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Lipid and Ultrastructural Changes in Brain After Induction of Essential Fatty Acid Deficiency During Development

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LIPID AND ULTRASTRUCTURAL CHANGES IN BRAIN AFTER INDUCTION

OF ESSENTIAL FATTY ACID DEFICIENCY

DURING DEVELOPMENT

by

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LIPID AND ULTRASTRUCTURAL CHANGES IN RAT BRAIN

AFTER INDUCTION OF ESSENTIAL FATTY ACID

DEFICIENCY DURING DEVELOPMENT

Biological membranes consist primarily of proteins and lipids and the proportions of these major constituents vary in membranes from different sources and are probably related to membrane function. The central nervous system (CNS) contains a high concentration of lipids with phospholipids constituting a major part of this component. Fatty acids play a significant role in the structure and function of phospholipids.

Alterations in the fatty acid composition of membrane lipids, such as caused by essential fatty acid (EFA) deficiency can alter membrane function and/or structure. The present study was an attempt to define some of the biochemical and structural alterations in CNS membranes during EFA deficiency, with emphasis on myelin membranes.

Alterations in whole brain phospholipids fatty acids during differing states of EFA deficiency were determined. The reversibility of these fatty acid alterations was determined by supplementing the essential fatty acids, linoleic and linolenic acid individually, to 120 day-old EFA deficient rats. The response of whole brain phospholipids to EFA deficiency and subsequent supplementation was compared to

that of liver phospholipids to determine if any responses were unique
to brain.

A progressive decrease in the dietary essential fatty acids of
the linoleic (N-6) and linolenic (N-3) acid families and an increase
in the nonessential fatty acids of the oleic (N-9) acid series occurred
during EFA deficiency. Linoleate and linolenate supplementation for
30 days returned the (N-6) and (N-3) fatty acids to control levels
while the (N-9) fatty acids approached control values. The response
of 22:5 (N-6) to EFA deficiency and subsequent supplementation differed
from the remainder of the (N-6) fatty acids. The response of liver
phospholipid fatty acids to EFA deficiency differed from that of the
CNS.

The fatty acid composition of the ethanolamine phosphoglycerides
(EPG) from subfractions of purified myelin was determined developmentally
and in states of EFA deficiency.

The fatty acid composition of EPG's from subfractions of myelin
from control animals differed developmentally. The monounsaturated
fatty acids increased with age while the polyunsaturates decreased. The
saturated fatty acids remained unchanged at the various ages studied.
The fatty acids of EPG's from "light" and "heavy" myelin also differed
at various ages. The heavy subfraction tended to consist of a higher
per cent of polyunsaturated fatty acids while the light fraction con-
tained a higher per cent of monounsaturated fatty acids.

Alterations in the EPG fatty acids of myelin subfractions during
EFA deficiency were detectable at 15 days and were similar to changes
found in whole brain phospholipids except the increase in 22:5 (N-6)
was not as apparent in the myelin fractions.

The morphological appearance of the optic nerve from 120 day-old control and EFA deficient rats were studied with light and electron microscopy.

Cross-sections of control and EFA deficient optic nerve at the light microscopic level were similar, except for the occurrence of a number of small vacuoles randomly dispersed throughout the EFA deficient nerves. Ultrastructurally no changes were found in glial cells, vascular components or the diameter of fibers within the optic nerve of EFA deficient rats. The vacuoles identified at the light microscopic level appeared as degenerating axons and intramyelinic splits at the electron microscopic level. Additional variations of the myelin sheath were observed in EFA deficient optic nerve. These consisted of formed organelles within the inner and outer mesaxon, isolated islands of cytoplasm within myelin sheaths and two concentric myelin sheaths surrounding a single axon.

The data appeared to demonstrate that alterations in the normal morphology of myelin could be demonstrated in EFA-deficient animals concomitantly with changes in fatty acid composition of phospholipids.

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I owe a special debt to Barney Dwyer whose biochemical knowledge and comradeship made this work easier and enjoyable. The training and suggestions of Hiro Tonaki and Robert Kaplan of the Electron Microscopy Laboratory of Hines V.A. Hospital were helpful in all morphological aspects of this work.

I also would like to thank my wife, Carol, for typing the many drafts of this dissertation and for always being there.

Bruce D. Trapp was born in Rockford, Illinois on May 3, 1950. He
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In September 1972, Bruce Trapp enrolled in the Graduate School
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Trapp, B.D. and Bernsohn, J. Changes in phosphoglyceride fatty acids of rat brain induced by linoleic and linolenic acids after pre- and post-natal fat deprivation. Accepted for publication. J. Neurochemistry.

Trapp, B.D. Subcellular distribution of 5' mononucleotidase in developing rat brain. In preparation.

Trapp, B.D. and Bernsohn, J. Ultrastructural effects of essential fatty acid deficiency on the optic nerve of the rat. In preparation.

Trapp, B.D. and Bernsohn, J. Alterations in the fatty acid composition of myelin subfractions during essential fatty acid deficiency. In preparation.

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INTRODUCTION

The present work was undertaken to elucidate effects of essential fatty acid (EFA) deficiency on the development of the central nervous system (CNS) of the rat. The work was multidisciplinary and involved a biochemical study of phospholipid fatty acids of the CNS and a morphological study of the optic nerve utilizing light and electron microscopy. The CNS was chosen due to its important role in the function and survival of an organism and due to the possible relationship of this study to the demyelinating diseases.

Membranes consist primarily of proteins and lipids and the proportion of these components vary from membrane to membrane (Chapman, 1975a). The CNS contains a high concentration of lipid and phospholipids are a major component of the total lipids (Norton & Poduslo, 1973a). Fatty acids play a major role in the function and structure of phospholipids (Chapman, 1975b). Thus changes in the fatty acid composition of membrane lipids (such as caused by EFA deficiency) may cause alterations in membrane function and/or structure. The present work is an attempt to define some of the structural and chemical alterations in CNS membranes during EFA deficiency.

Alterations in whole brain phospholipids during differing states of EFA deficiency were determined. The reversibility of these fatty acid alterations was determined by supplementing the essential fatty acids, linoleic and linolenic acid, to 120-day-old EFA deficient rats.

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The metabolism of the CNS is unique in several ways and it reacts differently than other organs under certain circumstances. Thus, the effects of EFA deficiency and subsequent linoleic or linolenic acid supplementation on the fatty acid composition of liver phospholipids was also determined to see if any responses were unique to brain.

The fatty acid composition of ethanolamine phosphoglycerides from subfractions of purified myelin isolated from control and EFA deficient rats at various ages was also determined. Myelin membranes have a high phospholipid content (Norton & Poduslo, 1973b) and therefore fatty acids probably play a major role in their function and structure. Function-ally myelin insulates axons by preventing diffusion of its potential into surrounding tissue and permits much higher conduction velocities (via "saltatory" conduction) than can occur in a comparably sized unmyelinated fibre.

Protein-calorie malnutrition initiated early in life causes a retardation in the nerve fibre growth of the optic nerve of the rat (Sima & Sourander, 1974; Sima, 1974b). The present study investigated the effects of EFA deficiency on the diameter of myelinated fibres and the morphological appearance of myelin utilizing light and electron microscopy. Chemical alterations in the fatty acid composition of myelin were correlated to morphological alteration found in optic nerve myelin.

In summary, it is hoped that data collected in these fields will allow correlations to be formed between chemical and morphological alter-ations induced by EFA deficiency with possible implications to the process of demyelination.

REVIEW OF LITERATURE

A.   Background

The role of dietary fat as an essential nutrient was first demon-
strated by Burr and Burr (1929). They described a new deficiency
disease produced by the exclusion of fat from the diet. Rats maintained
on the fat-free diet from weaning developed a scaly condition of the
skin between 70 and 90 days of age. The scaliness was particularly
evident in the caudal region of the animal and necrosis of the tail was
not uncommon as the disease progressed. The fat deficient animals demon-
strated decreased growth and maintained a weight approximately 25% under
control animals. The deficient animals remained at 75% of the control
body weight for several months, upon which time they began to lose weight
rapidly and expired. Death could be circumvented by the addition of fat
to the diet of the deficient animals. In the same paper Burr and Burr
demonstrated the fatty acids to be responsible for the effectiveness of
fat in curing or preventing the disease. Glycerol and the non-saponi-
fiable portion of fat had no beneficial effects when refed to deficient
animals. Thus in 1929 Burr and Burr presented the first evidence for
the essentiality of fatty acids. In the following year the same authors
(Burr and Burr, 1930) reported decreased ovulation and fertility in
fat deficient animals. They further showed the curative effects of
the fatty acids to be attributed to linoleic acid. Saturated fatty
acids had no effect on the mortality rates or symptoms of fat deficiency.

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It was hypothesized that warm blooded animals could not synthesize appreciable amounts of linoleic acid. The de novo synthesis of other unsaturated fatty acids, including linolenic acid, was also thought to be limited. Thus in 1930 Burr and Burr postulated that linoleic acid (and possibly other unsaturated fatty acids) was an essential fatty acid (EFA) since an exogenous source was mandatory for the survival of the animal. Since 1930 EFA deficiency has been induced in numerous species other than the rat; namely, mice (Tove and Smith, 1959, 1960; Sun and Sun, 1974; Sun et al., 1974), chickens (Bieri et al., 1957a; Jensen and Shuteze, 1963; Miller et al., 1963; Russell et al., 1940; Dam et al., 1952, 1956) guinea pigs (Bieri et al., 1957b; Reid et al., 1964), dogs (Patèl and Hansen, 1962; Wiese et al., 1957, 1962) swine (Leat, 1963; Caster et al., 1962, 1963; Hill et al., 1961), rabbits (Ahluwalia et al., 1967), calves (Lambert et al., 1954), subhuman primates (Greenberg and Wheeler, 1966; Portman et al., 1959; Fitch et al., 1961; Fiennes et al., 1973), fish (Lee et al., 1967; Castell et al., 1972; Yu and Sennhuber, 1975; Brenner et al., 1963; Kelly et al., 1958), insects (Chippendale et al., 1965) and humans (Wiese et al., 1958; Horwitt et al., 1959; Warwick et al., 1959; Hansen et al., 1963; Collins et al., 1971; Caldwell et al., 1972, Paulsrud et al., 1972; Jiejeebhoy et al., 1973; Press et al., 1974; Thompson et al., 1974; Richardson et al., 1975; Wene et al., 1975). Early reports emphasize external symptoms such as scaliness of the skin, necrosis of the tail, roughened hair coat as well as decreased weight gain and reproductive capacity. The external dermal symptoms varied with the humidity of the animals environment (Burr and Beber, 1937). With the development of advanced chemical and physiological techniques,

more precise criteria were used to define states of EFA deficiency.
One of the most reliable criteria developed for defining stages of EFA
deficiency was the fatty acid composition of tissue phospholipids.
Changes in the phospholipid fatty acids occur before the dermal signs
of EFA deficiency develop (Holman, 1968).

The development of gas chromatography allowed for the elucidation
of the metabolic transformation of the fatty acids. Three series or
families of polyunsaturated fatty acids (Figure 1) have been described
by Klenk (1965) and Mead (1961). Acids of the oleic series (n-9) are
non-essential fatty acids and can be synthesized de novo by mammalian
systems. The fatty acids of the linoleic (n-6) and linolenic series
(n-3) are dietary essential fatty acids in mammals as they cannot be
synthesized de novo and must be obtained from the diet. No intercon-
version between (n-6) and (n-3) families occurs. There are at least
three essential fatty acids; linoleic [18:2 (n-6)], linolenic
[18:3 (n-3)] and arachidonic [20:4 (n-6)]. Arachidonic acid is
synthesized from linoleic acid and usually is considered an essential
fatty acid as it reverses EFA deficiency symptoms when administered to
EFA deficient rats (Alfin-Slater and Kaneda, 1962).

The biosynthesis of polyunsaturated fatty acids involves two main
mechanisms: The addition of carbon atoms (elongation) and the intro-
duction of double bonds (desaturation) to existing fatty acid chains.
Mammalian cells add carbon atoms as acetate units to form longer chain
fatty acids by two different elongation pathways; one located in the
microsomes and the other in the mitochondria. The microsomes contain
an enzyme system which adds 2 carbon atoms to fatty acyl-CoA molecules
in the presence of malonyl-CoA and NADH (Mohrhauer et al., 1967).

Unsaturated fatty acids have a higher affinity for the enzyme system
than saturated fatty acids and the higher the degree of unsaturation of
the fatty acids the higher are its rates of elongation. Mitochondria
also contain an enzyme system for elongation of fatty acids. Acetyl–CoA
is the carbon donor and both NADH and NADPH are required (Wakil 1961,
1964). Unsaturated fatty acids are elongated by this system at a faster
rate than saturated fatty acids.

Desaturation or addition of double bonds into fatty acid chains
involves an enzyme system of the endoplasmic reticulum and occurs by
an aerobic mechanism in mammalian cells. The desaturation steps requires
oxygen and NADH or NADPH. The metabolic steps for polyunsaturated fatty
acid synthesis are outlined in Figure 1. The 18 carbon fatty acids
oleic, linoleic and linolenic are converted to their longer, less
saturated derivatives by a series of desaturation and elongation steps.
The preferred pathway is the most direct one represented by the horizon-
tal lines of Figure 1. This sequence begins with a C6–desaturation
(Brenner, 1974).

Klenk (1965) has shown there to be no interconversion of (n–3) and
(n–6) fatty acids. The lack of de novo synthesis of linoleic and
linolenic acids is due to the absence of an enzyme system within mamma-
lian cells. The synthesis of polyunsaturated fatty acids is ordinarily
accomplished in non-mammalian species by the insertion of a double bond
into the C12–13 position of oleic acid to form linoleic acid. Apparently
the enzyme system for converting oleic acid to linoleic acid has been
lost in metazoan evolution although linoleic acid is essential for
invertebrate and vertebrate nutrition (Hollaway et al., 1963). The loss
of this C9–desaturase enzyme does not prohibit the synthesis of other

FIGURE 1. PATHWAYS IN THE BIOSYNTHESIS OF FATTY ACIDS OF OLEIC, LINOLEIC AND LINOLENIC FAMILIES.

1. Oleic Acid Series (n-9)
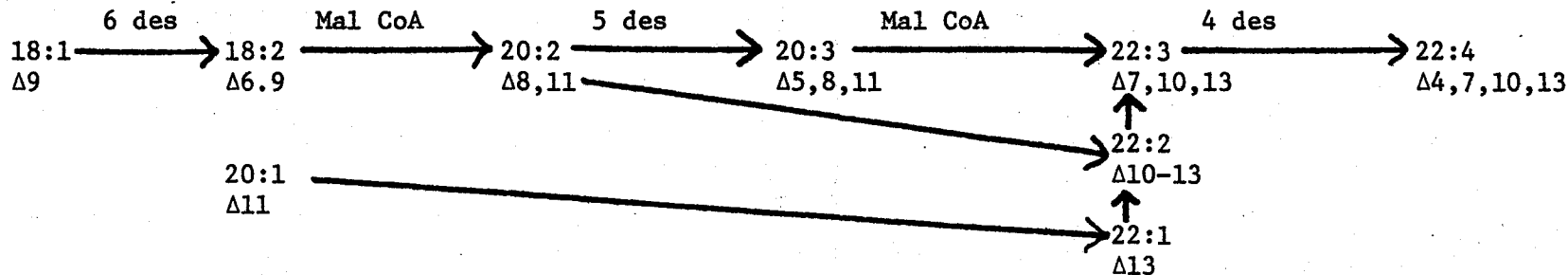
2. Linoleic Acid Series (n-6)
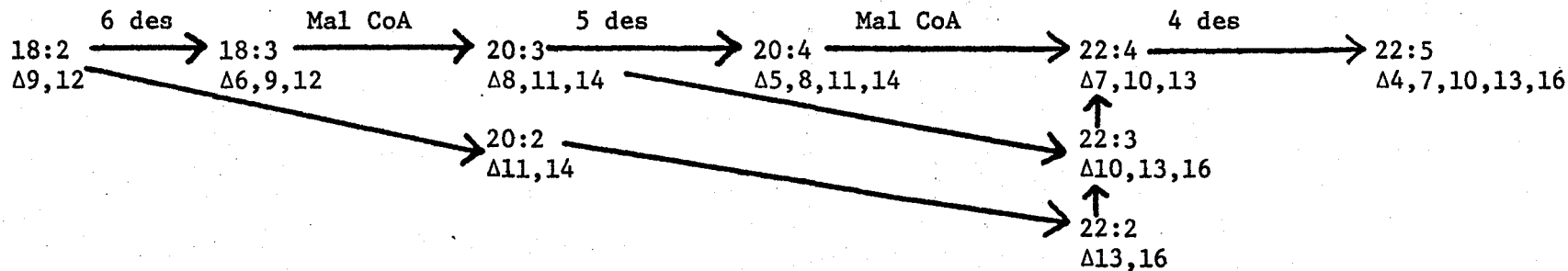
3. Linolenic Acid Series (n-3)
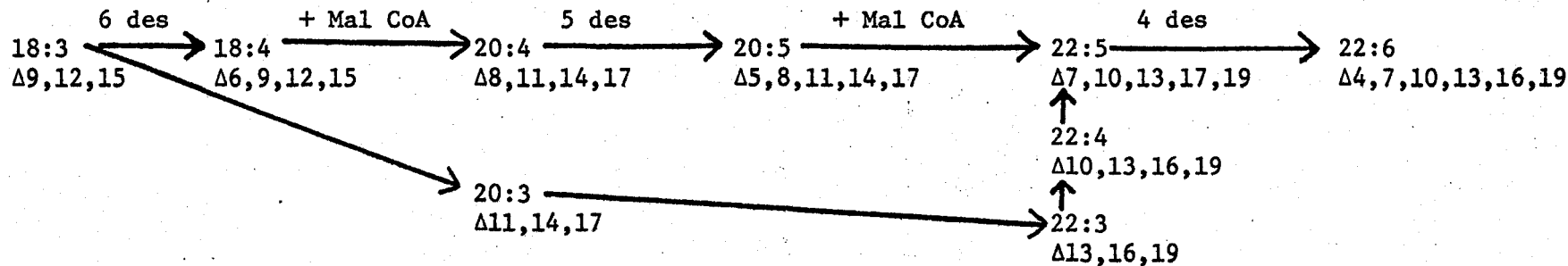
polyunsaturated fatty acids from oleic acid.

The fatty acid composition of tissue lipids is a sensitive tool for determining the dietary content of essential fatty acids (Mohrhauer and Holman 1963; Pudelkewicz and Holman 1968). The major changes found in the fatty acid composition of EFA deficient rats are an increase in the fatty acids of non-essential oleic (n-9) acid series and a decrease in the fatty acids of the essential linoleic (n-6) and linolenic (n-3) acid series.

The first report of changes in the fatty acid composition of tissue during EFA deficiency was that of Smedley-Maclean in 1943, utilizing the polybromide technique. She reported a decrease in the number of poly-unsaturated acids and in the degree of unsaturation of liver lipids. Reickenhoff et al. (1949) utilizing the more refined technique of alkaline isomerization demonstrated an increase in trienoic acids and a decrease in more unsaturated fatty acids during EFA deficiency. The development of gas chromatography allowed for the quantitative analysis of the fatty acid composition of small amounts of biological tissue. Thus the fatty acid pattern of a variety of tissues under different experimental conditions have been reported. The composition of fatty acids in lipids of various tissues show marked changes during EFA deficiency. These changes have been described in several species. Most work has been conducted with the rat and references will be restricted to this experimental animal unless specified. Changes in fatty acid composition of the rat during EFA deficiency have been described for: whole body (Holman & Taylor, 1950; Widmer & Holman, 1950; Witten & Holman, 1952a, 1952b), brain (Mohrhauer & Holman, 1963b; Biran et al., 1964; Rathbone, 1965, Galli et al., 1970; White et al., 1971; Alling et al., 1972,

1974), liver (Caster et al., 1966, Diller et al., 1961; Garcia & Holman, 1965; Holman & Taylor, 1950; Klien, 1958; Klenk & Oette, 1960; Mohrhauer & Holman, 1963c, 1963b; Morin et al., 1963; Peluffo et al., 1963; Rahm & Holman, 1964a, 1964b, 1964c, Rieckenhoff et al., 1949; Selinger & Holman, 1965; Widmer & Holman, 1950), heart (Aaes Jorgensen & Holman, 1958; Aaes Jorgensen et al., 1958; Caster & Holman, 1961; Holman 1960a; Holman & Taylor, 1950; Peluffo et al., 1963, Reickenhoff et al., 1949; Widmer & Holman, 1959), kidney (Garcia & Holman, 1965; Holman & Taylor, 1950; Klein & Johnson, 1954b; Widmer & Holman, 1959), lung (Panos et al., 1958), intestine (Enser & Bartley, 1962; Holman & Taylor 1950; Widmer & Holman, 1959), muscle (Century et al., 1961; Holman & Taylor, 1950; Rieckenhoff et al., 1959; Widmer & Holman, 1959), skin (Rieckenhoff et al., 1949; Widmer & Holman, 1959), adipose tissue (Macdonald, 1962; Mohrhauer & Holman 1963b; Rickenhoff et al., 1949; Widmer & Holman, 1950), testis (Aaes Jorgensen & Holman, 1958; Aaes Jorgensen et al., 1958; Garcia & Holman, 1965; Carney et al., 1968; Bieri et al., 1969), and blood components (Diller et al., 1961; Holman, 1960a; Klein, 1958; Mohrhauer & Holman, 1963d; Van Deenen et al., 1963; Caster & Holman, 1961; Walker & Kummerow, 1964; Widmer & Holman, 1950; Yu et al., 1966).

The first reported change in the fatty acid composition of lipids during EFA deficiency was an increase in trienoic acid in the liver (Smedley-Maclean, 1943; Rieckenhoff et al., 1949). Fulco and Mead (1959) identified this structure as 20:3 (n-9) (Δ 5,8,11 eicosatrienoic acid) an elongation product of oleic acid. When linoleate and linolenate are excluded from the diet, a decrease in these fatty acids occurs as well as in their elongation products in tissue lipids. There is one known exception, in brain, 22:5 (n-6) increases during EFA deficiency

(White et al, 1971). The level of 20:3 (n-9) in EFA deficient rats
decreases when the diet is supplemented with linoleic or linolenic acid
(Mohrhauer & Holman, 1963d; Pudelkewicz & Holman, 1968). The dietary
consumption of linoleic acid results in a parallel increase in the con-
tent of arachidonic acid in tissues. When linoleic acid is omitted
from the diet, the level of 20:4 (n-6) decreases and the level of 20:3
(n-9) increases in tissues. Holman (1960a) has used the ratio of trienes
to tetraenes [20:3 (n-9) / 20:4 (n-6)] as a parameter to describe the
severity of EFA deficiency (in fact, the triene/tetraene ratio measures
the extent of linoleic acid deficiency). A triene to tetraene ratio of
0.4 or less indicates normal EFA status in rats (Holman 1960a and
Mohrhauer & Holman 1963c). This ratio is maintained with the ingestion
of at least 1% calories of linoleate per day ($\simeq$ 40 mg/day). A diet
containing less than 1% calories of linoleate will decrease the conver-
sion of linoleate to arachidonate and enhance the synthesis of 20:3
(n-9) from oleic acid. Triene to tetraene ratios of 5.0 have been
reported for EFA deficient rats in liver (Holman 1960a). Arachidonate
is three times more effective as linoleate as an essential fatty acid
(Alfin-Slater and Kaneda 1962). Arachidonate supplied as 0.3% calories
maintains the triene to tetraene ratio of tissue lipids below 0.4
(Alfin-Slater and Kaneda 1962). Linolenic acid is elongated to 20:5
(n-3) and the dietary intake of linolenate has been estimated by the
ratio of 20:3 (n-9) / 20:5 (n-3). A triene to pentaene ratio smaller
than 0.4 (Mohrhauer & Holman 1963d) indicated a diet sufficient in
linolenic acids for rats. When linolenic acid is refed to EFA defi-
cient rats, the level of 20:3 (n-9) decreases and the level of 20:5

(n-3) increases (Mohrhauer & Holman 1963e). Arachidonate does not respond to linolenate supplementation.

Histological changes that developed during EFA deficiency were first investigated by Burr and Burr (1929, 1930). They noted changes in skin, kidney, ovary and testis. Since these initial observations pathological changes in many organs have been described during EFA deficiency. Skin lesions have been described in detail (Aaes Jorgensen et al., 1956; Funch et al., 1957; Hansen et al., 1951; Kolak & Rao, 1953; Panos & Finerty, 1953; Ramalingaswami & Sinclair, 1951, 1953a, 1953b; Williamson, 1941) and resemble hyperkeratosis, vitamin A deficiency and phrynoderma. Changes in liver histology usually consist of fatty infiltration (Alfin-Slater & Bernick, 1958; Guggenheim & Jurgins, 1944; Panos & Finerty, 1953, 1954; Rice & Jackson, 1934) and may indicate a breakdown of lipid transport or metabolism. EFA deficiency is often accompanied by decreased urine output (Sinclair, 1952; Ramalingaswami & Sinclair, 1953b) and kidney pathology is not uncommon (Borland & Jackson, 1931; Aaes Jorgenson et al., 1955, 1956a, 1956b; Panos & Finerty, 1953, 1954; McAmis et al., 1929; Sinclair, 1952). Renal tubules become calcified and tubular epithelium contains numerous lipid droplets. Necrosis of the renal medulla also occurs. Testicular degeneration has been described (Evan et al., 1934; Greenburg & Ershoff, 1951; Panos & Finerty, 1954; Kirschner & Harris, 1960) and decreased sperato-gensis has been reported (Aaes Jorgensen et al., 1957; Funch et al., 1957; Holman & Aaes Jorgensen, 1956). Histological abnormalities occur in the ovary resembling pituitary insufficiency (Aaes Jorgensen et al., 1956a, 1956b; Panos & Finerty, 1953; Evans & Burr, 1927, 1928; Maeder, 1937) and irregular ovulation is common in EFA deficient rats (Burr &

Burr, 1929, 1930; Evans & Burr, 1927, 1928; Maeder, 1937; Panos &
Finerty, 1953). Changes in the pituitary gland have also been reported
(Aaes Jorgensen & Holman, 1958; Greenberg & Ershoff, 1951; Panos &
Finerty, 1954; Panos et al., 1959). Histological investigation of
thyroid, heart, thymus, adrenal, pancreas, prostrate and seminal
vesicles either have indicated no changes during EFA deficiency or have
given conflicting results (Panos & Finerty, 1953, 1954; Alfin-Slater &
Bernick, 1958).

Changes in the fatty acid composition of tissue lipids and in the
microscopic appearance of tissues are accompanied by changes in the
function of organs and organ systems. In 1930, Burr and Burr reported
increased water consumption in rats deficient in essential fatty acids.
This was also demonstrated by Sinclair (1952) who showed no concomitant
increase in urinary output (Sinclair 1952 and Ramalingoswami & Sinclair
1953b). Wesson and Burr (1931) found an increased basal metabolic rate
in EFA deficient rats. This increase was believed to be due to increased
fat synthesis from carbohydrate. Increased evaporation of water through
the skin has also been postulated as a cause for the increased basal
metabolic rate (Basnayake & Sinclair 1956). The basal metabolic rate
returned to normal upon supplementation with 100 mg. of linoleate per
day (Panos et al., 1956). A decrease in capillary resistance is found
in EFA deficient rats before dermal signs develop (Kramar and Levine,
1953). EFA deficient rats demonstrate aberrant ventricular conduction
as a notch appears in the QRS complex in 100% of deficient animals tested
(Caster and Ahn, 1963).

Rats maintained on an EFA deficient diet exhibit decreased muscle
tone when compared to normal animals (Burr et al., 1932). The calcium

permeability of EFA deficient sacroplasmic reticulum isolated from muscle is reduced and gives rise to a higher rate of calcium uptake, a reduction of the ATP-ase activity and a higher calcium-concentrating ability (Sieler and Hasselbach, 1971). Pulmonary edema develops in EFA deficient rats and decreased plasma corticoids and urinary formal-dehydrogenic corticosteroids accompanied by decreased response of these steroids to ACTH suggest adrenal malfunction during EFA deficiency (Nogase and Hikasa, 1965). EFA deficiency decreases the resistance of rats to irradiation injury (Decker et al, 1950; Decker and Mead, 1949) and increases the mortality of mammals during hibernation (Holman, 1968). Liver lipoproteins are synthesized or released at a decreased rate (Fukayawa & Privett, 1972) and the ability of the liver to synthesize and secrete triglycerides and phospholipids is also reduced (Fukayawa et al., 1971). Although EFA deficiency does not effect the incorpora-tion of amino acids into intestinal mucosal cells, the retention of amino acids after incorporation is diminished (Johns and Bergen, 1974). Bone marrow and thymus exhibit a decrease in small lymphocytes in EFA deficient rats (Loilund et al., 1970).

These findings demonstrate the importance of the essential fatty acids in the structure and function of various organ systems. These findings also show the functional role of essential fatty acids in membrane phospholipids of various tissues.

Several conditions or chemical substances increase the require-ments for EFA's or accelerate EFA deficiency: humidity (Brown & Burr, 1936), water restriction (Thomasson, 1953), alloxan (Peiffer & Holman, 1955), cholesterol (Aaes-Jorgensen & Holman 1958; Aaes-Jorgensen et al., 1958; Hauge & Nicolaysen, 1959; Holman, 1964; Nath et al., 1959; &

Peiffer & Holman, 1955), thiouracil (Holman, 1960), dessicated thyroid (Greenberg & Deud, 1950; Bosshardt & Huff, 1953), Triton WR 1339 (Holman & Aaes-Jorgensen, 1958), amino nucleoside (Holman & Aaes Jorgensen, 1958) tri-o-cresy phosphate (Greenburg & Deuel, 1950), mineral oil (Bacon et al., 1952), hydrogenated fats (Aaes-Jorgensen, 1961; Aaes-Jorgensen & Dam 1954, Aaes-Jorgensen et al., 1957, 1956; Evans & Lipkovsky, 1932; Devel et al., 1951; Funch et al., 1957; Sinclair, 1936; Williams et al., 1972), acetostearins (Alfin-Slater 1963; Coleman et al., 1963), oleic acid (Dhopeshwarkar & Mead, 1961); triolein (Thomasson et al., 1960), androgens, saturated fats, B-sitosterol, bile salts (Alfin-Slater, 1963), tetrabromostearic acid (Kunkel & Williams, 1951), succinyl sulfathiazole, streptomycin, atebrin, triacetin (Bosshardt & Huff 1953) and the transisomer of linoleate (Holman & Aaes-Jorgensen, 1956).

   B.   Membranes, Phospholipids and Fatty acids

   Membranes are composed primarily of lipids and proteins in addition to water, cholesterol, glycosides and various ions. The chemical composition and the proportion of the components mentioned above vary from membrane to membrane. The chemical variation is probably due to specificity of function that membranes from various sources possess. Myelin, which has primarily an insulating function and very low enzymantic activity, has a very low ratio of protein to lipid (1:4) whereas mitochondrial membranes, which are involved in a great number of enzymatic reactions requiring cooperativeness among portions of the membrane, contain a very high ratio of protein to lipid (1:1). The proteins within a membrane exhibit great heterogeneity and few membrane

proteins have been chemically characterized due to the difficulty of extracting them intact and free of lipid. Membrane proteins are usually classified into two types; peripheral and integral. Peripheral proteins are weakly bound to their membranes and can be disassociated from a membrane with mild treatments such as high ionic strength or metal ion chelating agents. Peripheral proteins do not appear to interact with membrane lipids and once disassociated from the membrane they are soluble in neutral aqueous buffers. Integral proteins are strongly bound to membranes and detergents, organic solvents or chaotropic agents are usually required to disassociate them from a membrane. They are usually associated with membrane lipid and often depend on lipids for optimal function. Integral proteins are usually bound to lipids when solubilized and usually aggregate or are insoluble in neutral aqueous buffers. Peripheral and integral proteins vary primarily in the manner in which they are attached to membranes (Singer, 1974).

