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The Metabolism of Linolenic Acid in Developing Rat Brain

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THE METABOLISM OF LINOLENIC ACID IN DEVELOPING RAT BRAIN

by

Barney Dwyer

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

May 1978

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VITA

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LIST OF ABBREVIATIONS

EFA	essential fatty acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
PI	phosphatidylinositol
SPH	sphingomyelin
CoA	coenzyme A
FAS	fatty acid synthetase
DHAP	dihydroxyacetone phosphate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
CTP	cytidine triphosphate
CDP	cytidine diphosphate
S.E.M.	standard error of the mean

INTRODUCTION

Linolenic acid (18:3n-3) is an essential fatty acid (EFA) in that it must be supplied in the diet. Tinoco et al. (1971) questioned the essentiality of linolenic acid for normal growth and reproduction in rats. Results from later studies suggest a dietary role for linolenic acid (Rivers and Davidson, 1973). Weanling mice from dams fed a safflower seed oil (linolenate poor) diet weighed 17% less and their metabolic rate was increased over mice from females fed a diet supplemented with soya bean and linseed oil (linolenate rich). Also, Fiennes et al. (1973) reported that capuchin monkeys which were fed diets containing adequate amounts of linoleic acid, but were deficient in linolenic acid, showed classical symptoms of essential fatty acid deficiency as were described by Holman (1968).

A possible functional role for dietary linolenic acid in development has been suggested by Lamptey and Walker (1976). Female rats were placed on linolenate poor diets (10% safflower oil) or linolenate rich diets (10% soybean oil). Measurement of physical development, onset of reflexogenic responses, or onset of neuromotor coordination was not influenced by the nature of the dietary fat. In mature progeny weaned to the diet of the dam, testing of discrimination learning in a Y-maze showed animals fed the soybean oil diet performed in a superior manner to those fed safflower oil.

Benolken et al. (1973) demonstrated that the fatty acid composition of rat photoreceptor membranes was altered by dietary

manipulation. A decrease in the concentration of docosahexaenoic acid (22:6n-3),* the major metabolic product of linolenic acid in brain, from 45 mole% to 19 mole% of ethanolamine phosphoglyceride fatty acids, was seen in second generation rats raised on a fat free diet. The fat free diet of the females was supplemented with 0.85% linoleate to facilitate breeding and lactation, and the pups were weaned to a completely fat free diet. In addition, a functional alteration was also observed in the component of the electroretinogram which is generated by the photoreceptors.

Bernsohn and Stephanides (1967) postulated a dietary deficiency of linolenic acid as a possible contributing factor to the onset of Multiple Sclerosis.

It was reported that rats on EFA deficient diets were more susceptible to Experimental Allergic Encephalomyelitis than controls (Clausen and Moller, 1967). Polyunsaturated fatty acids have also been reported to inhibit lymphocyte transformations and cell mediated response (Mertin and Hughes, 1975; Mertin, 1976). In these latter instances the specific roles, if any, for particular fatty acids were not described. Perhaps future studies will elucidate specific roles for linolenic acid and its metabolites by utilizing experimental design which will separate the roles played by the linolenic acid family of fatty acids from that of the linoleic acid (18:2n-6) family of fatty acids.

*Fatty acid nomenclature - number of carbon atoms in fatty acid chain: number of double bonds n-position of first double bond numbering from the methyl terminal of the fatty acid.

Docosahaenoic acid is particularly concentrated in nervous tissue. Brain lipid and fatty acid composition has been extensively reported in humans (O'Brien and Sampson, 1965; Svennerholm, 1968; Rouser and Yamamoto, 1969; and Sun, 1973), in rats (Biran and Bartley, 1961; and Kishimoto et al. 1969), and in mice (Sun and Horrocks, 1968; Sun and Yau, 1976a; and Sun and Horrocks, 1970). Linolenic acid, itself, is found in very low concentrations in brain. However, 22:6n-3 is a major fatty acid of the ethanolamine and serine phosphoglycerides of whole brain gray matter, microsomes from gray matter and white matter, and synaptic endings. In contrast, there are only very small amounts of 22:6n-3 in the choline phosphoglycerides of the aforementioned fractions or in any of the phosphoglycerides of myelin. This pattern is also seen in the ethanolamine, serine, and choline phosphoglycerides of retina (Anderson, 1970).

Unquestionably, long chain polyunsaturated fatty acids have a role in normal functioning of cell membranes and their considerable concentrations in excitable tissue point up the possibility of a specialized function in the central nervous system. While the metabolism of linoleic acid (18:2n-6) an essential fatty acid, has been described in the literature in considerable detail, the metabolism of linolenic acid has been largely neglected, possibly because the essentiality of linolenic acid has been questioned, though evidence to the contrary exists. Thus it appeared of interest to examine the metabolic transformations which linolenic acid undergoes in the brain during critical periods of development. At 21 days of age a rapid

accretion of membrane lipid is occurring in rat brain. It was also of interest to examine the metabolism of linolenic acid in brain of rats which were born of dams deprived of essential fatty acids in their diets. A special requirement for essential fatty acids in general and linolenic acid in particular during development might manifest itself in an altered pattern of metabolism of linolenic acid. This was our interest, to examine the metabolism of linolenic acid in the developing brain of normal and essential fatty acid deprived rats.

LITERATURE REVIEW

MEMBRANE STRUCTURE AND FUNCTION: Presumably the function of 22:6n-3 in membrane phospholipids is related to its structure. The 22-carbon chain contains six methylene interrupted double bonds all of which are in the cis configuration. The unsaturation of fatty acids of membrane phospholipids is the major determinant of membrane fluidity.

The current concept of membrane structure is that membranes are composed of a fluid lipid bilayer which forms a matrix for various protein components which are associated with the lipid surface, are imbedded to varying degrees in the lipid matrix, or extend entirely through it. This is the fluid-mosaic model of Singer and Nicolson (1972). The proteins in membranes are classified as peripheral (extrinsic), integral (intrinsic), or as a third intermediate fraction based on their solubility properties (Singer, 1974; Steck and Yu, 1973). Peripheral proteins can be dissociated from the membrane by mild treatment, e.g. use of solutions of high ionic strength and metal chelating agents. Integral proteins require hydrophobic bond breaking reagents such as detergents or organic solvents. The intermediate class of protein is removed by protein perturbants such as sodium hydroxide or lithium diiodosalicylate without disrupting the membrane core. Both the protein and lipid components of the membrane are relatively free to diffuse laterally in the plane of the membrane. Transverse diffusion (flip flop) of

lipid molecules from one half of the bilayer to the other has also been observed. The latter appears to be a relatively slow process (Cherry, 1976). There are some areas of the membrane that have a highly organized structure such as synapses and gap junctions. It is suggested that in these structures the proteins themselves form a highly ordered matrix with specific protein-protein interactions while lipids might exist in the interstices (Singer, 1974).

A more elaborate model of membrane structure was introduced by Bretscher (1972). Experiments using a reagent which was specific for labelling the amino group of ethanolamine or serine phosphoglycerides demonstrated that few lipid amino groups were labelled on intact red blood cells, but instead labelling was more effective when red blood cells were lysed and the red blood cell membranes (ghosts) were exposed to the reagent; presumably both sides of the membrane are accessible to the reagent. A second set of experiments utilizing a phospholipase relied on the fact that in ruminants there is very little phosphatidylcholine in the erythrocyte membrane, but instead the difference is made up with sphingomyelin. There was very little phospholipase activity noted when intact sheep erythrocytes were treated compared to when ghosts were presented to the enzyme. As a consequence of these experiments, Bretscher (1972) postulated that the structure of the lipid bilayer from erythrocytes, and other biological membranes as well, contained phosphatidylcholine and sphingomyelin on the external surface and phosphatidylserine and phosphatidylethanolamine on the internal surface.

Using an approach based on the solid angles formed by lipid molecules, Israelachvilli and Mitchell (1975) attempted to explain the lipid packing the membrane. Their calculations indicate that phosphatidylcholine (PC), phosphatidylserine (PS), and probably sphingomyelin (SPH) are tapered lipids, ie., their surface head group areas are greater than their non-polar end areas. On the other hand, cholesterol and possibly phosphatidylethanolamine (PE) are frayed, ie., the non-polar end areas are greater than the surface head group areas. This may explain why cholesterol is found with phosphatidylcholine and sphingomyelin on the exterior surface of the bilayer while phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (PI) are found on the interior surface of the bilayer of plasma membrane (Emmelot and Van Hoesven, 1975). To avoid packing strains the frayed and tapered lipids should be found together.

Having reviewed the basic lipid structure of biomembranes, we can now look at the question of fluidity, which, as was said before, is determined to a great extent by the nature of the fatty acyl groups of the phospholipids. The more unsaturated the fatty acid composition, the more fluid the membrane will be. Cholesterol in the membrane can have a dual effect. Below the transition temperature (T_c : the temperature where fatty acid chains "melt" and display rapid random motion) cholesterol will fluidize the membrane, while above the transition temperature cholesterol tends to inhibit fatty acid chain motion. Hence cholesterol confers upon the membrane an intermediate degree of fluidity. One example of this is the myelin

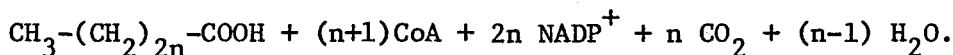
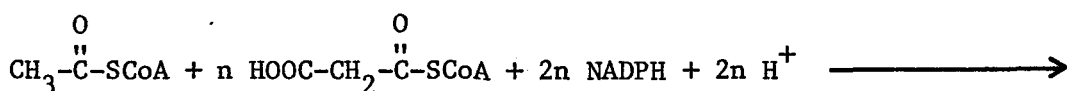
membrane for which no lipid thermal transitions are observed (Chapman, 1975). Thermal transitions are the result of heat absorption on melting of the fatty acyl chains in the membrane core. The interpretation for this result is that the cholesterol maintains the myelin in an intermediate state of fluidity at all temperatures. At body temperature, in the absence of cholesterol, myelin lipids can crystallize.

Chapman (1975) also discusses the possibility of phase separations of lipids in the membrane resulting in areas of the membrane being in the gel state while other areas are fluid. This has possible relevance to membrane functions such as transport mechanisms or regulation of membrane bound enzymes (Coleman, 1973; Farias et al. 1975).

The concept that there is an asymmetric distribution of phospholipids in membranes leads to the possibility of an asymmetry of fluidity of the membrane bilayers (Chapman, 1975; Emmelot and Van Hoeven, 1975). The fluidity of the inner lipid bilayer would be assured since the ethanolamine, serine, and inositol phosphoglycerides are the most unsaturated. Cholesterol has been suggested to be associated with the outer leaflet of lipid, and would impact fluidity to the fatty acids of sphingomyelin and phosphatidylcholine which are considerably less saturated than those of PE, PS, and PI (Emmelot and Van Hoeven, 1975). Indeed, an inverse relationship was demonstrated between the degree of unsaturation of fatty acids and the cholesterol content of the plasma membrane, the two factors possibly functioning to control the proper range of fluidity.

This concept opens up new avenues of approach for the study of enzyme mechanisms and regulation. Some vectorial enzymes such as $(\text{Na}^+ + \text{K}^+)$ ATPase and the hormone activated adenylyl cyclase span the entire membrane. They can be reactivated from delipidated membrane by phosphatidylserine or phosphatidylinositol in particular (Coleman, 1973). At a finer level of control, Farias et al. (1975) showed that the cooperativity (as measured by the Hill coefficient) of some membrane bound enzymes eg., $(\text{Na}^+ + \text{K}^+)$ ATPase and Acetylcholinesterase, was correlated with the fluidity of the membrane structure. It was suggested that the intact membrane structure itself could well be one of the modifying factors.

FATTY ACID METABOLISM: The stoichiometry of fatty acid synthesis is as follows:



This reaction has been demonstrated in the brain (Brady, 1960). Palmitate (16:0) is the major product of the reaction noted above and for which $n = 7$.

Acetyl CoA can be derived from β -oxidation of fatty acids, from the oxidative decarboxylation of pyruvate produced by glycolysis, and from the metabolism of certain amino acids. Malonyl CoA is formed by the action of acetyl CoA carboxylase (acetyl CoA ligase (ADP), EC 6.4.1.2).

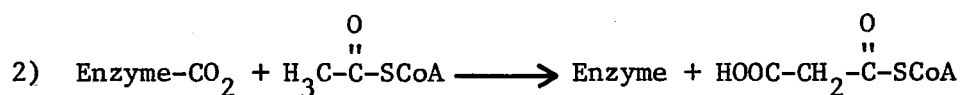
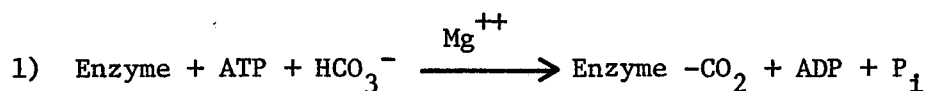
Nicotinamide adenine dinucleotide phosphate (reduced), NADPH, which provides the necessary reducing equivalents can arise from the oxidation of D-glucose 6-phosphate in the hexose monophosphate shunt, or via the combined effort of cytoplasmic malate dehydrogenase (L-malate:NAD-oxidoreductase EC 1.1.1.37) and malic enzyme (L-malate:NADP-oxidoreductase (decarboxylating) EC 1.1.1.40) (Porter et al. 1971). Lowenstein (1961) has also reported an extramitochondrial source of isocitrate dehydrogenase (L_s -isocitrate:NADP-oxidoreductase (decarboxylating) EC 1.1.1.42) in rat liver which he suggests is a source of NADPH.

Enzymes for the hexose monophosphate shunt are present in rat brain (Glock and McLean, 1954), though the catabolism of glucose via this pathway has been reported to be low (DiPietro and Weinhouse, 1959; Bloom, 1955; O'Neill et al. 1965). In addition, the levels of NADPH and $NADP^+$ was reported to be low while the ratio of $NADPH/NADP^+$ was greater than one (Glock and McLean, 1955). This further points to a low level of activity. Hostetler et al. (1970) estimated the maximum contribution of the pentose shunt to glucose metabolism at 5-8% in perfused monkey brain. Appel and Parrot (1970) reported that synaptosomes isolated from rat cerebral cortex showed pentose shunt activity. They suggested a possible role for the NADPH in maintaining the integrity of the synaptic membrane. In a developmental study, Baquer et al. (1973) reported an increase in glycolytic enzymes in rat brain, while the pentose shunt enzymes remained relatively constant. Hence, it is still unclear what role the hexose

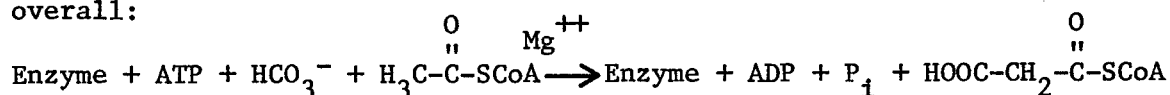
monophosphate shunt plays in brain during lipogenesis. Baquer (1973) reported on the developmental profiles of the NADP^+ dependent malic enzyme and isocitrate dehydrogenase from the soluble fraction of brain homogenate. The activity of the isocitrate dehydrogenase most closely parallels that of fatty acid synthetase which declines with development. It is likely that some interplay of these three pathways provide the necessary NADPH reducing equivalents for fatty acid biosynthesis in brain.

Two enzyme systems are responsible for de novo fatty acid synthesis: acetyl CoA carboxylase and the fatty acid synthetase complex (FAS), both of which are located in the cytoplasm of the cell.

Acetyl CoA carboxylase is a biotin containing enzyme which catalyzes the following reaction sequence:



overall:



Acetyl CoA carboxylase was initially characterized by Waite and Wakil (1962). The enzyme was purified from chicken liver. It was stimulated by citrate, isocitrate, and other Krebs' cycle intermediates. Gross and Warshaw (1974) have reported on acetyl CoA carboxylase activity in developing rat brain in vitro. The enzyme was maximally activated in the presence of Mg^{++} and citrate, and was markedly inhibited by

palmitoyl-CoA. The activity of the enzyme was maximal during the fetal period to 14 days post-natal. It decreased to about 50% of its maximum value in the adult.

All the acetyl CoA carboxylases studied have been shown to contain definable subunits (Volpe and Vagelos, 1976). The enzyme complex of *E. Coli* is readily dissociated into active subunits, three proteins in all. Biotin carboxylase catalyzes the carboxylation of the second protein, Biotin carboxyl carrier protein. The third protein, the transcarboxylase, catalyzes the reversible transfer of CO_2 from the biotin- CO_2 complex to acetyl-CoA forming in the process malonyl-CoA.

Fatty acid synthetase catalyzes the condensation of seven moles of malonyl-CoA with one mole of acetyl-CoA to form one mole of palmitic acid. The enzyme complex isolated from pigeon liver was initially characterized by Bressler and Wakil (1961). In a series of papers, the binding sites of acetyl-CoA and malonyl-CoA to the synthetase (Phillips et al. 1970), the binding of intermediates to the synthetase (Nixon et al. 1970) and a mechanism for fatty acid synthesis (Phillips et al. 1970) were described. Briefly, there are three binding sites: 1) 4'-phosphopantetheine site, 2) a site probably containing cysteine, and 3) a hydroxyl site in which either serine or threonine is responsible for binding. It is proposed that acetyl-CoA is initially bound to the hydroxyl site. By a series of nucleophilic displacements the acetyl moiety is transferred first to the 4'-phosphopantetheine site and then to the cysteine site, being

bound to these groups by a thioester bond. Malonyl-CoA can then bind to the hydroxyl site and be transferred to the 4'-phosphopantetheine site. Further reactions occur on the 4'-phosphopantetheine site. It was postulated that binding of acetyl-CoA in some way altered the synthetase so that thereafter malonyl-CoA would be preferentially bound. The residues condense with loss of CO_2 from the malonyl moiety. The resulting β -ketoacyl-4'-phosphopantetheine-Enzyme is reduced by NADPH to the β -hydroxyacyl-4'-phosphopantetheine-Enzyme which is then dehydrated to the α,β unsaturated acyl-4'-phosphopantetheine-Enzyme. This is then reduced by a second molecule of NADPH. The acyl moiety is transferred back to the cysteine residue enabling another molecule of malonyl-CoA to bind to the 4'-phosphopantetheine site thus initiating a new cycle of reactions. The synthetase complex has a deacylase activity which shows specificity for acyl chain lengths of sixteen and eighteen carbons (Dorsey, 1968). The free fatty acid is released.

Volpe and Kishimoto (1972) reported the developmental pattern of fatty acid synthetase from the brain and liver. In brain, the synthetase activity was greatest during the fetal period falling off gradually after birth. This was in stark contrast to the liver enzyme which had low activity during fetal development and the suckling period, but showed a striking increase in activity at weaning. The brain enzyme was more refractory to changes of diet and hormonal influence than was the liver enzyme.

Studies regarding the regulatory mechanisms for acetyl CoA

carboxylase and fatty acid synthetase are equivocal. Bortz and Lynen (1963) demonstrated the inhibition of a purified preparation of Acetyl CoA carboxylase from rat liver by long chain acyl-CoA derivatives. Gross and Warshaw (1974) reported a severe decrease in the activity of acetyl-CoA carboxylase and fatty acid synthetase in brain with increasing concentrations of palmityl-CoA. Jacobs and Majerus (1973) employing cultured human fibroblasts found that while palmitic acid or palmityl-CoA, complexed to albumin, did inhibit acetyl CoA carboxylase, the concentrations of the long chain acyl compounds remained unchanged. Citrate levels also remained unchanged. The authors didn't rule out the possibility that localized concentration changes could occur in the cells. Dorsey and Porter (1968) suggest that the inhibition of fatty acid synthetase is due to an irreversible detergent effect, and is observed, most likely, only in vitro. More recently, Flick and Bloch (1975) described the reversible inhibition of fatty acid synthetase from *M. smegmatis*. Palmityl-CoA dissociated the complex into inactive subunits. The binding could be prevented or reversed by fatty acyl-CoA complexing agents which occur in the organism. This system could operate as a negative feedback control of fatty acid synthetase.

