weight (g)</th>
<th>spinal cord length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>17</td>
<td>6.8±0.6</td>
<td>0.164±0.015</td>
<td>0.034±0.004</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>5.6±0.5</td>
<td>0.157±0.010</td>
<td>0.050±0.007</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>5.9±0.7</td>
<td>0.156±0.010</td>
<td>0.034±0.005</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>8.4±0.8</td>
<td>0.252±0.017</td>
<td>0.047±0.032</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>11.1±0.4</td>
<td></td>
<td>0.054±0.007</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>15.8±1.0</td>
<td>0.447±0.042</td>
<td>0.055±0.012</td>
<td>3.3±0.0</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>19.8±2.1</td>
<td>0.642±0.050</td>
<td>0.100±0.030</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>13.9±1.2</td>
<td>0.540±0.061</td>
<td>0.075±0.014</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>17.3±0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>14.8±1.4</td>
<td>0.667±0.039</td>
<td>0.092±0.113</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>40.5±1.9</td>
<td>0.751±0.043</td>
<td>0.131±0.020</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>26.9±2.3</td>
<td>0.822±0.038</td>
<td>0.118±0.027</td>
<td>4.5±0.0</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>42.4±1.1</td>
<td>1.015±0.044</td>
<td>0.149±0.003</td>
<td>5.1±0.1</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>31.5±3.1</td>
<td>0.974±0.055</td>
<td>0.161±0.010</td>
<td>4.4±0.1</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>56.7±6.0</td>
<td>1.133±0.049</td>
<td>0.208±0.018</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>62.9±4.8</td>
<td>1.034±0.082</td>
<td>0.228±0.020</td>
<td>5.2±0.3</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>68.1±5.3</td>
<td>1.023±0.061</td>
<td>0.215±0.016</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>114.6±7.2</td>
<td>1.081±0.094</td>
<td>0.285±0.014</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td>58</td>
<td>4</td>
<td>289.2±16.9</td>
<td>1.160±0.078</td>
<td>0.508±0.056</td>
<td>7.6±1.0</td>
</tr>
<tr>
<td>90</td>
<td>3</td>
<td>148.0±16.8</td>
<td>1.230±0.073</td>
<td>0.584±0.056</td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>3</td>
<td>430.0±31.2</td>
<td>1.366±0.075</td>
<td>0.743±0.038</td>
<td>12.0±0.0</td>
</tr>
<tr>
<td>190</td>
<td>5</td>
<td>456.6±12.7</td>
<td>1.321±0.075</td>
<td>0.656±0.069</td>
<td>9.8±0.3</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>453.3±18.8</td>
<td>1.321±0.075</td>
<td>0.656±0.069</td>
<td></td>
</tr>
</tbody>
</table>

n= number of samples
values are mean± S.D.
FIGURE 14 Cholesterol concentration of rat cerebrum during maturation.
FIGURE 14
FIGURE 15 Cholesterol concentration of rat spinal cord during maturation.
FIGURE 15
FIGURE 16 Galactolipid concentration of rat cerebrum during maturation.
FIGURE 16  Galactolipid concentration of rat cerebrum during maturation.
FIGURE 16

GALACTOLIPID
A moles / g tissue

AGE, DAYS (log scale)
FIGURE 17 Galactolipid concentration of rat spinal cord during maturation.
FIGURE 17
FIGURE 18 Phospholipid concentration of rat cerebrum during maturation.
FIGURE 19 Phospholipid concentration of rat spinal cord during maturation.
FIGURE 19

LIPID PHOSPHORUS
µmoles/g tissue

AGE, DAYS (log scale)
FIGURE 20  Ethanolamine plasmalogens from rat cerebrum during maturation.
 FIGURE 20
FIGURE 21 Ethanolamine plasmalogens from rat spinal cord during maturation.
FIGURE 21
FIGURE 22 Acid-stable ethanolamine phosphoglycerides from rat cerebrum during maturation.
FIGURE 23 Acid-stable ethanolamine phosphoglycerides from rat spinal cord during maturation.
FIGURE 23
FIGURE 24  Choline phosphoglycerides from rat cerebrum during maturation.
FIGURE 24

% OF ADULT LEVEL

AGE, DAYS (log scale)
FIGURE 25 Choline phosphoglycerides from rat spinal cord during maturation.
FIGURE 26  Sphingomyelin from rat cerebrum during maturation.
FIGURE 26
FIGURE 27  Sphingomyelin from rat spinal cord during maturation.
FIGURE 27
FIGURE 28 Serine phosphoglycerides from rat cerebrum during maturation.
FIGURE 28
FIGURE 29 Serine phosphoglycerides from rat spinal cord during maturation.
FIGURE 29
FIGURE 30 Inositol phosphoglycerides from rat cerebrum during maturation.
FIGURE 30
FIGURE 31 Cholesterol concentration in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 31
FIGURE 32 Cholesterol concentration in the microsomal fraction purified from rat spinal cord during maturation.
Figure 32
FIGURE 33  Galactolipid concentration in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 33

GALACTOLIPID

nmol/g tissue

AGE, DAYS (log scale)

5 7 11 15 20 30 40

0 1 2 3 4 5 6 7
FIGURE 34  Galactolipid concentration in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 34
FIGURE 35 Phospholipid concentration in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 35

LIPID PHOSPHORUS
µ moles /g tissue

AGE, DAYS (log scale)
FIGURE 36 Phospholipid concentration in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 36

LIPID PHOSPHORUS
μ moles /g tissue

AGE, DAYS (log scale)

1 2 3 5 7 11 15 20 30 40 58 90 127

1 2 3 5 7 11 15 20 30 40 58 90 127
FIGURE 37 Ethanolamine plasmalogens in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 37
FIGURE 38 Ethanolamine plasmalogens in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 38
FIGURE 39 Acid-stable ethanolaminephosphoglycerides in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 39
FIGURE 40 Acid-stable ethanolamine phosphoglycerides in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 40

**% of Phospholipid**

**Age, Days (log scale)**

- Axis labels and values are not provided in the image.
FIGURE 41 Sphingomyelin in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 42 Sphingomyelin in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 42
FIGURE 43 Choline phosphoglycerides in the myelin fraction purified from rat spinal cord during maturation.
FIGURE 43

% OF PHOSPHOLIPID

30  40  50  60

3  5  7  11  15  20  30  40  58  90  127

AGE, DAYS (log scale)
FIGURE 44 Choline phosphoglycerides in the microsomal fraction purified from rat spinal cord during maturation.
FIGURE 45  Serine phosphoglycerides in the myelin fraction purified from rat spinal cord during maturation.
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