Various lipids are found within membranes. The most abundant lipid within membranes are phospholipids and these constitute a substantial portion of all membranes so far analyzed. Phospholipids are structurally constituted on a glycerol framework which generally has two fatty acid molecules and a polar group attached to the 3 glycerol carbon atoms. The polar group often carries or can accept an electrical charge and is soluble in water (hydrophilic). Fatty acid chains are insoluble in water (hydrophobic) and project in the opposite direction of the polar group.

```
                         X      (polar group)
                         |
                         O
                         |
                     O —P —O
         α       β       O
         |       |       |
         C—————— C ————→ C
         |       |
         O       O
         |       |
(saturated)     FA      FA    (unsaturated)
```
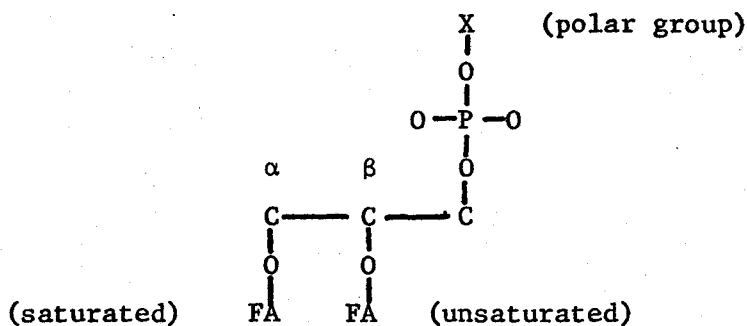
Fatty acids project into the interior of the membrane while the polar group remains near the surface. A saturated fatty acid is usually attached to the first carbon atom (α-position) of the glycerol molecule while an unsaturated fatty acid is usually attached to the second carbon (β-position) and the polar group is associated with the third carbon atom.

A theoretical model of a cellular membrane is presented in Figure 2. This model has been postulated by Singer (1975) and consists of globular proteins floating in a sea of lipid. The polar groups of the lipid molecules are located at the surface of the membrane while the fatty acid molecules project into the interior. The proteins are primarily globular and amphipathic with their hydrophilic ends protruding from the membrane and their hydrophobic ends embedded in the bilayer of lipids. Some proteins span the entire membrane and may possess two hydrophilic ends and a hydrophilic center. The proteins shown in Figure 2 would be considered integral proteins. Peripheral proteins would be attached to the hydrophilic ends of the integral proteins. Phospholipids can vary according to their fatty acids, to their polar groups, or both. The fatty acid composition of phospholipids varies according to the type of tissue within species, but there is little variation in fatty acid

Fig. 2. Model membrane taken from Singer (1975). Proteins are globular, with hydrophilic ends protruding from the membrane and hydrophilic portions within the lipid bilayer of the membrane.

patterns between mammalian species when identical tissues are studied.
The tissue specific pattern of phospholipid fatty acids is likely due
to the similar functions that membranes of the same tissue from differing
species share.

Changes in the fatty acid composition of membrane lipids can alter
the functional properties of a membrane. At physiological temperature
membranes usually exist in a liquid-crystal state. In this state the
glycerol and polar groups retain a rigid organization although the polar
group has limited mobility. The fatty acids chains melt and have a
considerable mobility with the methyl end of the chain, having the
greatest mobility. Thus in a membrane bilayer the interior of the membrane
is more fluid than the exterior portion. The membrane retains this
liquid-crystal state as long as it exists in an environment above the
transition temperature for maintaining the lipids in a liquid rather
than a gel state. The transition temperature of membrane lipids is
modulated by several factors; one being the type of fatty acids present.
The length of fatty acid chains and the degree of saturation or unsatura-
tion of those chains has a direct effect on the transition temperature
or fluidity of biological membranes (Chapman, 1975b). Short chain
length and increasing unsaturation will decrease the transition temper-
ature while fully saturated long chain fatty acids will raise the transi-
tion temperature. The amount of cholesterol in a membrane also effects
the transition temperature. Increased cholesterol lowers the transition
temperature. Thus changes in the fatty acid composition of membrane
lipids can change the fluidity of that membrane by altering the transi-
tion temperature. Changes in the fluidity of a membrane can alter the
function of the membrane in several ways. The permeability of the

membrane will depend on the degree of fluidity within the membrane.
Changes in fluidity may alter the elasticity of a membrane. Interaction
between protein and lipid may also be affected by changes in the fatty
acid composition of membranes (Chapman, 1975a).

C. The CNS and EFA Deficiency

The present work was undertaken to elucidate the effects of EFA
deficiency on the CNS. The work is multidisciplinary and involves both
biochemical and morphological tools. The central nervous system serves
a unique role as an integrator of afferent stimuli. It is this inte-
gration that enables an organism to respond to and interact with its
environment to provide optimal function and survival. The majority
of functions performed by the CNS involve specific interactions of
membranes. Membranes are essential to the function of the central
nervous system, as they play vital roles in the conduction and trans-
mission of information. As mentioned previously, phospholipids and
fatty acids play a major role in the structure and function of membranes.
The CNS contains more lipid than any other tissue with the exception
of adipose tissue. The dry weight of the brain is over 50% lipid.
Approximately 25% of the dry weight of the brain is phospholipid (Norton
& Poduslo, 1973b). No phospholipid is exclusive to CNS tissue although
a high per cent of ethanolamine phosphoglycerides are present. Ethano-
lamine phosphoglycerides contain a high per cent of polyunsaturated
fatty acids.

Changes in the fatty acid composition of whole brain phospholipids
(Mohrhauer & Holman, 1963; Biran et al., 1964; Rathbone, 1965; White

et al., 1971; Galli et al., 1970; Alling et al., 1972, 1974) have been reported during EFA deficiency. No marked changes are found in the concentration of the lipid classes during EFA deficiency (Galli et al., 1971; Alling et al., 1972, 1974; Montamat, 1974). The changes found in the fatty acid composition of brain phospholipids during EFA deficiency consists primarily of a decrease in the dietary essential fatty acids, linoleic and linolenic acid and their elongation products. These changes are compensated by an increase in the nonessential fatty acids of the oleic family. Alterations in the fatty acid composition of CNS phospholipids are reversible by refeeding a control diet containing adequate amounts of linoleic and linolenic acid (Galli et al., 1971a). Although questions as regards the essentiality of linolenic acid have been raised (Tinoco et al., 1971), this finding has been criticized (Crawford & Sinclair, 1972) and as essential role for (n-3) fatty acids has been demonstrated in primates (Fiennes et al., 1973), for superior learning capacity in the rat (Lamptey & Walker, 1976), for maintenance of normal electrical response of photoreceptor membranes of rat retina (Benolken et al., 1973) and for the normal development of insects (Chippendale et al., 1965) and trout (Lee et al., 1967, Castell et al., 1972 and Yau & Sennhuber, 1975). Previous reports of fatty acid supplementation after induction of EFA deficiency involved utilization of control diets containing both linoleic and linolenic acid (White et al., 1974; Galli et al., 1971a) or oils from various sources which are mixtures of (n-3) and (n-6) acids to varying degrees (Biran et al., 1964; Galli et al., 1971b). Klenk (1965) has shown that no interconversion of (n-6) and (n-3) fatty acids occur. Due to the fact that these fatty acid series have biosynthetic pathways that are independent

from one another, the effects of supplementing linoleic or linolenic acid independently, on the fatty acid composition of whole brain phospholipids after the induction of EFA deficiency was investigated. The metabolism of the CNS is unique in several ways and it reacts different than other organs under certain circumstances. Thus, the effects of EFA deficiency and linoleic or linolenic supplementation on the fatty acid composition of liver phospholipids was determined to see if any responses were unique to brain.

Upon gross inspection CNS tissue can be divided into two distinct types: white and gray matter. White matter is composed of myelinated axons, glial cells and vascular elements. Gray matter consists of nerve cell bodies with their dendritic and synaptic complexes in addition to the components found in white matter. Chemical differences between gray and white matter are primarily due to the presence of myelin in white matter. The dry weight of white matter consists of approximately 50% myelin. Myelin is characterized by low H_2O and protein content but contains a high per cent of lipid. The solids of myelin, depending on the species from which it is derived, are 70-80% lipid and 20-30% protein; 40-45% of the lipid is phospholipid (Norton, 1972).

Functionally myelin insulates axons and allows for increased conduction of propagated impulses down the axon. Myelin is formed by oligodendroglia within the CNS and the myelin sheath is continuous with the plasma membrane of the oligodendroglia cell (Bunge, 1968). A process of the oligodendrocyte becomes connected to the outer surface of axon, this process is separated from the axon by 120 Å. The process elongates and encircles the axon. The myelin sheath is expanded at its internal

and lateral mesaxon. The concentric lamella are formed by this expansion which may involve the slippage of adjacent myelin lamella to allow for the advancement of the inner mesaxon (Hirano & Dembitzer, 1967). The spiral lamella of the myelin sheath start at the inner mesaxon. An interperiod line is produced by opposition of the outer surface of the oligodendrocyte plasma membranes. The major dense line which alternates with the interperiod line is formed by the opposition of the two cytoplasmic surfaces of the oligodendrocyte plasma membrane. The outer mesaxon is formed by the separation of the major dense line of the outer-most lamella of myelin.

The distance between two major dense lines of fresh CNS myelin as determined by X-ray diffraction is 160 Å (Hildenbrand & Müller, 1974). The interperiod distance of myelin prepared for electron microscopy varies according to the preparation employed, the anatomical source, the number of lamella or thickness of the myelin sheath and ranges from 94Å- 126Å (Hildebrand & Müller, 1974). One oligodendrocyte can form more than one myelin sheath around differing axons and it has been estimated that oligodendrocytes in the rat optic nerve form on an average 42 myelin sheaths (Peters & Proskauer, 1969).

Myelin can be isolated in a highly purified state from rats of different ages (Norton & Poduslo, 1973a) and the chemical composition of myelin varies with age (Norton & Poduslo, 1973b). Several techniques have been developed which separates purified myelin into subfractions which are biochemically and morphologically unique utilizing density gradient centrifugation (Adams & Fox, 1969; Agrawal et al., 1974; Autillio et al., 1964; Benjamins et al., 1973; Cuyner & Davidson, 1968;

Eng & Begnami, 1972; Matthieu et al., 1973; McMillian et al., 1972; Morrel et al., 1972; Sabri et al., 1975; Waehneldt & Neuhoff, 1974; Zimmerman et al., 1975). Generally, three subfractions are obtained from these procedures and have been referred to as light, medium and heavy myelin in reference to their sedimentation characteristics on density gradients. Matthieu et al. (1973) have hypothesized that the heavy myelin is a membrane in transition from the oligodendrocyte plasma membrane to myelin. The light myelin fraction is believed to be mature myelin consisting of a compact multilamellar structure. A product-precursor relationship between heavy and lighter myelin fractions has been suggested (Agrawal et al., 1974; Sabri et al., 1975; Hofteig & Druse, 1976) although alternative explanations are possible (Benjamins et al., 1976a, 1976b).

The effects of EFA deficiency on the fatty acid composition of purified myelin phospholipids have been reported (Galli et al., 1972; Sun, 1972; Sun & Sun, 1974; Montamat et al., 1974; Karlsson, 1975). The fatty acid composition of phospholipids of myelin subfractions has not been reported. Thus the fatty acid composition of the ethanolamine phosphoglycerides from subfractions of myelin was determined developmentally and during EFA deficiency. The ethanolamine phosphoglycerides were isolated as they contain a high degree of polyunsaturated fatty acids. If the subfractions of myelin have unique functions, it may be reflected in their fatty acid composition or the fatty acid response to EFA deficiency.

Chemical changes induced in the CNS during EFA deficiency have received much attention and this is especially true for changes in the

fatty acid composition of membrane phospholipids. As pointed out, fatty acids play a major role in the structure and function of biological membranes. No reports on changes in the morphology of the CNS during EFA deficiency have been published. Morphological changes within the CNS have been reported during nutritional deficiencies produced by protein and protein-calorie malnutrition. Alterations during chronic undernutrition in the adult rat consisted of the formation of lamellar whorls and the aggregation of synaptic vesicles within presynaptic terminals in deep cerebellar nuclei (Yu et al., 1974). The undernourished developing rat showed a marked decrease in neuropil and the number of axon terminals found per neuron was reduced by 38-41% (Cragg, 1972). The morpholgical development of the cerebellar cortex is retarded during undernutrition of the developing rat (Sima & Persson, 1975). The number of neurons in differing areas of the cerebral cortex has also been found to be reduced in undernourished rats (Dobbing, 1968). Previous studies have shown protein-calorie undernutrition initiated in early life to cause retardation in nerve fibre growth of central and peripheral nerve tracts in the rat (Clos & Legrand, 1969, 1970; Hedley-Whyte & Meuser, 1971; Hedley-Whyte, 1973). Sima and co-workers have extensively studied the effects of early undernutrition on the degree of myelination in peripheral (Sima, 1974a) as well as CNS pathways (Sima & Sourander, 1974). They have reported decreased myelin deposition in ventral and dorsal root fibres of the fifth lumbar segments and in the nerve fibres of the optic nerve (Sima, 1974b). Thus it is apparent that changes in CNS morphology in response to nutritional deficiencies can occur.

To determine if EFA deficiency has any effects on CNS morphology, an ultrastructural study of the optic nerve of the rat was undertaken. This study focused on the diameter of myelinated fibres and the morphological appearance of myelin. Myelin, having a high lipid content, may be affected morphologically by EFA deficiency. Involvement of the optic nerve in demyelinating diseases, such as multiple sclerosis, is very common (Lumdsen, 1970). All fibres in the optic nerve of the rat are myelinated and the nerve is very accessible.

In summary, the present work is divided into 3 parts; 1) Changes in the fatty acid composition of whole brain phosphoglycerides during EFA deficiency and after supplementation of linoleic and linolenic acid to EFA deficient rats was investigated. These changes were compared to changes found in liver phosphoglyceride fatty acids. 2) The fatty acid composition of ethanolamine phosphoglycerides from subfractions of purified myelin was determined developmentally and in states of fat deprivation. 3) Changes in the ultrastructure of the optic nerve during EFA deficiency were also investigated.

MATERIAL AND METHODS

A.   Animals and Diets

Sprague-Dawley rats were placed on a fat-free diet (Nutritional Biochemicals, Cleveland, Ohio) 10-12 days after impregnation and were maintained on this regimen during gestation and lactation. The composition of the fat-free diet is given in Table I. Litter size was adjusted to between 9-11 pups at birth and weaning occurred at 21 days. Weaned animals were housed individually in stainless steel metabolic cages and had access to the fat-free diet and water ad libitum. A control group receiving standard laboratory chow (Purina Lab Chow) ad libitum was also maintained. The chemical composition of the control diet is given in Table II. Control and fat deficient animals were sacrificed by decapitation at 30, 60, 90 and 120 days postnatally. At 120 days, additional EFA deficient animals were divided into four groups: 1) Supplementation with linoleic acid (Nu-Check Pre., Inc., Elsyian, Minnesota, > 99% pure) for 10 days; 2) Similar supplementation for 30 days; 3) Supplementation with linolenic acid (Nu-Check Prep., Inc., Elsyian, Minnesota, > 99% pure) for 10 days; 4) Similar supplementation for 30 days. The fatty acids were administered orally via eye dropper in the form of the methyl esters at a level of 0.2 ml/day ($\simeq$ 1.5 Cal.). These supplemented animals were sacrificed at the specified time intervals. Following decapitation the brain was rapidly removed and the brain minus cerebellum was homogenized in 5 volumes of 0.9% NaCl

TABLE I

FAT-FREE DIET

"Vitamin-free" Casein*	21.10 %
Alphacel "cellulose"	16.45 %
Sucrose	58.45 %

Salt mixture 4.0 %:

Cupric sulfate 0.48 gm, Ferric ammonium citrate 94.33 gm, manganes sulfate 1.24 gm, ammonium alum 0.57 gm, potassium iodide 0.25 gm, sodium flouride 3 13 gm, calcium carbonate 68.8 gm, calcium citrate 308.3 gm, calcium biphosphate 112.8 gm, magnesium carbonate 35.2 gm, magnesium sulfate 38.3 gm, potassium chloride 124.7 gm, dibasic potassium phosphate 218.8 gm, sodium chloride 77.1 (all compounds are expressed as grams per kilogram).

<u>Vitamins</u> (gm/100 lb)

Choline chloride 272.5, nicotinic acid 27.25, inositol 13.75, A (200,000 /gm) 4.5, D (400,000 /gm) 3.0, α-tocopherol 10.225, menadione 0.102, thiamine 1.0, pyroxidine 1.0, riboflavin 1.0, calcium pantothenate 2.05.

** Vitamins A, D, B, and K are synthetic crystalline water soluble.

   B-complex are natural

* <u>Vitamins</u> (μg/gm)

Thiamine HCl	0.15	Pantothenic	0.15
Riboflavin	0.50	Biotin	0.0013
Niacin	0.30	Folic Acid	0.030
Pyridoxine HCl	0.65	B_{12}	0.003
Fat	< 0.01%		

TABLE II

CONTROL DIET

Protein % of total amino acids   23.20

Arginine	1.42	Lysine	1.40
Cystine	0.35	Methionine	0.44
Glycine	1.16	Phenylalanine	1.07
Histidine	0.62	Threonine	0.89
Isoleucine	1.28	Tryptophan	0.27
Leucine	1.85	Valine	1.17

Fat % of total   4.50

Fiber % of total   3.80

TDN   77.00

NFP (by difference)   52.50

Ash	6.00	Vitamins	
Calcium	1.01	Carotone (PPM)	5.60
Phosphorus	0.74	Menadione	–
Potassium	1.08	Thiamine (PPM)	10.90
Magnesium	0.21	Ribovlavin (PPM)	4.50
Sodium	0.42	Niacin (PPM)	60.00
Chlorine	0.52	Pantothenic (PPM)	12.50
Flourine (PPM)	65.00	Choline (PPMx100)	19.00
Iron (PPM)	1.97	Folic Acid (PPM)	1.70
Iodine (PPM)	1.17	Pyridoxine (PPM)	4.30
Manganese (PPM)	54.40	Biotin (PPM)	0.30
Copper (PPM)	15.10	B_2 (mcq/lb)	14.00
Cobalt	0.37	Vitamin A (IU/m)	12.00
		α-Tocopherol (IU/16)	18.00
		Ascorbic Acid	–

using a tight fitting teflon pestal. Aliquots of the homogenate were frozen at $-60°C$, lypholized and stored at $60°C$ for future use. The livers were removed from the 120 day control, fat deficient and EFA supplemented animals and were prepared in an identical manner. Lipids were extracted from the homogenized brain and liver. Phospholipids were separated from the lipids by column chromatography and the fatty acid composition of the phospholipids was determined by gas-liquid chromatography.

Another group of control and fat deficient animals were sacrificed at 15, 20, 30, 60 and 120 days of age. Whole brains were rapidly removed and homogenized in 5 volumes of 0.32 M sucrose and three subfractions of myelin were isolated. The lipids were extracted from the isolated myelin subfractions. The phospholipids were separated from the total lipids and phospholipid subclasses separated by thin layer chromatography. The fatty acid composition of the ethanolamine phosphoglycerides from the subfractions of myelin was determined by gas chromatography.

A third group of control and fat deficient animals was sacrificed by intracardiac perfusion for electron microscopic studies.

Both control and fat deficient animals were housed in the same quarters maintained with a 12-hour light dark cycle. The temperature was maintained between 71 and $72°C$ and the humidity was kept constant at 45%.

B.   Myelin Isolation

The number of brains used per myelin isolation varied with the age of the animals. At 15 days of age, six brains were pooled per separation

and the methyl esters of two separations were combined prior to fatty acid analysis. Thus each value for the 15 day animals represented the fatty acid composition of myelin subfractions from 12 pooled brains. The values for the 20, 30, 60 and 120 day animals represented 8, 6, 4 and 2 pooled brains respectively. Animals were sacrificed by decapitation and the whole brain was rapidly removed and homogenized in 5 volumes of 0.32 M sucrose at 4°C. The initial myelin isolation was essentially that of Norton & Poduslo (1973a) and all steps were carried out at 0.4°C. Fifteen ml of the homogenate was layered over 10 ml of 0.85 M sucrose. These gradients were spun at 75,000 xg for 30 minutes. The material that forms at the 0.32-0.85 M sucrose interface was collected and termed crude myelin. All other material was discarded. The crude myelin was homogenized and water-shocked for 30 minutes. This suspension was pelleted at 75,000 xg for 15 minutes and the supernatant is discarded. The pellet is dispersed and resuspended in 0.32 M sucrose, layered over 0.85 M sucrose and centrifuged at 75,000 xg for 30 minutes. The interface material was collected, dispersed in H_2O and centrifuged at 12,000 xg for 10 minutes. The supernatant was discarded and the pellet was collected and termed purified myelin.

The myelin purified by the procedure of Norton & Poduslo was further separated into 3 subfractions by the method of Matthieu et al. (1973). Purified myelin was homogenized in 0.32 M sucrose and layered on a discontinuous sucrose gradient. This gradient consisted of 6 ml of 0.70 M sucrose, 9 ml of 0.625 M sucrose and 15 ml of the suspended purified myelin in 0.32 M sucrose. These gradients are centrifuged at 75,000 xg for 30 minutes. Three myelin fractions were collected: one at the 0.32-0.625 interface, one at the 0.625-0.70 interface and a

pellet. These were termed light, medium and heavy myelin respectively.

These fractions were washed with H_2O twice and frozen at $-60°C$ for

future use. A flow sheet for the isolation of the three subfractions

of myelin is presented in Figure 3.

C. Lipid Methodology

Lipids were extracted by the method of Bligh and Dyer (1957) by

placing lypholized tissue in chloroform: methanol: H_2O (47:37:7) for

60 minutes. Nine ml of H_2O was then added to give a two phase system.

The lower phase was collected and the upper phase was washed with the

theoretical lower phase twice. All solvents were of A.R. grade or

better and were redistilled in glass. The lipid extract was taken to

dryness in a rotary evaporator under N_2 and dissolved in approximately

5 ml. of chloroform. Lipid subclasses were separated by column chroma-

tography by the method of Rouser et al. (1967) in a column 0.5 cm x 3.0 cm.

packed with Bio-Sil 350 mesh (Bio-Rad Lab., Richmond, California). Sea

sand was placed on top of the column packing to prevent disruption of

the mesh during application of the sample or solvents. The lipid extract

was applied to the column in 5 ml of chloroform. The neutral lipids

(cholesterol, mono, di and triglycerides and free fatty acids) were

eluted with 150 ml of chloroform. The glycolipids (cerebrosides and

sulfatides) were eluted with 150 ml of acetone and the phospholipids

with 150 ml of methanol. The phospholipids were brought to dryness in

a rotary evaporator under N_2 and redissolved in 5 ml of methanol. In

the myelin experiments the phospholipids were further subdivided by

thin layer chromatography. The phospholipids were evaporated to 0.25 ml

and applied to a thin-layer plate. The plate was covered by 0.6 mm

FIGURE 3.   PROCEDURE FOR ISOLATING MYELIN SUBFRACTIONS.

HOMOGENATE   0.32M SUCROSE (1 X 6 DILUTION)

0.32M

75,000XG/30M

0.85M
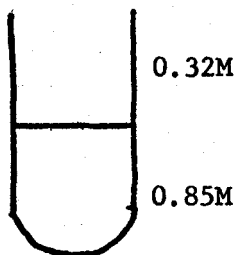
INTERFACE MATERIAL

WATER   75,000XG/15M
WASH   12,000XG/10M

RESUSPEND IN 0.32M SUCROSE - HOMOGENIZE
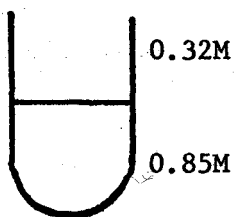
0.32M

75,000XG/30M

0.85M

INTERFACE MATERIAL

WATER   75,000XG/15M
WASH

RESUSPEND IN 0.32M SUCROSE - HOMOGENIZE

L   0.32M

0.625M   75,000XG/30M

M   0.70M

H
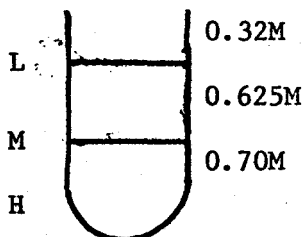
of silica gel H (Brinkmann Instruments Inc.) prepared by the method of Skipski (1964). The plates were activated at 120°C for 90 minutes before use. The thin layer plates were developed in chloroform: methanol: acetic acid: water (25:15:5:3) and then air dried at room termperature for 10 minutes. The dried plates were than sprayed with 1% 2,7-dichloro-florescein (Applied Science Laboratory) in methanol and detection of the lipid classes was conducted under a fluorescent lamp. Identification of the lipid classes was verified with authentic standards (Supelco Inc.). The ethanolamine, choline and inositol-serine phosphoglycerides were easily separated and were scraped into glass tubes with a teflon screw top. The EPG fraction was eluted from the silica gel H with chloroform: methanol (1:2) twice and chloroform twice.

Interesterification of whole brain and liver total phospholipids and for the ethanolamine phosphoglycerides of myelin subfractions was accomplished with BF_3 in Ch_3OH (14% w/v) by the method or Morrison & Smith (1964). The lipids were evaporated to dryness in a glass tube fitted with a teflon screw top. Three ml. of 14% BF_3 in methanol was added. The capped tubes were placed in a boiling water bath for 5 minutes. The tubes were colled, 5 ml of heptane was added and the tubes were boiled for an additional 5 minutes. The tubes were again cooled and 10 ml of NaCl saturated water was added. The tubes were shaken and the heptane containing the methyl esters was removed. The remaining solution was washed with 5 ml of heptane twice.

The methyl esters were injected into a Model 2100 Varian ionization gas chromatograph equipped with a flame ionization detector. Peak areas were determined by a Model 475 Integrator (Varian).

Separation of the fatty acids was achieved with a 15% DEGS-X (Applied Science Labs., State College, Pennsylvania) as the liquid phase in a glass column packed with Gas Chrom P (Applied Science Labs., State College, Pennsylvania) as the stationary phase at 190°C. The detector and injector temperature was maintained at 250°C. Helium was used as the carrier gas. Peaks were identified by retention times and comparison with authentic standards (Applied Science Labs., State College, Pennsylvania). Fatty acids were expressed as per cent of the total fatty acids and values were expressed as the average ± the standard error of the mean.

The unsaturation index (UI) is used as a means of determining the overall quantity of unsaturation present in a fatty acid mixture. The unsaturation index was calculated by totaling the per cent of the individual saturated classes (saturates, monoenes, dienes, etc.) and multiplying these by the number of double bonds present (0, 1, 2, etc.). Summing the products of the various classes gives the unsaturation index and this was expressed as absolute numbers.

D.   Electron Microscopy

1. Optic Nerve

Rats were sacrificed by intracardiac perfusion under light Sodium Pentabarbital (Abbot Labs, North Chicago, Illinois) anesthetic. The fixative consisted of 1% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4. The osmolarity of the fixative was 810 m OsM. Following fixation the brain and optic nerve were carefully removed and immersed in a fixative consisting of 2.5% glutaraldehyde and 1% acrolein in 0.1 M cacodylate buffer at pH 7.4 overnite. The

following day the optic nerves were dissected into 4 segments and then washed with the cacodylate buffer. The tissue was then post-osmicated with 2% aqueous O_sO_4 (Polysciences, Inc., Warrington, Pennsylvania) for two hours, washed with buffer and dehydrated through a series of ethyl alcohol and H_2O mixtures consisting of 70, 80, 95 and 100% at room temperature. The tissue was then placed into absolute propylene oxide for 20 minutes. Infiltration was carried out at room temperature for 18 hours in a 2:1 mixture of araldite and propylene oxide. Specimens were imbedded in a flat aluminum weighing dish and polymerizion of the resin was carried out for 36 hours at 56°C. Following polymerization of the resin the tissue was carefully trimmed from the araldite and re-embedded in a blank block of araldite. The optic nerves were oriented so a cross-section of the whole nerve could be obtained. This was accomplished by sawing into the tip of a polymerized block of araldite and placing a segment of optic nerve within the slit caused by the saw blade. A drop of araldite was placed on the top of the optic nerve and the block was placed in the oven at 56°C for 36 hrs to allow the inserted optic nerve to polymerize to the block. Following this final polymerization the blocks were trimmed and thick sections (1 μ) were cut on a Porter Blum MT II ultramicrotome. Thick sections were stained with toluidien blue. Thin sections were cut with a diamond knife on a Porter Blum MT II ultramicrotome, and stained with uranlyl acetate and lead citrate by the method of Venable & Coggeshall (1965). Thin sections were viewed with a RCA EMU-3G electron microscope.

2.   Isolated Myelin

The myelin fractions were removed from their respective sucrose gradients and immersed in 10 volumes of 3% glutaraldehyde in 0.1 M Millonigs buffer at pH 7.4. The isolated myelin was allowed to fix overnite upon which time it was pelleted in an International IEC Model CS centrifuge at one-half speed for 5 minutes. The sedimentation step followed all steps in the procedure. The fixative was removed and a buffer wash followed. The myelin was then post-osmicated for 1.5 hrs. in 1% O_SO_4 and rinsed with buffer again. Dehydration was accomplished at room temperature through a graded series of ethyl alcohols. The fractions were then placed in absolute propylene oxide for 30 minutes and then infiltrated for 36 hrs. in propylene oxide: araldite (1:1). Following infiltration the myelin was pelleted and the propylene oxide-araldite solution removed. The myelin fraction was then drawn into a plastic capillary tube with a syringe. The capillary tubing was occluded at one end and taped to a wooden swab stick. The tubing and stick was anchored to a centrifuge tube with masking tape and was spun at full speed on the IEC centrifuge. This step packed the myelin at the bottom of the capillary tube. The araldite was polymerized within the capillary tubing at 56°C for 36 hrs. Following this initial polymerization the isolated myelin concentrated at the bottom of the capillary tube was cut into small segments and re-embedded in araldite. These blocks were polymerized at 56°C for 36 hrs, trimmed and cut at 1 μ for thick sections. Thin sections were cut, stained and viewed similarly as the optic nerve.

E.   Fibre Diameter

A complete cross-section of the optic nerve was obtained.   To control for sampling error and to obtain a representative part of the total fibre population, a sector of nerve approximtely 40 degrees wide was photographed and a montage was assembled of this area.   The magnification of the montages was 30,000 times.   The total fibre diameter was measured with a millimeter ruler.   Irregular shaped fibres were measured according to Ramero and Skoglund (1965).   Pear shaped diameters were measured at their base and oval shaped fibres were measured at their minimium diameter.

RESULTS

A. GROWTH CURVE OF CONTROL AND EFA DEFICIENT RATS.

Animals maintained on the fat-free diet demonstrated typical signs
of EFA deficiency (Holman, 1968). There was a decreased growth rate and
animals demonstrated scaly skin, roughened hair coat and necrosis of the
tail. Animals were not scored according to degree of skin changes,
(Holman & Ener, 1954) as fatty acid composition of tissue lipids was
determined at various ages. Biochemical changes in fatty acids precede
dermal symptoms and are a better index of EFA deficiency status. The
growth curves for control and EFA deficient male and female rats are
presented in figure 4. The weight gain of both male and female rats
was decreased by EFA deficiency. The effects of EFA deficiency on the
growth curve of male rats was greater than on females at early ages.
The weight of 50-day-old EFA deficient male rats was decreased by 44%
when compared to control values whereas the female body weight was only
decreased 26%. This sexual dimorphism was not maintained at 120 days
where males were 38% of control weight and females 36% of controls.