A role for malonyl-CoA, the product of acetyl CoA carboxylase, in fatty acid synthesis has been proposed by Guynn et al. (1972). By studying the levels of various intracellular metabolites under different dietary conditions they demonstrated that with the exception of starved or fat-fed rats the availability of malonyl-CoA for fatty acid synthesis is rate limiting. They also suggested that acetyl

CoA carboxylase is regulated on a short term basis by long chain acyl-CoA. Malonyl-CoA has been reported to inhibit fatty acid synthetase, an effect which can be reversed by fructose 1,6-diphosphate (Plate et al. 1968). Wakil et al. (1966) have also reported the stimulation of fatty acid synthesis from acetyl-CoA and malonyl-CoA in crude extracts from pigeon liver by phosphorylated sugars. These results have been questioned, however (Gross and Warshaw, 1974).

It has been suggested that short term control over fatty acid synthesis may be mediated by a phosphorylation-dephosphorylation cycle of acetyl-CoA carboxylase (Inoue and Lowenstein, 1972; Carlson and Kim, 1973), as mediated by cyclic 3',5'-AMP (Bricker and Levy, 1972).

Adaptive changes in enzyme synthesis and degradation are likely to play a regulatory role in the long term control of fatty acid synthesis. Volpe et al. (1973) reported that changes in fatty acid synthetase activity in development of both brain and liver were due to changes in enzyme content. Responses to starvation and fat-free feeding in the liver enzyme were also shown to be due to enzyme content. Nakanishi and Numa (1970) reported that changes in the activity of acetyl CoA carboxylase from rat liver were due to changing quantities of carboxylase protein under varying dietary and hormonal conditions. These responses in enzyme content were also demonstrated by Volpe and Marasa (1975) in C6 glial cells grown in culture by varying the lipid content of the media, and in isolated liver cells from rat (Lakshmanan et al. 1975). Other reports indicate that these enzymes respond to dietary and hormonal influences, and that these

responses may vary between organs with the activity of the brain enzymes being relatively unaffected (Volpe and Kishimoto, 1972; Gross *et al.* 1975; Volpe and Vagelos, 1974).

The regulation of fatty acid synthesis has recently been reviewed by Volpe and Vagelos (1976). They indicate that control of acetyl CoA carboxylase while undergoing both short and long term control may be the rate limiting enzyme in short term control of fatty acid synthesis. Alternatively, they suggest that fatty acid synthetase is responsible for long term control.

The major product of the reaction sequence described above is palmitic acid (16:0) with lesser amounts of stearic acid (18:0) and myristic acid (14:0) being formed. The longer chain polyunsaturated fatty acids which are major components of tissue lipids are the result of the elongation and desaturation of fatty acids which are formed de novo or are derived from dietary sources. Amongst those derived from dietary sources are the essential fatty acids linoleic, linolenic, and arachidonic.

Fatty acid elongation had been initially described in rat liver mitochondria (Wakil *et al.* 1960; Harlan and Wakil, 1962) and microsomes (Lorch *et al.* 1963). The reaction sequence involves a reversal of the three steps of β -oxidation with the last step, the reduction of the α,β -unsaturated acyl-CoA carried out by a new enzyme, enoyl CoA reductase (Seubert and Podack, 1973). The enzymes catalyzing the first three steps, if not those for β -oxidation, are equivalent. Unlike de novo fatty acid synthesis where the acyl chain is bound

to the fatty acid synthetase complex, the form of the substrate for elongation is the acyl-CoA. The cycle adds a two carbon fragment to the existing acyl-CoA.

The enoyl-CoA reductase of microsomes and mitochondria from rat liver were separated and characterized by Podack and Seubart (1972). The enzymes showed different chain length specificities. The microsomal enoyl-CoA reductase showed maximal activity with hexenoyl-CoA as the substrate while the enzyme from mitochondria was more active with decenoyl-CoA. It appears that the microsomal chain elongation system shows a preference for unsaturated long-chain fatty acids which are present under physiological conditions (Seubert and Podack, 1973; Podack, 1971). Their results indicate that acyl carbon chains greater than C_6 are inhibitory. The physiological significance of this is that the polyunsaturated fatty acids such as linoleic, linolenic, and in particular α -linolenic ($18:3n-6$) with their chains "kinked" by the cis double bonds can approximate the structure of hexenoyl-CoA from the carboxyl end to the first double bond. This suggests that these fatty acids would be preferentially elongated by this system. This is an important physiological result. It appears that the microsomal chain elongation system is involved in biosynthetic reactions to form long-chain polyunsaturated fatty acids.

The mitochondrial system seems to play an altogether different role. Hinsch and Seubert (1975) demonstrated that both NADPH and NADH were required for optimal mitochondrial chain elongation activity in rat liver and pig-kidney cortex. Decenoyl-CoA (rat liver) or

octanoyl-CoA (pig-kidney cortex) showed optimal rates of elongation while the rates for palmitoyl-CoA or stearyl-CoA were greatly depressed. In addition, the CoA derivatives of linoleic and linolenic acids showed minimal activity. Thus the authors concluded that chain elongation of the polyunsaturated fatty acids is not a physiological function of the mitochondrial elongation system. They attributed experimental discrepancies in previous work to either contamination of mitochondrial fractions with microsomes or to the use of NADH as a hydrogen atom donor and the subsequent extraction of the 3-hydroxy fatty acid intermediate which accumulates when NADPH is not employed in the medium (Wakil, 1961; Barron, 1966; Quagliarello *et al.* 1968; Colli *et al.* 1969; Boone and Wakil, 1970; Mooney and Barron, 1970).

Several functions have been postulated for the mitochondrial chain elongation system (Whereat, 1971; Hinsch and Seubert, 1975; Seubert and Podack, 1973). In rat heart mitochondria conditions which caused an increase in the ratio of NADH/NAD^+ such as cytochrome chain inhibition or hypoxia caused increased synthesis of fatty acids, and in the process released NAD^+ which was available to generate ATP through substrate level phosphorylation. In this way the potential energy from substrate oxidation is retained in the fatty acids which can later be utilized. In addition, this system provides a means to shuttle reducing equivalents from the cell cytoplasm into the mitochondria. The NADH formed in the cytoplasm can be shuttled into the mitochondria via fatty acid synthesis at the outer mitochondrial membrane followed by transfer of the fatty acid into the mitochondria where the fatty acid can subsequently be oxidized to acetate and

NADH. The heart elongation system is unique in that it requires solely NADH as the pyridine nucleotide cofactor. In rat liver both NADH and NADPH are required for optimal activity. In the liver high ratios of NADH/NAD⁺ are not stimulatory indicating a different control mechanism may be at work. The system in liver could serve to transfer reducing equivalents from NADPH to the respiratory chain. The NADP⁺ could then be utilized in the oxidative deamination of glutamate which, in the liver, is NADP⁺ dependent. The α -keto glutarate thus produced is then available for transamination reactions to facilitate the flow of carbon atoms from amino acids to glucose (Tager and Papa, 1965).

The elongation systems of brain microsomes and mitochondria have been characterized by Aeberhard and Menkes (1968). They reported that malonyl-CoA was the donor of 2-carbon units in microsomes while either malonyl-CoA or acetyl-CoA could be utilized by mitochondria. The microsomal elongation system showed no preference for NADPH or NADH as a source of reducing equivalents, but NADH was preferred in the mitochondrial system. ATP was required for both. The microsomal system incorporated radioactivity into 18:0 (33%) and into 22:4 + 22:6 (60%). The percentages indicated are percentages of total radioactivity. The mitochondrial system synthesized 20:1 (38%), 18:0 (18%), and 22:4 + 22:6 (about 20%). The maximal activity in young rats was at 15-16 days of age, coincident with the period of rapid myelination in rat brain (Aeberhard *et al.* 1969). The microsomal system demonstrated increased production of saturated fatty

acids at 15-16 days of age while polyunsaturated fatty acid synthesis was relatively unchanged.

Boone and Wakil (1970) showed that rat brain mitochondria could elongate behenyl-CoA (22:0) and erucyl-CoA (22:1) to lignoceric acid (24:0) and nervonic acid (24:1) respectively. Both of these C-24 fatty acids are found in high concentrations in the sphingolipids of white matter and myelin. Hinsch and Seubert (1975) have suggested the mitochondrial preparations of the above authors may have been contaminated by microsomes.

Yatsu and Moss (1971) studied a mitochondrial elongation system for fatty acids during development in rat brain. In agreement with Aeberhard et al. (1969) they found maximal incorporation of acetate at 15 days of age into the 22:4 fatty acid. They also found a three-fold increase in 20:1 which the other authors didn't find. They suggested that the 20:1 could be a precursor of nervonic acid.

Fatty acid chain elongation has also been demonstrated using [$1-^{14}\text{C}$] Malonyl-CoA in the synaptosomes isolated from brain (Koeppen et al. 1973). Docosatetraenoic acid (22:4n-6) contained the most radioactivity. The fatty acid label appeared primarily in the choline phosphoglycerides. About 80% of the radioactivity was found associated with the intraterminal mitochondrial fraction while 20% was in the synaptosomal membrane. Synaptic vesicles weren't labeled.

To further complicate the picture, it has been suggested that multiple elongation systems exist in the microsomal fraction of brain (Goldberg et al. 1973) and liver (Sprecher, 1974).

In addition to chain elongation reactions, fatty acids also undergo desaturation reactions to form double bonds in the carbon chain. The oxidative desaturation of fatty acids has been reviewed by Brenner (1974).

Stritmayer et al. (1974) have reported the purification and properties of the stearyl desaturase from rat liver (a $\Delta 9$ desaturase: 18:0 $\Delta 9$ -18:1). (Δ indicates the position of the first double bond numbering from the carboxyl end of the fatty acid molecule). It is a single polypeptide, 53,000 daltons, containing 62% non-polar amino acid residues, and one atom of non-heme iron. Desaturase activity requires NADH although NADPH can be employed, molecular oxygen, stearyl-CoA, lipid, cytochrome b_5 reductase (EC 1.6.2.2; NADH:ferricytochrome b_5 oxidoreductase), cytochrome b_5 , and desaturase. The cytochrome b_5 is the direct electron donor to the desaturase which uses iron in the oxidation-reduction process. Jones et al. (1969) first demonstrated the lipid requirement for the desaturation of stearyl-CoA by hen liver microsomes. Oshino and Sato (1972) demonstrated the desaturase activity of rat liver could be modified by diet. Brenner and Peluffo (1969) have demonstrated that the desaturation of linoleic acid to γ -linolenic acid is dependent on molecular oxygen and is inhibited by cyanide. It was concluded that a similar electron transport chain functions with the different desaturases (Brenner, 1974). Recently, Catala et al. (1975) separated a protein factor which could be extracted from microsomes by low ionic strength solutions. This factor was needed for full activity of both the $\Delta 9$ desaturase and the $\Delta 6$

desaturase of their system. It, in itself, had no desaturase activity.

The $\Delta 9$ desaturase activity was initially described in rat liver homogenate and microsomes by Marsh and James (1962). This work was corroborated by Holloway et al. (1963) who also described the conversion of palmitic acid to plamitoleic acid ($16:0 \longrightarrow \Delta 9-16:1$). In addition they reported a $\Delta 6$ desaturase activity ($\Delta 9-18:1 \longrightarrow \Delta 6,9-18:2$). They also reported their preparations would not convert oleic acid to linoleic acid ($\Delta 9-18:1 \longrightarrow \Delta 9,12-18:2$). This inability of mammalian systems to desaturate fatty acids beyond the $\Delta 9$ carbon is the reason why linoleic, linolenic, and arachidonic acids must be supplied in the diet.

A number of factors are involved in regulating the $\Delta 9$ desaturase. The activity of the enzyme is depressed by starvation (Elovson, 1965; DeTomas et al. 1975). Refeeding of a control diet stimulates $\Delta 9$ desaturase activity to about three times that of control, while refeeding a high carbohydrate diet stimulates desaturase activity about five times that of control. The animals in this study were fasted 48 hours and then refed the various diets for a period of 24 hours. This effect of refeeding has been observed by others (Oshino and Sato, 1972; Inkpen et al. 1969). Refeeding a high protein diet to rats which had been starved for 48 hours brought the $\Delta 9$ desaturase activity back to control values (DeTomas et al. 1975). Mercuri et al. (1974) reported that dietary fructose, glycerol, or saturated fatty acids ($16:0$ and $18:0$) stimulated the $\Delta 9$ desaturase activity. They suggest this stimulation is due to adaptive synthesis of the $\Delta 9$

desaturase effected by the exogenous fatty acids or the endogenous fatty acids formed de novo. It was observed that the three test diets stimulated incorporation of ^3H -acetate into fatty acids. Glucose has also been reported to stimulate the $\Delta 9$ desaturase activity converting stearic acid to oleic acid (Brenner, 1974). A stimulation of activity five times above control values were observed in rats that had been starved 48 hours and then refed a high glucose diet (Inkpen et al. 1969). Rats fed a fat-free diet have 2.5 times greater desaturase activity than rats fed a control diet (Paulsrud et al. 1970).

The $\Delta 9$ desaturase activity was also found to be depressed in alloxan-diabetic rats (Gellhorn and Benjamin, 1964; DeTomas et al. 1973). It was reported that the difficulty was corrected by insulin administration.

Studies have been reported on the inhibition of $\Delta 9$ desaturation by other fatty acyl-CoA molecules. Brenner and Peluffo (1966) reported that the $\Delta 9$ desaturation of stearyl-CoA or of palmityl-CoA was unaffected by other fatty acids, including 20:4n-6 and 18:2n-6. In contrast to this, Ullman and Sprecher (1971) demonstrated that 18:2n-6, 20:3n-6, and 20:4n-6 all inhibited the desaturation of stearic acid. They suggested that the discrepancy in the results could be explained by the level of substrate used by Brenner and Peluffo being less than required for maximal conversion by their system.

The $\Delta 9$ desaturations of stearyl-CoA and palmityl-CoA have been described in brain slices (Seng and Debuch, 1975), homogenate (Cook and Spence, 1973a; b; 1974) and brain microsomes (Pullarkat and Reha, 1975). The brain and liver enzymes showed developmental differences

Cook and Spence, 1973b). The desaturase activity of 10 day old brain from rat was greater than that in liver. Brain desaturase activity was maximal during fetal life declining after birth to about 10% of the maximal values in the adult. In contrast, the liver enzyme activity was low during fetal life and the suckling period, but it increased dramatically at weaning. This pattern is similar to that found for fatty acid synthetase by Volpe and Kishimoto (1972).

Stearyl-CoA desaturase from rat brain microsomes was studied by Pullarkat and Reha (1975). The specific activity of the enzyme decreased to one third of its value at 8 days by 60 days, but the total activity per brain remained constant. The regional distribution pattern showed activity was in the order midbrain>medulla oblongata>cerebral hemispheres>cerebellum with the midbrain activity being about twice that in the cerebellum. The same developmental patterns in brain and liver were also observed by Seng and Debuch (1975).

Cook and Spence (1973b) reported that rats fasted for sixty hours had virtually no Δ^9 desaturase activity in the liver homogenate of the adult rat, while in ten day old rats it was markedly decreased. Sixty hours of fasting only slightly decreased the Δ^9 desaturase activity of rat brain homogenate from 10 day old rat and adult. Re-feeding the control diet for 48 hours stimulated desaturase activity particularly in the livers of adult animals. Results for brain from 10 day old and adult rat, and for liver of the 10 day old rat were varied. High concentrations of fatty acid were found to be inhibitory (Cook and Spence, 1973a).

In addition to the Δ^9 desaturase, there is also a Δ^6 desaturase which introduces a double bond between carbons 6 and 7 of the fatty acyl-CoA chain. This is the first metabolic step for the conversion of oleic, linoleic, and linolenic acids to their longer chain polyunsaturated metabolic products. Evidence has been presented which suggests the Δ^9 and Δ^6 desaturases are different enzymes (Brenner and Peluffo, 1966; Inkpen *et al.* 1969). This notion evolved from the observations that the desaturase activity of the two positions responded in different ways to different dietary challenges. The Δ^6 desaturase is considered the principal regulatory step in the biosynthesis of long chain polyunsaturated fatty acids in rat liver microsomes (Brenner, 1974).

A number of interactions between fatty acids have been reported. Brenner and Peluffo (1966) reported that saturated fatty acids 16:0 and 18:0 had no effect on the desaturase, while oleic, linoleic, and linolenic acids showed competitive inhibition, the order of inhibition being linolenic acid > linoleic acid > oleic acid. Additionally, the percent conversion of substrate to product increased with increasing unsaturation. Brenner (1971) demonstrated that elaidic acid (trans Δ^9 -18:1) and all trans linoleic acid weren't desaturated by this system.

The conversion of linoleic acid to γ -linolenic acid was stimulated by arachidonic acid (Nervi *et al.* 1968). The authors hypothesized that arachidonic acid competes with linoleic acid for esterification into lipids hence freeing more linoleyl-CoA for the desaturation reaction. They suggested this to be a possible means of regula-

tion. A later report from the same laboratory (Brenner et al. 1969) indicated that arachidonyl-CoA competitively inhibits the desaturation of linoleyl-CoA to γ -linolenyl-CoA. They also showed that the desaturation was also inhibited by α -linolenyl-CoA (18:3n-3) and by the product, γ -linolenyl-CoA. It thus appears that product inhibition may play a role in regulation of the desaturase.

The desaturated product of γ -linolenyl-CoA, 8,11,14-eicosatrienoic acid (20:3n-6) also inhibits the desaturation of linoleic acid (Brenner, 1969). He also reported that increasing the substrate concentration also inhibited the desaturation. The end product of linolenic acid metabolism, 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3) has been shown to inhibit the desaturation of linoleic acid to γ -linolenic acid and of α -linolenic acid to Δ 6,9,12,15-octadecatetraenoic acid (18:4n-3) (Brenner and Peluffo, 1967). Thus the possibility of a negative feedback mechanism for the regulation of the desaturase exists. Actis et al. (1970) demonstrated the inhibition of linoleic acid and α -linolenic acid desaturation by Δ 4,7,10,13,16-docosapentaenoic acid (22:5n-6) and Δ 7,10,13,16,19-docosahexaenoic acid (22:6n-3). Both desaturation reactions were inhibited by 22:5n-6 and 22:6n-3, but the 22:6n-3 inhibited the desaturation of α -linolenic acid to a greater extent than did the 22:5n-6.

Brenner and Peluffo (1969) studied the effect of 18 carbon fatty acids on the desaturation of linoleic acid to γ -linolenic. They found that all cis-linoleic acid, all trans-linoleic acid, γ -linolenic acid, α -linolenic acid, oleic acid, petroselinic acid

(cis $\Delta 6$ -octadecenoic acid), vaccenic acid (cis $\Delta 11$ -octadecenoic acid), and elaidic acid (trans $\Delta 9$ -octadecenoic acid) all were inhibitory.

To summarize then, possible mechanisms for the short term control of the $\Delta 6$ desaturase activity have been proposed. They are substrate and product inhibition, inhibition by intermediates in the metabolic pathway of polyunsaturated fatty acids, feedback inhibition by the end products of metabolism, and competition between the desaturation reaction and the transacylation of fatty acids into glycerolipids. In addition, long term regulation of the desaturase may be regulated via dietary control and hormonal influences (Brenner, 1974).

A $\Delta 5$ desaturase has been described in the microsomal fraction from rat liver (Brenner, 1969; Peluffo et al. 1970). The percent conversion of substrate 8,11,14-eicosatrienoic acid ($\Delta 5$ desaturation) to product was greater than that for linoleic acid by the $\Delta 6$ desaturase. Inhibition studies by Ullman and Sprecher (1971) suggest the possibility that the $\Delta 5$ desaturase is different from the $\Delta 6$ desaturase. This possibility was also suggested by the data of Castuma et al. (1972).

The $\Delta 4$ desaturase for fatty acids of 22 carbons appears to function since radioactive 22:5n-6 can be isolated after in vivo injection of (^{14}C) linoleate into testicles of rats. However, the direct conversion of 22:4n-6 \longrightarrow 22:5n-6 was not observed in vitro in desaturating microsomal systems from liver and testicles of rat (Ayala et al. 1973).