B. CHANGES IN FATTY ACID COMPOSITION OF WHOLE BRAIN PHOSPHOLIPIDS

WITH AGE, DURING EFA DEFICIENCY AND FOLLOWING LINOLEATE AND

LINOLENATE SUPPLEMENTATION.

1. FATTY ACID COMPOSITION IN AGE

There existed little variation in the phosphoglyceride fatty acid
composition of the brain of control animals at the various ages to 120
days (Table 3). The fatty acids; 18:0, 18:1 (n-9) and 16:0 each repre-
sented approximately 20% of the total phosphoglyceride fatty acids at
all ages studied. The next most abundant fatty acids were 20:4 (n-6)
and 22:6 (n-3), the major elongation products of linoleic and linolenic
acids respectively, which represent approximately 10% for the former and
14% for the latter of the total phosphoglyceride fatty acids. The
remaining fatty acids were minor components and no other single fatty
acid represented more than 4.0% of the total. The saturated fatty acids
constituted 43% of the total fatty acids while the monounsaturated and
polyunsaturated comprised approximately 22% and 33% respectfully (Table 3).
At all ages studied the unsaturation index showed no marked changes with
age in the control animals and varied between 165 and 180, differences
which were not significant (Table 4).

2. CHANGES DURING EFA DEFICIENCY

The effect of EFA deficiency on brain phosphoglyceride fatty acids
demonstrated a progressive change between 30 and 120 days of age. At
30 days the first noted changes were an increase in 22:5 (n-6) from 0.2%
to 3.1%, and a decrease in 22:6 (n-3) from 13.2% to 10.7%. These changes

Figure 4. Growth curve of control and EFA deficient male and female

rats.
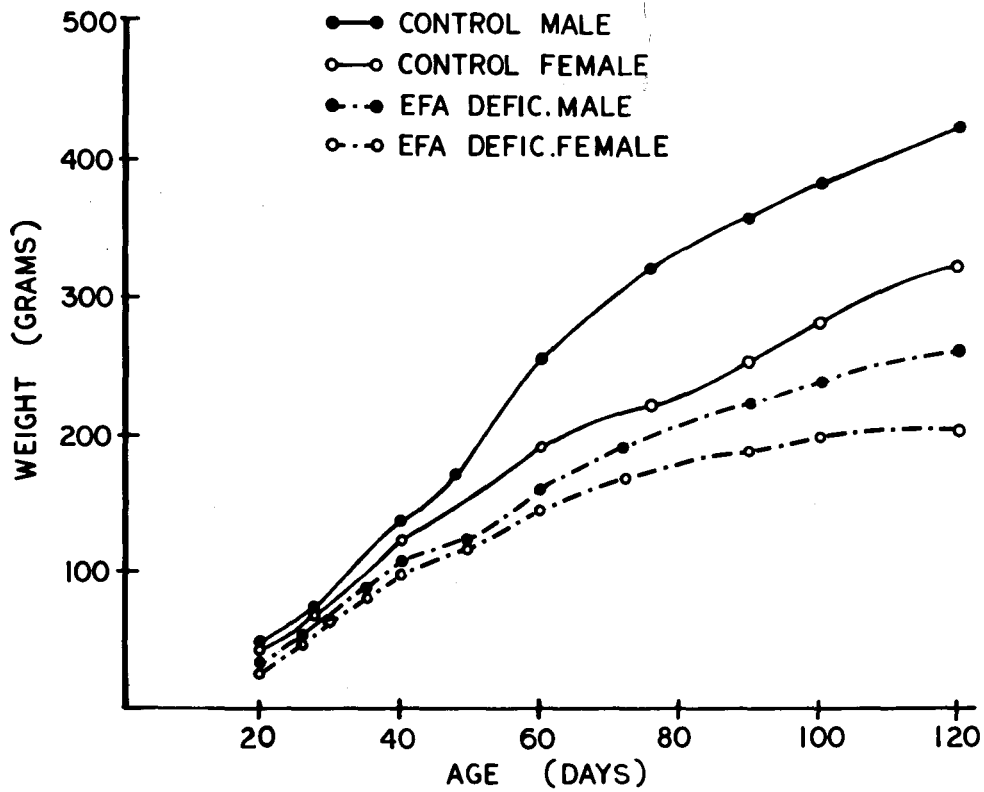
from control values became more marked as the EFA deficiency state continued. After 60 days an increase in the (n-9) fatty acids was observed as 20:3 (n-9) increased from 0.4% to 4.5% and 22:3 (n-9) increased from trace levels to 1.9%. A decrease in 18:2 (n-6) from 1.7% to 0.5% was also seen at 60 days. After 90 days 20:4 (n-6) was diminished to 7.9% from control values of 10.4%. All of these changes progressed to the extreme values reached after 120 days on the EFA-deficient diet. There were no marked changes in the total saturated, monounsaturated, or polyunsaturated fatty acids during EFA deficiency at any of the time periods studied. The unsaturated index remained unchanged during progressive states of EFA deficiency. If the unsaturation index for the individual fatty acid series was calculated, a continuous decrease in the unsaturation index of the (n-3) or linolenic series was found. This decrease was compensated for by an increase in the unsaturation index of the (n-9) or oleic acid series. The unsaturation index of the linoleic or (n-6) fatty acid series was slightly elevated at 30 and 60 days, but remained unchanged at 90 and 120 days.

3. SUPPLEMENTATION EFFECTS

Supplementation with linoleate and linolenate for 10 or 30 days to the 120-day EFA deficient animals had varying effects on the fatty acid composition of brain phosphoglycerides (Table 5). As expected, when linoleate was administered there was an increase in the (n-6) fatty acids, and when linolenate was fed there was an increase in the (n-3) fatty acids. A decrease in the (n-9) fatty acids occurred concomitantly and was of the same magnitude with either linoleate or linolenate supplemen-

tation. Supplementation with either linoleate or linolenate for either time period had no effect on the degree of saturation, or percent distribution of monounsaturates or polyunsaturates. The level of 20:4 (n-6) was increased from the deficient value of 6.3% to 8.1% with 10-day supplementation of linoleate, and approached the control value of 10% after 30 days; whereas 22:4 (n-6) remained unchanged after 10 days of linoleate but did reach the control value of 3.9% after 30 days. Supplementation with linolenate had no effect on the levels of 20:4 or 22:4 (n-6). Linoleate refeeding did not alter the value of 22:6 (n-3) from the deficient value of 8.3%, whereas 10 days of linolenate administration increased the level to 10.3% and after 30 days feeding the 22:6 (n-3) levels recovered to the control values of 14.0%. The (n-9) fatty acids increased during EFA deficiency, and after 120 days of fat deprivation 20:3 (n-9) increased from 0.8% to 6.0% and 22:3 (n-9) increased from 0.6% to 2.5%. Ten days supplementation with either linoleate or linolenate decreased the level of 20:3 (n-9) to approximately 4.7%, whereas after 30 days this fatty acid decreased to 2.8%, still above control levels. Ten days refeeding with linoleate had no effect on 22:3 (n-9) but 30 days of linoleate and 10 or 30 days of linolenate decreased the level of 22:3 (n-9) to approximately 1.7%. Thus, the return of the (n-6) and (n-3) fatty acids to normal levels following linoleate and linolenate supplementation preceded the decrease of the (n-9) fatty acid to their control values. The response of 22:5 (n-6) was unique in that, being an elongation product of linoleic acid, it increased during EFA deficiency. Upon supplementation with linoleate for 10 and 30 days, there occurred a progressive increase of the already elevated levels of

22.5 (n-6) to 6.4% of the total fatty acids. Refeeding linolenate for 10 days had no effect on 22:5 (n-6) whereas after 30 days the fatty acid decreased from 4.5% to 1.6%, which approached the control value of 0.6%.

The unsaturation index for the total phosphoglyceride fatty acids remained unchanged during linoleate or linolenate supplementation (Table 6) whereas the response of the individual fatty acid families differed. The unsaturation index of the (n-3) fatty acids was not affected by linoleate feeding and was approximately 58; 10 days of linolenate supplementation increased the unsaturation index to 66 and the control value of 89 is exceeded after 30 days supplementation where a value of 97 was obtained.

The unsaturation index of the (n-6) fatty acid series remained unchanged during EFA deficiency and was increased from 63 to 71 after 10 days and to 87 after 30 days supplementation with linoleate. Ten days of feeding linolenate had no effect on the unsaturation index of (n-6) series whereas after 30 days the value decreased to 43. The response of the unsaturation index of the (n-9) series was similar with either linoleate and linolenate supplementation. Ten days feeding of either fatty acid had little effect whereas feeding for 30 days decreased the level from 45 to 34, which approached the control value of 26.

TABLE 3. EFFECT OF FAT FREE DIET ON FATTY ACIDS IN RAT BRAIN PHOSPHOGLYCERIDES DURING BRAIN DEVELOPMENT

Fatty Acid	30 DAY		60 DAY		90 DAY		120 DAY	
	C (4)	D (4)	C (3)	D (3)	C (2)	C (2)	C (5)	D (4)
14:0	1.1± .1	1.1	0.8± .1	1.1	0.9	0.9	0.9± .1	0.8± .1
16:0+16:1	22.9± .5	24.0± .7	21.8± .7	20.7±1.2	22.0±1.1	24.6±1.0	21.1±1.2	21.8±1.3
18:0	22.0± .8	19.8± .2	20.5± .1	20.5± .3	21.5±1.2	22.1±1.6	20.1± .6	22.9± .7
18:1(n-9	20.2± .3	19.6± .4	19.5± .4	18.9± .2	19.5± .4	17.6±1.7	20.0± .7	16.1± .6
18:2(n-6)	1.9	1.2± .1	1.7± .2	0.5± .1	1.1± .1	0.3	1.4± .1	--
18:3(n-6)	0.8± .1	0.7± .1	0.7± .1	0.6	0.5	0.3± .1	0.5	0.6± .2
20:1(n-9)	2.1± .1	1.7	2.2± .1	2.2	2.4± .1	2.0± .1	2.6± .1	2.5
20:2(n-6)	0.5± .1	1.0± .2	0.8± .1	1.0± .1	0.3± .1	0.5	0.8± .1	2.1±1.0
20:3(n-9)	--	1.6± .2	0.4	4.5± .3	0.6± .1	4.8	0.8± .1	6.0± .3
20:4(n-6)	11.0± .2	10.8± .2	10.5± .2	9.0± .1	10.4± .1	7.9± .3	10.1± .2	6.3
20:4(n-3)	--	--	0.6	0.8± .1	0.4± .1	0.4	0.3± .1	0.8
20:5(n-3)	--	--	--	--	--	--	0.3± .1	0.9± .1
22:3(n-9)	--	0.7± .3	--	1.9± .2	--	1.7± .1	0.6± .2	2.8± .3
22:4(n-6)	3.6± .1	3.4± .2	3.8± .1	3.1± .3	3.6± .4	2.5± .1	3.9± .1	2.7± .1
22:5(n-6)	0.2± .1	3.1± .6	0.9± .1	5.0± .3	1.0± .4	4.7	0.6	4.5± .1
22:6(n-3)	13.2± .9	10.7± .9	14.5± .3	9.8± .4	14.2± .2	9.0± .5	14.0± .3	8.3± .2
Saturates	45.9±1.2	43.6± .8	43.1± .7	42.2±1.4	44.5±2.4	47.5±2.6	42.1± .8	45.5±2.0
Monounsat.	22.3± .3	22.6± .4	21.7± .5	21.1± .2	21.9± .5	19.6±1.8	22.7± .8	18.6± .7
Polyunsat.	30.9±1.2	33.1±1.3	34.2± .8	36.3±1.3	32.9±1.8	32.1±1.0	32.9± .7	35.2±1.2

C = Controls

D = Deficient

( ) = No. of Determinations

TABLE 4. DISTRIBUTION OF UNSATURATION INDEX BETWEEN DIFFERENT FATTY ACID SERIES IN FAT DEFICIENCY STATES

DURING BRAIN DEVELOPMENT

Unsaturation Index	30 DAY		60 DAY		90 DAY		120 DAY	
	C (4)	D (4)	C (3)	D (3)	C (2)	D (2)	C (5)	D (4)
(n-3)	78.9±5.1	64.3±5.3	90.0±2.2	62.4±2.5	90.4±4.0	55.8±3.2	88.8±1.9	57.6±2.1
(n-6)	62.7±1.4	76.6±3.4	67.0±1.5	76.6±2.0	63.9±4.0	66.6±1.8	62.1±0.8	63.1±2.7
(n-9)	22.6± .5	28.0±1.0	23.1± .2	40.1±1.3	23.7±0.7	39.1±2.1	26.2±0.3	44.8±2.4
TOTAL	164.2±4.9	169.0±5.2	180 ±3.5	179.2±5.7	178 ±8.7	161.4±7.1	177.2±2.6	165.5±3.0

C = Controls

D = Deficient

( ) = No. of Determinations

TABLE 5. CHANGES IN BRAIN PHOSPHOGLYCERIDE FATTY ACIDS UPON SUPPLEMEN-
TATION WITH LINOLEATE AND LINOLENATE TO EFA DEFICIENT RATS*

Fatty Acid	10 Days† (1)	30 Days (2)	10 Days (2)	30 Days (2)
14:0	1.0	0.8	0.7	1.1± .2
16.0 16:1	22.0	20.7±1.1	22.4± .8	23.3± .8
18:0	19.1	20.9± .8	21.0± .4	21.7±2.1
18:1(n-9)	19.8	17.0± .6	20.3± .2	18.7±2.0
18:2(n-6)	0.5	0.8± .2	0.6± .2	0.4
18:3(n-6)	0.6	0.4	0.4± .1	0.4
20:1(n-9)	2.4	2.4± .1	2.3	2.3± .1
20:2(n-6)	0.7	0.7± .1	0.8± .1	0.6± .1
20:3(n-9)	4.9	2.8± .2	4.5	2.9± .1
20:4(n-6)	8.1	9.4± .2	7.1	6.2± .3
20:4(n-3)	1.1	0.8	0.4± .1	0.6± .1
20:5(n-3)	0.3	1.2± .2	0.3	1.2± .1
22:3(n-9)	2.5	1.9	1.7	1.7± .3
22:4(n-6)	2.5	3.7± .4	2.3± .2	2.2± .3
22:5(n-6)	5.3	6.4± .3	4.5	1.6± .2
22:6(n-3)	8.7	8.2± .1	10.3± .3	14.4± .3
Saturates	42.1	42.4±1.9	44.1±1.7	46.0±2.7
Monounsat.	22.1	19.4± .7	22.6± .3	17.2±3.6
Polyunsat.	32.8	36.5±1.1	33.1± .9	32.4± .5

*Rats were maintained on fat free diet for 120 days prior to supplemen-
tation
†No. of days of fatty acid supplementation

( ) = No. of Determinations

TABLE 6. DISTRIBUTION OF UNSATURATION INDEX BETWEEN DIFFERENT FATTY ACID

SERIES IN BRAINS PHOSPHOGLYCERIDES AFTER SUPPLEMENTATION WITH

LINOLEATE AND LINOLENATE TO EFA DEFICIENT RATS*

	Linoleate		Linolenate	
Unsaturation Index	10 Days† (1)	30 Days (2)	10 Days (2)	30 Days (2)
(n–3)	58.3	58.5±1.8	66.1±1.8	97.4
(n–6)	71.5	87.3±4.4	62.9±1.2	42.9±1.3
(n–9)	44.3	33.7±0.2	41.2± .5	34.0±1.8
TOTAL	174.2	179.5±6.4	170.2±3.5	174.2±3.2

*Rats were maintained on fat-free diet for 120 days prior to supple-
mentation
†No. of days of fatty acid supplementation

( ) ≑ No. of Determinations

C. CHANGES IN FATTY ACID COMPOSITION OF LIVER PHOSPHOLIPIDS DURING EFA
   DEFICIENCY AND FOLLOWING LINOLEATE AND LINOLENATE SUPPLEMENTATION.

Liver phospholipids of the 120-day-old rat contained 41% saturated,
6% monounsaturated and 52% polyunsaturated fatty acids (Table 7). The
major fatty acids are 18:0 and 20:4 (n-6) each of which comprised 24% of
the total fatty acid composition. The next abundant fatty acids were
16:0 and 18:2 (n-6). Each represented approximately 15% and 22:6 (n-3)
and 18:1 (n-9) comprised 8% and 5% respectfully of the total fatty acids.
All other fatty acids were detected below 2% of the total.

Unlike CNS phospholipids, marked changes in the fatty acid sub-
classes of liver phospholipids occurred in 120 day EFA deficient rats.
The total polyunsaturated fatty acids decreased from 52% to 37%, the
monounsaturated fatty acids increased from 5.9% to 19%. The saturated
fatty acid remained unchanged and thus the ratio of saturated to un-
saturated fatty acids was not altered during EFA deficiency. The response
of the individual fatty acids during the deficiency state was as expected.

The major elongation products of linoleic and linolenic acid were
significantly decreased and the non-essential fatty acids of the oleic
family were increased. Linoleic acid and 20:4 (n-6) were decreased from
14% and 24% to 3% and 7% respectfully and 22:6 (n-3) decreased from 8%
to 2%. Oleic acid increased from 5% to 18% and the level of 20:3 (n-9)
in the EFA deficient liver was 20% as compared to 1% in the control
animals. Only minor variations occured in the remaining fatty acids.
Thus, in comparison to brain there was no increase in 22:5 (n-6) or 22:3

TABLE 7. CHANGES IN RAT LIVER PHOSPHOGLYCERIDE FATTY ACIDS DURING FAT DEPRIVATION AND SUBSEQUENT REFEEDING WITH LINOLEIC AND LINOLENIC ACID

	90 DAY		120 DAY		Supplementation Linoleate**		Linolenate	
	C (2)	D (2)	C (4)	D (4)	10 Day+ (4)	30 Day (2)	10 Day (3)	30 Day (3)
14:0	0.3	0.7± .1	0.5±.1	.4±.1	0.8± .1	--	0.7± .1	--
*16:0	18.1± .3	17.1± .9	16.4± .9	19.8± .8	18.7±1.2	16.3± .1	17.8±2.1	16.5± .7
18:0	21.0± .2	20.5±1.3	23.8±1.1	21.7± .6	21.6± .5	24.6±2.5	23.0±2.6	24.9±1.1
18:1	5.5± .5	18.5±2.4	5.0± .6	18.2±1.0	11.7± .7	12.2± .2	10.5±1.2	14.6± .8
18:2(n-6)	14.2± .4	2.23± .3	14.4± .7	3.2± .6	6.7± .5	5.9± .1	1.3± .2	1.9± .5
18:3(n-6)	0.5± .1	1.5± .2	0.9± .1	1.2± .2	0.7± .1	0.7± .2	1.0± .2	0.4± .1
20:1(n-9)	0.6± .3	--	0.9± .1	0.7± .1	0.9± .2	0.6± .3	1.0± .1	0.5± .2
20:2(n-6)	0.7± .1	1.2± .2	0.6± .1	--	0.9± .2	0.4± .2	1.0± .3	0.3± .1
20:3(n-9)	2.2± .1	22.6±1.3	1.3± .2	20.2± .8	6.4± .7	5.8± .9	6.9± .8	5.8± .5
20:4(n-6)	21.0± .9	9.7± .5	23.9±1.2	6.8± .8	19.3±1.1	24.5±2.0	4.3± .3	2.8± .3
20:5(n-3)	2.4± .2	0.9± .4	1.9± .4	0.6± .1	0.7± .2	--	11.5±1.0	13.6± .5
22:3(n-9)	--	0.8± .2	--	1.3± .7	0.8± .2	--	--	--
22:4(n-6)	1.4± .5	0.7± .2	0.6± .1	0.5± .1	0.6± .1	--	--	--
22:5(n-6)	--	2.0± .7	--	.7± .2	0.9± .2	0.7± .4	0.8± .3	--
22:5(n-3)	2.2± .2	--	1.6± .3	0.7± .2	3.8± .6	3.5± .7	1.2± .1	2.0± .5
22:6(n-3)	8.5± .7	1.6± .3	7.9± .8	1.8± .3	2.4± .4	2.4±1.0	16.2± .6	15.9
Saturates	39.4± .5	38.2± .5	40.6± .9	42 ±1.1	41.3± .6	41.2±2.8	41.5±1.9	41.8± .2
Monounsat.	6.1± .8	18.6±2.4	5.9± .7	18.9±1.0	12.6± .7	12.8± .4	11.5±1.2	15.1± .6
Polyunsat.	53.4± .2	43.2±1.9	52.4± .8	37.0± .4	43.2±1.0	44.6±1.4	44.6±1.4	43.0± .5

*Includes 16:1
  C = Controls
  D = Deficient
  ( ) = No. of Determinations
**Rats were maintained on the fat-free diet for 120 days prior to supplementation
+ Number of days of fatty acid supplementation

TABLE 8.   UNSATURATION INDICES OF DIFFERENT FATTY ACID SERIES OF RAT LIVER PHOSPHOGLYCERIDES DURING FAT DEPRIVATION AND SUBSEQUENT SUPPLEMENTATION WITH LINOLEIC AND LINOLENIC ACID.

| | 90 DAY | | 120 DAY | | Supplementation | | | |
| | | | | | Linoleate | | Linolenate | |
Unsaturation Index	C (2)	D (2)	C (4)	D (4)	10 Day+(4)	30 Day (2)	10 Day (3)	30 day (3)
(n-3)	73.4±6.1	14.3±4.1	64.2±6.7	17.2±2.6	37.1±4.9	33.5±10.5	24.4±3.1	173.9±4.5
(n-6)	120.9±3.6	58.2±7.4	128.1±4.6	37.6±4.0	99.3±3.1	113.9± 6.2	32.2±3.3	16.0±2.5
(n-9)	13.1± .6	88.6±5.5	10.0±1.1	83.6±3.7	34.1±1.8	31.6± 4.3	56.1±1.6	32.5± .9
TOTAL	207.4±3.2	161±.1±6.0	202.4±3.5	138.4±2.4	170.5±5.2	180.0± 8.6	163.1±6.9	222.5±1.2

C = Control

D = Deficient

( ) = No. of Determinations

**Rats were maintained on the fat-free diet for 120 days prior to supplementation

+ Number of days of fatty acid supplementation

(n-9) in liver. Also, a marked increase in 18:1 (n-9) was noted in liver as compared to a slight decrease in brain.

The total fatty acid unsaturation index in liver was decreased from 202 to 138 during EFA deficiency (Table 8). Both the (n-3) and (n-6) unsaturation index was decreased and although the (n-9) unsaturation index increased it did not compensate for the (n-3) and (n-6) decreases. Thus unlike CNS, a decrease in the total unsaturation index occurred in liver.

Supplementation with linoleate or linolenate decreased the total monounsaturated fatty acids while the total polyunsaturated fatty acids were increased. The saturated fatty acids were not effected by supplementation. Linoleate or linolenate refeeding for 10 or 30 days significantly decreased the levels of oleic acid and 20:3 (n-9), although control levels were not reached. Linoleate supplementation increased its own levels to 50% of control levels whereas its major elongation product, 20:4 (n-6), was returned to control levels. Refeeding linolenate had a rebound effect on its major elongation products. The values of 22:6 (n-3) increased from the deficient value of 1.8% to double the control value of 8%. In control and EFA deficient animals 20:3 (n-3) was a minor peak. Following supplemation of linolenate for 10 and 30 days the level of 20:3 (n-3) was increased to 11.5% and 13.6%. Thus in liver, there was a rebound effect on the (n-3) fatty acids following linolenate supplemation that did not occur in brain.

Thirty days supplementation of linoleate increased the UI of the (n-6) series from 37 to 114 which approached the control value of 128. Refeeding linolenate for 10 days had little effect on the (n-3) unsatura-

tion index whereas 30 days increased it to 174 which was almost three times greater than the control value of 64.   The (n-9) unsaturation index was decreased from 83 in EFA deficient liver to 32 following 30 days refeeding of either linoleate or linolenate.   The total unsaturation index was significantly increased by both linoleate and linolenate supplementation but only 30 days refeeding of linoleate returned the total unsaturation index to control levels.

D.   ELECTRON MICROSCOPIC APPEARANCE OF MYELIN SUBFRACTIONS.

The procedure employed for the isolation of myelin subfractions (Matthieu et al., 1973) from purified myelin supplied two bands of material at the 0.32-0.625M and the 0.625-0.70M interface. A pellet was also obtained. In the present studies, only the band at the 0.32-0.625M interface and the pellet was used for biochemical investigation. These two fractions were called light and heavy myelin, with the pellet representing the heavy subfraction.

Morphological comparison of the light and heavy myelin revealed two distinct subfractions of membranes. The light subfraction (Fig. 5) was primarily composed of multilayered membranes possessing the lamellar structure and periodicity of myelin. These membranes were loosely arranged and were of various shapes. Rarely, axonal fragments were identified within larger membrane profiles. The heavy subfraction (Fig. 6) consisted primarily of small vesicular membrane profiles, multilamellar membranes were rarely present in the heavy subfraction.

Figure 5.   Electron micrograph of light myelin subfraction isolated from

120-day-old control rats.   Large multilayered myelin fragments are present.
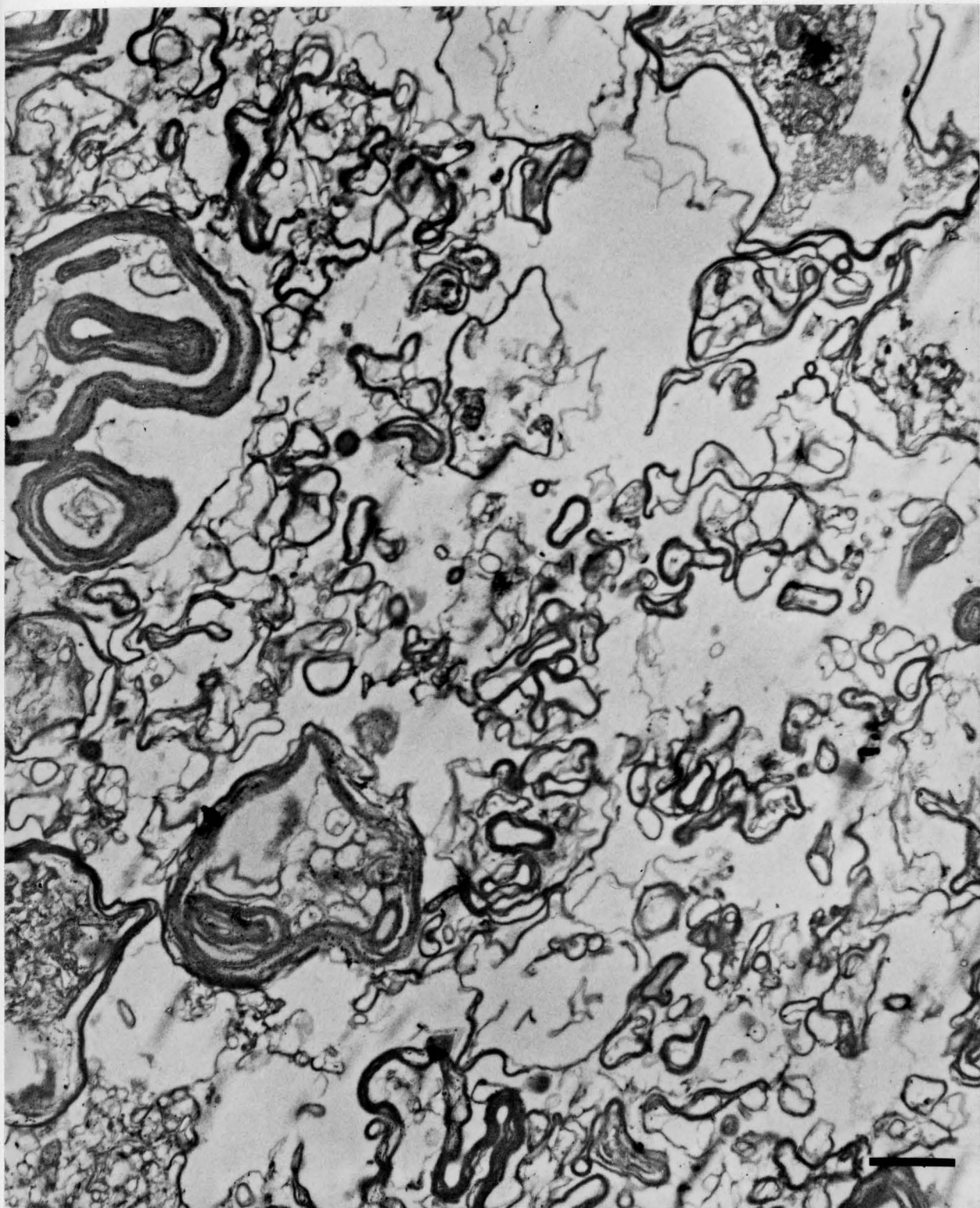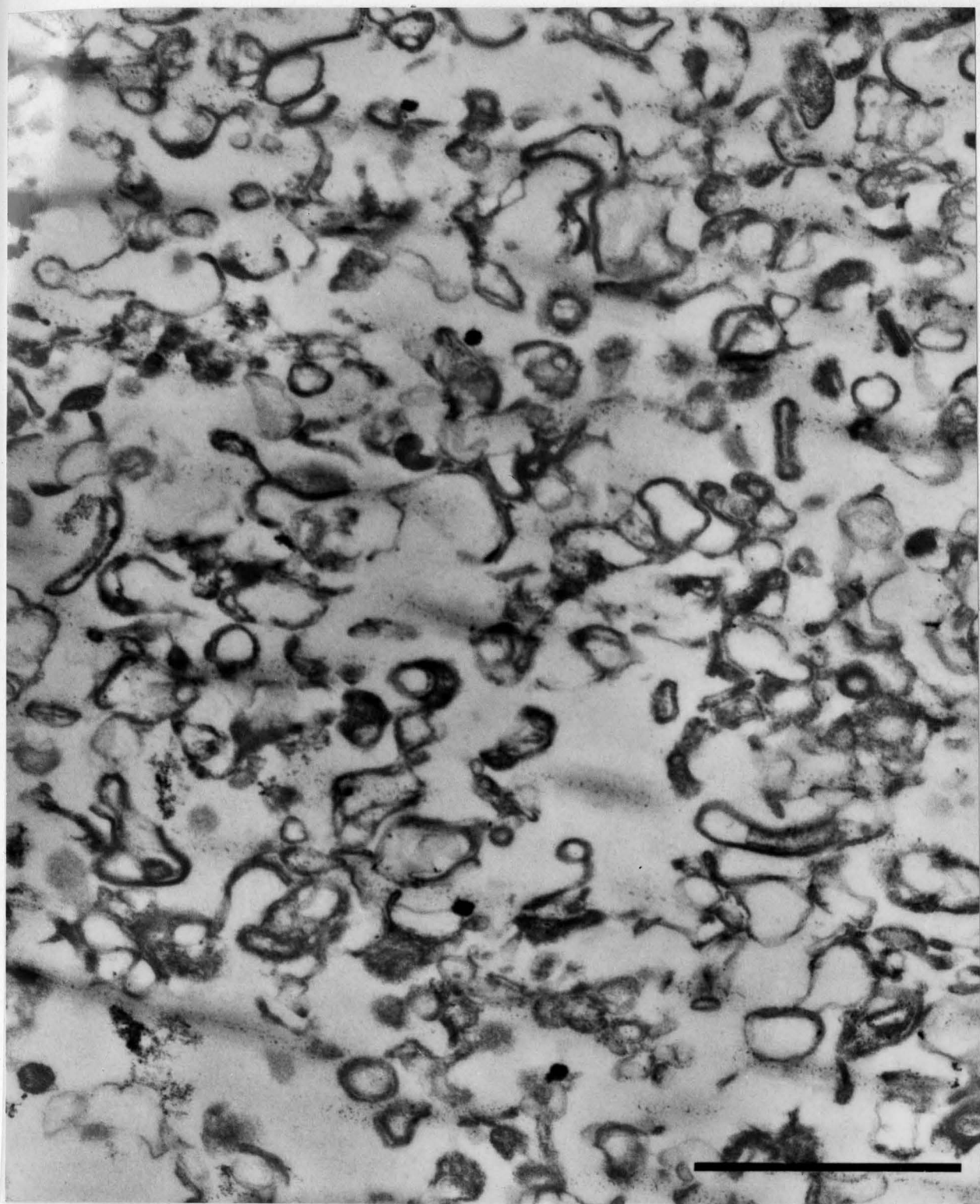
Bar in lower right corner represents one micron.