The initial elongation of linoleic, linolenic, or oleic acid and the subsequent desaturation would require the presence of a $\Delta 8$

desaturase. This enzyme is absent from liver (Sprecher and Lee, 1975) and brain (Dhopeswarkar and Subramanian, 1976a;b), and hence these alternate pathways are not operational. A general scheme for the elongation and desaturation of fatty acids is presented in Fig 1.

PHOSPHOLIPID METABOLISM:

The major brain phospholipids (including their plasmalogen forms) are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), with lesser amounts of phosphatidylinositol (PE), sphingomyelin, and small amounts of lysophosphatides, phosphatidic acid, cardiolipin, and the di- and tri-phosphoinositides.

Phosphatidic acid (1,2-diacyl-sn-glycero 3-phosphate), a key intermediate in phospholipid biosynthesis can arise in situ in a number of ways. There is the two step acylation of sn-glycero 3-phosphate (Husbands and Lands, 1970; Martensson and Kanfer, 1968). A glycerol kinase and dihydroxyacetone kinase activity has recently been reported in the brain (Jenkins and Hajra, 1976). Lapetina and Hawthorne (1971) have reported a diglyceride kinase activity in brain. Monoglycerides have been shown to be phosphorylated to lysophosphatidic acid (Pieringer and Hokin, 1962a), and the lysophosphatidic acid subsequently acylated to phosphatidic acid (Pieringer and Hokin, 1962b; Webster, 1965).

It has been suggested that dihydroxyacetone phosphate (DHAP) can be converted to phosphatidic acid (Agranoff and Hajra, 1971). Hajra and Agranoff (1968) reported the enzymatic reduction of acyl DHAP by guinea pig mitochondria utilizing NADPH as a cofactor. These

Linolenic Acid Series (n-3)

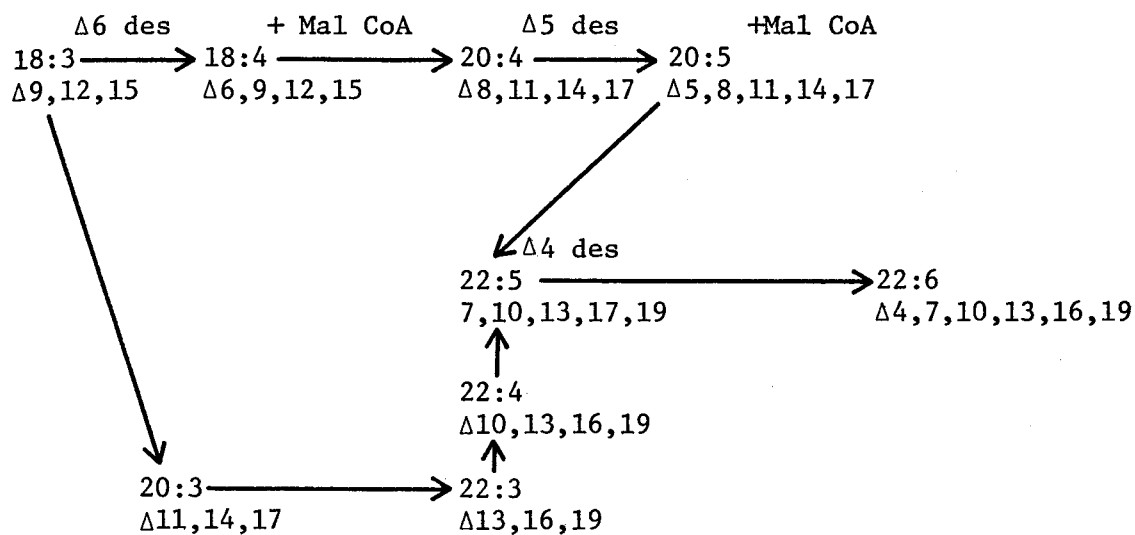


FIG. 1: Metabolic Pathways for the (n-3), (n-6), and (n-9) Fatty Acid Families.

results were disputed by Okuyama and Lands (1970). It was shown later (Manning and Brindley, 1972) that a tritium (^3H) isotope effect was responsible for the results of Okuyama and Lands (1970). Manning and Brindley (1972) estimated that as much as 50% of the glycerolipid backbone arises via DHAP. Recently Pollack et al. (1975) demonstrated that the DHAP pathway was active in addition to the acylation of glycerol 3-phosphate in cultured fibroblasts.

The acylation reactions involved in the formation of phosphatidic acid have been investigated. Particular attention has been paid to their specificity since tissue glycerolipids display an asymmetrical distribution of their fatty acyl groups.

The work of Yamashita and Numa (1972) and Yamashita et al. (1972) demonstrate that there are two different enzymatic acylating activities in rat liver microsomes. One catalyzes the formation of 1-acyl-sn-glycerol 3-phosphate from sn-glycerol 3-phosphate and palmitoyl-CoA while the second enzyme catalyzes the formation of phosphatidic acid. Specificities for either enzyme weren't clearly shown. This lack of specificity was also reported by Sanchez de Jimenez and Cleland (1969), Tamai and Lands (1974), and Okuyama and Lands (1972). Furthermore, no preference for saturated acyl-CoA was shown in the acylation of 2-acyl-sn-glycerol 3-phosphate which occurs at 1/10 to 1/5 the rate of the acylation of the 1-acyl isomer (Okuyama et al. (1971). Yamashita et al. (1973) did report a specificity for mono and dienoic fatty acyl-CoA by the 1-acyl glycerophosphate acyltransferase and for saturated fatty acyl-CoA by the 2-acyl glycerophosphate acyltransferase in rat liver microsomes. This result supported the

conclusions of Possmayer et al. (1969).

A mitochondrial acylation system was described for which 1-palmityl-sn-glycero 3-phosphate was the major product Daae (1972). Monroy et al. (1972) described an acyl transferase system from the outer mitochondrial membrane of rat liver whose products were mono and diacyl glycerophosphate. Palmitic acid was found esterified exclusively to the one position whereas linoleyl-CoA competed effectively with palmityl-CoA in the acylation of 1-palmityl-sn-glycero 3-phosphate. Monroy et al. (1973) reported the partial purification of a substrate and position specific acyl-CoA: sn-glycero 3-phosphate acyltransferase from rat liver mitochondria. All the sn-glycero 3-phosphate acylated in the presence of palmityl-CoA was identified as the 1-acyl isomer.

Intact tissue studies demonstrated a fair degree of selectivity for the transferase reaction. In contrast to the relatively unspecific acylations in their rat liver microsomal preparations, Hill et al. (1968) reported that rat liver slices showed a high degree of specificity. Over 75% of the phosphatidic acid produced was of the monoene and diene type with saturated fatty acids at position one and the unsaturated fatty acids at position two. Akesson et al. (1970a) demonstrated that after (^3H) glycerol was injected intraportally, most of the radioactivity was present in the saturated-monoene and saturated-diene fractions. Additionally, after the intraportal injection of ($9,10\text{-}^3\text{H}_2$) palmitic acid (Akesson et al. 1970b) or of ($1\text{-}^{14}\text{C}$) linoleic acid (Akesson, 1970) the palmitic acid radioactivity was found almost

exclusively at the one position while linoleic acid radioactivity was found almost exclusively at the two position. Baker and Thompson (1972) demonstrated selectivity in the incorporation of fatty acids into phosphatidic acid in the brain. In general, saturates were incorporated into position one, arachidonic acid into position two, and oleic and linoleic were about equally divided between the two positions. It appears possible that some control mechanism present in intact tissue is made inoperable in cell free preparations. Cytidine nucleotides have been suggested to play a regulatory role (Possmayer and Mudd, 1971; Possmayer et al. (1973).

Phosphatidic acid can be hydrolyzed by phosphatidate phosphohydrolase (EC 3.1.3.4) to sn-1,2-diacylglycerol and inorganic phosphate. Phosphatidic acid and the 1,2-diacylglycerol are precursors for triglyceride and glycerophosphatide biosynthesis. The enzyme has been studied in brain (McCaman et al. (1965). It appears there is no selectivity toward the acyl groups by the hydrolase (Akesson et al. 1970; Hill et al. 1968).

The resultant 1,2-diglyceride can react with cytidine diphosphate (CDP) choline or CDP-ethanolamine to form the major phospholipids. Studies on the CDP-choline: 1,2-diglyceride choline phosphotransferase (EC 2.7.8.2) by McCaman and Cook (1966) and the CDP-ethanolamine: 1,2-diglyceride ethanolaminephosphotransferase (EC 2.7.8.1) by Ansell and Metcalfe (1971) have been reported. In vitro studies with rat liver microsomes indicate there is no specificity for the enzyme with regards to the fatty acyl portion of the 1,2-diglyceride

(DeKruyf et al. 1970; Mudd et al. 1969), except possibly for the ethanolamine phosphotransferase which shows a slight preference for hexaenoic species of diglyceride (Kano, 1970). In vivo studies indicate that specificity for the fatty acids of the diglyceride could be exerted at the level of the phosphotransferase reaction (Sundler, 1973; Holub and Kuksis, 1971; Arvidson, 1968).

The biosynthesis of phosphatidylserine in animal tissues appears to occur via a Ca^{++} stimulated, energy independent base exchange process only (Porcellati et al. 1971). By this process serine, choline, and ethanolamine may be incorporated into phospholipids by exchange of the base portion of the molecule. There is no net synthesis of phospholipid by this process. The system has been reported to be active in brain, particularly in the microsomal fraction (Porcellati et al. 1971; Kanfer, 1972; Gaiti et al. 1974). The solubilization and properties of an enzyme from rat brain catalyzing the base exchange reaction was reported by Saito and Kanfer (1973). They reported that ethanolamine phosphoglycerides when added to the incubation medium were more stimulatory than either serine or choline phosphoglycerides. Dioleyoyl EPG was more stimulatory than the distearoyl species. This effect was also seen in the liver. The incorporation of choline into phosphatidylcholine subspecies (Bjerve, 1971) and ethanolamine into phosphatidylethanolamine subspecies (Bjerve, 1973) was greater for unsaturated species, in particular, the hexaenoic subspecies. Studies in the liver concluded that the base exchange reaction was relatively unimportant for the synthesis of phosphatidyl-

choline or phosphatidylethanolamine (Nagley and Hallinan, 1968; Salerno and Beeler, 1973). Bjerve (1973) estimated that the complement of phosphatidylserine in the liver could in total be accounted for by the base exchange reaction.

The inositol phosphoglycerides are formed via a CDP-diglyceride intermediate which then reacts with inositol (Possmayer and Strickland, 1967; Benjamins and Agranoff, 1969; Bishop and Strickland, 1970). It was observed that the CDP-diglycerides with unsaturated fatty acids were preferred in the transferase reaction. It might be mentioned in passing that the majority of the phosphatidylinositol molecules have stearic acid esterified in the one position and arachidonic acid esterified in the two position.

Plasmalogens, primarily the ethanolamine and serine species, are found in the brain with particular enrichment in the myelin fractions (O'Brien and Sampson, 1965; Sun and Horrocks, 1968). Structurally they differ from other phospholipids in that they have an alkenyl ether linkage at the one position. On acid hydrolysis the ether link is broken and the aldehyde is released. The fatty aldehydes of the ethanolamine and serine plasmalogens are primarily the 16 carbon and the 18 carbon saturated or monounsaturated species. The metabolism of plasmalogens has been extensively reviewed by Horrocks (1972).

The enzymatic synthesis of sphingomyelin from CDP-choline and N-acylsphingosine was demonstrated by Sribney and Kennedy (1958). Sphingolipid metabolism has been thoroughly reviewed (Morell and

Brown, 1972; Stoffell, 1973). Sphingomyelin contains primarily saturated and monounsaturated fatty acids with a considerable amount of the α -hydroxy fatty acids. This fraction contains a very small amount of the polyunsaturated fatty acids.

The fatty acyl portions of intact phospholipid molecules can be tailored by deacylation-reacylation cycles. The enzymatic acylation of lysolecithin was first demonstrated by Lands (1960). Esterification of saturated fatty acids at the one position and unsaturated fatty acids at the two position was preferred for the acylation of both acyl glycerophosphorylcholine (Lands and Merkl, 1963) and acyl glycerophosphorylethanolamine (Merkl and Lands, 1963). Experiments conducted with *cis* and *trans* isomers indicated that acyl transfer to the one position is sensitive to configurational differences while acyl transfer to the two position is not, but instead prefers π bonds at positions $\Delta 9$, $\Delta 12$, and $\Delta 5$ (Okuyama *et al.* 1972).

It was reported that stearate and arachidonate were incorporated into phosphatidylcholine of liver *in vivo* (Elovson, 1965) and *in vitro* (Van Golde *et al.* 1969) via acylation of endogenous lysolecithins, while 1-palmityl-2-oleoyl and 1-palmityl-2-linoleyl-phosphatidylcholines were synthesized via the CDP-choline pathway *in vivo* (Arvidson, 1968; Trehella and Collins, 1969). Yamashita *et al.* (1973) reported that their partially purified 1-acylglycerophosphorylcholine acyltransferase is highly specific for arachidonyl-CoA.

Hexaenoic species of phosphatidyl choline appear to arise from the N-methylation of hexaenoic species of phosphatidyl ethanolamine

(Rytter et al. 1968; Arvidson, 1968). Lyman et al. (1975) reported that the relative affinities for the incorporation of labeled methyl groups into phosphatidylcholine subspecies was of the order 22:6 > 20:4 > 18:2. This N-methylation reaction is thought not to occur in brain (Ansell and Spanner, 1971) though recently limited activity has been reported (Morganstern and Abdel-Latif, 1974).

In contrast to phosphatidylcholine, the hexaenoic species of phosphatidylethanolamine are formed via de novo synthesis utilizing CDP-ethanolamine (Kanoh et al. 1969; Arvidson, 1968; Akesson et al. 1970b; Vereyken et al. 1972), while the acylation-deacylation cycle is responsible for forming the arachidonic acid containing species of PE (Van Golde et al. 1969; Kanoh, 1969; Vereyken, 1969; De Tomas and Brenner, 1970). Baker and Thompson (1972) suggest that in brain, arachidonic acid appeared to be esterified via acyltransferase reactions while stearate was incorporated probably by both acyltransferase and de novo synthesis.

ISOLATED CELLS: In the past ten years a number of methods have been published for the bulk isolation of brain cells (Satake and Abe, 1966; Rose, 1967; Blomstrand and Hamberger, 1969; Fewster et al. 1967; Sellinger et al. 1971; Norton and Poduslo, 1970; Poduslo and Norton, 1972). The morphology of the isolated cells obtained from the method of Norton and Poduslo (1970) and Poduslo and Norton (1972) has been described (Raine et al. 1971; Trapp et al. 1975) though extensive morphological investigation has been hindered due to the fragile nature of the cells following the isolation procedures.

Metabolic studies have been carried out on these cells both in vitro and in vivo. Protein synthesis appears to be more active in the neuronal fraction than in the glia (Blomstrand and Hamberger, 1969; 1970; Rose, 1968; Rose and Sinha, 1974; Blomstrand et al. 1975). As might be expected, RNA metabolism was reported to be more active in the neuronal fraction compared to glia (Flangas and Bowman, 1970; Jarlstedt and Hamberger, 1971; and Yanagihara, 1974).

Lipid metabolism has also been investigated in vitro and in vivo. Sterol formation from mevalonate, acetate, and glucose in 11 day old rats was greater in the glial fraction than the neurons (Jones et al. 1971; 1975). The synthesis of monogalactosyl diglyceride was greater in the oligodendroglia compared to the neuronal soma and astroglia (Deshmukh, 1974). Kohlschutter and Hershkowitz (1973) reported that sulfatide synthesis measured by the incorporation of $^{35}\text{SO}_4$ in neurons isolated from the brains of "jimpy" mice was only 14% of control values in vivo and 24% of controls in vitro. Benjamins et al. (1974) reported that cerebroside sulfotransferase activity was enriched in white matter and in the oligodendroglial fraction from calf brain compared to the grey matter and neuronal fraction. The incorporation of (3- ^{14}C) serine into gangliosides was greater in the neuronal fraction of 11 day old rat than in the glia (Jones et al. 1972). The base exchange reaction has been reported active in the isolated cells (Raghavan et al. 1972; Goracci et al. 1973). Fatty acid incorporation into the isolated cells has been investigated (Cohen, PhD thesis, Loyola University of Chicago, Ill.). The

synthesis of phosphatidylethanolamine and ethanolamine plasmalogen has been studied in vitro and in vivo (Goracci et al. 1975; Binaglia et al. 1974; Roberti et al. 1975). It appears the neurons are more active than the glia. Freysz et al. (1969) using ^{32}P reported that the rate of turnover of neuronal phospholipids was greater than that of glial phospholipids. The order of individual phosphatides was PI>PC, PE, PS.

Interpretation of results must be undertaken with some caution. For in vitro metabolic studies, the cells are traumatized considerably during the isolation procedure, and sometimes are exposed to proteolytic enzymes which could effect their activity (Cohen and Bernsohn, 1974; Guarnieri et al. 1976).

MATERIALS AND METHODS

Animals: Sprague-Dawley rats of either sex were purchased from Locke-Erikson Laboratories (Melrose Park, Ill.). For experiments involving rats on essential fatty acid deficient diets, timed pregnant female rats were purchased, and were maintained on "Fat Free" Test Diet (ICN Nutritional Biochemicals, Cleveland, Ohio) from the 10th day of pregnancy. The composition of this diet which was formulated per Wooley and Sebrell (1945) is given in Fig. 2. Only pups from litters of 9-11 animals were used. In studies involving rats on the fat free regimen, pups were taken from the dams at 21 days of age for injection, and were then returned to metabolic cages where they were given the fat free diet and water ad libitum until the time of sacrifice. All other animals were maintained on a standard laboratory rat chow diet. For whole brain studies, at least four rats were injected for each determination. For studies involving subcellular fractions 3 brains were pooled for each determination. The number of determinations to obtain the final values are given in the appropriate tables.