Figure 6.   Electron micrograph of heavy myelin subfraction from 120-day-old

control rats.   Numerous small membrane fragments, many in vesicular form,

are present.

E. CHANGES IN THE FATTY ACID COMPOSITION OF ETHANOLAMINE PHOSPHOGLYCERIDES

 OF MYELIN SUBFRACTION WITH AGE AND DURING EFA DEFICIENCY.

The fatty acid composition of the ethanolamine phosphoglycerides (EPG's) from light and heavy myelin subfractions isolated from control and EFA deficient rats is presented in Tables 9 to 12 and summarized in Figures 7 to 12. All values are the average of two determinations. The number of brains pooled for each determination varied with the age of the animals and is given in the materials and methods.

The percentage of total unsaturated fatty acids showed little variation during any of the experimental conditions and varied between 25% and 30% (Fig. 7). Due to this stability the ratio of saturated to unsaturated fatty acids was not altered with age, between light and heavy fractions or during EFA deficiency. The level of 18:0 randomly varied between 13% and 20% whereas 16:0 represented from 4% to 7% of the total fatty acids (Table 9). The remaining unsaturated fatty acids, 14:0 and 20:0, were minor components and showed little variation.

There existed little variation in the total (n-6) fatty acids between the light and heavy subfraction in control animals and values usually ranged between 20 and 25% (Table 10). The (n-6) fatty acids in both the heavy and light subfractions were decreased in EFA deficient animals at 20,30,60 and 120 days. At 20 days the (n-6) fatty acids of EFA deficient light myelin were decreased from 24.8% to 15.4% and (n-6) fatty acids in the heavy myelin was 20.5% as compared to 26.2% in control animals. At 120 days these decreases reached a maximum, total (n-6) fatty acids in the light fraction decreased from 16.5% to 7.1% and the heavy

fraction decreased from 20.0% to 16.1%. The level of 20:4 (n-6) decreased from 11.6% to 7.1% in the light fraction at 120 days whereas the heavy fraction decreased from 13.9% to 8.4%. This diminution of 20:4 (n-6) was progressive with age and at 120 days the light and heavy fraction was 3.2% and 3.6% respectfully compared to the control values of 8.0% and 9.9%. The level of 22:4 (n-6) was decreased in a similar manner to 20:4 (n-6) except the decrease was apparent at 15 days. The levels of 18:2 (n-6) and 22:5 (n-6) showed minor increases during EFA deficiency at various times up to 120 days.

The total (n-3) fatty acids increased between 15 and 20 days and then decreased between 20 and 120 days in control animals. The heavy fraction had a higher percentage of (n-3) fatty acids than did the light fraction at all ages in control animals. The (n-3) fatty acids were decreased during EFA deficiency with the exception of the heavy fraction at 30 days. The percent decrease was maximum at 120 days where the light subfraction was 2.9% as compared to the control value of 6.9% and the heavy subfraction decreased from 8.9% to 2.1% (Table 11). The levels of 22:6 (n-3) in control animals varied with age in both the light and heavy subfractions. The light fraction increased from 7.5% to 9.2% between 15 and 20 days and then progressively decreased to 3.8% at 120 days. The heavy fraction followed a similar pattern increasing from 8.0% to 14.5% between 15 and 20 days and decreasing to 5.3% at 120 days. The remaining (n-3) fatty acids were minor components.

The total (n-9) fatty acids tended to be greater in the light fraction than in the heavy fraction in control animals (Fig. 11). The heavy fraction decreased from 38.3% at 15 days to 31.7% at 20 days upon which

time it increased to 46% at 60 days (Table 12). The heavy fraction

followed a similar pattern decreasing from 36.5% to 24.8% at 20 days and

then increasing to 39.4% at 60 days. The (n-9) fatty acids were increased

at all ages, except for the 15 day heavy fraction, during EFA deficiency.

The levels of 20:3 (n-9) increased from 0.7% to 7.3% in the heavy and

3.0% to 7.6% in the light subfraction at 15 days. The levels of 22:3

(n-9) also increased at 15 days. In control animals the levels of 18:1

(n-9) and 20:1 (n-9) decreased between 15 and 20 days and then increased

between 20 and 60 days. Oleic acid was the most abundant fatty acid

found in the ethanolamine phosphoglycerides of myelin subfractions and

varied from 21.0% to 30.0%.

The saturated fatty acids showed little variation at the time points

measured (Fig. 7). The total monounsaturated fatty acid (Fig. 8), tended

to be greater in the light myelin than in the heavy in control animals.

The monounsaturates decreased between 15 and 20 days and then increased

between 20 and 60 days. The response of the monounsaturates to EFA

deficiency varied at different ages with a decrease of 35% in the 15 day

heavy fraction the only major change. Changes in the polyunsaturates

tended to mirror those of the monounsaturates. The total polyunsaturated

fatty acids tended to be greater in the heavy than in the light fraction

(Fig. 9). The polyunsaturates in both the heavy and light myelin increased

between 15 and 20 days. The heavy fraction polyunsaturates decreased

between 20 and 30 days and then remained constant whereas the light

myelin polyunsaturated fatty acids decreased between 20 and 120 days.

Changes in the unsaturation index (Fig. 10) were similar to those of the

polyunsaturated fatty acids. The unsaturation index tended to be greater

in the heavy fraction. It increased between 15 and 20 days in both

fractions and decreased between 20 and 30 days in the heavy fraction

whereas the light fraction decreased between 20 and 120 days.

The major responses of the fatty acids during EFA deficiency are

demonstrated in figures 11 and 12. The total (n-3) + (n-6) fatty acids

(Fig. 11) showed marked decreases in both light and heavy fractions.

These decreases were progressive with age. The total (n-9) fatty acids

(Fig. 12) demonstrated marked increases during EFA deficiency and these

also progressed with age.

TABLE 9. CHANGES IN THE SATURATED FATTY ACIDS OF ETHANOLAMINE PHOSPHOGLYCERIDES ISOLATED FROM MYELIN SUBFRACTION DURING DIFFERENT STATES OF FAT DEPRIVATION.

		15*		20		30		60		120	
		L	H	L	H	L	H	L	H	L	H
14:0	C	1.6	2.9	4.4	4.1	2.7	0.5	2.4	2.2	4.6	4.1
	D	1.7	2.3	2.7	5.3	1.6	2.2	1.0	2.2	2.8	4.2
16:0	C	4.2	6.7	4.6	6.8	5.6	5.7	5.3	5.8	5.8	6.4
	D	5.7	12.0	5.0	5.4	3.1	4.9	3.1	7.6	3.6	4.1
18:0	C	18.8	15.9	17.6	17.7	15.6	20.2	13.1	15.5	16.6	14.9
	D	14.5	14.2	19.1	15.8	16.8	13.2	19.0	11.9	20.5	19.8
20:0	C	1.3	1.1	1.8	2.1	1.1	1.8	1.8	1.8	1.8	1.2
	D	3.1	2.6	1.5	2.3	1.5	2.4	1.6	2.2	0.8	0.3
Total	C	25.9	26.6	28.4	30.7	25.0	28.2	22.6	25.3	28.8	26.6
	D	25.0	31.1	28.3	28.8	23.0	22.7	24.7	23.9	27.7	28.4

L = Light myelin

H = Heavy myelin

C = Control

D = Deficient

* Age in days

Values are the mean of two determinations

TABLE 10. CHANGES IN THE (n-6) FATTY ACIDS OF ETHANOLAMINE PHOSPHOGLYCERIDES ISOLATED FROM MYELIN SUBFRACTIONS DURING DIFFERENT STATES OF FAT DEPRIVATION.

		15*		20		30		60		120	
		L	H	L	H	L	Y	L	H	L	H
18:2(n-6)	C	1.3	5.6	2.2	2.7	1.4	3.8	1.8	3.1	1.4	1.5
	D	4.7	5.5	1.4	4.5	2.1	4.5	2.6	5.2	0.6	0.4
20:3(n-6)	C	2.8	1.5	2.8	2.5	2.1	2.6	1.6	2.1	1.8	1.8
	D	2.9	2.9	1.1	2.0	2.0	-	-	-	0.3	0.3
20:4(n-6)	C	11.7	9.0	11.6	13.9	8.8	9.3	8.5	8.8	8.0	9.9
	D	8.1	9.2	7.1	8.4	6.7	9.2	5.3	6.5	3.2	3.6
22:3(n-6)	C	0.2	0.3	0.2	.6	0.2	-	1.1	0.7	0.4	0.6
	D	-	-	-	-	0.7	-	-	-	-	-
22:4(n-6)	C	7.3	3.7	7.0	6.2	6.7	5.1	7.4	6.7	4.4	4.5
	D	3.6	2.5	3.5	2.7	3.5	4.1	3.4	3.2	1.9	1.0
22:5(n-6)	C	0.2	-	1.0	0.3	0.3	-	0.4	-	0.5	1.7
	D	1.6	1.3	2.3	2.9	2.0	2.3	1.5	2.0	1.1	0.8
Total	C	23.5	20.1	24.8	26.2	19.5	20.8	20.8	21.4	16.5	20.0
	D	20.9	21.4	15.4	20.5	17.0	17.1	12.8	16.9	7.1	6.1

L = Light myelin

H = Heavy myelin

C = Control

D = Deficient

* Age in days

Values are the means of two determinations.

TABLE 11. CHANGES IN THE (n-3) FATTY ACIDS OF ETHANOLAMINE PHOSPHOGLYCERIDES ISOLATED FROM MYELIN SUBFRACTIONS DURING DIFFERENT STATES OF FAT DEPRIVATION.

		15*		20		30		60		120	
		L	H	L	H	L	H	L	H	L	H
20:4(n-3)	C	0.1	0.9	1.1	–	1.0	–	0.5	1.2	1.9	2.0
	D	–		0.7	1.8	–	1.5	–	–	1.1	0.5
20:4(n-3)	C	0.3	0.2	0.2	0.9	2.0	0.5	0.8	1.6	–	1.3
	D	1.4	1.9	1.2	2.2	2.0	5.5	1.6	3.6	0.9	0.7
22:5(n-3)	C	0.4	–	0.9	0.3	0.3	–	0.3	–	0.3	0.3
	D	–	–	–	–	–	–	–	–	0.3	–
22:6(n-3)	C	7.5	8.0	9.2	14.5	5.1	9.0	3.5	7.4	3.8	5.3
	D	5.5	7.0	4.7	8.9	3.7	5.3	1.7	3.6	0.6	0.9
Total	C	8.3	9.1	11.4	15.7	8.4	9.5	5.1	10.2	6.9	8.9
	D	6.9	8.9	6.6	12.9	5.7	12.3	3.3	7.2	2.9	2.1

L = Light myelin

H = Heavy myelin

C = Control

D = Deficient

* Age in days

Values are the mean of two determinations.

TABLE 12. CHANGES IN THE (n-9) FATTY ACIDS OF ETHANOLAMINE PHOSPHOGLYCERIDES ISOLATED FROM MYELIN SUBFRACTIONS DURING DIFFERENT STATES OF FAT DEPRIVATION.

		15*		20		30		60		120	
		L	H	L	H	L	H	L	H	L	H
18:1(n-9)	C	29.8	23.2	23.5	17.5	27.6	27.8	29.1	26.0	28.4	24.8
	D	24.5	17.1	29.8	18.3	28.9	21.0	27.9	21.5	30.1	29.7
20:1(n-9)	C	6.6	9.0	5.1	3.4	8.0	7.0	13.1	7.7	8.2	5.8
	D	6.5	5.9	3.8	5.9	8.1	7.0	10.7	7.2	9.1	4.7
20:2(n-9)	C	1.0	1.0	2.0	2.4	5.6	1.5	2.2	3.3	1.4	3.7
	D	3.8	2.9	2.6	2.8	2.9	3.0	2.9	3.1	3.0	2.0
20:3(n-9)	C	0.8	3.0	0.9	1.3	1.0	2.4	0.9	1.7	1.7	1.8
	D	7.3	7.6	5.2	6.8	6.7	9.8	9.5	10.8	9.1	12.9
22:3(n-9)	C	0.2	0.3	0.2	0.2	0.3	–	1.2	0.7	0.5	2.9
	D	2.0	2.3	2.7	1.4	6.7	3.2	5.7	5.3	5.9	5.2
Total	C	38.3	36.5	31.7	24.8	42.5	38.7	46.5	29.4	40.2	39.0
	D	44.1	35.8	44.1	35.2	53.3	44.0	56.7	47.9	57.2	54.5

L = Light myelin

H = Heavy myelin

C = Control

D = Deficient

* Age in days
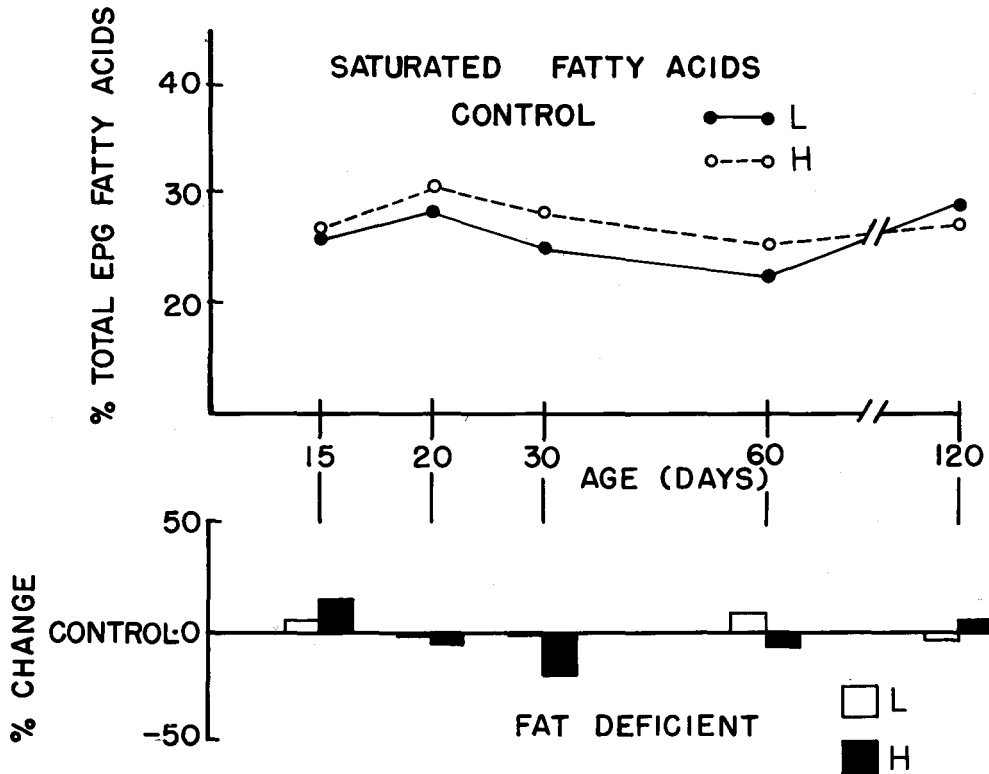
Values are the mean of two determinations.

Figure 7. Top: Saturated fatty acid content of EPG's from light and heavy myelin isolated from control animals at various ages. Bottom: The percent change from control values during fat deficiency. Values expressed as percent of the total fatty acids.
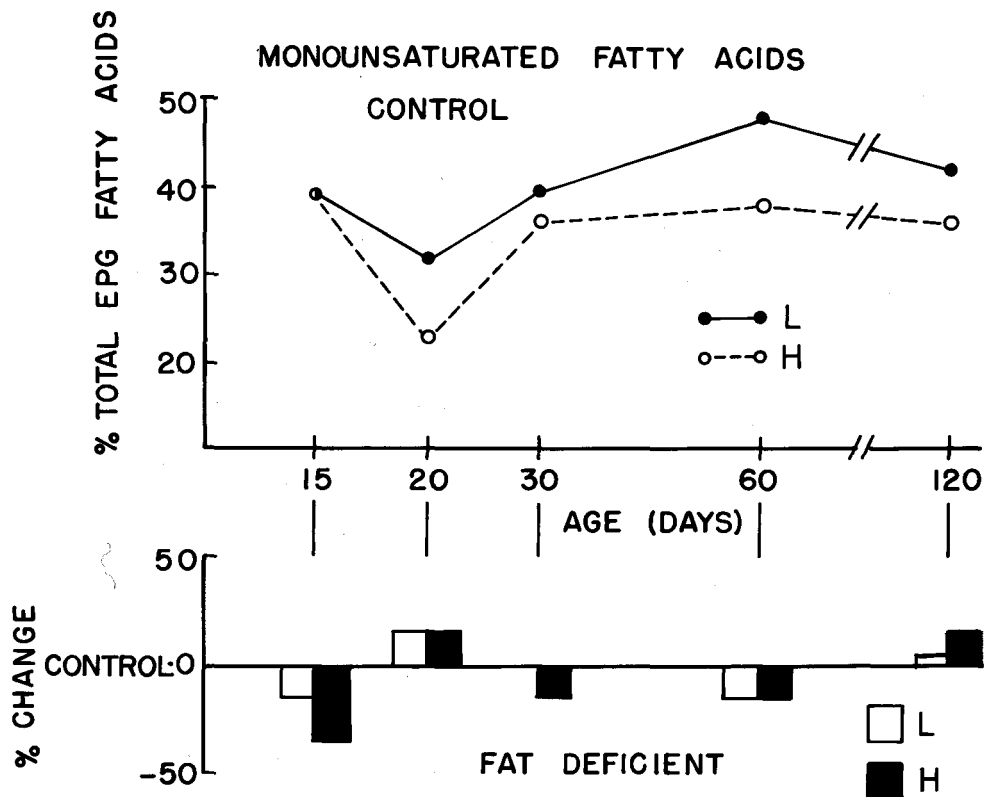
Figure 8. Top: Monounsaturated fatty acid content of EPG's from light and heavy myelin isolated from control animals at various ages. Bottom: The percent change from control values during fat deficiency. Values expressed as percent of total fatty acids.
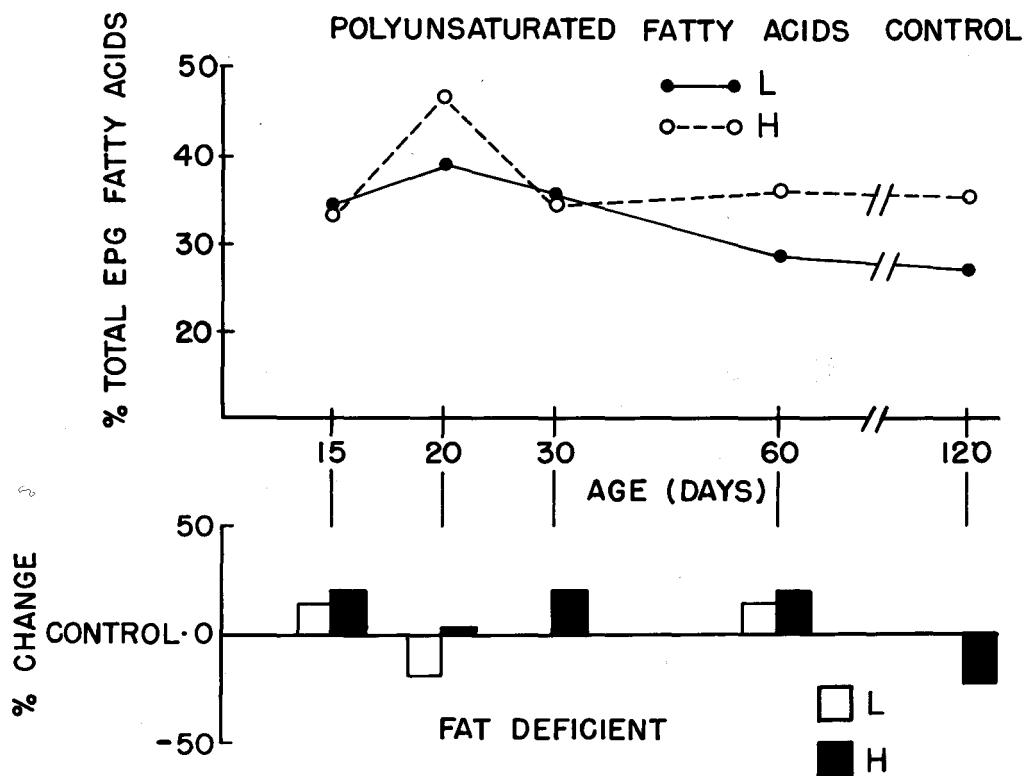
Figure 9. Top: Polyunsaturated fatty acid content of EPG's from light and heavy myelin isolated from control animals at various ages. Bottom: The percent change from control values during fat deficiency. Values expressed as percent of the total fatty acids.
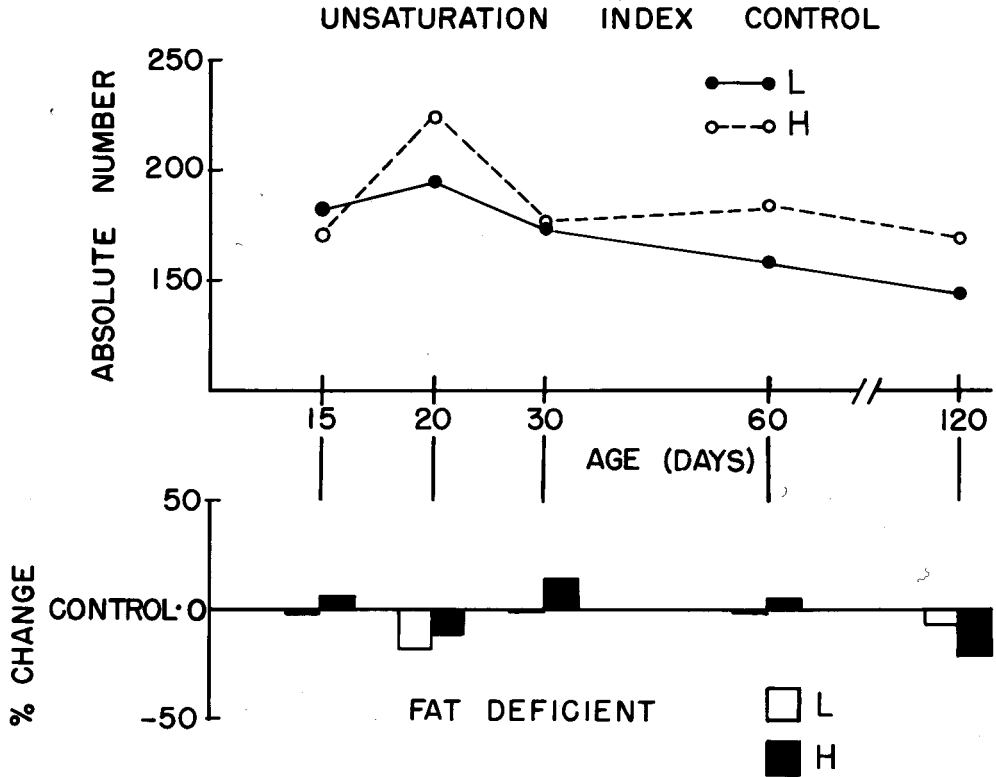
Figure 10. Top: Unsaturation index of EPG's from light and heavy myelin isolated from control animals at various ages. Bottom: The percent change from control values during fat deficiency. Values are expressed as absolute numbers and calculated as described in the materials and methods.
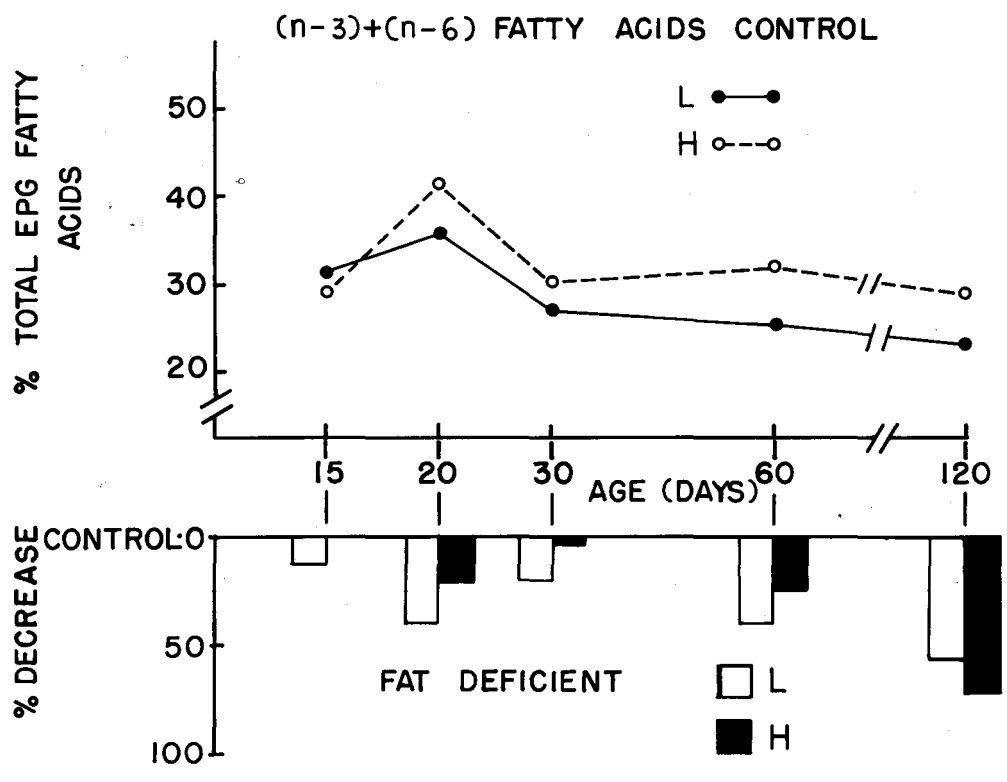
Figure. 11. Top: (n-3) + (n-6) fatty acid content of EPG's from light
and heavy myelin isolated from control animals at various ages.
Bottom: The percent decrease from control values during fat deficiency.
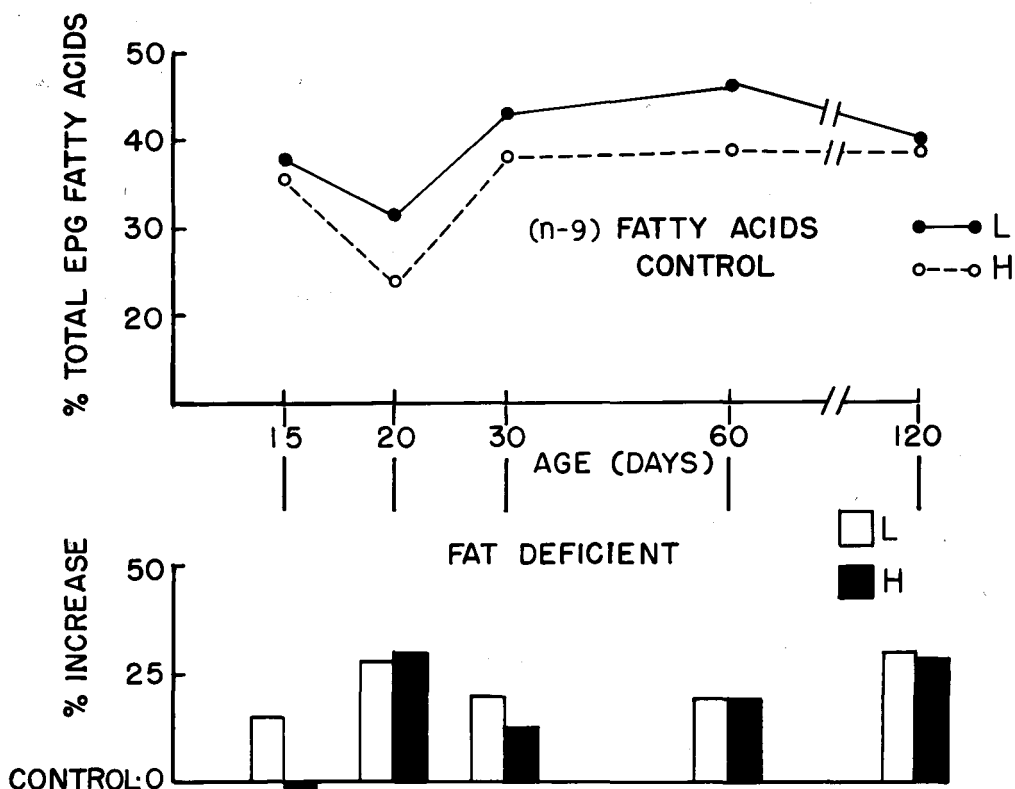Values expressed as percent of the total fatty acids.

Figure 12. Top: (n-9) fatty acid content of EPG's from light and heavy myelin isolated from control animals at various ages. Bottom: The percent increase from control values during fat deficiency. Values expressed as percent of total fatty acids.

F. MORPHOLOGICAL EFFECTS OF EFA DEFICIENCY ON RAT OPTIC NERVE.

1. LIGHT MICROSCOPY

Examination of one micron thick cross-sections of control optic nerve (Fig. 13a) revealed myelinated fibers which were round or oval and compactly arranged. Most fibers were small and all fibers appeared to be myelinated. Similar sections prepared from EFA deficient optic nerves (Fig. 13b) demonstrated myelinated fibers of the same shape and apparent number and size as found in control optic nerve. In addition the EFA deficient optic nerve had a number of small vacuoles either totally or partially bound by apparent myelin sheaths or myelin fragments. These vacuoles were of various shapes ranging from circular or oval to irregular. Some appeared empty while others contained various amounts of membranous debris. No abnormalities were observed in oligodendrocytes, astrocytes or vascular elements in EFA deficient optic nerve at the light microscopic level.

Figure 13.   Cross-section of control optic nerve (a.) myelinated fibers

are compactly arranged and appear normal.   Similar area in EFA

deficient optic nerve (b.) reveals several small vacuoles

(arrows) surrounded by myelin fragments.

13a

13 b
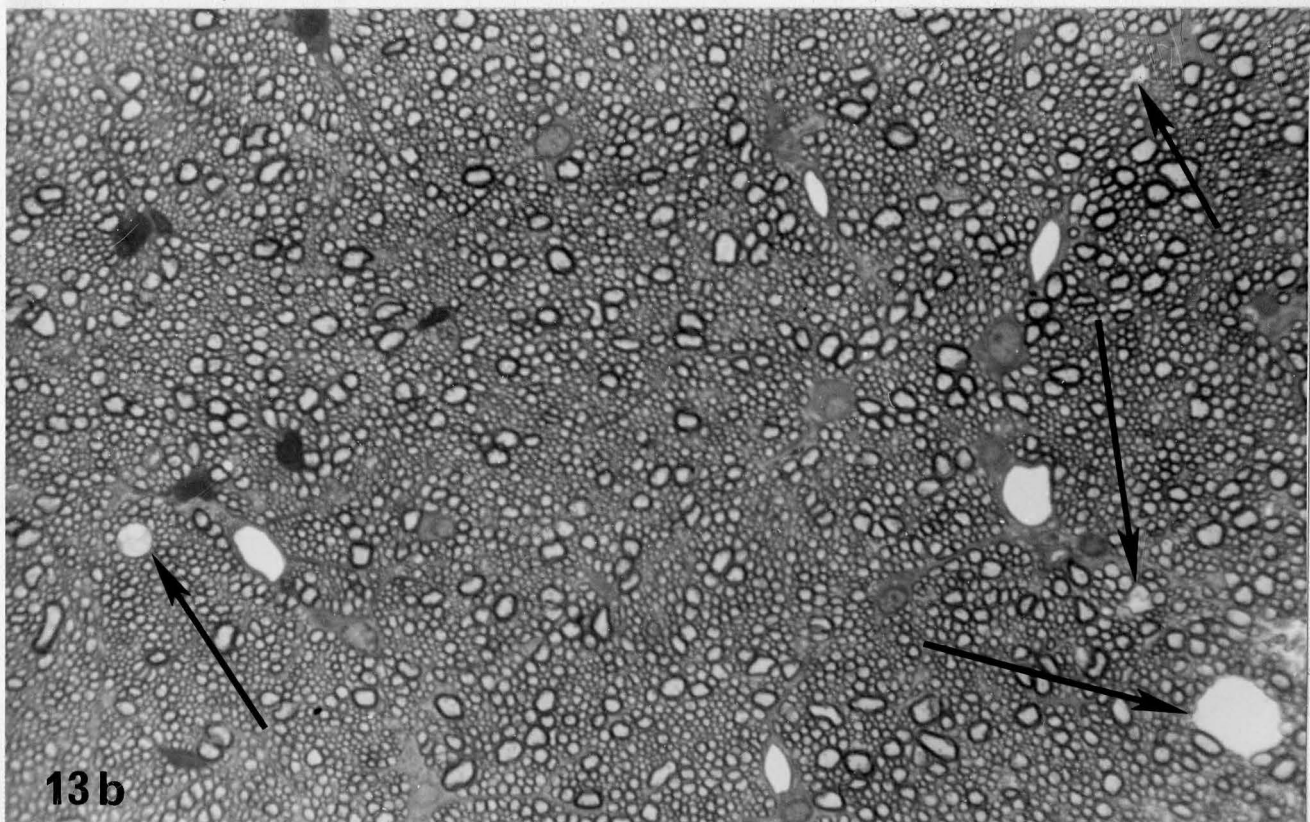
2. ELECTRON MICROSCOPY

A representative micrograph of the optic nerve from a 120-day-old control rat is presented in Fig. 14. The manner in which irregular shaped fibre diameters were measured as discussed in the materials and methods is demonstrated by the arrows. Essentially, all axons were surrounded by a myelin sheath and those which were not were assumed to be sectioned through a nodal region. Microtubules, neurofilaments and mitochondria were found within the axons. Inner and outer mesaxons were present as were glial cells and processes.

The fibre size distribution of control and EFA deficient optic nerves was compared in Fig. 15. No marked difference existed between control and EFA deficient fibre diameter, although there may have been a greater percentage of fibres less than 0.5μ in the EFA deficient optic nerves. Approximately 60% of the fibres in both control and EFA deficient optic nerve are between 0.50 and 1.00 microns with the peak distribution (35%) between 0.50 and 0.75μ. Less than 4% of the fibres were greater than 1.75μ in either control or EFA deficient optic nerve.

Although the EFA deficient optic nerve did not demonstrate a decrease in fibre diameter, alterations were found in some of the myelinated fibres. Alterations of fibres in the optic nerve of 120 day EFA deficient rats consisted of three general types; intermyelinic splits within the myelin sheath, degeneration of myelinated fibres and unusual configurations of the myelin sheath. Intermyelinic splits and degenerating fibres are the ultrastructural representation of the vacuoles which were present in light microscopy (Fig. 13b).

Figure 14.   Electron micrograph of optic nerve from 120-day-old control

rat.   All axons are myelinated and are compactly arranged.   Arrows denote

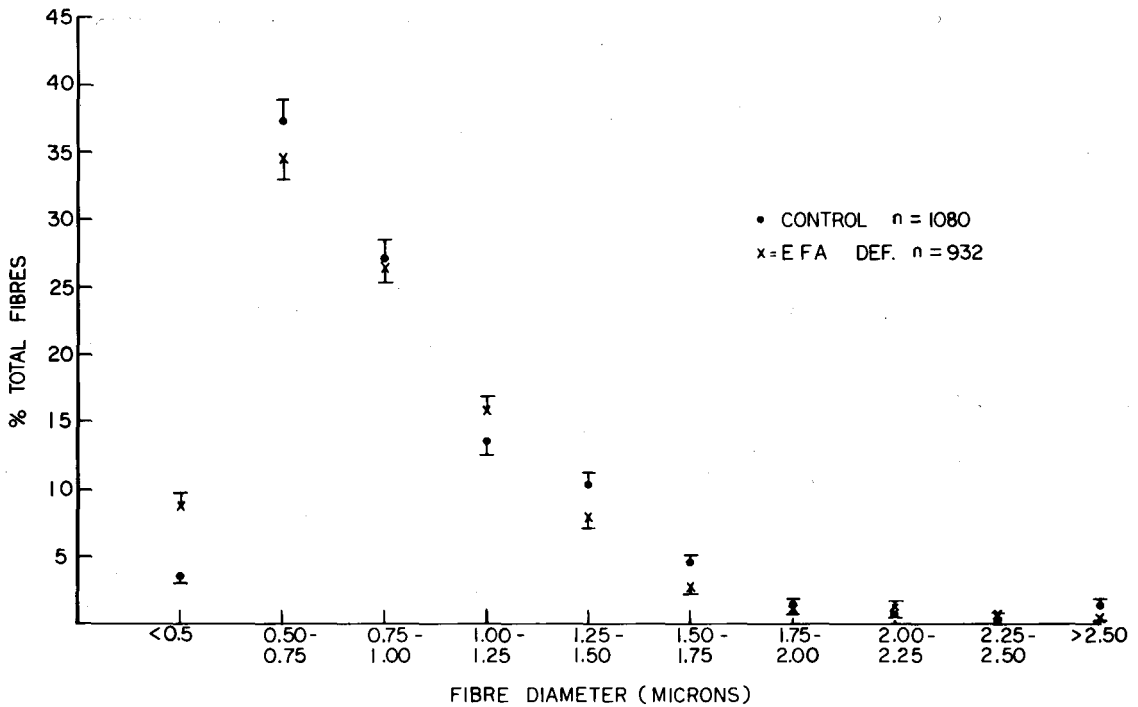mode of measurement of irregular shaped figures.
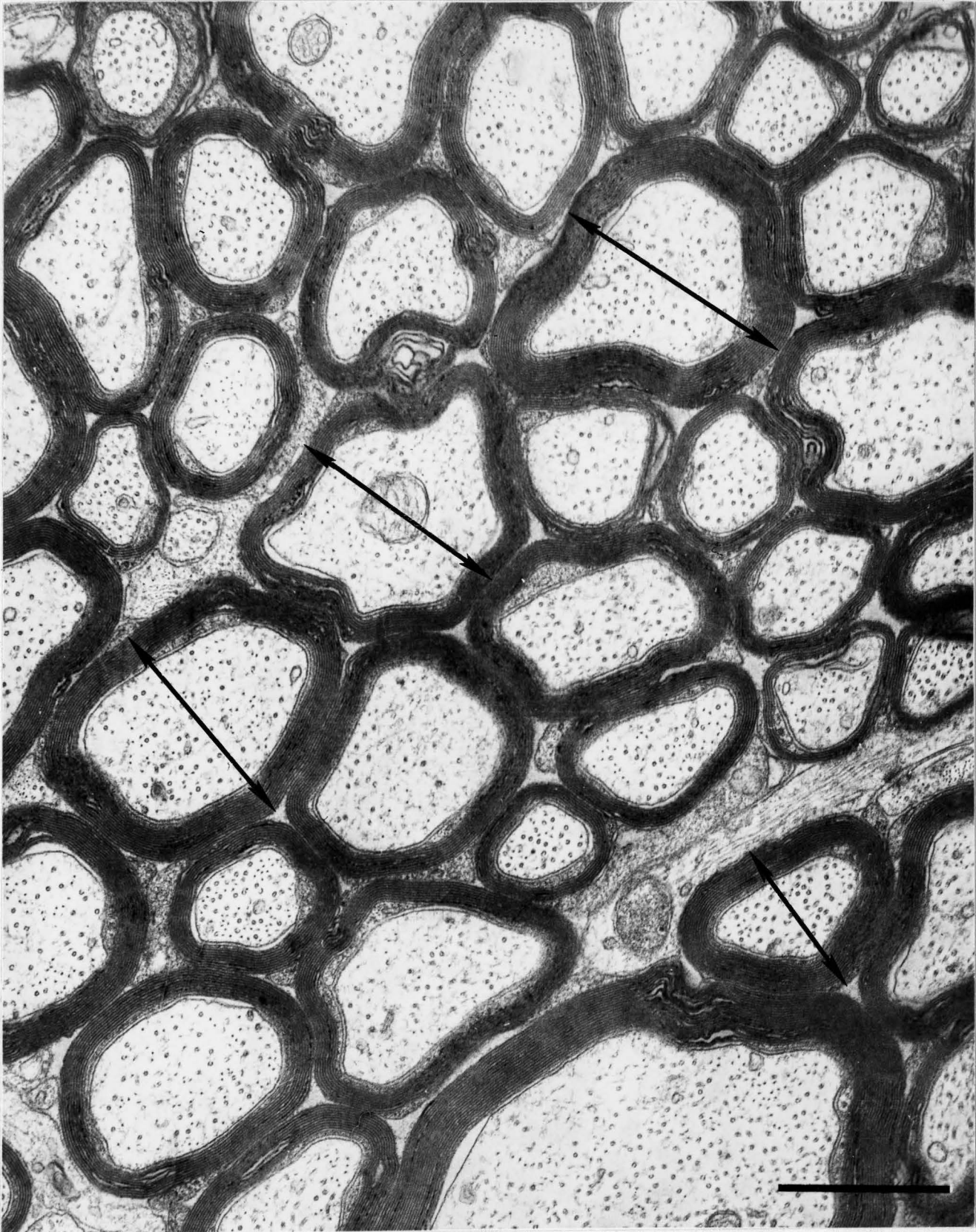
Figure 15.   Cabiber spectra of optic nerves from 120-day-old control and

and EFA deficient animals.   Values are expressed as % of total

fibres ± standard error of proportions.

Many fibres displayed characteristics of degenerating axons. These consisted of swelling of the axon and an accumulation of mitochondria, dense bodies and vesicles (Fig. 16). Although the axons appear vastly distended, they are surrounded by myelin lamellae which demonstrated no apparent abnormalities. There appeared to be a granular disentegration of neurofilaments and vesicular elements within the axon. These responses are typical of fibres undergoing Wallerian degeneration (Lampert, 1967). Other degenerating axons appeared to be collapsed (Fig. 17) and axonal as well as myelin debris were apparent in the resulting vacuole. Eventually the axoplasm disentegrates and the myelin sheath collapses upon itself (Fig. 18). These degenerating axons were surrounded by normal appearing fibres.

Several fibres of EFA deficient optic nerves demonstrated alterations in the appearance of the myelin sheaths. Large vacuoles were found within the lamellae of the myelin sheath (Fig. 19). These typically occurred by the splitting of the myelin sheath at the intraperiod line, although one such split did occur at the major dense line (inset, Fig. 19). The splits usually occurred in the outer myeline lamellae rather than close to the axon. The vast majority of intramyelinic splits appeared to have an electron lucent appearance to the resulting vacuole. The myelin surrounding the vacuole generally consisted of 2-4 lamellae of myelin and was morphologically intact. The myelin displayed the usual density and periodicity (inset, Fig. 19) and although they are markedly elongated to surround the vacuole, they appeared normal. The axons of the fibres exhibiting myelin splitting also appeared normal. Microtubules, mitochondria and neurofilaments were present within the axon. A small number

of myelin splits demonstrated electron dense or reticulated substance within the split (Fig. 20). This material is not typical of edematous fluid or cytoplasm of the oligodendroglial cell. Other types of vacuoles found at the ultrastructural level were swollen processes (Fig. 21) presumably astrocytic in origin. These contained a reticulated ground substance although no discernable organelles were present. The vacuoles described (Figs. 16 to 21) were surrounded by myelinated fibres which appeared normal. The extracellular space appeared normal as did oligodendrocytes, astrocytes and vascular elements.

The second type of alteration that occurred, unusual configurations of the myelin sheath, were of three distinct types. The first was the occurrence of formed organelles within the inner or outer mesaxon. In the normal myelinated axon (Fig. 14) no formed organelles are present in the inner or outer mesaxon except for a few microtubules. The presence of a formed organelle within the inner mesaxon (Fig. 22) greatly increased the size of the inner mesaxon. The second type of unusual configuration of the myelin sheath was the occurrence of an isolated island of cytoplasm within a myelin sheath (Fig. 23). This cytoplasmic island was of similar density to the inner and outer mesaxon. The third unusual configuration was the presence of two concentric myelin sheaths surrounding a single axon (Fig. 24). Each sheath was associated with its own inner and outer mesaxon, giving the axon four mesaxons. In addition to double myelin sheaths there also occurred a formed organelle in the outer mesaxon of the internal myelin sheath. In addition to these unusual configurations in the myelin sheath, several myelin sheaths appeared to surround two axons (Figs. 24 and 25). The axons were separated from each other by cyto-

plasm of the inner mesaxon and one axon was always much smaller than the other. This may represent a split in the axon. Whether this was a functional aplit is not known. In one such fibre a formed organelle was present in the inner mesaxon (Fig. 26).

Upon close examination of the fibres of the optic nerve of 120-day-old EFA deficient rats several unusual relationships between the axon and the oligodendrocyte plasma membrane were observed. One such occurrence was the presence of oligodendrocyte cytoplasm within the axon (Fig. 27). This was bound by plasma membrane and the cytoplasm had the same density as the cytoplasm of the inner mesaxon. If this cytoplasmic island is connected to the inner mesaxon was not known. How such a connection could have been formed was demonstrated by Fig. 28. This axon has an isolated island of cytoplasm that was surrounded by axonal membrane on three sides which formed a canal for continuity of the island to the inner mesaxon. This continuity was demonstrated by Fig. 29 as the inner island of cyto-plasm within this axon is connected to the inner mesaxon. The cytoplasm of the inner mesaxon is continuous with that of the island of cytoplasm. The island of cytoplasm in Figs. 27 and 29 surrounds a vesicle which apparently is axonal in origin. The occurrence of this circular island of cytoplasm within the axon (Fig. 27) could be explained by the intrusion of the mesaxon into the axon as demonstrated by Figs. 27, 28, and 29.

Another type of unusual configuration found in EFA deficient optic nerve was the presence of concentric membranes within the axon (Figs. 30, 31, and 32). These membranes were always present in multiples of two. The membrane surrounds an inclusion which is similar to that surrounded by the oligodendrocyte cytoplasm in Fig. 27. Whether these membranes can

be related to the evagination of the inner mesaxon into the axon cannot

be determined at the present time and remains to be clarified by serial

sectioning.
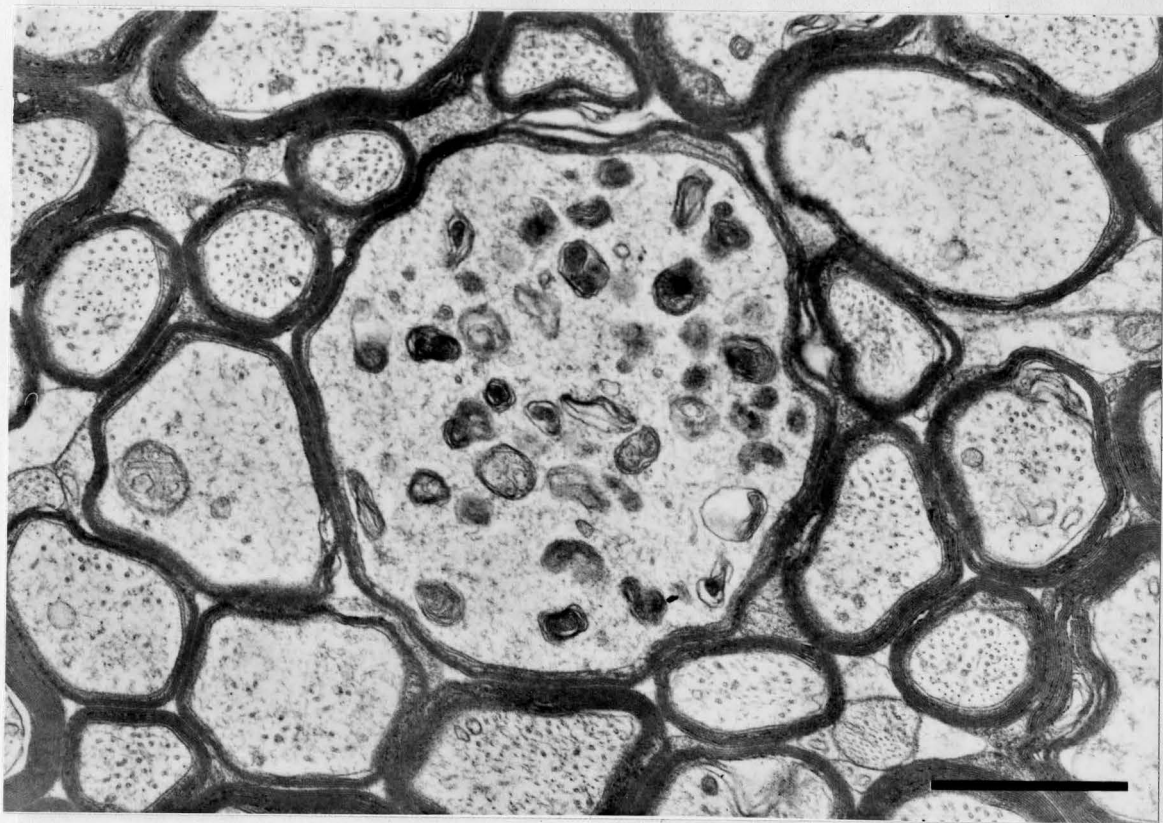
Figure 16.   Electron micrograph of optic nerve from 120-day-old EFA de-
ficient rat.   Swollen axon contains numerous dense bodies, mitochondria
and membranous profiles.
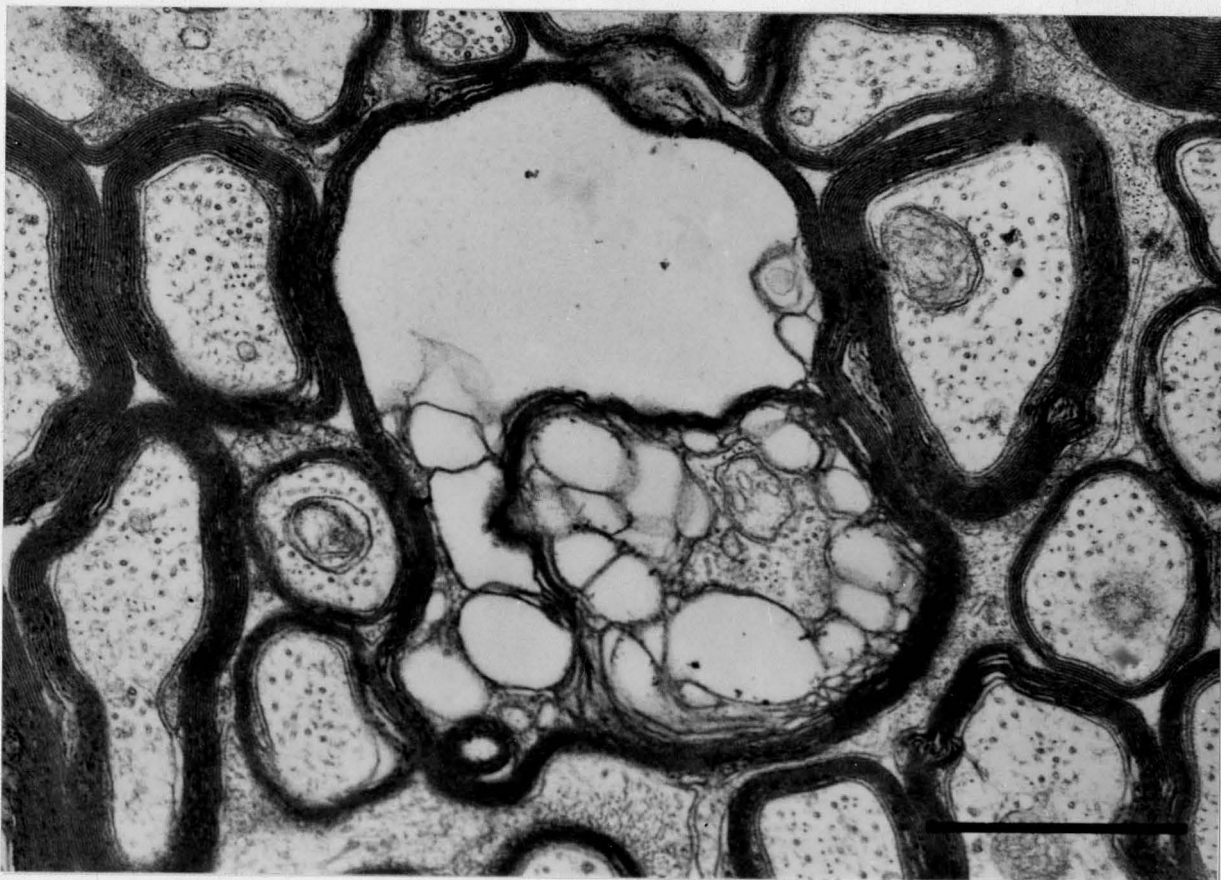
Figure 17. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Vacuole surrounded by myelin sheath. Myelin and axonal debris occurs within the vacuole.

Figure 18. Electron micrograph of optic nerve from 120-day-old EFA de-

ficient rat. Enlarged vacuole with degenerating axon surrounded by

collapsed myelin sheath.

Figure 19. Electron micrograph of optic nerve from 120-day-old EFA de-

ficient rat. Intramyelinic split is present. Split is devoid of any

   discernable material. Split occurs at major dense line (inset).

   Myelin surrounding vacuole retains normal periodicity. Axon of

            fibre containing myelin split appears normal.