Preparation of tracer and injection: Linolenic acid ($1-^{14}\text{C}$) (specific activity 60 mCi/mmol) was purchased from Amersham Searle (Arlington Heights, Ill.). Purity was assessed at close to 100% by gas liquid chromatography. Linolenic acid was prepared as the potassium salt, and was suspended in a 10% solution of bovine serum albumin

COMPOSITION

"Vitamin Free" Casein	21.10%
Alphacel "Cellulose"	16.45%
Sucrose	58.45%
Salt Mixture U.S.P. XIV	4.00%

Plus the following Vitamin Supplements:

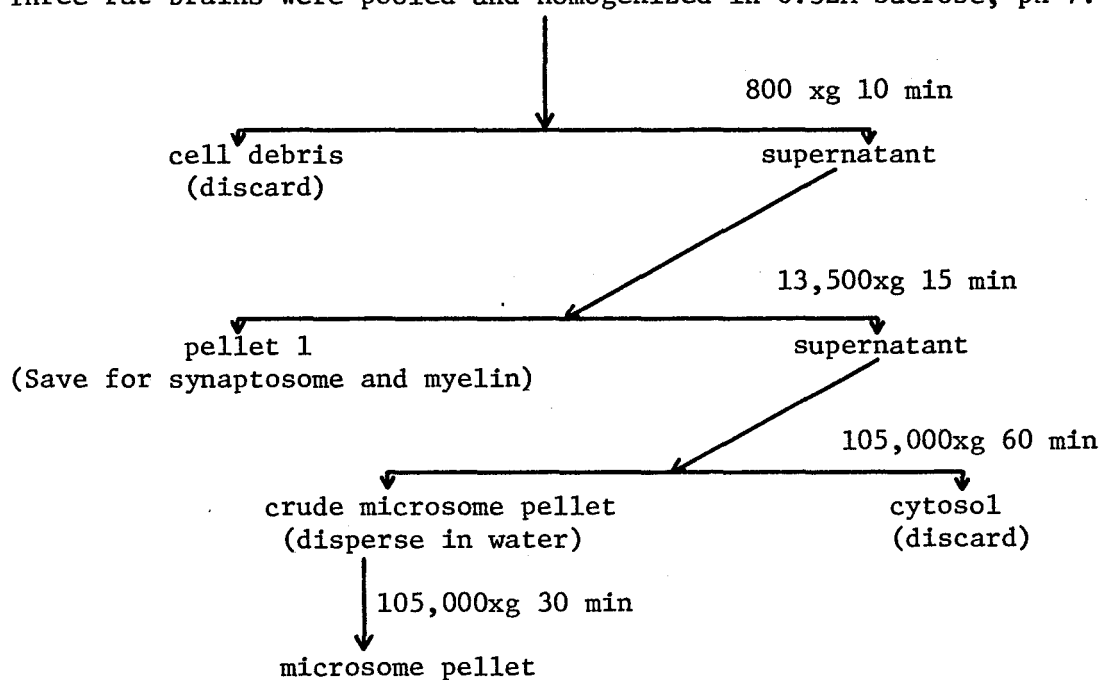
	Grams per 100 lbs.
Choline Chloride	272.500
Nicotinic Acid	27.250
Inositol	13.750
Vitamin A Concentrate	4.500
(200,000 units per gram)	
Vitamin D Concentrate	3.000
(400,000 units per gram)	
Alpha Tocopherol	10.225
Menadione	0.1025
Thiamine Hydrochloride	1.000
Pyridoxine Hydrochloride	1.000
Riboflavin	1.000
Calcium Pantothenate	2.050

Fig. 2

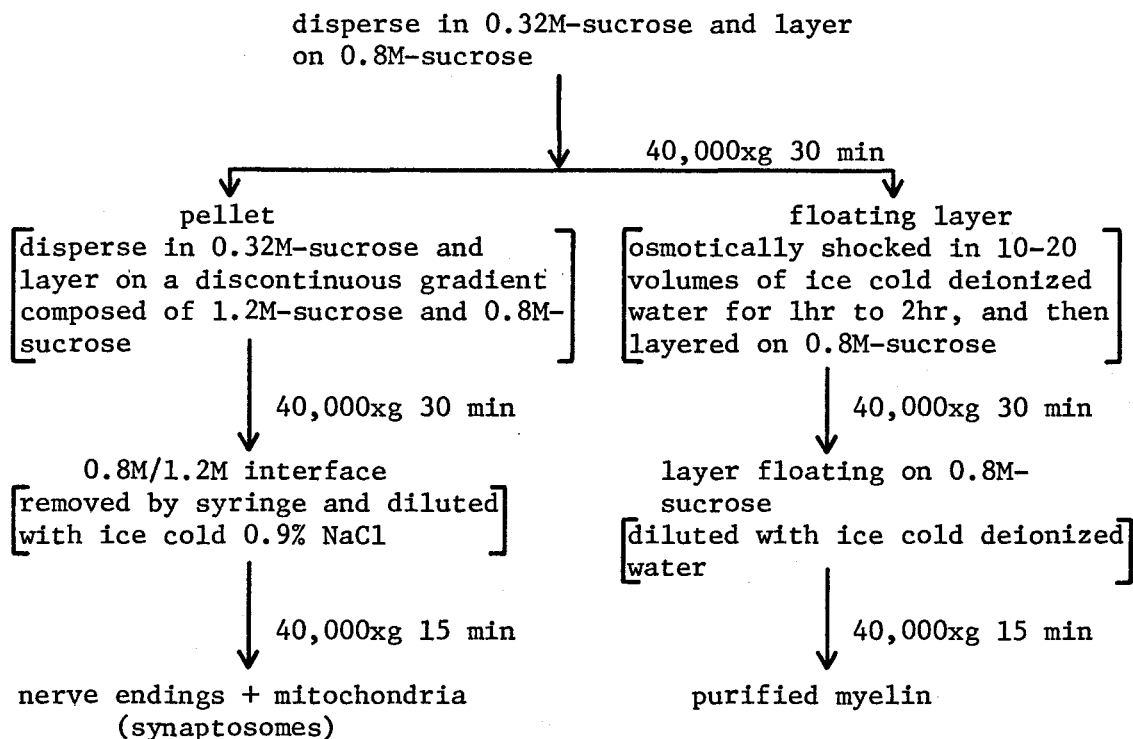
by sonication. For experiments involving whole brain and isolated brain cell fractions, each rat received 0.75 μ Ci of (1-¹⁴C) linolenate in a total volume of 15 μ l intracerebrally. For experiments involving preparation of subcellular fractions, each rat received 1.0 μ Ci of (1-¹⁴C) linolenate in a total volume of 15 μ l intracerebrally. Except for 14 day old animals, the injections were carried out under light ethyl ether anesthesia. Rats up to 28 days of age were injected directly through the skull. For 90 day old and 140 day old animals, an incision was made in the scalp, and after clearing away muscle, the bone was partially drilled through with a small dental burr prior to injection. After injection the hole was closed with bone wax, and the incision was closed with wound clips. This manner of injection distributed (¹⁴C) radioactivity over the surface of the brain. In some studies there appeared to be a slight gradient of increasing radioactivity from front to back. This possibly was the result of cerebrospinal fluid flow. Slight variations in the injection site did not seem to alter the radioactivity distribution. When the injection drew a large quantity of blood or if the needle was disturbed during injection by head movement the rat was discarded. This was a rare occurrence. At selected times after injection the rats were sacrificed by decapitation. The forebrain minus the cerebellum was rapidly removed, and placed in ice cold saline (0.9%, w/w) for whole brain studies, 0.32M-sucrose for preparation of subcellular fractions, or Hexose-albumin, phosphate (HAP) buffer for the preparation of isolated cells.

Preparation of subcellular fractions: Subcellular fractions were prepared according to the procedure of Sun and Horrocks (1973), as amended by Sun, Winniczek, Go, and Sheng (1975). A brief scheme is outlined below:

Three rat brains were pooled and homogenized in 0.32M-sucrose, pH 7.4



Pellet 1



Preparation of isolated brain cell types: All buffers were prepared fresh the night before the experiment, chilled to 0-4°C, and adjusted to pH 6.0 before use. The hexose-albumin-phosphate (HAP) buffer consisted of 5% (w/v) glucose, 5% (w/v) fructose, and 1% bovine serum albumin (Cohn fraction V; Sigma Chemical Co., St. Louis, Mo.). The solutions for the density gradients were prepared from HAP and 2M-sucrose made up in HAP, mixed in the proper proportions. Trypsin was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio)(twice crystallized, salt free, from beef pancreas). Nylon bolting cloth (153 mesh) and stainless steel screen (200 mesh) were obtained from Tobler, Ernst, and Traber (DesPlaines, Ill.).

Calf serum (fetal, lyophilized) was obtained from Calbiochem (La Jolla, Calif.). 1.7 grams of powder is used for 50ml water, the pH being adjusted to 6.0. Fractions enriched in neuronal and astroglial cells were prepared by the method of Norton and Poduslo (1970). Six rat brains were minced finely by scalpel, and were incubated for one hour at 37°C with shaking in 0.5%(w/v) trypsin made up in HAP.

Trypsinization presumably acts to disrupt the intercellular matrix allowing for more facile tissue disruption during the meshing steps. The trypsin solution was filtered prior to use, and was adjusted to pH 6.0. After incubation, 0.2 volumes of calf serum is added to the mince, and the flask is chilled. The mince is washed twice with HAP to remove trypsin, and then it is placed in 0.85M-sucrose for 10 min prior to meshing. The tissue is first passed through the nylon bolting cloth which is held in place in a Millipore filter funnel (No. XX20-047-20). The tissue is prodded through with a glass stirring rod, without suction, and with the aid of 0.85M-sucrose. The suspension is then passed through the stainless steel screen a number of times in order to break up any clumping of tissue.

The cell suspension is layered over a discontinuous sucrose gradient consisting of 2M-, 1.55M-, 1.35M- (5ml each), and 0.9M- (10ml) steps. The tubes are centrifuged at 3300xg for 10 min in a SB-110 rotor in an IEC model B-60 ultracentrifuge. The crude myelin band floating on the 0.85M-sucrose is removed and saved for determination of the dilution factor (see below). The neuronal band at the 1.55M/2.0M interface and the crude astroglial fraction at the 0.9M/

1.35M interface are removed. The astroglial fraction is slowly diluted at 1/10th its volume per minute with HAP, and then is relayered on a second discontinuous sucrose gradient consisting of 1.45M- and 0.9M-sucrose steps of 5 ml each. The tubes are centrifuged at 3300xg for 20 min and the purified astroglia band is removed from the 9.9M/1.45M interface.

Due to the small numbers of oligodendroglia in rat brain, calf brain white matter has been employed as a carrier for these cells. Oligodendroglia cell soma are prepared by the method of Poduslo and Norton (1972). Freshly removed calf brain was obtained at a local abattoir, and was transported on ice to the lab. The cell isolation usually began within 90 min of death. White matter (40gm) was dissected from the corpus callosum and centrum semiovale and was minced finely with a scalpel. Six whole brains minus cerebelli, previously injected, were minced in with the calf white matter, except where otherwise stated when white matter was first dissected from whole rat brain. The mince was incubated for 60 min at 37°C with shaking in 0.1%(w/v) trypsin made up in HAP, filtered, and adjusted to pH 6.0. The trypsin was neutralized by adding 0.2 volumes of calf serum after incubating, and the flask was chilled. The mince was washed twice with HAP to remove trypsin, set in 0.85M-sucrose for 10 min, and meshed as described previously, except in this case a slight suction was employed. The white matter suspension was layered on a discontinuous sucrose gradient consisting of 1.55M (5ml), 1.45M (8ml), and 0.9M (5ml) steps, and was centrifuged at 3300xg for 10 min. The

mixed rat + calf myelin fraction floating on the 0.85M-sucrose was removed for determination of the dilution factor (see below). The mixed rat + calf oligodendroglia fraction was removed from the 1.45M/1.55M interface by syringe.

All the purified cell fractions were diluted slowly with HAP, and were pelleted by centrifugation at 630xg in a Sorvall refrigerated centrifuge. The rat myelin fraction and the mixed rat + calf myelin fraction were water shocked in 10-20 volumes of ice cold deionized water for at least 30 min. These two fractions were then centrifuged at 12,000xg in a SW25.1 rotor in a Beckman Spinco Model L ultracentrifuge.

The dilution factor referred to above is used to calculate the specific activity of the rat oligodendroglia. It is applied to the mixed rat + calf oligodendroglia to correct the obtained specific activity for the dilution by cold oligodendroglia from the calf white matter. Briefly, the dilution factor is determined by the degree of dilution of radioactive rat myelin by cold, that is, non radioactive calf myelin.

$$\frac{\text{specific activity rat myelin}}{\text{specific activity mixed rat + calf myelin}} = \text{dilution factor}$$

(specific activity of mixed rat + calf oligodendroglia x dilution factor = true specific activity of rat oligodendroglia.

The validity of this factor rests on the assumptions that the dilution of radioactive rat myelin by calf myelin and radioactive rat oligodendrocytes by calf oligodendrocytes is the same. We have

routinely obtained values between 30 and 40 for the dilution factor. Even if the initial assumption were wrong by a factor of 2, the calculated specific activities of the oligodendroglia would still be considerably greater than that found for the neuronal or astroglial fractions. The magnitude is what one would expect of the estimation by Norton and Poduslo (1973) that 25% of rat brain is white matter. This would yield the following calculation:

$$50\text{g calf white matter} \div 1.25\text{g/rat brain} \times 6 \text{ rat brain} \div 4.0$$

$$(\text{the proportion of gray matter/white matter in rat brain}) = 27.$$

Lipid extraction and fractionation: Tissue samples were lyophilized and subsequently extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2/1,v/v) (Folch et al. 1957). 0.9% NaCl (w/v) was added to bring the aqueous volume to 20%. After the phases separated, the upper phase was removed by suction, and the lower phase was washed twice with Folch's theoretical upper phase to remove any water soluble contaminants remaining in it. The lower phase was brought to dryness by vacuum distillation. A nitrogen atmosphere was maintained at all times. The lipid residue was dissolved in a small volume of CHCl_3 for silicic acid chromatography.

Silicic acid chromatography was performed as described by Rouser et al. (1967). Lipid was applied to the silicic acid column (1x5cm) (Bio Rad BioSil A 100-200 mesh), elution was performed by the sequential addition of CHCl_3 150ml, acetone 300ml, CH_3OH 150ml, eluted neutral lipids, and acetone the glycolipids. The methanol fraction containing the phosphatides was adjusted to 25ml, and

aliquots were taken for determination of radioactivity and phosphorus. Suitable aliquots from the neutral lipid fraction and the glycolipid fraction were taken for determination of radioactivity.

Thin layer chromatography: Thin layer chromatography was performed essentially by the method of Skipski *et al.* (1964). Forty grams of Silica Gel H (without CaSO_4 binder) (Brinkman Instruments, DesPlaines, Ill.) were slurried with 95ml 1mM- Na_2CO_3 to prepare four plates (200mmx200mm). The slurry was spread using a Desaga applicator adjusted for a gel thickness of 600mm. Phospholipid samples in CHCl_3 were streaked with the aid of a 25ul Hamilton syringe. The developing solvent system consisted of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ (25/15/5/3, by volume). Lipid bands were detected with iodine vapor, identified by comparison with known standards, and the appropriate areas were scraped from the plates into 15ml conical glass test tubes. Lipid was recovered from the silica gel by elution with 7ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1/1,v/v), followed by two elutions with 7ml of methanol. Suitable aliquots of the eluate were removed for determination of radioactivity and phosphorus.

Preparation of fatty acid methyl esters: Methanolysis of the phospholipid fraction was performed by the method of Morrison and Smith (1964), with $\text{BF}_3\text{-CH}_3\text{OH}$ (14% w/v) which was purchased from Applied Science Laboratories Inc. (State College, Pennsylvania). Briefly, the phospholipid fractions were evaporated to dryness under a stream of nitrogen, and three ml of the esterifying reagent was added, the reaction vessel being glass culture tubes fitted with a

Teflon lined screw top. The tubes were placed in boiling water bath for 5 min, cooled, and after adding 5ml of hexane, were set in the boiling water bath for an additional 5 min. The tubes were again cooled, and saturated NaCl solution was added to effect phase separation. The upper hexane layer was removed and the lower aqueous layer was reextracted twice with hexane. The hexane solution containing the fatty acid methyl esters was stored under nitrogen with a drying agent, anhydrous Na_2SO_4 , added at -20°C until needed.

Gas liquid chromatography: GLC of the fatty acid methyl esters was performed on a Varian Aerograph model 90-P gas chromatograph equipped with a thermal conductivity detector. The column packing consisted of 10% Hi-Eff 1BP (diethylene glycol succinate) by weight on Gas-Chrom P (80/100 mesh) (Applied Science Laboratories Inc.). Helium (high purity, 99.995%) was employed as the carrier gas. Peaks were identified by comparison of retention times with those of known standards. The gas chromatograph was equipped with a Packard Tri Carb Gas Chromatography fraction collector, model 850 (Packard Instrument Company, LaGrange, Ill.) (Karmen, Giuffrida, and Bowman, 1962). The fatty acid methyl esters were collected on glass wool (Pyrex brand, fine grade, Corning Glass Works, Corning, New York) which was inserted into glass cartridges (Tri-Carb cartridges No. 6001039, Packard Instrument Company Inc.) (Bennett and Coon, 1966). Recovery of radioactivity applied to the column was essentially 100%. A typical fatty acid methyl ester tracing is included in the appendix.

Determination of radioactivity and phosphorus: Radioactivity is expressed as disintegrations per minute (dpm). It was determined in a Mark II Liquid Scintillation System model 6847 (Nuclear-Chicago, DesPlaines, Ill.). Quench was determined utilizing the external standard ratios method with ^{133}Ba as the external gamma source. Samples were prepared by evaporating the appropriate aliquots of lipid solution to dryness in the scintillation vials, and redissolving the lipid material in 15ml of scintillation fluid. The scintillation fluid contained Packard Permablend II (98% diphenyloxazolyl (PPO) - 2% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)) in toluene (5gm/liter).

Phosphorus was determined by the method of Bartlett (1959). This involves digestion of the organic material with H_2SO_4 , the development of an absorbing molybdate-inorganic Phosphorus complex, and spectrophotometric determination of absorbance at 800nm.

RESULTS

Table 1 shows the percent distribution of radioactivity among the brain lipid classes. At 5 min, 64% of the lipid radioactivity is associated with the neutral lipid fraction while 31% is associated with phospholipid fraction in EFA deprived rats. In the control rats, 61% and 34% of the radioactivity is found in these respective fractions. Between 5 min and 30 min an inversion occurs. At 30 min the neutral lipid fraction contains 24% of the radioactivity compared to 72% in the phospholipid fraction of deprived animals. This compares with 26% and 69% of radioactivity in the respective fractions of controls. At later time points deprived rats attain a maximum of 92% of radioactivity in their phospholipid fraction while the percent of radioactivity in the neutral lipid fraction decreases to 5.5%. At later time points between 85% and 88% of radioactivity is associated with the phospholipid fraction of control rats while radioactivity in the neutral lipid fraction decreases to between 7% and 10%. The glycolipid fraction from both EFA deprived and control rats contains only about 2% to 5% of the lipid radioactivity. In general, at all time points, except 5 min, the percent of radioactivity in the phospholipid fraction is greater for deprived animals compared to controls, while the percent distribution of radioactivity in the neutral lipid and glycolipid fractions is less in deprived animals compared to controls.

Table 1.

Percent distribution of radioactivity in lipid fractions from whole brain after the administration of [1-¹⁴C] linolenate to essential fatty acid deprived and control rats.

Percent Distribution ± S.E.

Time	Neutral lipid		Glycolipid		Phospholipid	
	Control	EFA Deprived	Control	EFA Deprived	Control	EFA Deprived
5 min.	61.0±0.47	64.1±1.15	5.1±0.41	5.2±0.00	33.9±0.83	31.1±0.74*
30 min.	25.5±0.98	24.4±1.68	5.3±0.14	3.7±0.18‡	69.0±0.98	71.8±1.84
60 min.	22.5±0.47	15.5±0.50‡	4.6±0.16	3.2±0.40*	72.3±0.79	81.4±0.45‡
4 hr.	13.0±0.80	9.2±0.23†	4.6±0.29	3.1±0.19†	82.4±0.89	87.9±0.52†
12 hr.	8.5±0.57	5.4±0.08†	3.0±0.09	3.0±0.31	88.5±0.57	91.4±0.51†
24 hr.	10.6±0.60	5.6±0.12‡	5.2±0.39	2.5±0.07†	84.1±0.92	91.9±0.15‡
36 hr.	7.7±0.28	5.4±0.46†	4.5±0.42	2.8±0.35*	88.1±0.66	91.8±0.78†
72 hr.	10.2±0.93	5.6±0.11†	4.5±0.64	2.1±0.08*	84.9±0.55	92.3±0.19‡

21 day old rats were injected with 0.75 µCi of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. Each value represents the mean of 3 or 4 determinations.

The significance of the difference between the means of deprived and control values is shown by:
*P<0.05, †P<0.01, and ‡P<0.001.

Table 2 shows the specific activity of whole brain phospholipids from EFA deprived and control animals. Maximum incorporation in both was at 30 min after injection. At 72 hr the specific activity of the phospholipids from the deprived animals decreased to 40% of the value at 30 min, while for control animals the specific activity decreased to 37% of the value at 30 min. The ratio of specific activity values for deprived and control animals is also shown in Table 2. At all time points the specific activity of the phospholipids of deprived animals is greater than for controls.

Table 3 shows a developmental study of the specific activity of brain phospholipids following injection of (1-¹⁴C) linolenate into EFA deprived and control rats. The greatest specific activity was seen in 10 day old deprived and controls. The specific activity of both groups decreased with development. The specific activity of phospholipids from EFA deprived animals at 21 days of age was 42% of that at 10 days, while at 120 days it was 35% of the day 10 value. In control rats the phospholipid specific activity at 21 days of age was 16% of that at 10 days, while at 120 days the value was 11% of that at 10 days. Except for 10 days, the incorporation of linolenate into whole brain phospholipids was greater for deprived than control animals. It should be noted that the ratio of specific activities of deprived to control is 2.04 at 21 days of age, and that it increases to 2.63 in 120 day old rats.

Table 4 shows the specific activity of the individual phosphatides obtained from whole brain phospholipid. In general, the phos-

Table 2.

Specific activity of whole brain phospholipids after administration of [1-¹⁴C] linolenate to essential fatty acid deprived and normal control rats.

Time of Sacrifice After Injection	Specific Activity \pm S.E.M*		EFA Ratio Deprived Control
	EFA Deprived	Control	
5 min.	1775 \pm 153(3) [†]	1086 \pm 46(3) [§]	1.63
30 min.	3143 \pm 253(3)	1571 \pm 347(3) [‡]	2.00
60 min.	2197 \pm 106(3)	1039 \pm 258(3) [§]	2.11
4 hrs.	1073 \pm 475(4)	651 \pm 143(3)	1.65
12 hrs.	2163 \pm 277(6)	497 \pm 144(4) [§]	4.35
24 hrs.	1238 \pm 176(7)	538 \pm 149(3) [‡]	2.30
36 hrs.	1743 \pm 159(8)	875 \pm 177(4) [§]	1.99
72 hrs.	1249 \pm 91(3)	580 \pm 133(4) [§]	2.15

21 day old rats received 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally. Lipids were extracted, phospholipids fractionated, and specific activity determined as stated in the Methods section.

*Specific activity expressed as d.p.m./ μ M phospholipid P.

[†]Number in parentheses indicates number of individual determinations.

The significance of the difference between the means of deprived and normal control values is shown by: [‡]P<0.05 and [§]P<0.01.

Table 3.

Specific activity of whole brain phospholipid after the administration of [1-¹⁴C] linolenate to essential fatty acid deprived and control rats of varying ages.

Age (days)	Specific Activity \pm S.E.M*		EFA Ratio Deprived Control
	EFA Deprived	Control	
10	5186 \pm 781	6432 \pm 133	0.81
15	4189 \pm 480	3375 \pm 830	1.24
21	2197 \pm 106	1039 \pm 258†	2.04
120	1791 \pm 544	682 \pm 42	2.63

Each rat received 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally and was sacrificed one hour after injection. For 10, 15 and 21 days of age each value is the mean of 3 determinations, while the values at 120 days are the means of 2 determinations.

*Specific activity expressed as d.p.m./ μ M Phospholipid P.

†P<0.01

Table 4.

Specific activity of individual phosphatides from whole brain of essential fatty acid EFA deprived and control rats following administration of [1-¹⁴C] linolenate intracerebrally.

Specific Activity (d.p.m./ μ M Phospholipid P) \pm S.E.M.

Time	PC		PS + PI		PE*	
	Control	Deprived	Control	Deprived	Control	Deprived
5 min.	1380 \pm 280	2650 \pm 230 [†]	770 \pm 60	1480 \pm 160 [‡]	480 \pm 140	745 \pm 100
30 min.	2200 \pm 470	4390 \pm 270 [‡]	1000 \pm 200	2700 \pm 35 [§]	580 \pm 200	1640 \pm 80 [‡]
60 min.	3900 \pm 600	3720 \pm 170	1300 \pm 640	1920 \pm 75	1680 \pm 180	1600 \pm 150
4 hrs.	820 \pm 170	1320 \pm 580	370 \pm 80	430 \pm 250	360 \pm 80	860 \pm 400
12 hrs.	700 \pm 140	2050 \pm 400	230 \pm 43	1840 \pm 350 [†]	310 \pm 63	2500 \pm 500 [†]
24 hrs.	690 \pm 180	1160 \pm 190	460 \pm 80	1200 \pm 170 [†]	520 \pm 140	1770 \pm 270 [†]
36 hrs.	850 \pm 120	1390 \pm 110 [†]	650 \pm 60	1710 \pm 170 [‡]	820 \pm 140	2320 \pm 210 [§]
72 hrs.	710 \pm 250	890 \pm 100	890 \pm 380	1480 \pm 110	1460 \pm 440	1830 \pm 200

21 day old rats were injected with 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally and sacrificed at the indicated times. Each value is the mean of 3 or 4 determinations, except for deprived animals sacrificed at 12, 24 and 36 hours where 8 determinations were employed.

*Includes the plasmalogen form.

The significance of the difference between the means of deprived and control values is shown by: [†]P<0.05, [‡]P<0.01 and [§]P<0.001.

phatides isolated from brain of EFA deprived animals showed a greater specific activity than those from brain of control animals throughout the time interval studied. Initial incorporation at 30 min and 60 min was considerable in all three fractions. Following this initial incorporation, the activities of all three fractions decreased. While the specific activity of PC remained low at later time points, that of PS+PI and PE increased, the PE fraction of both EFA deprived and control animals reaching its maximum activity then. These trends are seen in both deprived and control animals also.

The interrelationships of PC, PS+PI, and PE are illustrated in Figures 3-5. Fig. 3 depicts the ratio of specific activities of PE to PC from EFA deprived and control brain. At early time points in both deprived and control animals the PC fraction is more active than PE. With increasing time after injection, the PE becomes more active relative to PC. In deprived animals PE becomes more active than PC between 4 hr and 12 hr after injection. PE ultimately becomes 2.2 times more active at 72 hr. The increase in activity of PE relative to PC is less rapid in the controls. The activity of PE surpasses that of PC between 24 hr and 36 hr. A table with the ratio data is provided in Appendix A. Fig. 4 shows the ratio of specific activities of PS+PI to PC. An initial dip in the curves for both EFA deprived and control animals is followed by a steady increase out to 72 hr, indicating that in both cases PS+PI is becoming more active relative to PC. At early time points PC is more active relative to PS+PI in both deficient and control. With

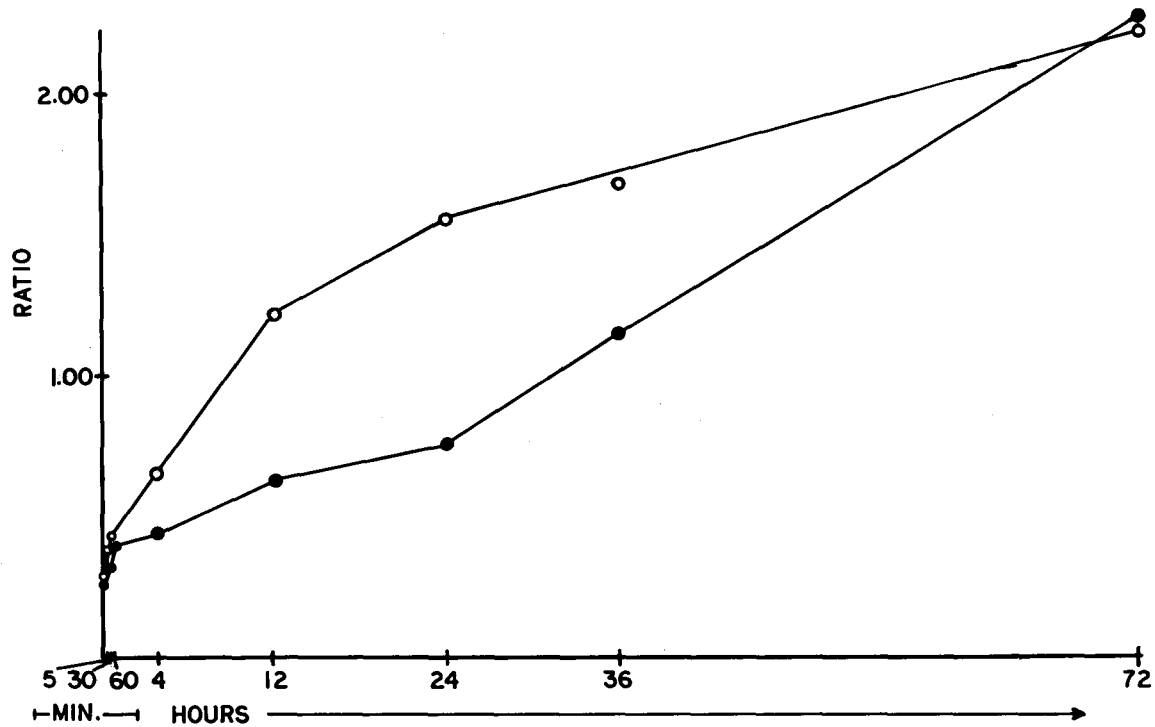


FIG 3: Specific Activity Ratio - PE/PC

○—○ EFA Deprived

●—● Control

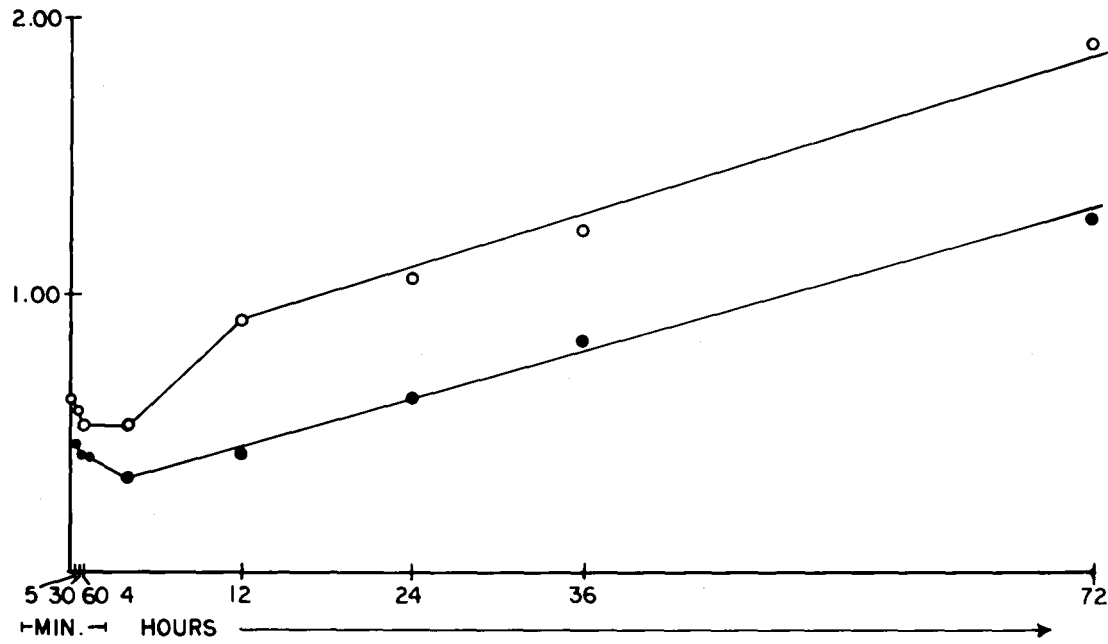


FIG 4: Specific Activity Ratio PS+PI/PC

○—○ EFA deprived

●—● Control

increasing time a reciprocal relation holds. The PS+PI of deprived animals becomes more active than PC between 24 hr and 36 hr. At 72 hr the activity of the PS+PI is about 1.8 times that of PC. In controls the time of crossover is again delayed. In controls the PS+PI becomes more active than PC between 36 hr and 72 hr. At 72 hr, the activity of PS+PI is about 1.3 times that of PC. Fig. 5 depicts the ratio of specific activities of PE to PS. Up to 36 hours the curves from deprived and control rats are comparable. The large difference at 72 hours is probably due to variability in determining the specific activity at this time point.

The percent distribution of radioactivity in the various fatty acids from whole brain phospholipids is shown in Table 2A (1-4) in Appendix A. Some results are presented here. Table 5 shows the percent distribution of radioactivity in the saturates, monounsaturates, and polyunsaturates from whole brain phospholipid. At all time points studied the polyunsaturated fatty acid (PUFA) fraction contained the majority of the radioactivity followed by the saturated fatty acid fraction. The monounsaturated fatty acids contained the least amount of radioactivity. In general, the fatty acids from deprived animals showed a greater proportion of radioactivity present in the PUFA fraction compared to controls. In contrast, a greater percentage of the radioactivity was found in the saturates and monounsaturates of the controls as compared to the deprived. This trend was particularly marked for palmitic acid (16:0). In control animals there was a steadily increasing percentage of

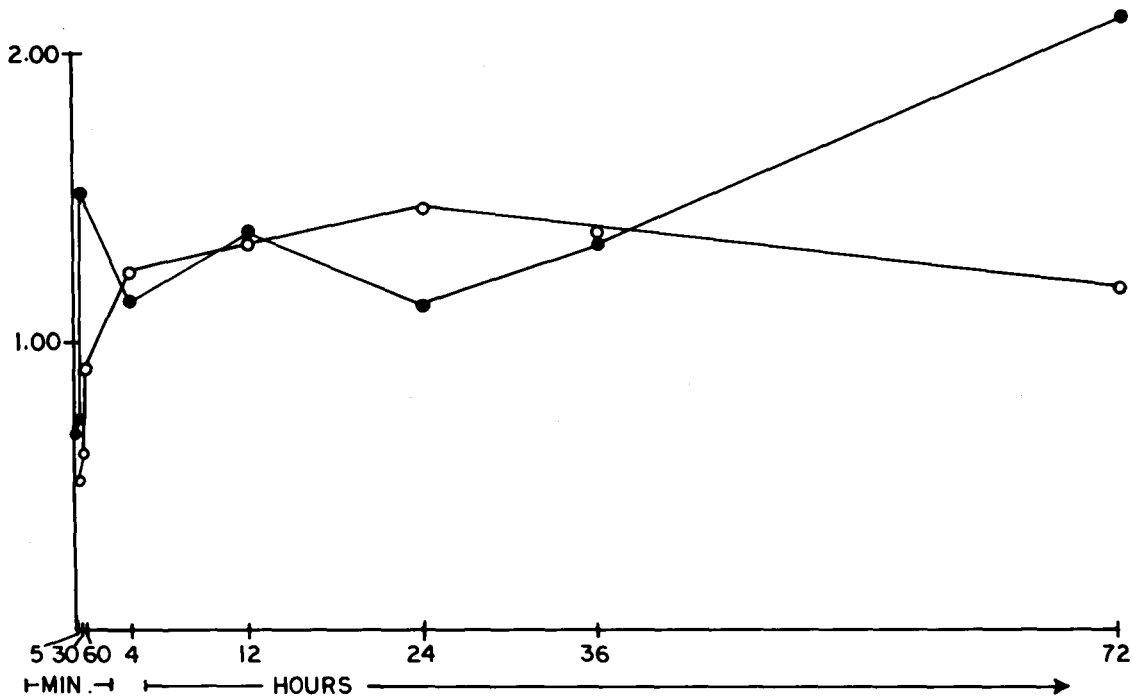


FIG 5: Specific Activity Ratio - PE/PS+PI
 ○—○ EFA deprived
 ●—● Control

Table 5.

Percent distribution of radioactivity in fatty acids from whole brain phospholipids of essential fatty acid deprived and control rats following administration of [1-¹⁴C] linolenate intracerebrally.

% Distribution of Radioactivity §

Time	<u>Saturates*</u>		<u>Monounsaturates†</u>		<u>Polyunsaturates‡</u>	
	Control	Deprived	Control	Deprived	Control	Deprived
5 min	5.0	2.9	1.6	0.5	12.2	14.3
30 min.	9.4	5.4	2.4	1.0	19.9	35.6
60 min.	10.9	7.0	2.5	2.0	21.7	33.1
4 hrs.	15.4	10.0	3.2	2.4	29.0	44.8
12 hrs.	27.6	9.0	2.9	2.8	41.2	51.8
24 hrs.	20.5	8.2	4.9	3.2	41.2	64.1
36 hrs.	22.5	8.6	6.0	3.0	48.7	68.3
72 hrs.	22.3	10.8	7.0	4.4	46.8	67.7

21 day old rats were injected with 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally, and were sacrificed at the indicated time points.

* 14:0, 16:0, 18:0

† 16:1, 18:1

‡ n-3 family: (20:4n-3+20:5n-3+22:3n-9), 22:5n-3, 22:6n-3

§ n- family fatty acids and minor fatty acids were not included in the totals, but % distribution calculations took their contribution into consideration.

radioactivity found in 16:0 with time. At 72 hr 14% of the radioactivity of the total fatty acid fraction was found in 16:0. In deprived animals, however, the proportion of radioactivity found in 16:0 leveled off rapidly after the injection with only about 6.5% being found in 16:0 at 72 hrs. It should be added that the source of radioactivity found in the saturates, monounsaturates, and some (n-6) family polyunsaturates is radioactive acetate derived from the oxidation of radioactive (n-3) fatty acids. The ratio of percent distribution of radioactivity is shown in Table 6. The most consistent difference is seen in the ratio of $18:3n-3/[20:4n-3 + 20:5n-3 + 22:3n-9]$ which is lower at all time points in deprived animals relative to controls. This indicates a greater percentage of radioactivity is converted to the C-20 acids from 18:3n-3 in deprived rats. Less dramatic differences are seen for the ratio of the $[20:4n-3 + 20:5n-3 + 22:3n-9]/22:5n-3$ though they are marginally significant at 30 min, 24 hrs, and 36 hrs. Similarly, the ratio of $22:5n-3/22:6n-3$ is significantly different only at 30 min and 24 hrs.

Table 7 shows the specific activity of the phospholipids of subcellular fractions derived from whole brain. The microsome and synaptosome fractions showed the greatest activity at early time points, and both decreased with time. The myelin fraction, on the other hand, showed low activity initially, but with increasing time it became more active till it was comparable in activity to the microsome and synaptosome fractions.

Table 6.

Ratio of percent distribution of radioactivity in fatty acids of the n-3 series following administration of [1-¹⁴C] linolenate intracerebrally.

Ratios of Percent Distribution

Time	$\frac{18:3n-3}{20:4n-3 + 20:5n-3 + 22:3n-9}$		$\frac{20:4n-3 + 20:5n-3 + 22:3n-9}{22:5n-3}$		$\frac{22:5n-3}{22:6n-3}$	
	Control	Deprived	Control	Deprived	Control	Deprived
5 min.	6.35	5.90	5.92	7.00	1.79	2.43
30 min.	3.20	1.94	3.19	3.00*	2.27	4.16*
60 min.	3.12	1.66†	2.27	1.83	2.43	2.78
4 hrs.	1.89	0.94‡	1.23	1.18	2.08	2.08
12 hrs.	0.85	0.34*	0.72	0.69	1.79	1.75
24 hrs.	0.75	0.20‡	0.55	0.67*	1.11	0.81*
36 hrs.	0.49	0.22†	0.43	0.52*	0.95	0.98
72 hrs.	0.37	0.28	0.59	0.36	0.56	0.55

21 day old rats were injected with 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. The significance of the difference between the means of deprived and control values is shown by: *P<0.05, †P<0.01 and ‡P<0.001.

Table 7.

Specific activity of phospholipids of subcellular fractions
derived from brain following administration
of [1-¹⁴C] linolenate intracerebrally.

Specific Activity (d.p.m./ μ M Phospholipid P) \pm S.E.M.

<u>Time</u>	<u>Microsome</u>	<u>Synaptosome</u>	<u>Myelin</u>
5 min.	935 \pm 243	932 \pm 155	278 \pm 21.0
30 min.	1405 \pm 198	1138 \pm 226	380 \pm 352
60 min.	854 \pm 120	572 \pm 105	162 \pm 33.0
4 hrs.	837 \pm 64.0	710 \pm 64.0	217 \pm 8.0
12 hrs.	716 \pm 76.0	505 \pm 15.0	209 \pm 8.0
24 hrs.	933 \pm 159	418 \pm 120	918 \pm 32.0
36 hrs.	505 \pm 245	392 \pm 2.8	448 \pm 44.0
48 hrs.	873 \pm 79.0	498 \pm 183	780 \pm 88.0
72 hrs..	501 \pm 171	307 \pm 146	659 \pm 134
96 hrs.	653 \pm 16.0	431 \pm 266	315 \pm 155

21 days old rats were injected with 1.0 μ Ci of [1-¹⁴C] linolenate intracerebrally and sacrificed at the indicated times. Three rat brains were pooled for each determination. The values given represent the mean of 2 or 3 determinations.