0.3 u
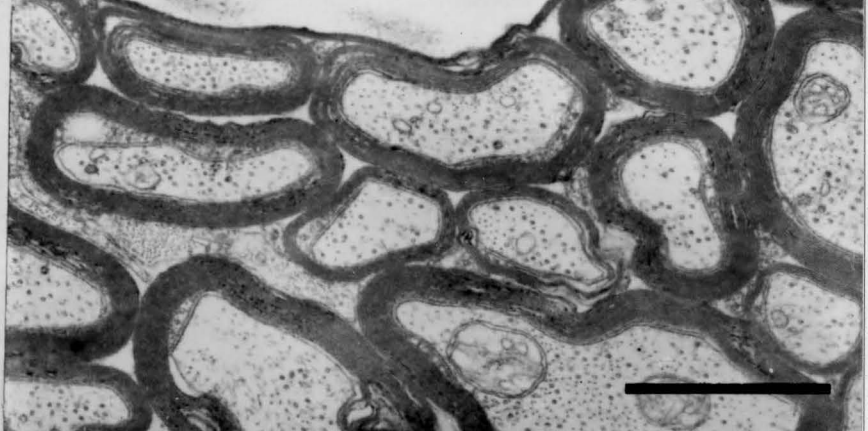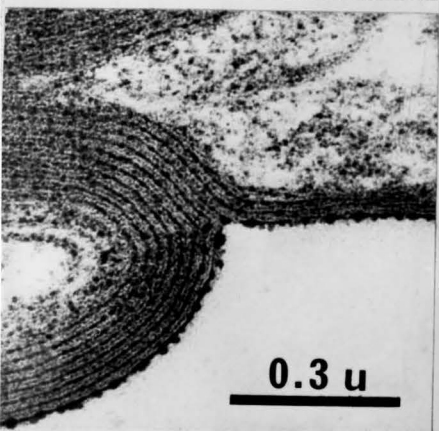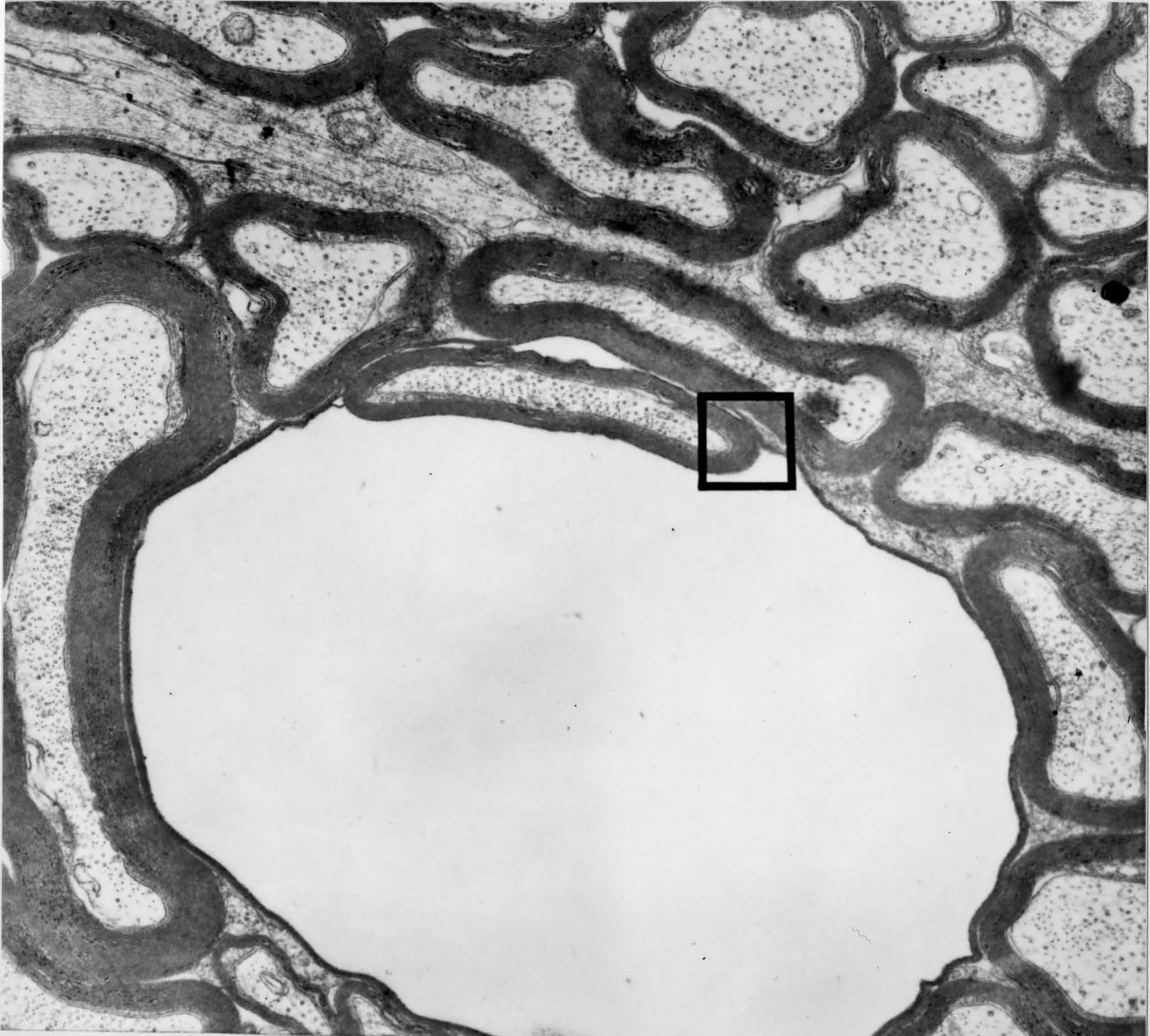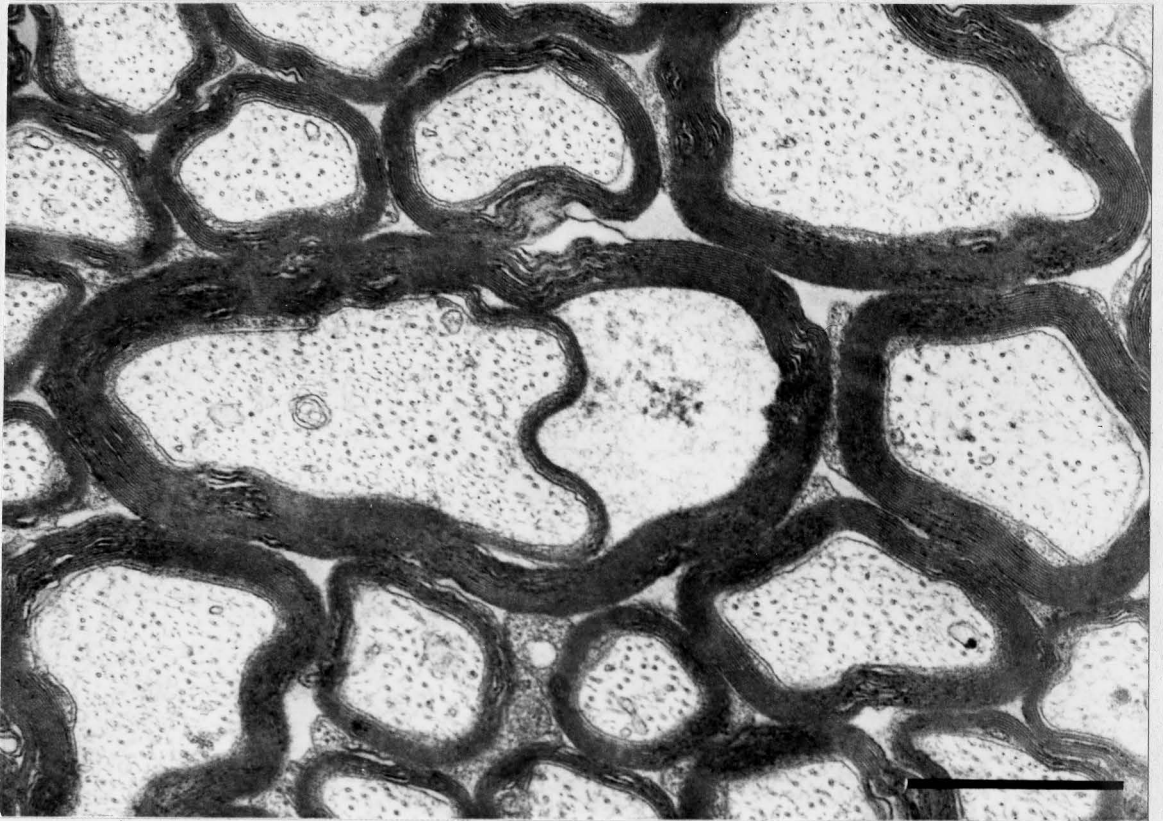
Figure 20. Electron micrograph of optic nerve from 120–day–old EFA de-
ficient rat. Intramyelinic split is present. Split contains some
electron dense reticulated material. Axon of the fibre demonstrating
the intramyelinic split appears normal.
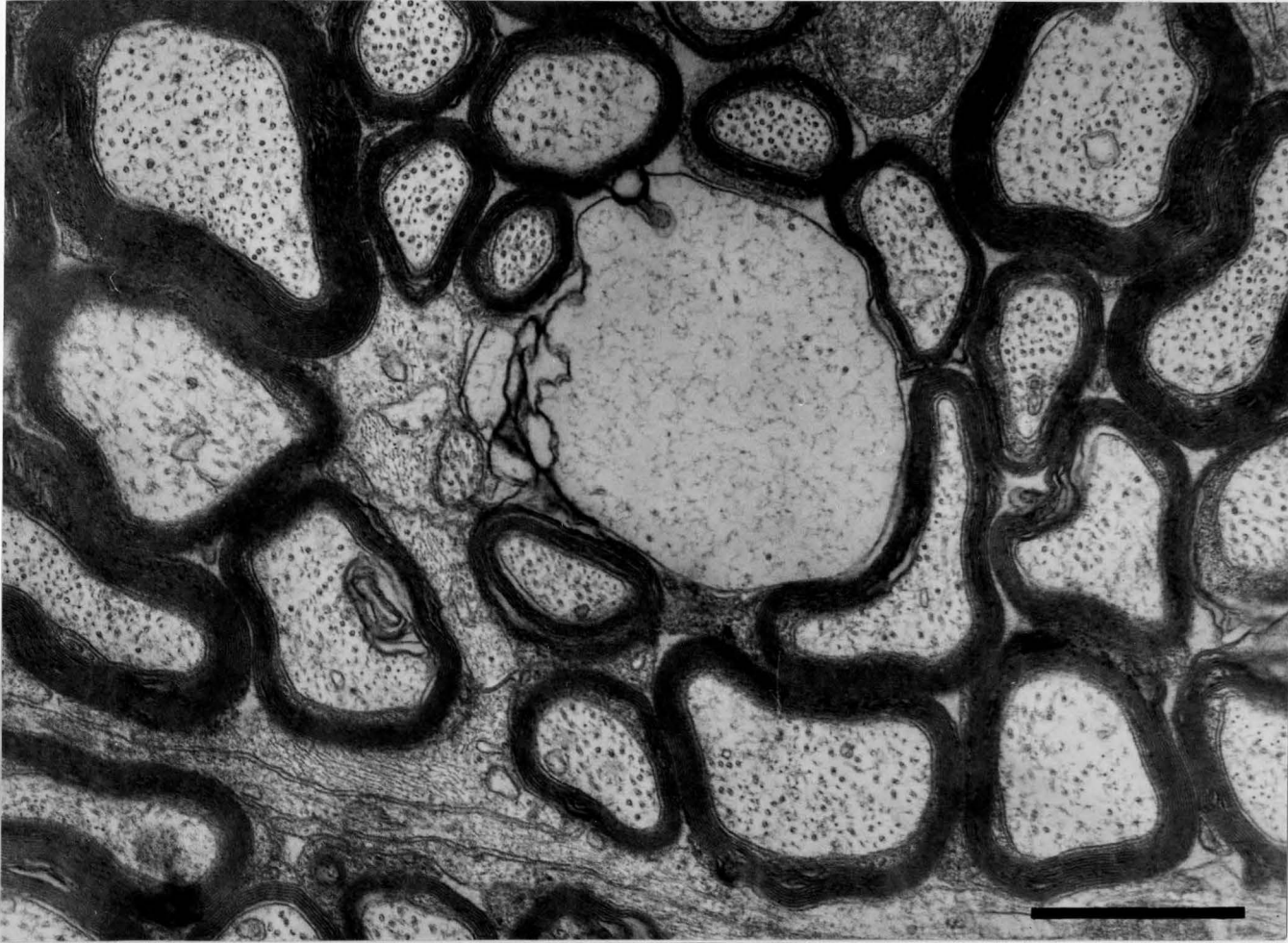
Figure 21. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Central process is swollen and contains electron dense reticulated substance.
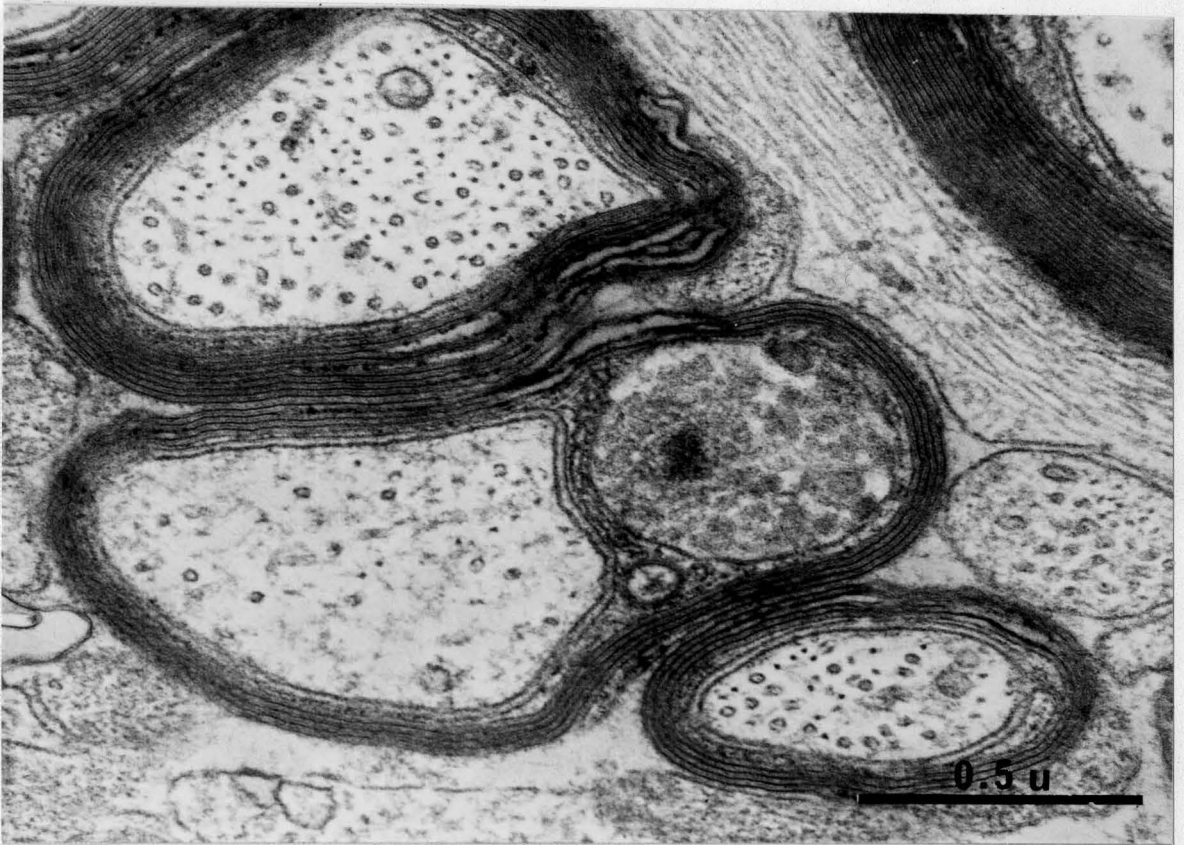
Figure 22. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. The inner mesaxon is substantially increased in size by the presence of a dense body.
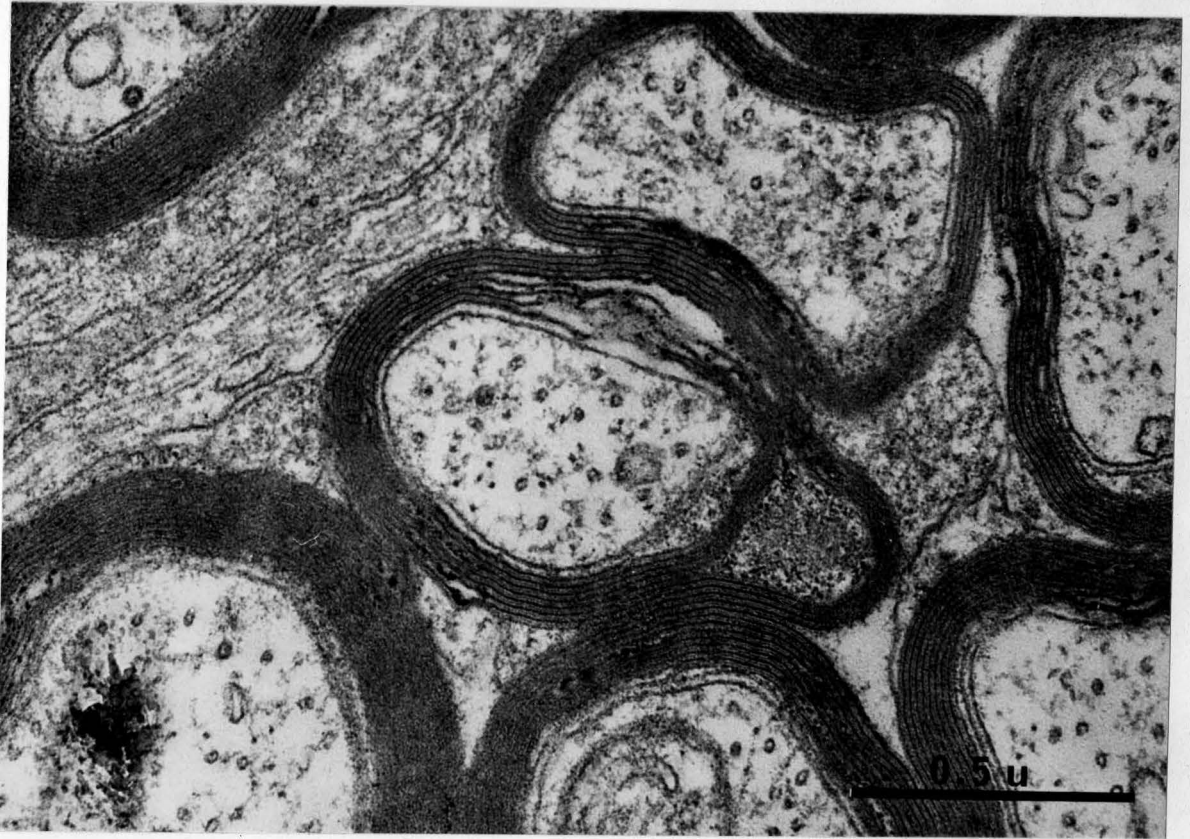
Figure 23. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. An isolated island of cytoplasm is present within myelin lamella.
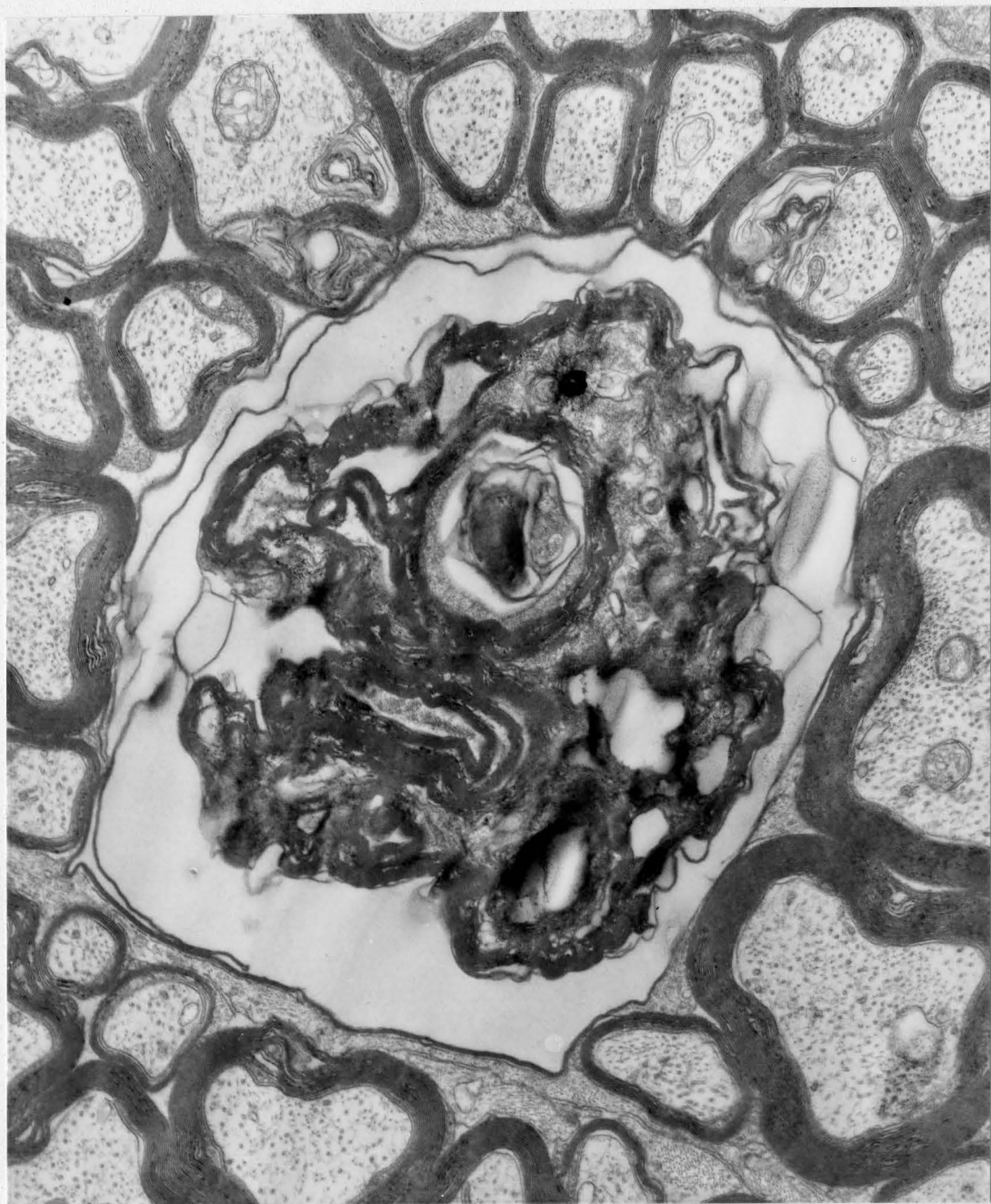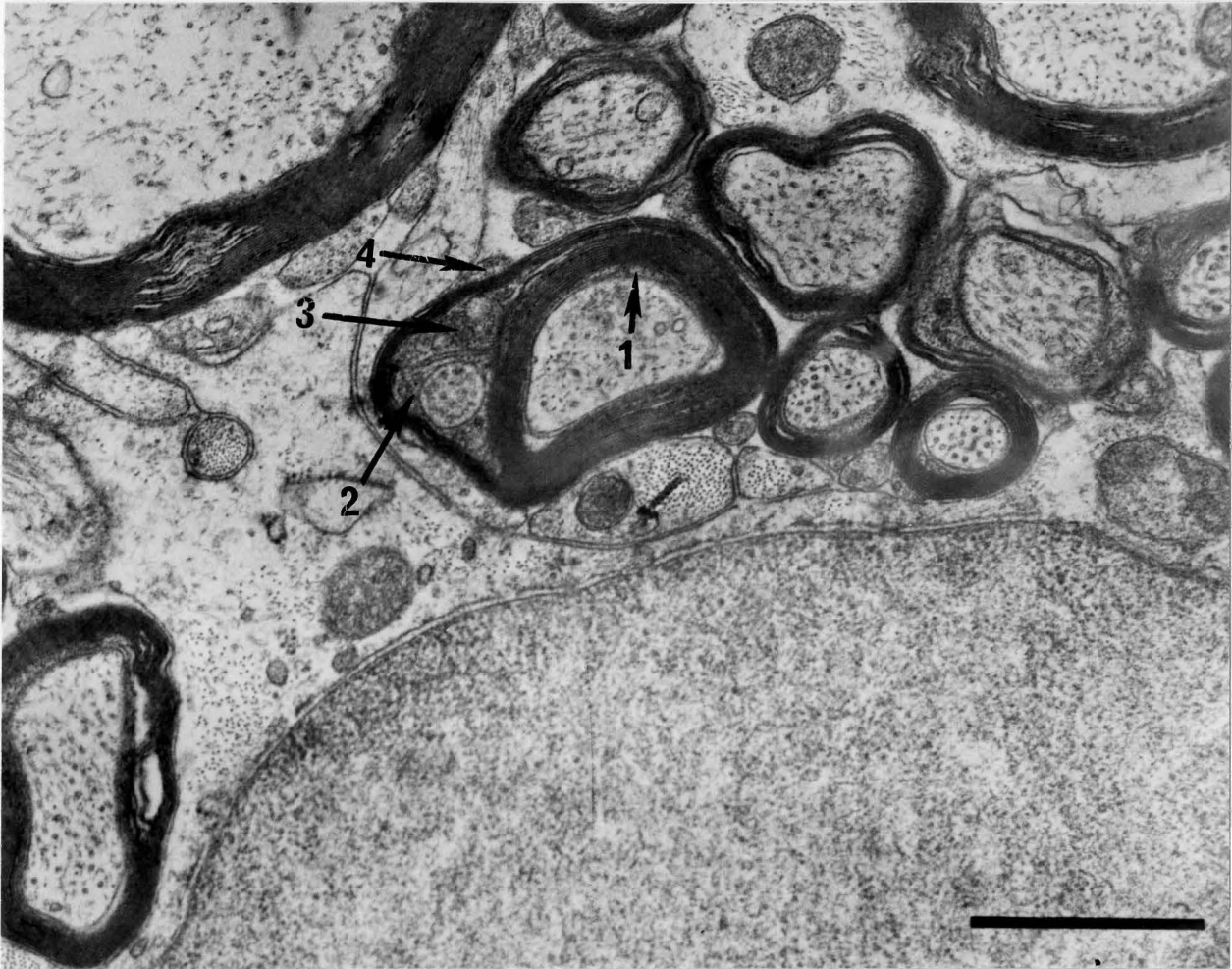
Figure 24. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Central axon has two distinct concentric myelin sheaths. The inner (1) and outer (2) mesaxon of the internal sheath as well as the inner (3) and outer (4) mesaxon of the external sheath are visible. The outer mesaxon of the inner sheath (2) also contains a formed organelle.
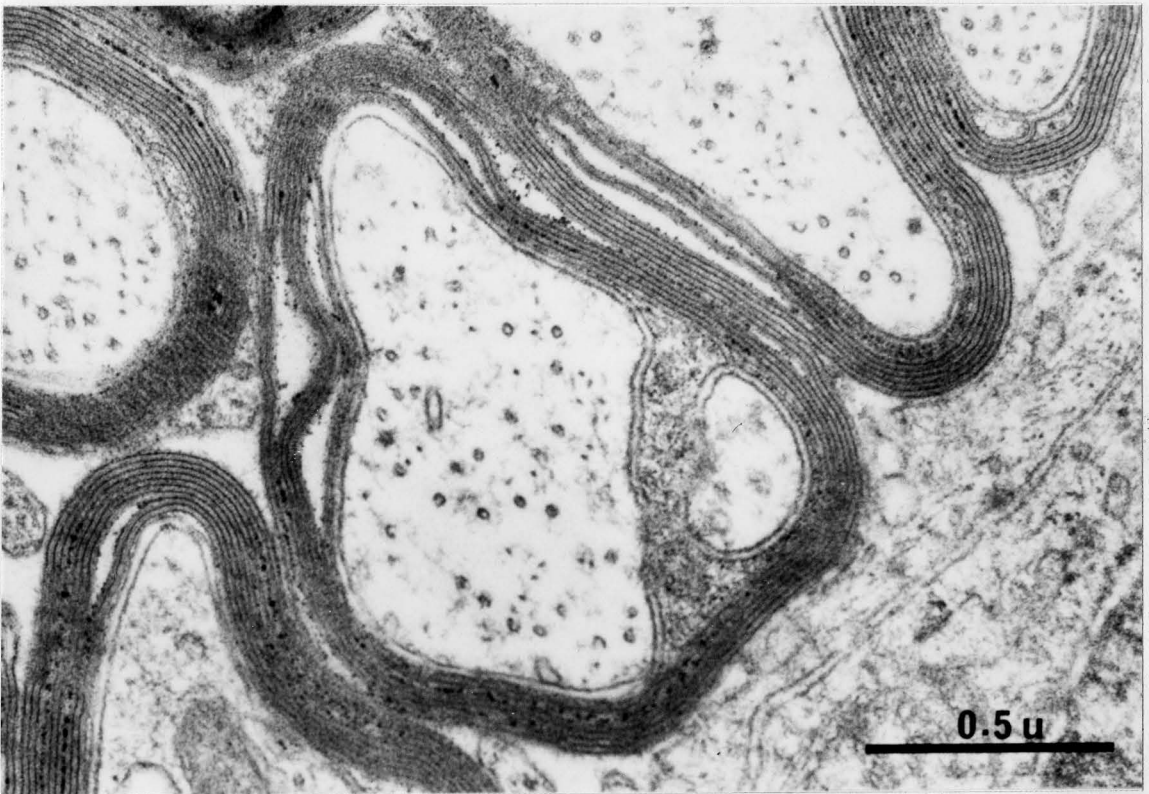
Figure 25. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Myelin sheath surrounds two axons, one much smaller than the other.
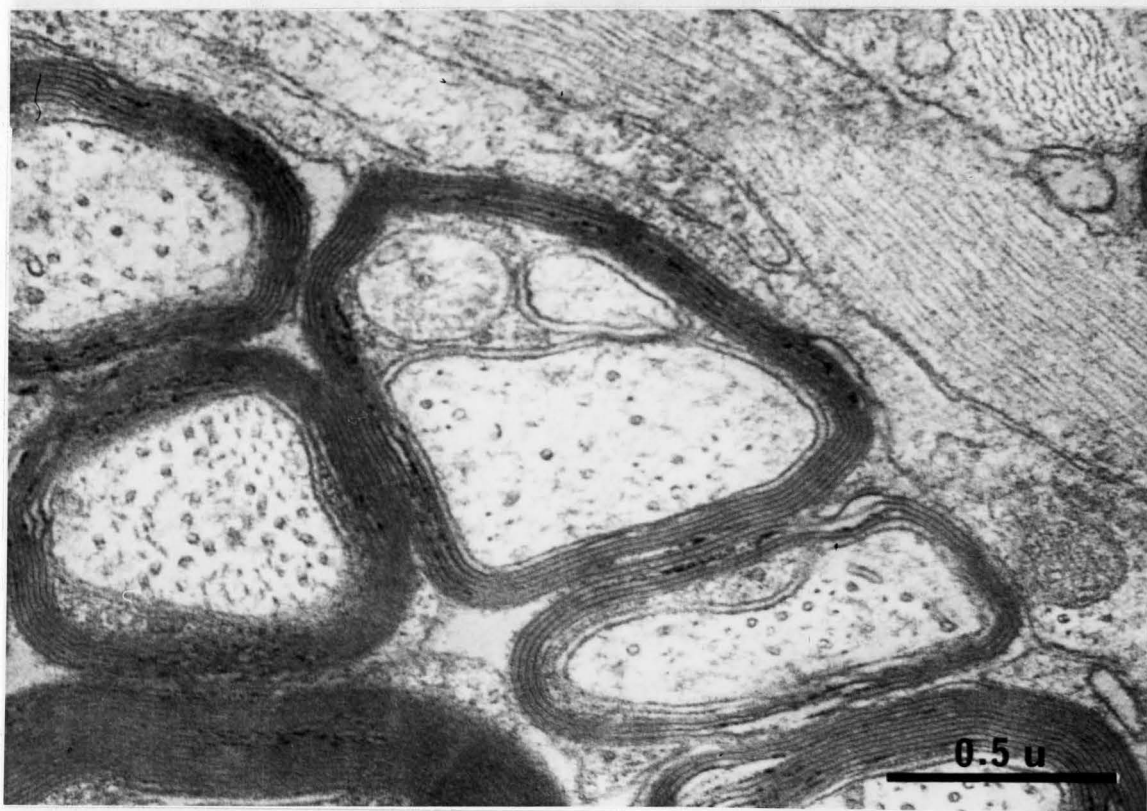
Figure 26. Electron micrograph of optic nerve from 120-day-old EFA de-
ficient rat. Fibre similar to that in Fig. 23. In addition a formed
organelle is present in the inner mesaxon.
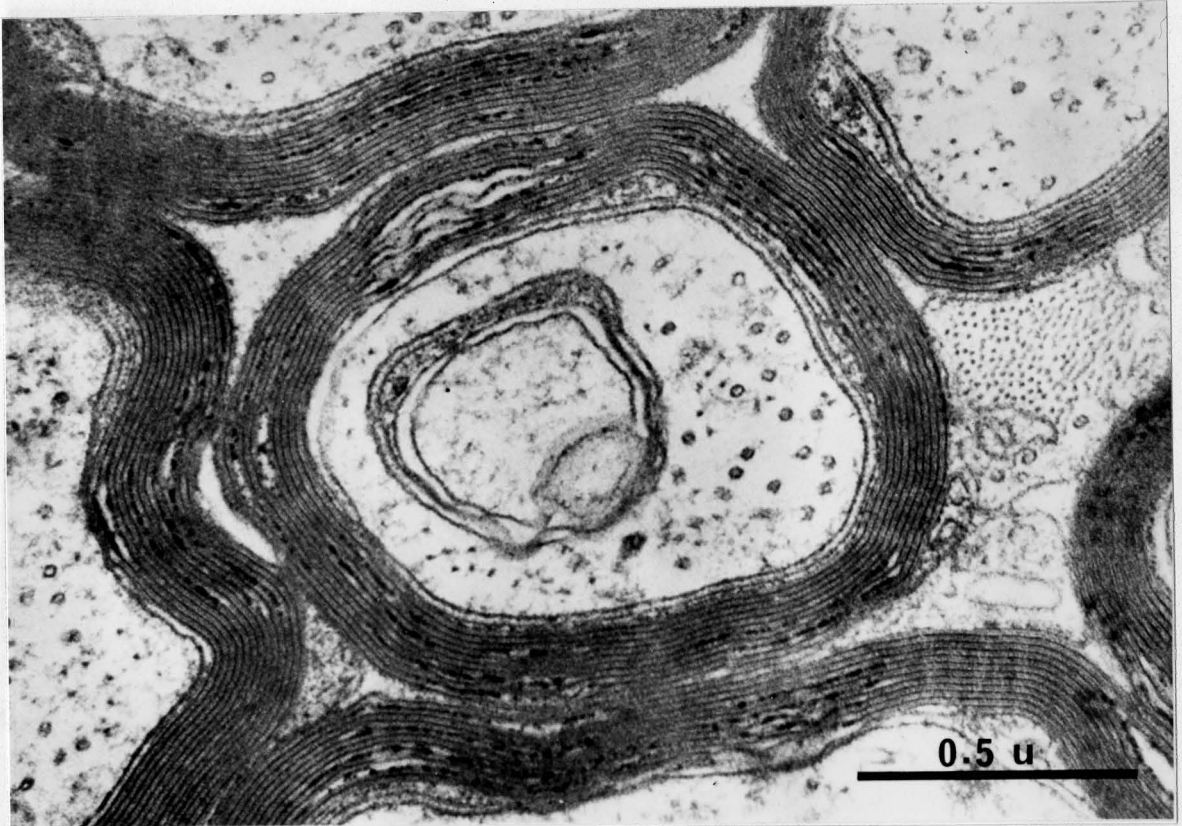
Figure 27. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Axon contains a circular isolated island of cytoplasm.

Figure 28. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Isolated island of cytoplasm surrounded by axon on three sides. Inner mesaxon approaches island of cytoplasm.

Figure 29. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Axon contains circular section of cytoplasm, similar to that in Fig. 27, which is connected to the inner mesaxon.
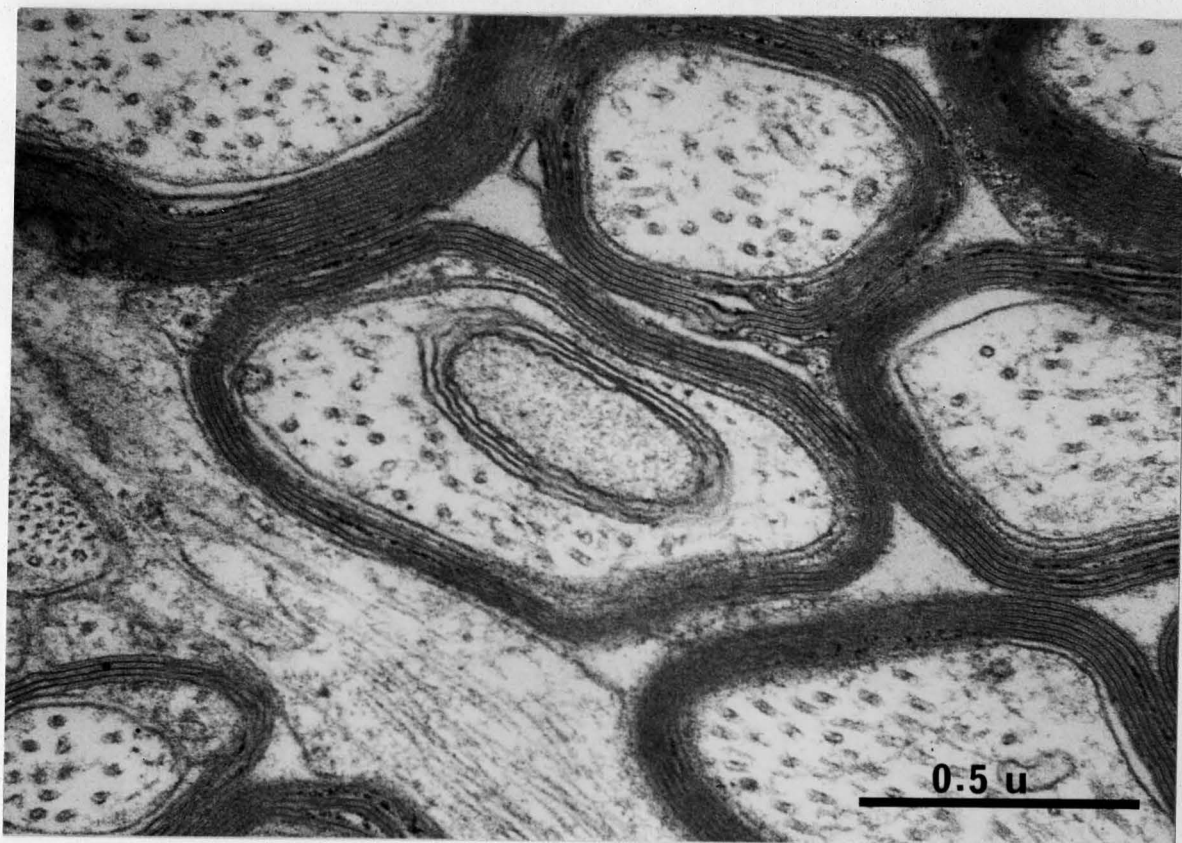
Figure 30. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Axon has central inclusion surrounded by two concentric membranes.
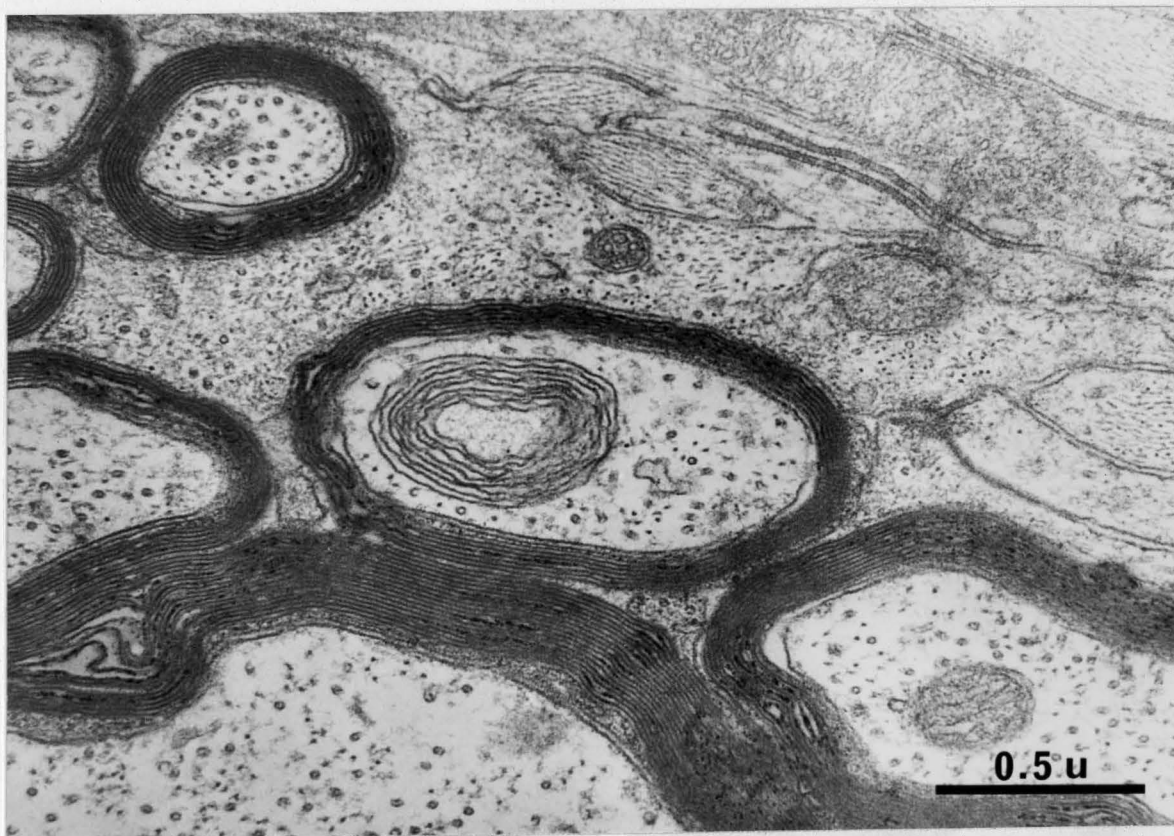
Figure 31. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Axon has inclusion surrounded by six concentric membranes.