Tables 8-10 show the specific activity of the individual phosphatides derived from the microsome, synaptosome, and myelin fractions. Table 8 shows the specific activity of phosphatidylcholine. The activity of PC, at early time points, is greater in the microsome and synaptosome fractions as compared to myelin. With time, the specific activity of the PC in the former two fractions decreases to about 25% of its value at 30 min while the activity of myelin PC slowly increases to values comparable to those of microsomal and synaptosomal PC.

Table 9 shows the specific activity of the phosphatidylserine + phosphatidylinositol fraction. The activity of PS+PI remains relatively constant from 60 min to 72 hr in both microsomes and synaptosomes. Initially the PS+PI of microsomes and synaptosomes is more active than that of myelin, which reaches comparable values at 48 and 72 hr.

Table 10 considers the specific activity of PE from the three subcellular fractions. The activity of microsomal PE tends to increase during the time interval studied. That from synaptosomes remains relatively constant after 30 min. The PE fraction from myelin shows the most dramatic change. From initially low values it gradually increases with time until its activity surpasses that of PE from synaptosomes and is comparable to that of PE from the microsomes.

In Figures 6-8 the ratios of specific activities of the individual phosphatides from microsomes, synaptosomes, and myelin

Table 8.

Specific activity of phosphatidyl choline (PC) from subcellular fractions of rat brain following administration of [1-¹⁴C] linolenate intracerebrally.

Specific Activity* ± S.E.M.

<u>Time</u>	<u>Microsomes</u>	<u>Synaptosomes</u>	<u>Myelin</u>
5 min.	1280±380	1000±182	258±2
30 min.	2040±168	1323±45	666
60 min.	1152±90	717±90	299±2
4 hrs.	971±59	851±185	233±11
12 hrs.	763±74	610±71	210±66
24 hrs.	845±40	418±106	-----
36 hrs.	534±86	396±6	245±32
48 hrs.	687±86	440±112	442±59
72 hrs.	492±3	280±39	402±10
96 hrs.	481±135	384±260	150±9

21 day old rats were injected with 1.0 µCi [1-¹⁴C] linolenate intracerebrally, and were sacrificed at the indicated times. Each value is the mean of 2 or 3 determinations, except that for myelin at 30 min., which is a single determination.

* Specific activity expressed as dpm/µM phospholipid P.

Table 9.

Specific activity of phosphatidyl serine + phosphatidyl inositol (PS + PI) from subcellular fractions of rat brain following administration of [1-¹⁴C] linolenate intracerebrally.

<u>Specific Activity* ± S.E.M.</u>			
<u>Time</u>	<u>Microsomes</u>	<u>Synaptosomes</u>	<u>Myelin</u>
5 min.	762±291	776±446	166±60
30 min.	1128±53	766	219
60 min.	615±21	400	52±26
4 hrs.	405±40	384	121±2
12 hrs.	425±82	288±18	109±12
24 hrs.	634±47	306±46	----
36 hrs.	367±47	241±27	265±139
48 hrs.	675±112	355±178	586±148
72 hrs.	384±157	173±54	521±210
96 hrs.	655±17	331±91	240±38

21 day old rats were injected intracerebrally with 1.0 µCi of [1-¹⁴C] linolenate and were sacrificed at the indicated times. Each value is the mean of 2 or 3 determinations except where no value is given for S.E.M., which is a single determination.

* Specific activity expressed as dpm/µM phospholipid P.

Table 10.

Specific activity of phosphatidyl ethanolamine* (PE) from subcellular fractions of rat brain following the administration of [1-¹⁴C] linolenate intracerebrally.

<u>Specific Activity ± S.E.M.†</u>			
<u>Time</u>	<u>Microsomes</u>	<u>Synaptosomes</u>	<u>Myelin</u>
5 min.	322±124	432±40	64±23
30 min.	854±274	659±211	462
60 min.	440±115	293±28	95±10
4 hrs.	652±148	413±112	189±23
12 hrs.	565±28	364±68	173±13
24 hrs.	667±216	360±132	----
36 hrs.	408±1	334±48	396±53
48 hrs.	909±198	488±319	826±36
72 hrs.	684±184	208±36	795±48
96 hrs.	786±54	365±250	205±28

21 day old rats were injected with 1.0 µCi of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. Each is the mean of 2 or 3 determinations except where no value is given for S.E.M., which is a single determination.

*Includes plasmalogen form

†Expressed as dpm/µM phospholipid P.

respectively are given. Tables of ratios are presented in Tables 3A, 4A, and 5A in Appendix A. In microsomes the specific activity of PC relative to PE and PS+PI is greater at early time points. With increasing time the specific activities of PE and PS+PI become greater and eventually surpass that of PC, PE doing so before PS+PI. In the synaptosome fraction, the same trends are observed (Fig. 7). Though PC is more active relative to PE and PS+PI at early time points, both PE and PS+PI activity increases with time relative to PC, and at 96 hr the ratio of specific activity in all cases is about 1.0. Figure 8 depicts the specific activity ratios of myelin phosphatides. Again, somewhat similar trends compared to the other subcellular fractions are observed. PC is more active relative to PE and PS+PI at early time points. After 60 min the activities of PE and PS+PI increase relative to that of PC, and the activity of PE surpasses that of PC between 12 hr and 36 hr, while the activity of PS+PI surpasses that of PC around 36 hr.

The incorporation of linolenate into isolated brain cell fractions has also been studied. Table 11 shows, developmentally, the specific activity of the phospholipids derived from neuronal-, astroglial-, and oligodendroglial-enriched fractions from rat brain. For all age groups studied, the specific activity of the oligodendroglial-enriched fraction far surpassed that of either the astroglia or neuronal fraction, and though it decreased with age, it remained substantially active even in 140 day old animals. The astroglia fraction is more active than the neuronal fraction in rats 14 and 21

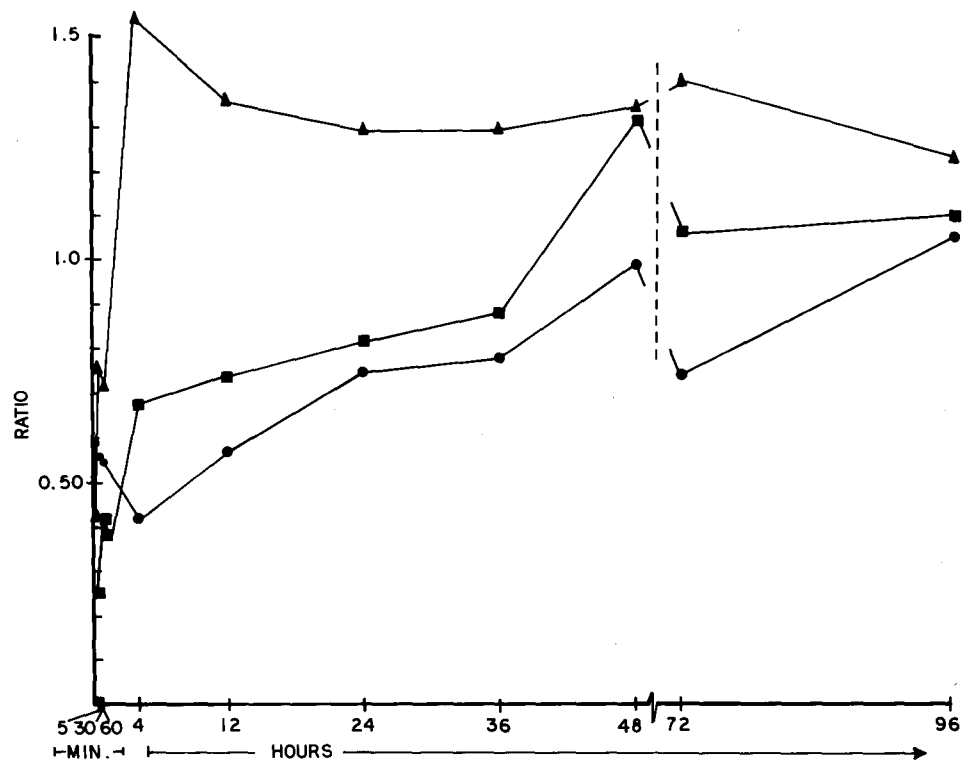


FIG 6: Specific activity ratios of phosphatides from microsomes

- PE/PC
- PS+PI/PC
- ▲—▲ PE/PS+PI

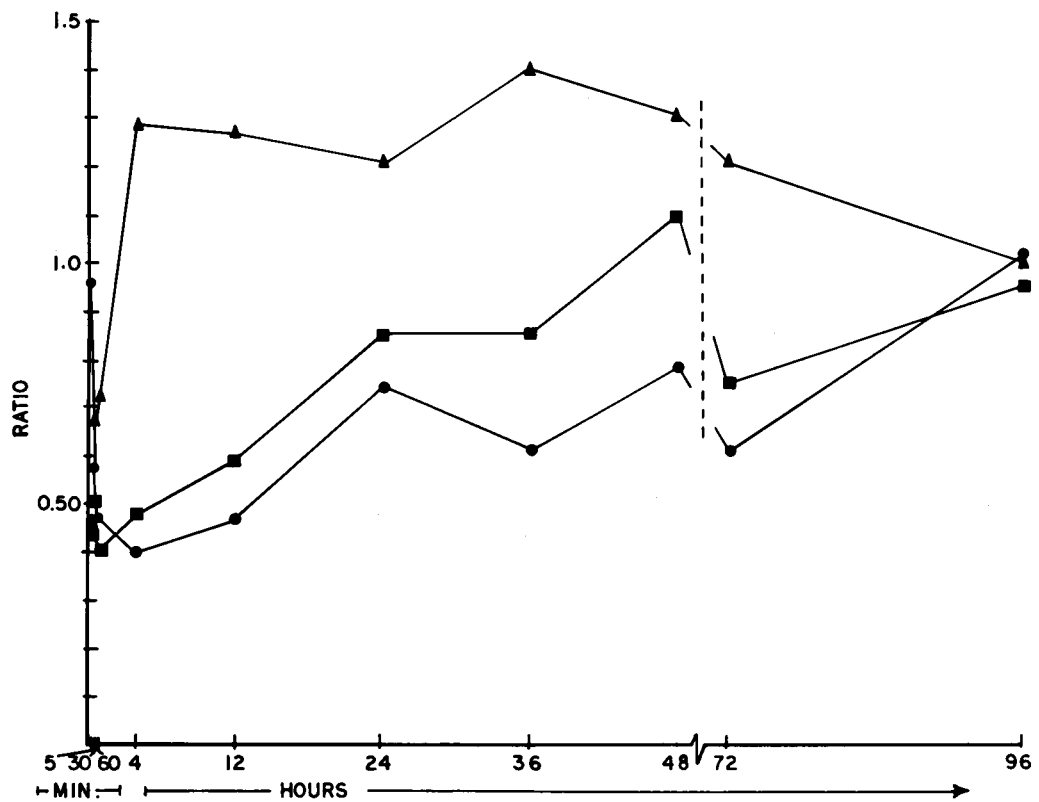


FIG 7: Specific activity ratios of phosphatides from Synaptosomes

- PE/PC
- PS+PI/PC
- ▲—▲ PE/PS+PI

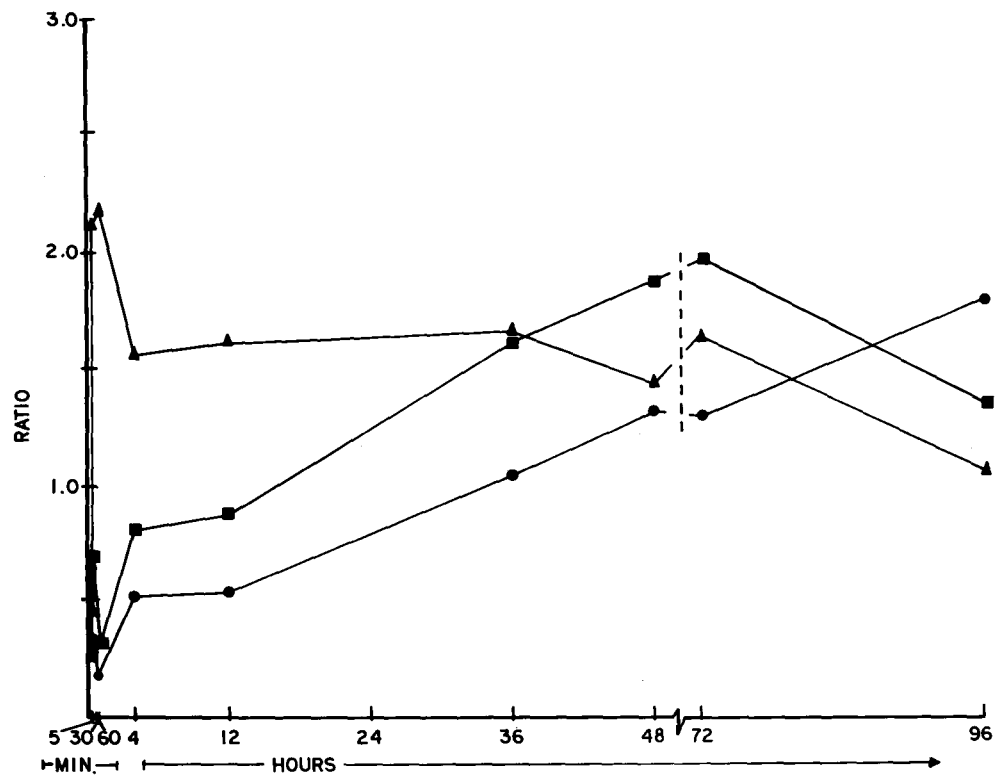


FIG 8: Specific activity ratios of phosphatides from Myelin

- PE/PC
- PS+PI/PC
- ▲—▲ PE/PS+PI

Table 11.

Specific activity of phospholipids from neuronal-, astroglial-, and oligodendroglial enriched fractions isolated from rat brain following intracerebral injection of [1-¹⁴C] linolenate.

<u>Specific Activity* ± S.E.M</u>			
<u>Age (days)</u>	<u>Neuron</u>	<u>Astroglial</u>	<u>Oligodendroglial</u>
14	2067±538(4)†	3012±749(4)	172,200±8560(2)
21	1432±106(2)	1873±222(2)	106,800±49,500(2)
28	4012±561(2)	2352±332(2)	124,700±71,400(2)
90	2778(1)	1025(1)	59,600(1)
140	2165±853(2)	733(1)	43,400±48,400(2)

Rats of varying ages received 0.75 µCi of [1-¹⁴C] linolenate intracerebrally, and were sacrificed after 1 hour.

*Specific activity expressed as dpm/µM phospholipid P.

†Number of individual determinations.

days of age. Between 21 and 28 days of age the activity of the neuronal fraction increases, and becomes greater than that of the astroglia, remaining so throughout the other age groups studied.

Tables 12-14 show the percent distribution of the radioactivity in the individual lipid classes of the three cell types. The percent distribution of the lipid classes appears to be relatively constant with increasing age in all three cell types. In neurons, about 86% of the radioactivity one hour after injection is in the phospholipid fraction compared to about 13% in the neutral lipids. There is some difference noted in the astroglial lipids. After a one hour pulse, about 79% of radioactivity is in the phospholipid fraction while about 20% is associated with neutral lipids. The radioactivity distribution in the oligodendroglial lipids tends toward values intermediate to the other two fractions. About 81% of the lipid radioactivity is in the phospholipids, and about 16% of radioactivity is in the neutral lipids. The glycolipid fraction from the three cell types contained no more than 1%-2% of the lipid radioactivity at any of the ages studied. The glycolipid fraction from the oligodendroglia showed a tendency to higher radioactivity compared to the neuronal and astroglial fractions.

Table 12.

Percent distribution of radioactivity in the individual lipid classes of the neuronal-, enriched fraction from rat brain following administration of [1-¹⁴C] linolenate intracerebrally.

Percent Distribution of Radioactivity \pm S.E.

<u>Age (days)</u>	<u>Neutral Lipid</u>	<u>Glycolipid</u>	<u>Phospholipid</u>
14	13.0 \pm 0.88(3)*	0.9 \pm 0.13(4)	86.2 \pm 0.87(3)
21	12.7 \pm 1.20(2)	1.5 \pm 0.35(2)	85.9 \pm 0.85(2)
28	13.1 \pm 1.77(2)	1.7 \pm 0.21(2)	86.1 \pm 2.69(2)
90	13.5(1)	1.0(1)	85.5(1)
140	14.0 \pm 3.82(2)	2.2(1)	84.9 \pm 2.19(2)

Rats of varying ages received 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally, and were sacrificed 1 hour after injection.

*Number of determinations

Table 13.

Percent distribution of radioactivity in the individual lipid classes of the astroglial-enriched fraction from rat brain following administration of [1-¹⁴C] linolenate intracerebrally.

Percent Distribution of Radioactivity ± S.E.

<u>Age (days)</u>	<u>Neutral Lipid</u>	<u>Glycolipid</u>	<u>Phospholipid</u>
14	22.1±1.03(3)*	0.6±0.075(4)	77.4±1.07(3)
21	19.0±0.71(2)	1.3±0.21(2)	79.3±1.63(2)
28	15.6±3.58(3)	0.9±0.32(3)	83.4±3.68(3)
90	21.4(1)	1.5(1)	77.1(1)
140	21.2(1)	-----	78.8(1)

Rats of varying ages were injected with 0.75 µCi of [1-¹⁴C] linolenate intracerebrally, and were sacrificed after 1 hour.

*Number of determinations

Table 14.

Percent distribution of radioactivity in the individual lipid classes of the oligodendroglial-enriched fraction from rat brain following administration of [1-¹⁴C] linolenate intracerebrally.

Percent Distribution of Radioactivity ± S.E.

<u>Age (days)</u>	<u>Neutral Lipid</u>	<u>Glycolipid</u>	<u>Phospholipid</u>
14	18.8±5.51(2)*	1.0±0.35(2)	77.9±8.70(2)
21	15.1(1)	3.7(1)	81.1(1)
28	16.0±0.71(2)	1.8±1.13(2)	82.2±0.42(2)
90	18.0(1)	2.0(1)	80.0(1)
140	15.3±2.76(2)	2.9(1)	84.0±1.70(2)

Rats of varying ages were injected with 0.75 µCi of [1-¹⁴C] linolenate intracerebrally, and were sacrificed after 1 hour.

*Number of determinations.

DISCUSSION

Fatty acids can arise in the brain by three mechanisms: Synthesis de novo (Brady, 1960), direct uptake from the vascular compartment (Dhopeswarkar and Mead, 1973), and through the elongation and desaturation of the intermediate length fatty acids arising by the first two options (Aeberhard and Menkes, 1968). The third route is required for the biosynthesis of the long chain polyunsaturated fatty acids of the (n-3) and (n-6) families which are found in large quantities in brain phospholipids which are important membrane constituents.

In studying the dynamic metabolism of biological compounds two major considerations come to mind. First, metabolic studies are being carried out more and more with compounds that have radioactive atoms incorporated into them. Amongst the more common isotopes employed are ^{14}C , ^3H , and ^{32}P . It is important to know precisely which atom of a compound is labelled, and the metabolic fate of that atom. Long half lives ($t_{1/2}$) for phospholipids from rat cerebral cortex of about 38 days using (^{14}C) acetate compared to 5.1 days using (^3H) glycerol (Lapetina et al. 1969) could be explained by the loss of ^3H in the metabolism of glycerol and its subsequent equilibration with total body water which effectively removes the isotope from the system. This in contrast to a constant recycling of the (^{14}C) acetate into the acetate pool following lipid catabolism.

The second consideration is the route of administration of the radioactive compound. Often employed routes include oral administration, intraperitoneal injection, intracarotid injection, and intracerebral injection. Intracerebral injection seems to be the method of choice in the study of cerebral metabolism since it allows the brain to directly metabolize the compound free from the contributions of other tissues, particularly the liver, and it allows a much higher percentage of the administered dose to get into the brain. A drawback to its use is that it is a non-physiological way to introduce the compound being studied into the brain. With these considerations in mind, the study of linolenic acid metabolism in brain was undertaken.

Linolenic acid was found to be rapidly incorporated into the brain lipids of 21 day old control and essential fatty acid (EFA) deprived rats. Control rats incorporated about 8.4% of the administered dose while deficient rats incorporated about 16.3% of the dose. These results are comparable to those reported by Yau and Sun (1973; 1974) for (^{14}C) oleic acid and arachidonic acid.

At five minutes after injection the neutral lipids retained the greatest proportion of the radioactivity (61-64%) in both deficient and control animals (Table 1). Between 5 min and 30 min an almost complete inversion occurred. At 30 min almost 70% of the radioactivity was found in the phospholipid fraction of both groups. This pattern of radioactivity partition with increasing time following intracerebral injection was reported by Sun and Horrocks (1971) for

(^{14}C) oleic acid, and by Yau and Sun (1974) for (^{14}C) arachidonic acid. Similar results were seen in cultured cells from cerebral hemispheres of rat embryos utilizing (^{14}C) stearic acid (Yavin and Menkes, 1973), and following intraventricular injection of (^{14}C) acetate (Bonser and Lunt, 1976).

In general, at all time points the phospholipids from deficient rats contain a greater percentage of radioactivity compared to controls while the neutral and glycolipid fractions from deficient rats contain a lesser proportion of radioactivity than the respective fractions from control rats. Though these differences (Table 1) are in most cases statistically significant, whether the actual differences are physiologically significant is not clear. It seems that the factors involved in transferring radioactivity from the neutral lipid fraction to the phospholipid fraction is operating at near maximal levels in the normal 21 day old rat brain. The increase in the percent of radioactivity in the phospholipids may be due to increased activity of the acylation-deacylation cycle (Lands, 1960) in EFA deficient rats. An increased activity of acylation of phospholipids has been suggested by DeTomas and Mercuri (1973) after injection of (^3H) glycerol and (^{14}C) linoleic acid into EFA deficient and control rats.

Data on the metabolism of fatty acids in the brains of EFA deficient animals in general, and of linolenic acid in particular is sparse. Lyles et al. (1975) studied the uptake of (^3H) oleic acid or of (^3H) arachidonic acid by brain in EFA deficient and control

rats that were 45 days of age. The fatty acids were administered by stomach tube and values were obtained over a time course of 24 hr. They reported that the plateau values for oleic acid and arachidonic acid taken up into the total brain lipids of deficient rats was 1.5 and 1.7 times that of the respective values in controls. Their calculations suggested that the deficiency state did not alter the rate of uptake of fatty acids into the brain. They postulated that the flux of fatty acid out of the brain is reduced in EFA deficiency. A decreased efflux was observed for docosahexaenoic acid (22:6n-3) in brain polar lipids by Dhopeswarkar and Subramanian (1975a) following intraperitoneal injection of (^{14}C) linolenic acid.

The data of Dhopeswarkar and Subramanian (1975a) are difficult to interpret in terms of the actual metabolism of linolenic acid by the brain. Results obtained following intraperitoneal injection of (^{14}C) linolenic acid must include the considerable metabolism of linolenic acid in the liver. This is evidenced by their data which show that at early time points (8hr and 48hr) brain palmitate had the highest specific activity. The relative carboxyl activity indicated that it had been formed by de novo synthesis from radioactive acetate derived from linolenic acid. That linolenic acid is taken up by the liver after oral administration was demonstrated by Sinclair (1975). In the same paper it was shown that 22:6n-3 was taken up by liver following oral administration, and that the subsequent uptake of both fatty acids by the brain was the order 22:6n-3 > 18:3n-3. The data we present in Table 2A (1-4) indicates there is a rapid conver-

sion of 18:3n-3 to 22:6n-3 in brain. The time course of labelling of 22:6n-3 of brain polar lipids (Dhopeswarkar and Subramanian, 1975a) shows a rise in activity between 8hr and 48hr followed by a decrease between 48 hr and 15 days. It subsequently declines slowly from 15 days to 45 days. This pattern suggests that 18:3n-3 is being metabolized to 22:6n-3 in the liver, and that the 22:6n-3 is being supplied to the brain via the bloodstream.

Lyles et al. (1975) have suggested a decreased efflux of arachidonic acid from brain occurs during EFA deficiency. Our results do not indicate that there is a sparing effect on brain fatty acids. Maximum incorporation of C¹⁴ into brain phospholipids was seen at 30 min (Table 2). At 72 hr the specific activity of the phospholipids decreased to 40% and 37% of the maximal values in the deprived and control animals respectively. This is not to say there isn't a sparing effect on the fatty acids in the brain during the deficiency. The data for the phospholipid fraction indicates there might be a slight retention of radioactivity during deficiency. The duration of our experiments is 72 hr. At this time point the radioactivity is still increasing in the 22:6n-3 fraction. The loss of radioactivity at this early stage might obscure a sparing effect on 22:6n-3 which could become evident at longer time intervals. Dhopeswarkar and Subramanian (1975b) follow linolenate incorporation out to 45 days.

Lyles et al. (1975) utilize animals that have been on an essential fatty acid deficient dietary regimen from 5 days pre-natal until

they were used in the experiment at 45 days of age. In our experimental procedure dams were placed on the EFA deficient diet from the 10th day of pregnancy, and the pups were taken from their mothers at 21 days of age for injection. Those that weren't immediately sacrificed were placed on the EFA deficient diet. The maximum time interval any pup would be on this diet then, was 72 hr. Hence, the degree of deficiency induced in this case may be a factor. In addition, these authors employ arachidonic acid, the major metabolic product of linoleic acid metabolism, while we are using linolenic acid, the initial precursor of all the (n-3) family of long chain polyunsaturated fatty acids. It is possible that during the process of elongation and desaturation of linolenic acid to docosahexaenoic acid the linolenic acid and its intermediate metabolites are esterified to various lipids and unesterified from them several times thus making them more frequently exposed to degradative reactions. In contrast, arachidonic acid once esterified may be spared.

In spite of the fact that no sparing effect is observed, the radioactivity content in lipids from deficient animals is always about twice that found in controls. The effect is not entirely due to an increased esterification of fatty acid to phospholipid which Dhopeswarkar and Subramanian (1975a) suggest could protect these fatty acids from catabolism since this increased incorporation was found at 5 min after injection at which time about 2/3 of the radioactivity of both deficient and control rats is associated with the neutral lipid fraction. Since between 5 min and 30 min there is a

rapid shift in radioactivity from the neutral lipid fraction to the phospholipid fraction, it appears that some component of the neutral lipid fraction acts in a storage capacity for fatty acids prior to esterification into phospholipids. This pool is present in normal rats and it seems to be expanded in deficient rats. Since the neutral lipids were not subfractionated, it is not possible from our work to state the nature of the pool. Other workers have reported that following the intracerebral injection of labelled fatty acids the triglyceride fraction of neutral lipids becomes labelled rapidly and to an appreciable extent (Sun and Horrocks, 1971; Yau and Sun, 1973; 1974). This has also been observed in cultured cells dissociated from rat cerebrum (Yavin and Menkes, 1973; 1974a) and in the protozoan, Tetrahyema pyriformis (Borowitz and Blum, 1975). Yavin and Menkes (1973) have suggested that triglycerides act in brain tissue as a reservoir for fatty acids. Borowitz and Blum (1976) have suggested that the triglyceride in Tetrahymena pyriformis is not catabolized during times of energy need, but instead, when phospholipid synthesis is required. Elsbach and Farrow (1969) suggested that triglyceride in granulocytes could serve as a reservoir of fatty acids that may be used, during phagocytosis, for increased phospholipid synthesis rather than as an energy source.

Increased levels of brain triglycerides have been observed in some pathological conditions. Davison and Wajda (1962) reported an increased level of triglyceride in brains from multiple sclerosis patients. An increase in triglyceride levels was found by Wood and

Dawson (1974) in sciatic nerve undergoing Wallerian degeneration. Increased triglyceride levels have also been reported in several intracranial tumors (Smith and White, 1968). An increased activity was observed for phospholipid synthesizing enzymes in peripheral nerve undergoing Wallerian degeneration (McCaman et al. 1965; McCaman and Cook, 1966). An increased activity of phosphatidic acid phosphatase was reported by McCaman et al. (1965) and an increased activity of choline phosphokinase and phosphorylcholine: 1,2-diglyceride choline phosphotransferase by McCaman and Cook, 1966).

The above results could possibly be construed to suggest that a triglyceride pool exists in the brain which serves to provide, in some way, precursors for phospholipid synthesis. This notion is consistent with the fatty acid profile for brain triglycerides which was reported by Sun (1970). A high content of 18:1 (28%) and 22:6n-3 (10%) was present. A similar compositional pattern was reported for triglycerides isolated from cultured cells from rat cerebral cortex by Yavin and Menkes (1973).

Data for the distribution of radioactivity in fatty acids demonstrate that linolenic acid undergoes rapid metabolism in brain of both control and deficient rats. It is seen that the polyunsaturated fatty acids contain the greatest percentage of radioactivity at all time points measured. This is somewhat at odds with the results of Strouve-Vallet and Pasquad (1971) who report that the desaturation of linoleic acid to γ -linolenic acid is occurring at only trace levels at 11 days of age in vitro. They report that the

activity in liver is however, unchanged with development. It seems unlikely that the brain would lose the ability to synthesize long chain polyunsaturated fatty acids at a time when brain growth is still occurring to a considerable extent.

In general, the fatty acid radioactivity patterns show about a 50% decrease in proportion of radioactivity associated with the saturated fatty acids of deficient rats compared to controls at 72 hr. A decrease, though not as great, is also seen in the monounsaturated fatty acids. As would necessarily follow, a greater proportion of radioactivity is found in the polyunsaturated fatty acids of deficient animals (68%) compared to controls (47%) at 72 hr. Since the radioactivity associated with the saturates and monounsaturates is derived from (^{14}C)-acetate following degradation of linolenic acid, it appears that the rate of degradation of linolenic acid and its metabolites may in some way be affected by the deficiency state.

Linolenic acid can be catabolized by β -oxidation in the mitochondrial compartment. Access to the mitochondrial compartment doesn't seem to be a factor. Christophersen and Bremer (1972) showed linolenylcarnitine was almost 70% as effective as palmitylcarnitine as a substrate for Palmityl-CoA: carnitine O-palmityltransferase in vitro. The effect of the deficiency state on this enzyme is unknown. Once the polyunsaturated fatty acids are exposed to the oxidizing enzymes in the mitochondria it appears that there is no sparing of them with a preference of the enzymes for saturated or monounsaturated fatty acids (Brown and Tappel, 1959; Mead, 1968).

Coniglio et al. (1964) reported a sparing of arachidonic acid (20:4n-6) in EFA deficient rats following oral administration of ethyl arachidonate (1-¹⁴C). Coots (1965) reported a sparing of arachidonic acid relative to linoleic acid following administration of both of the (¹⁴C) fatty acids. The explanation put forth was that the sparing effect was due to the rapid incorporation of arachidonic acid into tissue phospholipids. This is reasonable in light of our results. The incorporation of radioactivity into palmitate is slightly lower in deficient rats relative to controls from 5 min to 60 min. After 60 min the radioactivity in the control animals increases at a steady rate, though not as rapidly as between 5 min and 60 min, out to 72 hr. In the deficient rats after 60 min the radioactivity in palmitate remains relatively constant out to 72 hr. This corresponds to a period where a greater proportion of the (¹⁴C) is associated with the longer chain polyunsaturated fatty acids.

Looking more closely at the data, at 4 hr after injection 82.4% of lipid radioactivity is associated with the phospholipid fraction of control rats while 29% of the fatty acid radioactivity is associated with the longer chain polyunsaturated fatty acids (20:4n-3 + 22:3n-9), 22:5n-3, and 22:6n-3. At 60 min after injection 81.4% of the total lipid radioactivity is associated with the phospholipid fraction while 32% of the fatty acid radioactivity is associated with the longer chain polyunsaturates. If one then considers the increased proportion of radioactivity in the saturated and mono-unsaturated fatty acids from 4hr and 60 min in the control and defic-

ient rats respectively and compares these values with those observed at 72 hr, it is evident that there is an increase of 10.7% for these fatty acids in control rats in contrast to an increase of only 6.2% deficient rats.

A number of possible explanations arise. One explanation might be that there is a larger pool of acetate present in the brains of deficient rats compared to controls which dilutes the radioactive acetate derived from P-oxidation of linolenate. That such an increased pool exists is not known, but it seems unlikely. One piece of data reported here argues against it. In EFA deficiency there is an unusual response of docosapentaenoic acid (22:5n-6). While other (n-6) fatty acids decrease in deficiency, the 22:5n-6 level increases (Sun et al. 1974; White et al. 1971). There is a slight but detectable increase in the radioactivity associated with the 22:5n-6 in deficient animals while all the other (n-6) fatty acids show a decrease in the incorporation of radioactivity compared to controls. At this state of deficiency there is very little change in the fatty acid profiles in rat brain, and there is no detectable change in the level of 22:5n-6. If one assumes that the turnover of 22:5n-6 is the same in deficient and controls then dilution of radioactive acetate by a larger acetate pool in deficient rat brain would tend to yield a value lower than control for the radioactivity proportion in 22:5n-6.

A second explanation could be that the (^{14}C)-acetate is being used preferentially to elongate the already radioactive polyunsatu-

rated fatty acids in deficient animals. That such a separate pool would exist is unlikely.

Rats on a EFA deficient dietary regimen have been shown to have a higher basal metabolic rate (Wesson and Burr, 1931; Burr and Beber, 1937). The cause of this is possibly due to hyperthyroidism, but this still remains unclear (Holman, 1968). The possibility arises that more (^{14}C)-acetate is employed for metabolic needs and hence is unavailable for fatty acid synthesis in the deficient pups. This appears unlikely for a number of reasons. In these studies adult mice are used which have been on deficient diets for extended periods of time while our rats are weanlings, and have had extremely limited exposure to the deficient diet. Also the metabolic rates reported above were the basal metabolic rates for the whole animal which might very easily not reflect the metabolic rate of the brain. In these animals the respiratory quotient, RQ (the ratio of CO_2 excreted to the O_2 taken up), is greater than 1.0. This indicates that fat is being formed from ingested carbohydrate. Hence it appears that a sufficient supply of carbohydrate is supplied in the diet to satisfy energy needs. One last note, if the increased basal metabolic rate is indeed caused by hyperthyroidism, it has been shown that some of the lipid synthetic enzymes of brain are resistant to certain dietary and hormonal alteration including hyperthyroidism (Volpe and Kishimoto, 1972).

A possible reason is that differences in the fat content of the EFA deficient and control diets is responsible. Lynn and Brown

(1959) showed that rats on diets containing either 20% lard or 20% corn oil showed an increased oxidation of fatty acids relative to rats on a control diet (2% fat). Again, the age of the animals used is different from ours, these being adult rats. Also 20% is a very high content of fat and it may be inducing other changes in situ. Newborn rats have a considerable store of triglycerides in their livers (Sinclair, 1974). Galli et al. (1975) have showed that (n-6) polyunsaturates in liver are not depleted in rat pups fed an EFA deficient diet until about 40 days after birth. It seems then that in spite of the fact that the dams were maintained as indicated previously on a fat-free diet and the pups, when necessary, after injection, the fat stores in the body appear adequate for the short duration that the fat-free diet is employed.

Finally, a decrease in the degradation of fatty acids during deficiency could also account for the decreased availability of (^{14}C)-acetate for fatty acid synthesis. This could come about in two ways. First, the enzyme activities are diminished during deficiency. The opposite effect seems to be true. Mead et al. (1956) placed mice on a deficient diet for three months and then fed them (^{14}C) stearate, oleate, and linoleate. In 6hrs 20-30% of the fatty acids had been oxidized. However, stearate and linoleate were oxidized to a greater extent by deficient rats compared to controls, while the reverse was true for oleate.

The fatty acids might be protected from exposure to catabolic enzymes. In experiments referred to before (Coniglio et al. 1964;

Coots, 1965) an apparent sparing effect was seen for arachidonic acid, and this was attributed to its incorporation into phospholipids of the tissues. This conclusion is not unreasonable, and most likely it is a factor. From our data, however, it appears that there is a sparing effect present in the EFA deprived pups which is not present, or not present to the same extent, in control pups. By inference from the data on initial uptake at 5 min, it is suggested that this sparing mechanism resides in the domain of the neutral lipid fraction, possibly being localized in the triglyceride fraction of the neutral lipids. A hypothetical sparing mechanism might be as follows: Acylation of polyunsaturated fatty acids to triglyceride present in membrane fractions, perhaps concentrated in the proximity of the enzymes for elongation and desaturation of fatty acids, protects these fatty acids by making them less readily available for catabolic reactions. The triglycerides would also act as a reservoir for fatty acids, a function previously suggested (Yavin and Menkes, 1973; Borowitz and Blum, 1976). As a general mechanism the reservoir would increase during times when lipid is being deposited rapidly, or when, for example, membrane degeneration might signal for an increased synthetic effort to replace the membrane which was being lost.

The metabolic sequence which linolenic acid undergoes was presented in the introduction (Fig 1). Fig 9,10 show the percent distribution of fatty acid radioactivity in control and deficient animals. Radioactivity is rapidly transferred from 18:3n-3 to the C-20 (n-3) family fatty acids. The distribution curves are qualitatively

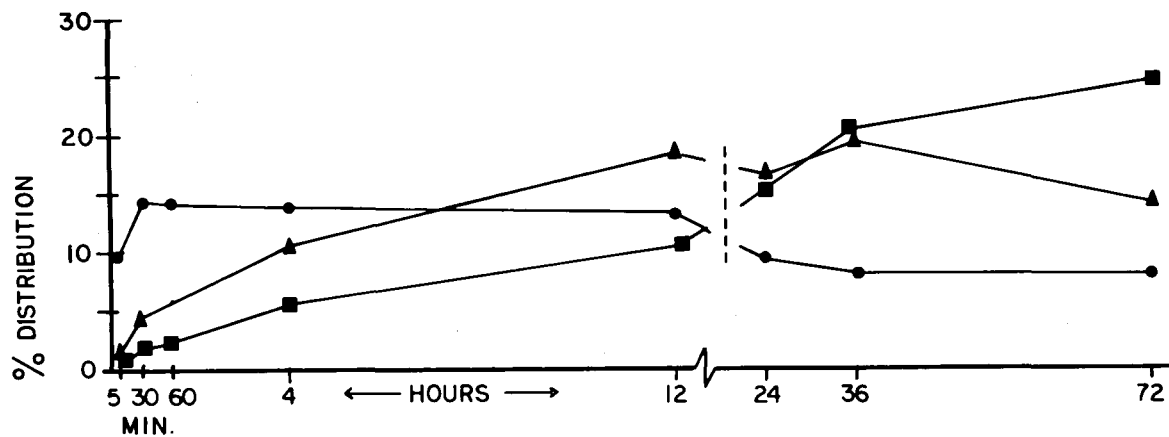


FIG 9. Percent distribution of radioactivity in (n-3) family fatty acids from whole brain phospholipids of 21 day old control rats.