Figure 32. Electron micrograph of optic nerve from 120-day-old EFA deficient rat. Axon has inclusion surrounded by four concentric membranes.

DISCUSSION

A. ANIMALS AND DIETS

Animals placed on the EFA deficient diet demonstrated reduced weight gain comparable to previous published results (Burr & Burr, 1929; Panos & Finerty, 1954; Galli et al., 1970; White et al., 1971). The deficient animals displayed dermal signs of EFA deficiency. These consisted of development of scaly skin, roughened hair coat and necrosis of the tail as described by Burr and Burr in their original papers (Burr & Burr, 1929, 1930). Although, decreased weight gain and dermal symptoms have been two of the major criteria used for determining degree of EFA deficiency caution should be taken in relying too extensively on these variables. The dermal symptoms of EFA deficiency have been shown to vary with changes in the humidity of the animals' environment. Low relative humidity alone, can cause dermal symptoms similar to EFA deficiency. Similarly, high relative humidity can mask dermal signs of EFA deficiency (Evans & Lepkovsky, 1932). Differences in the fatty acid composition which are related to sex have been described for plasma (Monsen et al., 1962), sexual organs and liver (Okey et al., 1961). Sex differences have been reported in the tissue fatty acid response to EFA deficiency (Lyman et al., 1966; Aftergood & Alfin-Slater, 1965). Female rats have been reported to be less sensitive than male rats to EFA deficiency (Loeb & Burr, 1947) as the requirement for essential fatty acids are greater in the male than in the female (Greenberg et al., 1950). Despite these reported differences, the fatty acid composition of CNS lipids are similar

in control male and female rats and EFA deficiency has identical effects on male and female fatty acids (White et al., 1971; Galli et al., 1970, 1971a; Alling et al., 1972; Sun, 1972). Thus sex was not controlled for in the present study.

The methodology for inducing EFA deficiency in experimental animals has differed for different laboratories. Various protocols appear appropriate for studying the effects of fat deprivation on various stages of CNS development. Alling et al.; (1974) has objected to studies (Berg-Hansen and Clausen, 1968; Galli et al., 1970; 1971a; White et al., 1971; Paoletti and Galli 1972; Sun, 1972) in which pregnant dams are placed on a fat-free diet. Under this regimen the fatty acids of the mother's fat stores are released gradually to the offspring during the intrauterine and suckling periods and a deficiency state is hard to produce in young animals. Alling et al., (1974) has employed a nutritional design which supplies a diet low in essential fatty acids (0.07% calories) to animals for a time period before mating. This allows for a depletion of EFA's in the mother's fat stores and decreases the transfer of EFAs to the offspring during the intrauterine and suckling periods. This protocol is probably the one of choice when studying the effects of EFA deficiency on prenatal and newborn rats. Studies utilizing this design (Alling et al., 1974; Karlsson, 1975) produce maximum changes in the fatty acid composition of CNS lipids in the immature or young rat ($\leq$ 45 days). Lyles et al., (1975) has shown that although EFA deficiency does not alter the uptake of fatty acids into the brain, EFA deficient animals retain fatty acids, especially essential fatty acids, in brain lipids to a greater extent than control animals. Thus, animals fed a low fat diet

retain what EFAs they receive and an acute stage of EFA deficiency in adult animals cannot be produced. This is demonstrated in the work of Alling et al., (1974) and Karlsson (1975) where the fat deficient diet contained 0.07 calories % of EFAs. The older animals (120 days) in their study did not demonstrate an alteration in the deficiency state, when a comparison of fatty acid composition is made with the younger animals ($\leq$ 45 days). Thus, while the nutritional design employed by Alling et al., (1974) and Karlsson (1975) is suitable for a study of the effects of EFA deficiency on prenatal and very young rats, it does not appear appropriate for a study of the effects of acute nutritional deprivation, since the deficiency is not progressive. Also, mating animals over two and three generations as Alling et al., (1974) and Karlsson (1975) have done may allow for selective breeding of animals less susceptible to EFA deficiency, as a high mortality rate occurs in the litters on these low EFA diets (Alling et al., 1972). The variable mortality rates of animals on identical deficient diets suggest differing individual resistance to fatty acid deprivation (personal observation).

In the present study the deficient diet was initiated 10-12 days after impregnation. This design allowed for a progressive increase in the deficiency state when defined by changes in the fatty acid composition. While a large percentage of the animals do not survive the experimental period under the conditions described in this work, those that do, appear to exhibit no severe clinical signs.

B. WHOLE BRAIN FATTY ACIDS

Although in normal animals changes with age in the ethanolamine phosphoglyceride fatty acids of whole brain (White et al., 1971) and in subcellular fractions (Sun et al., 1976), have been reported, we found no marked changes in controls in the fatty acid pattern of whole brain phosphoglycerides between 30 and 120 days. Thus, changes induced by EFA deficiency are not interacting with age changes during this period. Since little change in total phospholipids occurs during this time, variations in fatty acid composition in EFA deficiency may be attributed to different rates of turnover of existing lipids. The degree of saturation and the saturation/unsaturation ratio of brain fatty acids remained constant during all experimental periods. The stability of these fatty acid subclasses may be a mechanism of maintaining the physiological and functional integrity of CNS membranes.

The expected increase in the (n-9) trienes (20:3 + 20:3) and the decrease in the (n-6) tetraenes (20:4 and 22:4) and in 22:6 (n-3) was progressive from 30 to 120 days. The anamalous increase of 22:5 (n-6) during EFA deficiency has been reported previously (White et al., 1971, Galli et al., 1971a). White et al., (1971) has shown the peak identified as 22:5 (n-6) to be uncontaminated and that the response cannot be explained by the presence of two fatty acids of differing families co-chromatographing as suggested by Sun (1972). This increase in 22:5 (n-6) during EFA deficiency may be a unique response of CNS tissue as it does not occur in liver (Alling et al., 1972). It appears that 22:5 (n-6) can function at variance with the remainder of the (n-6) fatty acids. The source of the

(n-6) fatty acids used as precursors in deficiency states for 22:5 is unknown, the increase in 22:5 may represent some sparing mechanism utilized by CNS tissue. Galli et al. (1975) has shown an accumulation of (n-6) fatty acids in brain occurring concomitantly with depletion of liver stores of (n-6) fatty acids. Thus, the brain may receive (n-6) fatty acids from the liver, and elongate them subsequently to 22:5 (n-6) to maintain the highest degree of unsaturation in the (n-6) family during EFA deficiency. It is interesting that the per cent of 22:6 (n-3) plus 22:5 (n-6) shows very little variation during EFA defiency and subsequent supplementation. This may be a mechanism whereby a constant per cent of these highly unsaturated pentaenes and hexaenes are maintained. There may exist a biological requirement for a minimal per cent of fatty acids with double bonds in the Δ, 4, 7, 10, 13, 16 positions (or any combination of these) as these double bonds are common to both 22:5 (n-6) and 22:6 (n-3). The position of the double bonds appears to play a role in the replacement of 20:4 (n-6) by 20:3 (n-9) in EFA deficiency, both acids having Δ, 5, 8, 11 bonds.

The elongation product of palmitoleate 20:4 (n-7), is a non essential fatty acid and is incorporated into phospholipids (Beerthuis et al., 1968). This fatty acid has the same degree of unsaturation and chain length as 20:4 (n-6), however, 20:4 (n-7) does not increase to any appreciable amount during EFA deficiency (Fulco and Mead, 1959, and Klenk and Oette, (1960) and 20:3 (n-9) is the principle "substitute" for 20:4 (n-6). The double bonds of the palmitoleate series are shifted one carbon atom toward the carboxyl end of the fatty acid when compared with the linoleate series. In contrast, oleic acid and 20:3 (n-9) has the double bonds in

the same location as 20:4 (n-6) relative to the carboxyl end but contain one less double bond. Thus the position of the first three double bonds in relation to the carboxyl end appears to be a more important structural consideration than the presence of the degree of unsaturation in the replacement of archidonic acid during EFA deficiency.

The triene: tetraene ratio has been used by Holman (1960) as a biochemical index of EFA deficiency, a ratio greater than 0.4 is indicative of EFA deficiency. This ratio is exceeded in the 60-day deficient animal (0.5) and reaches a maximum of 1.0 at 120 days, a reflection of the progressive course for the deficiency.

Supplementation with linoleate for 30 days returns its major elongation products 20:4 (n-6) and 22:4 (n-6) to control levels. At this time the increase of 22:5 (n-6) after linoleate supplementation above its already elevated levels indicates that some regulatory mechanism may be operating in EFA deficient animals that allows for this unsaturated fatty acid to be retained in membrane phosphoglycerides. When control animals are supplemented with linoleate, no increase in 22:5 (n-6) occurs.

The higher the degree of unsaturation of the fatty acid, the greater affinity it has for the enzyme system involved in its elongation (Brenner and Peluffo, 1966). Upon supplementation with either linoleate or linolenate there occurs a decrease in the elongation products of oleic acid and an increase in the elongation products of the more unsaturated EFA. Linoleate and linolenate decrease the level of 20:3 (n-9) and 22:3 (n-9) to the same degree, which demonstrates that the degree of unsaturation has no effect on the inhibition of oleic acid elongation. Supplementation with linolenate for 30 days causes a return of the level of 22:6 (n-3) to

control values. Linolenate supplementation also alters the level of 22:5 (n-6). The level of 22:5 (n-6) was significantly decreased by 30 days supplementation of linolenate. The decrease in this (n-6) fatty acid after supplementation of a (n-3) fatty acid was surprising in view of the results obtained with linoleate.

Galli et al. (1971a) have suggested that the increased level of 22:5 (n-6) during EFA deficiency is due to a decreased rate of degradation of 22:5 (n-6). The results with linolenate supplementation in the present study offers little support to this theory, as an increase in (n-3) fatty acids should not inhibit degradation enzyme reactions of the (n-6) series. It seems more reasonable that the increased level of 22:5 (n-6) in EFA deficiency is due to an increased rate of synthesis. This synthesis is enhanced by supplementation with linoleic acid. The decrease in 22:5 (n-6) with linolenate supplementation is not inconsistent with known competitive mechanisms between (n-6) and (n-3) pathways. Fatty acids of the (n-3) series are known to inhibit the desaturation of linoleic acid at the 6- and 5- desaturase step, (Brenner & Peluffo, 1966; Brenner et al., 1969 and Actis Dato & Brenner, 1970). Sun (1972) has suggested that the increase in 22:5 (n-6) may be due to contamination of the peak by an (n-9) fatty acid. From supplementation data of the present study it is apparent that if the response of 22:5 (n-6) is due to an (n-9) fatty acid, that this (n-9) fatty acid responds differently than the other (n-9) fatty acids 20:3 and 22:3) as it is not decreased by linoleate supplementation. Knowledge of the turnover rates of the individual fatty acids in control, EFA deficient and supplemented animals may provide a clue to the 22:5

(n-6) response. It is evident that further investigation into the metabolism of 22:5 (n-6) is needed.

The unsaturation index does not vary significantly during EFA deficiency. This is in accordance with previous published results (Galli et al., 1970, 1971a; White et al., 1971; Sun 1972 and Montamat et al., 1974). This constancy of the unsaturation index may be unique to brain as it decreases in liver during EFA deficiency (Alling et al., 1972). Although the total unsaturation index does not change, the unsaturation index for the individual fatty acid families differs. The unsaturation index does not decrease during the deficiency state for the n-6 series, due primarily to the increase in 22:5 (n-6) which compensates for the decrease in 20:4 (n-6) and 22:4 (n-6). During EFA deficiency there may be a mechanism within brain that maintains a constant percentage of polyunsaturated fatty acids with the first double bond six carbon atoms from the methyl end. An interrelationship between the (n-3) and (n-6) families is observed in the response of the unsaturation index after linolenate supplementation. The unsaturation index for (n-3) fatty acids increases beyond control levels after 30 days supplementation with linolenate. The unsaturation index for the (n-6) fatty acids decreases below control levels with linolenate supplementation due to 22:5 (n-6) diminution. This may be an additional means to demonstrate a deficiency in (n-6) fatty acids as it is the only manner in which the (n-6) unsaturation index is decreased.

C. LIVER FATTY ACIDS

The response of liver phospholipid fatty acids during EFA deficiency differed from that of CNS in several ways. There was a decrease in the percent of polyunsaturated fatty acids of liver phospholipids. This decrease caused the unsaturation index of the liver fatty acids to be significantly reduced during EFA deficiency. This decrease in total number of double bonds per fatty acid molecule may have had an effect on the fluidity of the liver membranes (Chapman, 1975a). The CNS did not demonstrate a decrease in the unsaturation index during EFA deficiency. Some sparing mechanism was employed in maintaining a constant number of double bonds in CNS phospholipids. This phenomena may insure a constant fluidity to CNS membranes during states of fat deprivation. The total unsaturated fatty acids of liver phospholipid were unchanged in the deficient animals as were the CNS unsaturates. This stability of saturated fatty acids has been reported in all tissues analyzed during EFA deficiency (Holman, 1968). The total monounsaturates were increased in liver phospholipids to compensate for the decreased polyunsaturates. The CNS monounsaturates did not differ in deficiency states, this is in agreement with results reported by Mohrhauer and Holman (1963a). The fatty acid subclasses are very stable during EFA deficiency in CNS phospholipids while liver phospholipids showed marked variation.

The response of the individual fatty acids to EFA deficiency was similar in liver and CNS although to varying degrees. The increase in 22:5 (n-6) is unique to brain as this fatty acid showed little variation in liver. Members of the oleic series responded differently in liver

than in brain.   There was no increase in 22:3 (n-9) in liver although a significant increase was found in CNS.   The liver compensated for this by increasing 18:1 (n-9) in the deficiency state; 18:1 (n-9) was unchanged in brain.   This lack of conversion of 18:1 (n-9) to 22:3 (n-9) in liver is a major cause for the decrease in the unsaturation index of liver phospholipids during EFA deficiency.

Differences between liver and brain in the effects of linoleate and linolenate supplementation were found.   In CNS linolenate supplementation returned the (n-3) fatty acids to control levels and decreased (n-6) fatty acids due to 22:5 (n-6) dimunition.   The (n-6) fatty acids of liver were unaffected by linolenate refeeding whereas the (n-3) fatty acids were increased to 3 times the control value.   This rebound of (n-3) fatty acids following linolenate supplementation is primarily due to the marked increase in 20:3 (n-3) to 13.6% as compared to 1.9% in controls and to 22:6 (n-3) which increased to 16% compared to 8% in controls.   The level of 20:5 (n-3) is not increased following linolenate supplementation. These results are similar to previous published results (Poovaiah et al., 1976) showing the rate limiting step in synthesis of 22:6 (n-3) to be the elongation of 20:5 (n-3) to 22:5 (n-3).   It has been shown that longer chained and more saturated fatty acids of the linoleic family [γ-linoleic, 20:3 (n-6), 20:4 (n-6), 22:5 (n-6)] have an inhibitory effect on the desaturation of linoleic acid to γ-linoleic acid (Brenner 1969; Brenner & Peluffo, 1969; Brenner et al., 1969; Actis Dato & Brenner, 1970).   The elevated levels of 22:6 (n-3) may inhibit the elongation of 20:5 (n-3). The desaturation steps of linolenate synthesis do not appear to be affected by linolenate supplementation.

D. MYELIN SUBFRACTIONS

The morphological appearance of the light and heavy myelin sub-
fractions were similar to previous published results (Matthieu et al.,
1973; Zimmerman et al., 1975). Electron microscopic examination of the
myelin subfractions was not performed at all ages studied. Utilizing
an isolation procedure identical to the one employed in the present study
Zimmerman et al., (1975) have shown the morphological appearance and
many of the biochemical properties of the isolated myelin subfractions
to be similar between 16 day-old and 60 day-old rats. The light subfrac-
tion consisted of multilamelar whorls of myelin and appeared as one would
expect mature compact myelin to appear. The heavy myelin subfraction
contained single membranous structures, vesicles, and small myelin frag-
ments. It has been postulated (Matthieu et al., 1973; Zimmerman et al.,
1975) that the light myelin fraction is mature myelin and that the heavy
myelin consists of loose, uncompacted myelin and the oligodendrocyte
plasma membrane and its region of transition into myelin. This hypothesis
has been supported by biochemical analysis of the subfractions. The
light myelin fraction contained a higher concentration of the myelin
specific or basic protein than the heavier fraction (Matthieu et al.,
1973; Eng & Bignami, 1972; Agrawal et al., 1974; Hofteig & Druse, 1976;
Benjamins et al., 1973). The light fraction also contained a higher
concentration of cholesterol (McMillan et al., 1972; Benjamins et al.,
1973) and had a greater lipid to protein ratio (Agrawal et al., 1974;
McMillan et al., 1972) which would be expected from its sedimentation
characteristics. The amount of light myelin isolated increased with

age in relation to the heavier fraction (Zimmerman et al., 1975), this would be expected if the light myelin is mature multilamellar myelin. Cerebrosides are deposited in the brain at the same rate as myelin (Norton & Poduslo, 1973b) and are considered a marker for myelin. Agrawal et al. (1974) have found cerebrosides to exist almost exclusively in the light fraction while McMillan et al. (1972) found cerebroside content of the light myelin to be greater than that of the heavier fractions. The total galactolipid and sulphatide content was also greater in light myelin fractions (Benjamins et al., 1973; Agrawal et al., 1974). Thus, many of the chemical parameters associated with myelin development and isolated mature myelin were localized in the lighter myelin fractions.

The heavy myelin fraction contained a higher concentration of protein in comparison to the light fraction expressed as per cent of dry weight (Matthieu et al., 1973). The amount of chloroform methanol insoluble protein was much greater in the heavy than in the light fraction (Matthieu et al., 1973). The heavy fraction had less basic protein (25-30%) than the light myelin (50%), whereas the heavy fraction had a higher concentration (40%) of higher molecular weight protein than the light fraction (22%) (Matthieu et al., 1973; Hofteig & Druse, 1976). The proteolipid protein did not seem to vary between light and heavy fractions (Matthieu et al., 1973; Hofteig & Druse, 1976). The heavy fraction had a lower lipid content than the light fraction and this is evidenced in its sedimentation characteristics. The heavy fraction did contain a higher concentration of phospholipid than the light fraction (Agrawal et al., 1974; McMillian et al., 1972; Benjamins et al., 1973).

The heavy fraction has been shown to contain a high concentration of the enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (Matthieu et al., 1973; Agrawal et al., 1974; Sabri et al., 1975) which is a well established myelin associated enzyme (Norton, 1972; Kurihara et al., 1970). Therefore it appears that the heavy fraction consists of myelin related membranes which contain a low lipid but high phospholipid content, a low content of basic protein and a high content of high molecular weight protein. The myelin-associated enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase is present in the heavy fraction. Thus, it has been hypothesized that the heavy fraction is enriched in loose myelin membranes, which may represent the outer or inner mesaxon, the lateral loop or portions of the oligodendrocyte plasma membrane and possibly the transition of the oligodendrocyte plasma membrane into myelin (Matthieu et al., 1973; McMillian et al., 1972; Benjamins et al., 1973; Zimmerman et al., 1975). It has been postulated (Hirano & Dembritzer, 1967) that the inner and outer mesaxon as well as the lateral loop membranes are continuous with and part of the oligodendrocyte plasma membrane.

Differences in the lipid composition of the myelin subfractions have been reported (Matthieu et al., 1973; Agrawal et al., 1974; McMillan et al., 1972; Benjamins et al., 1973). The characterization of the fatty acid composition of myelin subfractions has not been reported. McMillan et al. (1972) have quantified the major fatty acids (16:0, 18:0, 18:1) in three subfractions of myelin isolated from adult (300gm) rats. Lipid subclasses were not fractionated in their work. The fatty acid composition

of purified myelin phospholipids has been reported for human myelin

(O'Brein & Sampson, 1965), bovine myelin (MacBrinn & O'Brein, 1969),

and rat myelin (Galli et al., 1972; Karlsson 1975; Montamat et al., 1974;

Sun, 1972). The results of the present study were in agreement with

published results for the fatty acid composition of the phospholipids

of isolated myelin from the rat.

Results from the present study give evidence for a difference

between the ethanolamine phosphoglyceride fatty acids of light and heavy

myelin. The heavy fraction tended to contain a higher per centage of

polyunsaturated fatty acids while the lighter fraction contained more

monounsaturated fatty acids. As the degree of polyunsaturation increases

so does the unsaturation index. If the heavy fraction does represent

the inner and outer mesaxon, the lateral loops and portions of the oligo-

dendrocyte plasma membrane it would be in contact with the extracellular

space. Studies involving the injection of election dense tracers into

the CNS have demonstrated the lateral loops, the inner and outer mesaxon

as well as the periaxonal space to be penetrated by the tracer (Hirano

et al., 1969; LaVail & LaVail, 1974; Reier et al., 1976). The tracers

did not penetrate the myelin sheath. Thus the heavy myelin should be

more permeable to allow for the transfer of metabolic substances. There

is a marked increase in the permeability of membranes to water (Bittman &

Blau, 1972) and non-electrolytes such as glycerol and erythritol (de Gier

et al., 1968) when fatty acids within the membrane become more unsaturated.

Cholesterol has been shown to decrease the permeability of membranes

(Bittman & Blau, 1972). Heavy myelin has been shown to contain a lower

content of cholesterol than light myelin (McMillan et al., 1972; Benjamins

et al., 1973). Thus the fatty acid composition and cholesterol content
of the heavy myelin fraction supports the theory that this fraction is
derived from the inner and outer mesaxon, the lateral loops and from
portions of the oligodendrocyte plasma membrane.

We cannot, of course, rule out the possibility that the heavy
myelin fraction is contaminated by another membrane fraction. The fatty
acid composition of the ethanolamine phosphoglycerides from the heavy
fraction does rule out the possibility of contamination by any distinct
subcellular fraction; microsomes, synaptosomes or mitochondria. The
fatty acid composition of the ethanolamine phosphoglycerides of these
subcellular fractions isolated from the CNS have been reported (Karlsson,
1975; Galli et al., 1972; Sun & Yau, 1976). The heavy myelin consists
of a higher percent of 22:6 (n-3) than the light fraction. Synaptosomal
plasma membranes contain a high percent ($\approx$25%) of 22:6 (n-3) (Karlsson,
1975; Galli et al., 1972) and could be considered as a possible source of
contamination. Synaptosomes also contain a higher percent of 18:0 ($\approx$30%)
(Karlsson, 1975; Galli et al., 1972). If the heavy fraction was conta-
minated by synaptosomes one would find a similar increase in 18:0 as
found with 22:6 (n-3). No increase in 18:0 is found in the heavy fraction
compared to the light. Thus, the heavy fraction cannot be contaminated
by synaptosomes. Similar reasoning rules out contaminations by mitochon-
drial or microsomal membranes.

Due to the difficulty in isolating plasma membranes from the CNS
and due to the fact that the CNS consists of many cell types, little is
known about the chemical composition of plasma membranes within the CNS.
Poduslo (1975) has isolated oligodendrocytes from calf brain and has

further fractionated these isolated cells into a myelin and plasma membrane fraction. Many of the characteristics of the plasma membrane from oligodendrocytes were similar to the heavy myelin fraction. There exists a greater protein content and a lower lipid content in the plasma membrane fraction than in the myelin fraction. The amount of chloroform-methanol insoluble protein was much greater in the plasma membrane fraction than in the myelin. The cholesterol and galactolipid content of the myelin fraction was greater than the plasma membrane fraction. The greater content of galactolipids was reflected in both the cerebrosides and sulphatides. The phospholipid content of the plasma membrane fraction was greater than that in the myelin fraction. A high level of 2',3' -cyclic nucleotide 3' -phosphohydrolase was found in both the plasma membrane and myelin fractions. All of the above data supports the hypothesis that the heavy myelin fraction may be related to the oligodendrocyte plasma membrane or a transition of the plasma membrane into myelin.

There are alternative explanations for the morphological and biochemical differences between light and heavy myelin. The possibility that the myelin subfractions originate from different brain regions or are formed at different stages of myelinogensis must be considered. Also, the heavy myelin fraction may be contaminated by other membrane fractions.

The chemical composition of myelin is known to vary with age (Horrocks et al., 1966; Norton et al., 1967; Suzuki et al., 1967; Horrocks, 1968; Cuzner & Davison, 1968; Eng & Noble, 1968; Norton, 1971; Norton & Poduslo, 1973b). The major changes in myelin lipids are an increase in

the relative proportion of galactolipid, cholesterol and ethanolamine plasmalogen with an accompanying decrease of choline phosphoglycerides. The fatty acid composition of myelin phospholipids is also known to vary with age (Sun & Yau, 1976; Karlsson, 1975). Results from the present study are in agreement with these published results. The total mono-unsaturates increased between 20 and 120 days. This increase was primarily due to an increment in 18:1 (n-9) and 20:1 (n-9). The saturated fatty acids showed little variation with age. The polyunsaturated fatty acids decreased between 20 and 120 days. This decrease was primarily due to 20:4 (n-6) and 22:6 (n-3) although 22:4 (n-6) did decrease between 60 and 120 days. The total (n-6) and (n-3) fatty acids decreased between 20 and 120 days. These changes were very similar to those published by Sun & Yau (1976) and Karlsson (1975). The changes in the ethanolamine phosphoglyceride fatty acids between 15 and 20 days were often the opposite of the changes found between 20 and 120 days. For example, the mono-unsaturates decreased and the polyunsaturates increased. The time span between 15 and 20 days represents the most active stage of myelin forma-tion in the rat. It has been estimated that oligodendroglial cells are synthesizing over 3 times their own weight of myelin per day during this period (Norton & Poduslo, 1973b). It is not unusual to find many unique metabolic occurrences within the CNS between 15 and 20 days (Norton & Poduslo, 1973a).

The changes found in the myelin during development may primarily be due to an increase in the ethanolamine plasmalogen containing monounsatur-ated fatty acids and there may be no decrease in the actual amount of ethanolamine phosphoglycerides containing polyunsaturated fatty acids.

The proportion of ethanolamine plasmalogen of the total phospholipid was small in myelin from young animals (14 day) (Horrocks, 1968; Eng & Noble, 1968). The acyl groups of the ethanolamine plasmalogen were also lower in the proportion of monounsaturated fatty acids and higher in 22:6 (n-3) than myelin isolated from older animals (Sun & Yau, 1976). With increasing age there appeared to be an increase in the proportion of ethanolamine plasmalogen in myelin. It also appeared that monounsaturated fatty acids are preferentially incorporated into the ethanolamine plasmalogen. This increased incorporation could be due to an enzyme induction in the biosynthesis of monounsaturated fatty acids. Another possibility, is that the dietary supply of unsaturated fatty acids cannot meet the requirements of myelin synthesis at the rapid period of myelin deposition. Thus, due to the unavailability of essential fatty acids there was an increase in the monosaturated fatty acids which were supplied through de novo synthesis. With the large increase in myelin during development (Norton & Poduslo, 1973b) the mechanism for changes in the fatty acid composition of ethanolamine fatty acids might have been due to an increase in the phospholipids containing monounsaturates (alkenylacyl - GPE). There may have been no actual decrease in the amount of polyunsaturates.

Developmental changes in the light and heavy fractions were similar although differences between the two fractions existed.

Another possible explanation for the developmental difference in the ethanolamine phosphoglyceride fatty acids is that the myelin fractions from the early ages were contaminated by membranes of non-myelin origin.

In order to compare the biochemical properties of myelin isolated from animals of various ages two criteria must be met. The isolation must provide a similar per-centage of the total myelin at all ages and the amount of impurities must be maintained at a constant low per-centage. The initial isolation employed in the present study was that of Norton and Poduslo (1973a). This procedure isolates a constant percent of myelin based on the recovery of cerebroside (Norton & Poduslo, 1973b). The accumulation of cerebroside within the CNS parallels the deposition of myelin (Folch-Pi, 1955, Bass & Hess, 1969; Norton & Poduslo, 1973b). Thus, if a constant percent of the total cerebrosides are isolated in the myelin fractions at the various ages a constant percent of myelin is also being isolated. The procedure for the initial isolation of myelin employed in the present study does isolate approximately 42% of the total cerebroside at various ages. The purity of the myelin isolated by the procedure of Norton and Poduslo (1973a) has been shown to be over 95% pure at various ages as determined by chemical composition and enzyme content (Norton & Poduslo, 1973a). Thus in the present study, the con-tamination of the myelin subfractions should have been constant at all ages and should rule out the possibility of other membranes causing developmental differences.

Alterations in the fatty acid composition of ethanolamine phospho-glycerides of myelin occur during EFA deficiency (Galli et al., 1972: Sun, 1972; Montamat et al., 1974; Karlsson, 1975). These changes consist of a decrease in members of the linoleate and linolenate families and an increase in the members of the oleic acid family. The increase in the

(n-9) fatty acids (20:3 and 22:3) preceeds the decrease in (n-3) and

(n-6) fatty acids. In contrast to the data on whole brain phospholipids

we did not find a marked increase in 22:5 (n-6) during EFA deficiency.

This is in disagreement with the results of Karlsson (1975) who found a

marked increase in 22:5 (n-6) when feeding rats a diet containing 0.7%

essential fatty acids over two generations. The sum of 22:5 (n-6) plus

22:6 (n-3) also showed little variation in his study, while the sum of

these two fatty acids was greatly reduced in EFA deficiency in the

present study. The discrepancies between the data of Karlsson (1975)

and the present study can be explained by the difference in the feeding

regimen and the composition of the deficient diets. The diets used by

Karlsson contained small amounts of essential fatty acids (0.07% of diet).

Lyles et al., (1975) has shown EFA deficient animals to retain essential

fatty acids within CNS lipids to a greater extent than control animals.

Thus, via the continuous supply of (n-6) fatty acid from the diet an

increased level in 22:5 (n-6) was maintained. In the present study the

diet contained no essential fatty acids thus the increase in the levels

of 22:5 (n-6) was not marked, due to the lack of a constant supply of

(n-6) fatty acids. What small increase was found in 22:5 (n-6) at early

ages in the present study can be explained by the depletion of liver

stores (Galli et al., 1975). The response of 22:5 (n-6) during EFA

deficiency in the myelin fraction differed from that in whole brain.

These animals were on the same feeding regimen. Thus the increase in

22:5 (n-6) must be reflected in other membranes within the CNS or in

other phospholipid subclasses as the myelin fatty acids were from ethano-

lamine phosphoglycerides while the whole brain fatty acids are from total phospholipids. The data of Karlsson (1975) supports this theory as the increase in 22:5 (n-6) in the ethanolamine phosphoglycerides of synaptosomes is over twice that found in myelin. Thus data from the present study supports the theory of Galli et al., (1972) that myelin membranes are more susceptible to EFA deficiency than other brain subcellular fractions.

Karlsson (1975) has stated that the experimental design used in the present study will not produce EFA deficiency until after weaning due to the release of EFA's from the fat stores of the dam (Alling et al., 1974). The data from the 15 day deficient rats of the present study did demonstrate marked changes in fatty acids. The levels of 20:3 (n-9) were increased from 0.7% and 3.0% in the light and heavy myelin fraction to 7.3% and 7.6% in the deficient animals. There was also a 10 fold increase in the levels of 22:3 (n-9) in 15 day EFA deficient myelin. The levels of 22:4 (n-6) was decreased in 15 day light and heavy myelin during EFA deficiency. Also, the levels of 20:4 (n-6) and 22:6 (n-3) were decreased in 20 day EFA deficient myelin subfractions, which is prior to weaning.

Alterations in the fatty acids of ethanolamine phosphoglycerides during EFA deficiency were similar in light and heavy myelin. It was hoped that the response of the ethanolamine phosphoglyceride fatty acids from myelin subfractions during EFA deficiency and development might have demonstrated a response unique to one of the subfractions. This information would have been valuable in supporting any product - precursor role between the myelin subfractions. A produce - precursor role between

light and heavy myelin has been supported by several laboratories (Agrawal et al., 1974; Sabri et al., 1975; Hofteig & Druse, 1976). These studies involved the injection of radioactive precursors, followed by the isolation of myelin subfractions. The distribution of the radioactivity within the myelin subfractions was then determined at various time points. An increase with time in the ratio of light myelin/heavy myelin fractions as determined by specific activity is supportive of a product – precursor role. Based on these criteria a product – precursor role has been supported for protein (Agrawal et al., 1974; Sabri et al., 1975; Hofteig & Druse, 1976) and sulphatide (Benjamins et al., 1973). Recently, Benjamins et al., (1976a, 1976b) has supported the hypothesis of a product – precursor role between light and heavy myelin utilizing single label studies for choline phosphoglycerides, ethanolamine phosphoglycerides, cerebrosides, sulphatides and protein. When establishing a product – precursor role utilizing a single pulse one must assume that the product pools being measured are in equilibrium (not changing in size relative to one another). This assumption cannot be made when considering subfractions of myelin during development (Benjamins et al., 1976b). Thus the studies cited can only support a product – precursor relationship between myelin subfractions, it does not prove it.

Benjamins et al., (1976a, 1976b) has employed a double label technique involving staggered injections which internally controls many of the potential variables of single pulse studies. The relationship between subfractions of myelin can be determined by specific radioactivities at several time points in this design. Staggered injections of carbon-14 and tritium labeled precursors were used to determine whether lipids and

proteins enter subfractions of myelin simultaneously or in sequence. If

there is a product - precursor relationship from heavy myelin to light

myelin the tritium/carbon-14 ratio would be highest in the heavy fraction

and lowest in the lighter or more mature myelin. If the tritium/carbon-14

ratio was identical in all subfractions of myelin the precursors were

probably added to the myelin subtypes simultaneously. Only if the

tritium/carbon-14 ratio decreased from heavier to lighter fractions

would a product - precursor relationship be supported. Data from the

double label experiments supported a product - precursor relationship

for some chemical precursors while others appeared to be simultaneously

incorporated into the subfractions of myelin. Ethanolamine phosphogly-

cerides and proteo-lipids appeared to be added to myelin in a sequential

manner involving a product - precursor relationship between heavy and

light subfractions. Cerebrosides, sulphatide, galactosyl diglyceride,

choline phosphoglycerides and high molecular weight and basic protein

appeared to enter myeline subfractions simultaneously. Thus the results

of Benjamins et al. (1976a, 1976b) suggests that two mechanisms are

involved in the synthesis of myelin membranes. Precursors can enter

myelin subfractions simultaneously (in less than 15-30 min.) or sequen-

tially from the site of synthesis. The differences between the single and

double label experiments emphasizes the methodological problems of studying

membrane genesis and further demonstrates the complexity of the myelin

subfractions.

E. OPTIC NERVE MORPHOLOGY

The normal fibre distribution of rat optic nerve has been reported

(Forrester & Peters, 1967; Sima & Sourander, 1974). Our results concern-

ing the 120-day-old control rats were in good accordance with those of

these authors. We found the majority of nerve fibres (60%) ·to have a

diameter between 0.50 and 1.00 M. Forrester and Peters (1967) reported

55% of all fibres of the optic nerve of adult (250 gm.) rats to have a

diameter between 0.50 and 1.00 M. Sima and Sourander (1974) also found

55% of all fibres between 0.50 and 1.00 M. in 90-day-old rats. The peak

distribution (35%) in the present study was fibres with diameters between

0.75 and 1.00 M. This value is very similar (32%) to that found in

90-day-old control rats (Sima & Sourander, 1974).

The present study revealed no diminution in the diameter of myelin-

ated·fibres in the optic nerve of the rat during EFA deficiency. The

findings do not correspond to those of other nutritional deficiencies

such as protein-calorie malnutrition (Sima & Sourander, 1974; Sima, 1974).

When measuring the total fibre diameter two variables are involved, the

axonal diameter and the thickness of the myelin sheath. A linear rela-

tionship between axonal circumference and myelin sheath thickness has

been demonstrated (Friede & Samorajski, 1967, 1968; Clos & Legrand, 1970;

Headly-Whyte, 1973). However a non-linear relationship exists between

axonal diameter and myelin sheath thickness (Webster & O'Connell, 1970;

Webster, 1971). Thus either the axonal diameter and/or the myelin sheath

thickness could be affected by nutritional deprivation. In protein-calorie

malnutrition the expansion of the axon appeared to be more affected than

myelin deposition, although both are significantly decreased (Sima, 1974).

The decrease in total fibre size of protein-calorie deficient optic nerve

was not reversed by refeeding a control diet (Sima, 1974). The deposition

of myelin was restored to control levels following refeeding despite

the retarded total fibre diameter. Thus, the decrease in fibre diameter

in rehabilitated animals was primarily due to a decrease in axonal dia-

meter. A decrease in the axonal diameter could cause a decrease in the

total fibre diameter while a decrease in the myelin sheath would not,

due to the fact that the thickness of the myelin sheath in the optic

nerve only constituted a small percentage of the total fibre diameter

(Sima, 1974). It could be possible that EFA deficiency reduced the

degree of myelination but not the axonal diameter. This is feasible as

myelin deposition occurs after axonal development.

Although no changes were observed in the diameter of fibres from

EFA deficient optic nerve, structural alterations of optic nerve fibres

did occur. Fibres in various stages of degeneration (Lampert, 1967)

were common (Fig. 16, 17 and 18). It is not possible to determine if

the degeneration of these fibres was a primary effect of EFA deficiency

or a secondary effect on the perikarya of the fibres within the retina.

Futterman et al., (1971) have investigated the effects of EFA deficiency

on the morphology of the retina. In their study the only morphological

alteration reported within the retina was a decrease in the mural cells

or retinal capillaries. No alterations in the neural elements of the

retina were reported.

Intramyelinic splits similar to those found in the present study

have been described in pathological conditions of the CNS. Intramyelinic

splitting of CNS myelin resulted from exposure of the CNS to several

toxic substances such as triethyltin (Levine et al., 1963; Hirano et al.,

1968; Aleu et al., 1963; Lee & Bakay, 1965; Schneiberg et al., 1965;

Kolkmann et al., 1967; Torack et al., 1970; Graham et al., 1975) and

hexachlorophene (Kimbrought & Gaines, 1971; Lampert et al., 1973;

Pleasure et al., 1973; Rose et al., 1972; Matthieu et al., 1974; Webster

et al., 1974). The relationship between EFA deficiency and triethyltin

(TET) and hexachlorophene (HCP) intoxication is not known at the present

time. Several features of the myelin splitting were common to both EFA

deficiency and these intoxicating substances. The majority of the myelin

splits occur at the intraperiod line in EFA deficiency and in TET and

HCP intoxication. The intraperiod line is formed by the fusion of the

external surface of the oligodendrocyte plasma membrane. The morpholo-

gical appearance of the area within the myelin split is electron lucent.

The electron lucent appearance of the intramyelinic spaces do not resemble

the accumulation of electron dense material found in edema fluid (Hirano

et al., 1968). No breakdown of the blood-brain-barrier occurs in TET

(Aleu et al., 1963) or hexachlorophene intoxication (Lampert et al.,

1973) as tested by injection of trypan blue. Trypan blue failed to pene-

trate the CNS of EFA deficient rats in the present study. The myelin

splits were not related to vascular elements. Thus, the accumulation of

fluid in the intramyelinic spaces were not functionally related to

alterations of the blood-brain-barrier. The distribution of horseradish

peroxidase in the CNS of TET intoxicated rats revealed a failure of the

substance to penetrate the intramyelinic splits, although the horseradish

peroxidase was abundantly found within the extracellular space (Hirano et al., 1969). Chemical studies have reported no **increase** in levels of sulphate, albumin or thiocyanate (Katzman et al., 1963; Baklay, 1965; Streicher, 1962) in the CNS of TET intoxicated animals. These chemicals are used as a measurement of the extracellular space within the CNS. Thus, it can be assumed that the intramyelinic splits do not represent an increase in the extracellular space. Chemical studies have shown the intramyelinic spaces produced during TET intoxication to contain water and sodium chloride (Aleu et al., 1963; Katzman et al., 1963). These substances were probably derived as an ultrafiltrate of the plasma.

The cause of the intramyelinic splits during EFA deficiency is not known at the present time. There is no reason to discount a direct effect of EFA deficiency on the components of the myelin membrane as a cause of intramyelinic splits. It appears that TET and HCP both have direct effects on the myelin membranes. This was demonstrated by the focal occurrence of myelin splits when TET was implanted into CNS white matter (Hirano et al., 1968). Thus it appears that TET has a direct toxic action on the CNS. This is supported by in vitro studies where myelinated cultures of mouse spinal cord exhibited intramyelinic splitting when exposed to triethyltin (Graham et al., 1975). Isolated myelin has been reported to have a high affinity binding site for TET (Lock & Aldridge, 1975). Hexachlorophene has been shown to bind strongly to proteins (Haique & Buhler, 1972). The uptake of HCP into brain subcellular fractions showed the highest levels of HCP per mg. of protein in the myelin fraction (Webster et al., 1974). Since the highest concentration

of HCP appears in the subcellular fraction where the morphological alterations appear it seems reasonable to suggest that HCP has a direct effect on myelin membranes. Thus both TET and HCP cause intramyelinic splits and both have been shown to bind to myelin membranes.

The binding of HCP and TET to myelin proteins may cause intramyelinic splits by interfering with bonding forces which maintain the compactness of the myelin sheath. Local changes in the osmolarity around the intra-period line caused by HCP or TET could cause the accumulation of fluid within the split. The tissue surrounding the myelin split, including the axon within the myelin split, appear normal. This also supports a toxic effect of HCP and TET on myelin. Although HCP and TET cause similar morphological lesions and both bind to myelin protein, they apparently do not bind at the same sites within the myelin membrane. Hexachloraphene does not compete for the high affinity binding site of triethyltin (Locke & Aldridge, 1975). Therefore, there may be more than one site of binding which can cause intramyelinic splitting or the binding site may not be directly involved in myelin splitting. Triethyllead and trimethyltin compete with triethyltin for the high affinity binding site in myelin (Locke & Aldridge, 1975). Neither triethyllead or trimethyltin produce intramyelinic splits within the CNS. Thus, there are at least two binding sites which have an affect on intramyelinic splitting and binding to at least one of these (TET) is not always accompanied by intramyelinic split-ting.

Alterations in the fatty acid composition of membrane phospholipids can affect membrane elasticity, properties of membrane proteins and lipids as well as the permeability characteristics of membranes (Chapman, 1975b).

Thus, changes induced by EFA deficiency could affect myelin membranes in a similar manner as HCP and TET. It has been shown that tadpoles kept at low temperature demonstrate varying degrees of myelin abnormalities (Cullen & Webster, 1976). Goldfish kept at similar low temperatures demonstrated marked alterations in the fatty acid composition of ethanolamine phosphoglycerides from myelin (Selivonchick & Roots, 1976). This demonstrates other means of producing myelin abnormalities by altering membrane lipids.

The means of entry of fluid into the myelin split is not known. Examination of series of electron micrographs of myelin splits failed to demonstrate any continuity between the extracellular apace and intramyelinic space (Aleu et al., 1963; Hirano et al., 1968). If the fluid is an ultrafiltrate of plasma, some filtration process probably occurs at the myelin membrane. Cerebral edema produced simultaneously with myelin splitting distinguished between fluid accumulation in edema (electron dense) and that produced during TET intoxication (electron lucent) (Hirano et al., 1968). Thus a filtration process may occur which involves a functional change in the permeability of the myelin membrane. The most probable site for this alteration in membrane permeability is the intraperiod line since this is the location of most intramyelinic splits. Changes in the fatty acids of membrane phospholipids has been shown to alter membrane permeability (Bittman & Blau, 1972).

Electron dense material was found within some intramyelinic spaces in the present study. This has been reported by others (Hirano et al., 1968). The origin of this material is believed to be from the extracellular space (Hirano et al., 1968). The entry of this material could be

caused by the bursting of the myelin lamellae as the intramyelinic split expanded. Damage to the myelin lamella also could be caused by the initial stages of perfusion. It may also be possible that the filtration process mentioned above becomes defective.

The occurrence of unusual configurations of the myelin sheath as appears in figures 22-24 have been described in detail previously (Hirano & Dembitzer, 1967). The occurrence of double axons within a single myelin sheath (Fig. 25, 26) have not been reported in the optic nerve of the rat.

Hirano & Dembitzer (1967) have proposed a generalized model of the myelin sheath based on a hypothetical unrolling of the myelin (Fig. 33). Variations in this model (Fig. 34) can explain the three types of abnormalities of the myelin sheath described in the present study. If the myelin sheath is unrolled it can be considered as a shovel-shaped myelin membrane surrounded by a continuous rim of cytoplasm (Fig. 33 D). The inner and outer rim of cytoplasm forms the inner and outer mesaxon and the lateral rim of cytoplasm forms the lateral loops. The outer rim of cytoplasm should also be continuous with the cytoplasm of the oligoden- drocyte. Thus, the rim of cytoplasm surrounding the myelin sheath (inner and outer mesaxons and lateral loops) can be considered as part of the oligodendrocyte cytoplasm. The normal appearing myelin sheath is demonstrated by figure 34A, and when sectioned through the plane indicated by the dotted line appears as demonstrated by A'. The occurrence of formed organelles within the inner and outer mesaxon (Fig. 22) can be explained by their presence in cytoplasmic rim surrounding the myelin sheath (Fig. 34B). The occurrence of isolated islands of cytoplasm

Figure 33.   a) Diagram of a myelinated axon.   Part of the myelin sheath

is cut away to show relationships of inner, outer and lateral loops.

b-e) Demonstrates the unrolling of the myelin sheath, resulting in

a shovel-shaped myelin sheath surrounded by continuous rim of cyto-

plasm representing inner and outer mesaxons and lateral loops.

(Hirano and Dembitzer, 1967 J. Cell Biol. 34: 555.)

OUTER LOOP
INNER LOOP
LATERAL LOOP

a)

AXON

b)

c)

d)

OUTER
LATERAL
INNER

e)
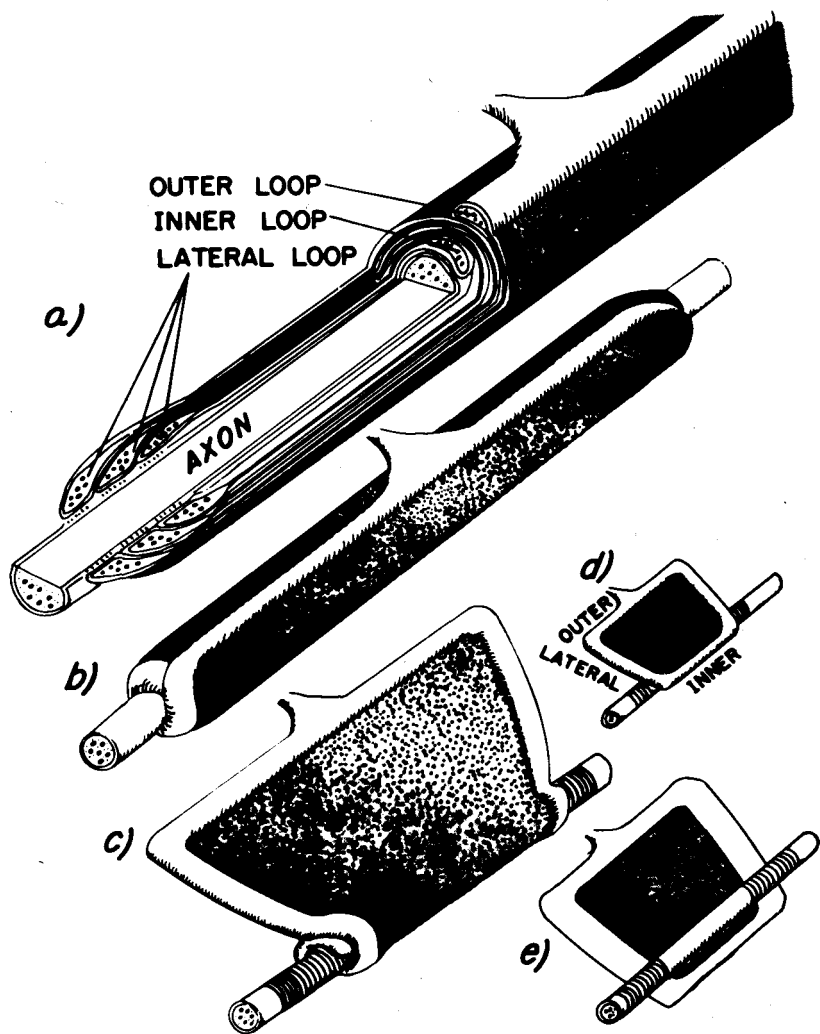
within the myelin sheath (Fig. 23) can be explained by the lateral rim

of cytoplasm extending into the interior of the myelin sheath (Fig. 34C).

The density of the island of cytoplasm (Fig. 23) within the myelin

lamellae is very similar to that of the inner and outer mesaxon. This

similarity in cytoplasmic density would be expected if the island of

cytoplasm is continuous with the rim of cytoplasm surrounding the myelin

sheath as shown in figure 34C. The presence of two myelin sheaths

concentrically surrounding a single axon (Fig. 24) can be explained by

folding of the lateral loop into the myelin sheath (Fig. 34E). These

myelin sheaths should spiral in the same direction. The outer mesaxon

of the inner sheath in figure 24 contains a formed organelle demonstrating

that more than one of these variations can occur in a single myelin seg-

ment.

Variations in the myelin sheath described by Hirano and Dembitzer

occurred during the recovery stage of white matter lesions. The lesions

were caused by cyanide intoxication, experimental allergic encephalomye-

litis,dibenzanthracene implantation, cold injury, cryptococcus neoformis

and cryptococcal polysaccharide implantation; and all variations of the

myelin sheath were evident in each of these lesions (Hirano & Dembitzer,

1967). The relationship of EFA deficiency to these lesions are not known

at the present time. The occurrence of these variations in the myelin

sheath in such a large number of experimental conditions does suggest

that these variations are a general response to experimental manipulation

of the CNS rather than a specific one.

The described variations of the myelin sheath represent deviations

of the normal relationship between the myelin membranes and the rim of

Figure 34. A. Normal myelin sheath, when wrapped around an axon and

sectioned in the plane of the dotted line, we see the normal cross-

section (A'). B. Formed organelles present within continuous rim of

cytoplasm surrounding myelin sheath. C. Extension of lateral rim of

cytoplasm extends into myelin sheath giving appearance of an

isolated island of cytoplasm within the myelin sheath (C'). E. The

lateral rim of cytoplasm is folded into the myelin sheath and appears

as two concentric myelin sheath surrounding single axon (E'). (Hirano

& Dembitzer, 1967 34: 555).
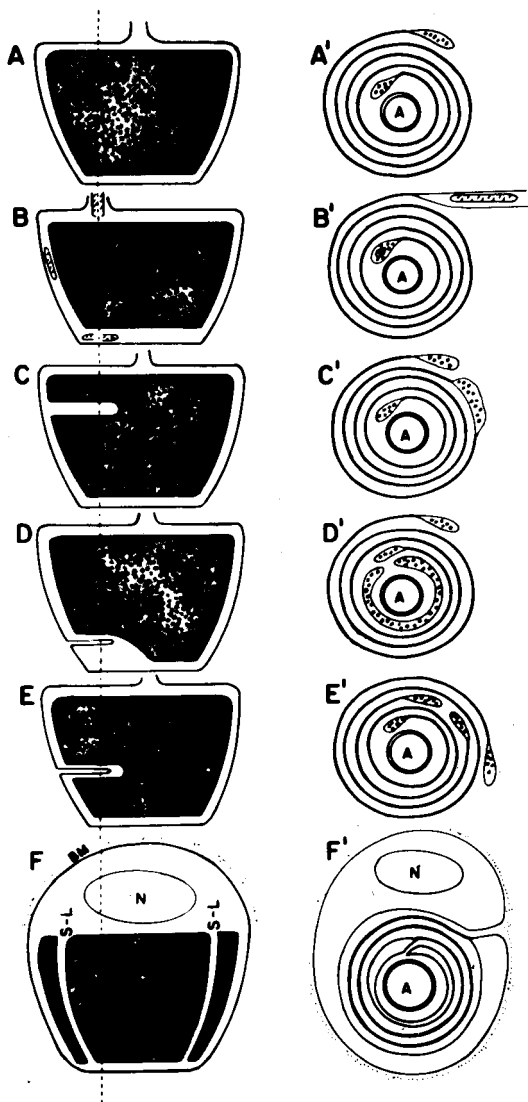
continuous cytoplasm which surrounds the myelin membranes (Fig. 33).
These deviations may be due to a defect of the myelin membranes, the
outer rim of cytoplasm or both. If the location of the isolation myelin
subfractions occurs as discussed earlier one may hypothesize that chemical
alterations in the heavy myelin fractions could be correlated to morpho-
logical changes in the outer rim of cytoplasm, whereas chemical alter-
ations in the light myelin fraction may be correlated to morphological
changes in the myelin membranes. Chemical alterations occur in the fatty
acid composition of both light and heavy myelin. Morphological changes
can be attributed to changes in both the myelin membranes and to the
surrounding rim of cytoplasm. Thus chemical alterations in the myelin
subfractions can be correlated to morphological changes occuring during
EFA deficiency.

The occurrence of two axons within a single myelin sheath has not
been reported in rat optic nerve. Whether these splits are functional
is not known. When two axons appeared within a single myelin sheath one
axon is much smaller than the other. It is most probable that the
smaller axon is a bud extending from the larger axon as demonstrated in
figure 35. The exact relationship between the two axons within a single
myelin sheath may be revealed by serial sectioning such an area.

Several unusual relationships between the inner mesaxon and the
axon proper were obseeved in EFA deficient rats (Fig. 27-29). These
consist of evaginations of the inner mesaxon into the axon. The occurrence
of these evaginations into the axon are not observed in normal CNS or
have they been observed by other researchers who are actively engaged in
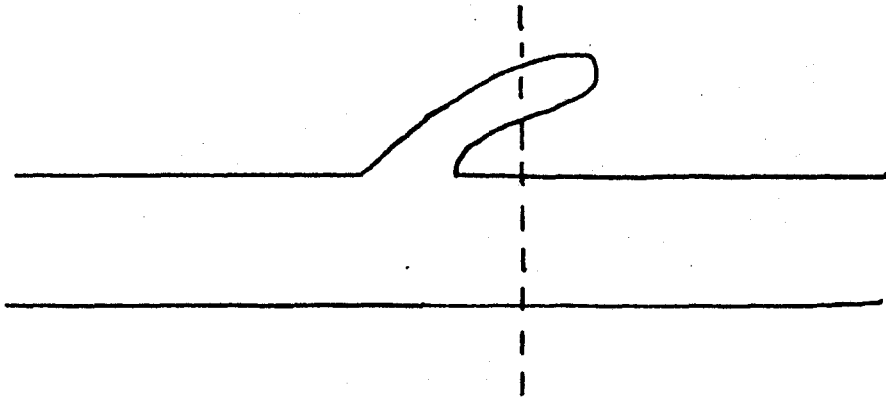myelin studies of the CNS (Hirano, 1976; Webster, 1976). These evagina-

Figure 35. A diagram to illustrate the occurrence of two axons within

a single myelin sheath. Dotted line represents plane of sectioning.

tions can result in the occurrence of isolated islands of cytoplasm within the axon (Fig. 27). The similarity between the density of the cytoplasm of the evagination with that of the inner mesaxon suggests that the two are continuous. One such island was found to be continuous with the inner mesaxon (Fig. 29). Some of these evaginations appear to surround portions of the axonal membrane (Figs. 27 and 29). The manner by which these evaginations form and the cause of their formation remains to be clarified. It cannot be determined if EFA deficiency has a direct effect on their formation. These evaginations could be the result of an active intrusion of the inner mesaxon into the axon. Another possibility is that the axons are somehow deformed or pitted and the oligodendrocyte plasma membrane follows the contours of the axon. The oligodendrocyte must be programmed to do so, expecially within a fibre tract which is completely myelinated such as the optic nerve. Thus these evaginations may be an axonal as well as a myelin abnormality.

Several axons contained concentric membranes which appeared to surround a vesicle which may be of axonal origin (Fig. 30-32). The origin of these membranes was not known and awaits clarification by serial sectioning. The membranes always occurred in multiples of two and surrounded vesicles similar to those surrounded by evaginations of the inner mesaxon. (Figs. 27 and 29). It is tempting to hypothesize some connection between these membranes and the evaginations of the inner mesaxon.

Biological membranes consist primarily of proteins and lipids and the proportions of these major constituents vary in membranes from different sources and are probably related to membrane function. The central nervous system (CNS) contains a high concentration of lipids with phospholipids constituting a major part of this component. Fatty acids play a significant role in the structure and function of phospholipids. Three important series of polyunsaturated fatty acids are present in the lipids of mammalian biological membranes. The oleic or (N-9)* fatty acid family are nonessential fatty acids and can be synthesized de novo by mammalian systems. The fatty acids of the linoleic (N-6) and linolenic (N-3) series are dietary essential fatty acids in mammals as they cannot be synthesized de novo since the mammalian organism has lost the ability to desaturate fatty acids from positions more distal than nine (9) carbon atoms from the carboxyl group.

Alterations in the fatty acid composition of membrane lipids, such as caused by essential fatty acid (EFA) deficiency can alter membrane function and/or structure. The present study was an attempt to define some of the biochemical and structural alterations in CNS membranes during EFA deficiency, with emphasis on myelin membranes.

*   This refers to the position of the first double bond proximal to the methyl end of the fatty acid chain.

Alterations in whole brain phospholipids fatty acids during differing states of EFA deficiency were determined. The reversibility of these fatty acid alterations was determined by supplementing the essential fatty acids, linoleic and linolenic acid individually, to 120 day-old EFA deficient rats. The response of whole brain phospholipids to EFA deficiency and subsequent supplementation was compared to that of liver phospholipids to determine if any responses were unique to brain.

A progressive decrease in the dietary essential fatty acids of the linoleic (N-6) and linolenic (N-3) acid families and an increase in the nonessential fatty acids of the oleic (N-9) acid series occurred during EFA deficiency. Linoleate and linolenate supplementation for 30 days returned the (N-6) and (N-3) fatty acids to control levels while the (N-9) fatty acids approached control values. The response of 22:5 (N-6) to EFA deficiency and subsequent supplementation differed from the remainder of the (N-6) fatty acids. The response of liver phospholipid fatty acids to EFA deficiency differed from that of the CNS.

The fatty acid composition of the ethanolamine phosphoglycerides (EPG) from subfractions of purified myelin was determined developmentally and in states of EFA deficiency.

The fatty acid composition of EPG's from subfractions of myelin from control animals differed developmentally. The monounsaturated fatty acids increased with age while the polyunsaturates decreased. The saturated fatty acids remained unchanged at the various ages studied. The fatty acids of EPG's from "light" and "heavy" myelin also differed

at various ages. The heavy subfraction tended to consist of a higher per cent of polyunsaturated fatty acids while the light fraction contained a higher per cent of monounsaturated fatty acids.

Alterations in the EPG fatty acids of myelin subfractions during EFA deficiency were detectable at 15 days and were similar to changes found in whole brain phospholipids except the increase in 22:5 (N-6) was not as apparent in the myelin fractions.

The morphological appearance of the optic nerve from 120 day-old control and EFA deficient rats were studied with light and electron microscopy.

Cross-sections of control and EFA deficient optic nerve at the light microscopic level were similar, except for the occurrence of a number of small vacuoles randomly dispersed throughout the EFA deficient nerves. Ultrastructurally no changes were found in glial cells, vascular components or the diameter of fibers within the optic nerve of EFA deficient rats. The vacuoles identified at the light microscopic level appeared as degenerating axons and intramyelinic splits at the electron microscopic level. Additional variations of the myelin sheath were observed in EFA deficient optic nerve. These consisted of formed organelles within the inner and outer mesaxon, isolated islands of cytoplasm within myelin sheaths and two concentric myelin sheaths surrounding a single axon.

The data appeared to demonstrate that alterations in the normal morphology of myelin could be demonstrated in EFA-deficient animals concomitantly with changes in fatty acid composition of phospholipids.

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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 Committtee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

___January 7, 1977___   _____
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