●—● 20:4n-3, + 20:5n-3 + 22:3n-9

▲—▲ 22:5n-3

■—■ 22:6n-3

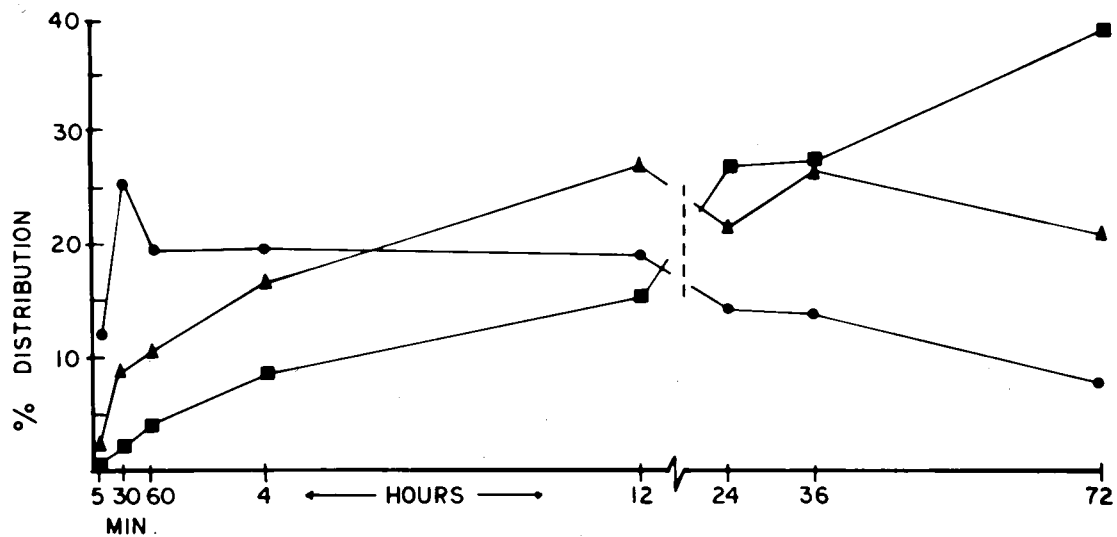


FIG 10. Percent distribution of radioactivity in (n-3) family fatty acids from whole brain phospholipids of 21 day old EFA deprived rats.

●—● 20:4n-3, + 20:5n-3 + 22:3n-9

▲—▲ 22:5n-3

■—■ 22:6n-3

similar in both EFA deprived and control rats. There is a rapid increase in the radioactivity of the C-20 fatty acids which then remains constant followed by a slower increase in the radioactivity in the 22:5n-3 and 22:6n-3 fatty acids. In general, the fatty acids from deficient rats have a greater proportion of radioactivity relative to controls. A qualitatively similar pattern of radioactivity incorporation was reported in cultured cells from rat cerebrum after incorporation of (^{14}C) linolenate (Yavin and Menkes, 1974a).

Product-precursor ratios of the percent distributions is given in Table 6. These indicate that the desaturation of 18:3n-3 by the $\Delta 6$ desaturase and possibly the desaturation of 20:5n-3 by the $\Delta 5$ desaturase are the steps affected by the dietary regimen. A decrease in the ratio, as is seen for deficient animals, indicates a more rapid transfer of radioactivity from precursor to product. This is most evident for the $\Delta 6$ desaturation. A slight decrease is also seen for the transfer of radioactivity from the C-20 fatty acids to 22:5n-3 in the deficient rats, the $\Delta 5$ desaturation. This latter desaturation might respond to a longer state of deficiency. Castuma *et al.* (1972) reported that a fat-free diet increases the $\Delta 6$ desaturase activity in rat liver microsomes while the $\Delta 5$ desaturase is unaffected. These results are consistent with Brenner (1974) that polyunsaturated fatty acid metabolism is regulated at the level of the $\Delta 6$ and $\Delta 5$ desaturases.

A question that presents itself, is, what is responsible for the enzyme response? Only minor changes are noted in the lipid and

fatty acid compositions of pups from dams fed an EFA deficient regimen which was continued for the pups at weaning even at 30 days of age (Galli et al. 1971). Paulsrud et al. (1970) report that the $\Delta 9$ desaturase activity from rat liver microsomes of EFA deficient rats was 2.5 times that from control microsomes. This activity was elevated, and it remained at a constant level whether the rats were on the EFA deficient diet 7 days or 18 months. The lipid and fatty acid compositions wouldn't change appreciably in 7 days. In addition, if rats on the deficient diet were then starved, the $\Delta 9$ desaturase activity decreased below that of controls. These results suggest that the desaturase activity responds rapidly to dietary factors. Dietary factors can alter the other desaturase activities (Brenner, 1974).

That there is a rapid response to the deficiency is illustrated in Table 3. This shows that the incorporation of (n-3) fatty acids is enhanced in rats on a fat-free diet. Maximum incorporation into both groups occurred early in life, at 10 days of age. At 21 days of age the pups on the fat-free dietary regimen were incorporating twice the radioactivity into brain phospholipids with respect to controls. At 120 days of age rats on the deficient diet were incorporating 2.6 times the radioactivity compared to controls.

From the data of Sinclair and Crawford (1972) it is estimated that almost $3/4$ of the brain's complement of $20:4n-6$ and $22:6n-3$ was laid down between conception and weaning, the period when the rat pups were dependent on maternal nutrition. Alling et al. (1974)

and Sinclair and Crawford (1973) have suggested that placing female rats on a fat-free diet during pregnancy does not insure that the pups are deficient since depot stores of essential fatty acids in the dam are slowly released. Depletion of liver stores of 20:4n-6 was shown to take 10 weeks in adult rats maintained on an EFA deficient diet (Sinclair and Collins, 1968). Rat pups are born with a high concentration of polyenoic fatty acids in their livers (Sinclair and Crawford, 1973) which increases during the suckling period. Additionally, Galli and Spagnuolo (1974) maintained female rats on diets with varying contents of linoleic acid and linolenic acid beginning 10 days before mating. It was found that while the polyunsaturated fatty acids of milk triglycerides varied with the dietary composition, the milk phospholipids tended to accumulate these fatty acids when the maternal diets were deficient in them.

It appears then, at least for the fetal and newborn rat pup, that two reservoirs of polyunsaturated fatty acids has been provided to insure an adequate supply during the period of rapid lipid deposition in brain i.e., fetal storage primarily in the liver during gestation, and during the suckling period from maternal milk (Crawford, 1976).

Crawford (1976), using guinea pig, examined the transfer of (^{14}C) linoleic, linolenic, and arachidonic acids from maternal liver to the fetal brain. A "biomagnification" process was reported for linoleic and linolenic acids i.e., increasing proportions of radioactivity were found in long chain polyunsaturated fatty acids

through the progression maternal liver<placenta<fetal liver<fetal brain. Arachidonic acid studies indicated it was taken up 10 times more actively than linoleic acid. They concluded that in addition to the maternal stores or the fetal stores of long chain polyunsaturated fatty acids, the fetal guinea pig was able to actively metabolize precursor linoleic and linolenic acids to their respective end products. This appears to be the level that the dietary influences are working at. That the increases in the metabolic activity that we've presented are of such a magnitude at a very early age reinforces the notion that long chain polyunsaturated fatty acids are required by cerebral lipids to insure the proper structural and functional integrity of the nervous system during development, and that any threat to the supply of long chain polyunsaturated or their precursors in brain results in a rapid response by the brain to counteract it.

The labelling pattern of the individual phospholipid species reflects this rapid response by the brain in deficient rats. The general pattern observed for the three fractions, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) (includes plasmalogen form), and phosphatidyl serine + phosphatidyl inositol (PS + PI) is as follows: PC is more active than PE or (PS + PI) at early time points. This reflects probably two factors. First, the enzyme systems forming PC de novo are more active than those forming PE; the enzyme system forming PS must rely on existing phospholipids so radioactivity incorporation will be slower. A preference for mono and dienoic species of diglyceride is shown for incorporation into PC (Kanoh, 1969;

Trehwella and Collins, 1969) while the hexaenoic fatty acid species of diglyceride are preferred for incorporation into PE (Kanoh, 1969; Kanoh, 1970; Trehwella and Collins, 1969). PS is formed by base exchange with existing phospholipids. The time course of labelling does not distinguish whether serine is preferentially exchanged with PC or PE, except that its latent increase in specific activity following the latent increase in specific activity of the PE fraction suggests there might be a preferential exchange of serine with existing molecules of PE. The PS fraction of brain gray matter contains a substantial amount of polyunsaturated fatty acids, and hence it might be expected that incorporation of serine into phospholipids might show a preference for the more highly unsaturated PE molecules.

With increasing time the PE and (PS + PI) fractions become more active than PC. The PE and (PS + PI) become more active than PC at earlier times in the EFA deprived animals relative to the controls. PE becomes more active than PC before (PS + PI) does in both groups of rats. This seems to reflect the increased proportion of radioactivity in the long chain polyunsaturated fatty acids at earlier times after injection in deficient rats compared to controls. PE becomes more active than PC in deficient rats when between 44.8% and 51.8% of the fatty acid radioactivity is associated with the long chain polyunsaturates (4-12 hours) while PE becomes more active than PC in control rats at around 36 hr when 48.7% of the radioactivity is associated with the long chain polyunsaturates. Similarly, the (PS + PI) from brain of EFA deprived rats becomes about as active as

PC around 12 hr when 51.8% of the fatty acid radioactivity is associated with the long chain polyunsaturates while in controls (PS + PI) surpasses the activity of PC between 36 and 72 hours when between 47-49% of the fatty acid radioactivity is associated with the long chain polyunsaturates. A similar metabolic pattern was reported by Yavin and Menkes (1974a) in cultured cells from rat cerebrum. These findings are consistent with the accepted fact that ethanolamine and serine phosphoglycerides contain the majority of the long chain polyunsaturated fatty acids in the brain.

The incorporation of (1-¹⁴C) linolenic acid into the subcellular fractions of brain was studied in the 21 day old rat. Substantial radioactivity is incorporated into the phospholipids of microsomes, synaptosomes, and myelin even at 5 min after injection, the specific activity of the microsomes and synaptosomes being about 5-6 times that of myelin with regards to the phospholipids. That both microsomes and synaptosomes are actively labelled very early indicates that synaptosomes are able to actively incorporate radioactivity derived from linolenic acid into phospholipids, though this does not rule out a rapid exchange process or axoplasmic transport which couldn't be distinguished by the time intervals studied. A similar rapid incorporation of radioactivity into the synaptosome fraction was reported by Sun and Yau (1976b) following the intracerebral injection of (¹⁴C) oleic and arachidonic acids. Lapetina et al. (1969b) reported that following subarachnoidal injection of (³²P) orthophosphate incorporation of the isotope into brain subcellular

fractions including microsomes and synaptosomes occurred at the same rate. Miller and Dawson (1972a) report that the synaptic endings from guinea pig brain are capable of phospholipid synthesis, a capacity they suggest is associated with the intraterminal smooth endoplasmic reticulum and synaptic vesicles. In addition, the acylation and deacylation of phospholipids of the synaptic membranes could be modulated by neurotransmitters (Gullis and Rowe, 1975a;b).

Over the course of the experiment radioactivity declines in the microsomes and synaptosomes while that in myelin increases. It should be noted that myelin deposition is actively occurring at this time and that no attempt is made to correct the myelin specific activity for dilution by new myelin. Hence the myelin specific activities are probably lower limits of the true specific activity. A possible explanation for these data is that the endoplasmic reticulum is synthesizing phospholipid which is somehow transported to the myelin fraction possibly via a transport lipoprotein similar to that described for sulfatide transport by Pleasure and Prockop (1972). That the synaptosome fraction is losing radioactivity in a parallel manner to the microsomes could suggest the possibility of a transport of radioactivity from the endoplasmic reticulum to the synaptic terminal possibly by a slow exchange process as described by Miller and Dawson (1972b). However, Abdel-Latif and Smith (1970) have reported that the turnover rate of microsomes and synaptosomes was equivalent, as measured following the incorporation of three different lipid precursors into the phospholipids of these subcellular fractions.

If the turnover is equivalent, and if the endoplasmic reticulum is synthesizing phospholipids for the myelin and possibly other cellular structures, then an independent synthetic activity might be postulated to exist in the synaptic endings in order to make up for the difference. This is supported by the turnover studies of phosphatidylcholine in rat cerebral cortex membranes following the intraventricular injection of (Me-¹⁴C) choline by Lapetina et al. (1970) which indicate that if anything, the turnover of the microsomal PC is slower than that of nerve terminal components.

The phosphatidylcholine specific activity of both synaptosomes and microsomes is about 5-6 times that of myelin (Table 8) at five minutes after injection. While the specific activity in the former two fractions declines with time, that of myelin increases to a limited extent. Such a pattern is compatible with the slow transfer of radioactivity from the endoplasmic reticulum to myelin while synaptic terminals can readily incorporate fatty acids into phosphatidylcholine possibly via an acylation deacylation cycle. The actual subsynaptic fractions labeled, and the degree of labelling are not known. The rapid incorporation of fatty acids into PC has been demonstrated by Koeppen et al. (1973) in isolated synaptic membranes following incubation with (¹⁴C) malonyl-CoA.

The pattern of radioactivity incorporated into the phosphatidylethanolamine is different from what might be expected. Similar to the pattern of incorporation seen in whole brain studies, the specific activity of PE is low at early time intervals after injection, and

at greater time intervals after injection increases in both the microsomes and the myelin. Again, as in whole brain, this is consistent with the transfer of fatty acids from PC to PE as the elongation and desaturation reactions proceed, and the transfer then of phospholipid synthesized in the endoplasmic reticulum to the myelin. An interesting result is that unlike whole brain, there is no increase in the specific activity of synaptosomal PE, but rather the specific activity of PE remains relatively constant throughout the duration of the experiment. Since the PC fraction from synaptosomes was about 3 times as active as PE, and the synaptosome fraction isolated by this procedure has about equivalent amounts of PC and PE (including plasmalogen) as determined by lipid phosphorus (Sun and Horrocks, 1973), it would be expected that as the fatty acid from PC were desaturated and elongated the PE would become more active, as is seen for whole brain. It is unlikely that an increase in the specific activity of PE would be obscured by the variability of the data. The relatively constant specific activity for PE argues for a steady state turnover of the PE at the nerve terminal.

That the specific activity of PE doesn't increase suggests the possibility that the synaptic terminal has only a limited ability to carry out lipid metabolism i.e., the metabolic transformations of linolenic acid to its long chain polyunsaturated products, at least, may not be in its repertoire. Koeppen *et al.* (1973) reported that isolated synaptosomes could not synthesize fatty acids *de novo* from Malonyl-CoA, but could elongate 20:4n-6 to 22:4n-6 which was

incorporated into phospholipids with 70% of the radioactivity associated with phosphatidylcholine. Over 80% of the total radioactivity was associated with the synaptosomal mitochondria while about 20% was associated with the synaptic membrane. This elongation system could be similar to that described by Seubert and Podack (1973) and hence, the desaturation and possibly the elongation of fatty acids for incorporation into structural lipids may not take place. If this be the case, the long chain polyunsaturated fatty acids characteristic of the synaptosomes (Kishimoto et al. 1969) must be derived from direct uptake from the extracellular space which is unlikely due to their very low concentration, or through the transfer of lipid from membranes with the synthetic capacity, namely the endoplasmic reticulum possibly by lateral diffusion of phospholipids through the membrane structure due to a gradient effect, or possibly via axoplasmic flow as suggested by Miani (1963).

This data for PE is supported in part by the data for (PS + PI). The (PS + PI) of the synaptosome fraction as the PE did not show an increase in specific activity over the duration of the experiment. The microsomal fraction itself was relatively constant as regards specific activity of (PS + PI). The reason that the microsomal fraction is relatively in a steady state of turnover is not clear, but it may be that the (PS + PI) is being rapidly transported from the endoplasmic reticulum. The heterogeneity of the microsomal fraction may be a factor and the exact nature of the precursor lipid which reacts with serine in the base exchange is unknown. The

labelling pattern of the synaptosomal (PS + PI) suggests that the long chain polyunsaturates are not available for incorporation into (PS + PI) or PE as they are in whole brain. That the synaptosomal (PS + PI) activity paralleled that of the microsomal (PS + PI) might be construed as evidence to support the transport of lipid from the endoplasmic reticulum to the synaptic ending. A complicating factor in this situation is the fact that PS and PI co-chromatograph in our thin layer chromatographic system. Sun and Horrocks (1973) reported that the inositol phospholipid could make up about one third of the combined (PS + PI) fraction as measured by lipid phosphorus. While the serine phosphoglyceride is rich in 22:6n-3 the inositol phosphoglyceride is primarily the species with stearic acid esterified to the one position, and arachidonic acid esterified to the two position. In addition the inositol phosphoglycerides have been implicated in the process of nerve transmission. Hence there may be a number of different metabolic events occurring and attempts to elucidate them must come. It should be concluded here that the data concerning (PS + PI) while supporting that for PE in the synaptosomes should not be weighed too heavily at this time. It might be pointed out that the data for the synaptosome fraction does not rule out the possibility that there is a rapid loss of the incorporated radioactivity from the PE and the (PS + PI) so that the incorporation of long chain polyunsaturated fatty acids into these fractions in the synaptosomes is evenly balanced by the loss from the compartment. It seems unlikely though that the loss of radioactivity from the

phosphatidylcholine does not show up somewhere. It appears that there is no recycling of the radioactivity in this circumstance, and that it is just lost from the compartment.

To summarize, somewhat, two processes seem to be occurring. Initially microsomes and synaptosomes, particularly the PC fraction, are rapidly labelled. The decrease in the specific activity of PC in the nerve terminals without a concomitant rise in the specific activity of PE and (PS + PI) suggests the possibility that the desaturation and possibly the elongation of the fatty acids derived from the injected linolenic acid does not occur since it appears that the incorporation, in this case, of radioactivity into PE and (PS + PI) is dependent on the formation of long chain polyunsaturated fatty acids. This is also reported by Yavin and Menkes (1974). Hence, the fatty acid released from the phosphatidylcholine may be oxidized by intraterminal mitochondria, or just simply lost from the nerve terminal compartment. On the other hand, it appears that there is a continual exchange of phospholipid between the endoplasmic reticulum and the myelin compartment, and possibly between the endoplasmic reticulum and the nerve terminal also.

Possible roles for phospholipids and fatty acids have been postulated in the events occurring during nerve transmission (Yagihara et al. 1973; Hawthorne and Bleasdale, 1975; Gullis and Rowe, 1975a). This is a factor which might complicate the interpretation of experimental results in the nerve terminal.

We have looked at the incorporation of linolenic acid at the cellular level also. It was shown that (1-¹⁴C) linolenic acid was incorporated to the greatest extent into the phospholipid of the oligodendroglial enriched fraction as compared to the neuronal and astroglial enriched fractions. These data are presented in Table 11. The oligodendroglial cells are responsible for the formation of myelin and its maintenance. It was estimated that 3.5 mg of myelin are synthesized per day per brain in the 20 day old rat (Norton and Poduslo, 1973). Based on their calculations for the number of oligodendroglial cells present in rat brain white matter these authors estimated that each oligodendroglial cell synthesized greater than three times its weight per day of myelin. Hence the vastly greater incorporation of linolenic acid into oligodendroglial phospholipids is not surprising.

The specific activity of the astroglial fraction is greater than that of the neuronal fraction at 14 and 21 days. The neuronal fraction becomes more active than the astroglial fraction between 21 and 28 days and remains elevated in the oldest animal used, 120 days. This could reflect changes associated with the maturation of the brain (Wells and Dittmer, 1967), and increased neuronal activity.

Astroglial activity decreases with age, it being greatest during the period when rapid lipid deposition was occurring. In addition the neutral lipid fraction has associated with it almost twice the proportion of radioactivity that the neuronal fraction has. It is conceivable, as was discussed earlier, that the radioactivity

could, in the main, be associated with the triglyceride fraction. If so, it would support the notion that the astroglial cell could function, in part, in a support capacity for other components of the nervous system.

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APPENDIX

Table 1A.

The incorporation of [1-¹⁴C] linolenate into individual phosphatides of essential fatty acid deprived and control rats: Ratios of phosphatide specific activities.

Time	<u>Phosphatide Ratios</u>					
	<u>PE/PC</u>		<u>PE/PS+PI</u>		<u>PS/PC</u>	
	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>
5 min.	0.28±0.015	0.30±0.008	0.68±0.070	0.52±0.002*	0.47±0.005	0.62±0.040*
30 min.	0.32±0.018	0.37±0.005*	0.73±0.017	0.62±0.007†	0.44±0.016	0.59±0.039†
60 min.	0.41±0.007	0.42±0.022	1.51±0.247	0.90±0.073*	0.43±0.123	0.52±0.014
4 hrs.	0.44±0.013	0.66±0.016‡	1.14±0.064	1.23±0.029	0.35±0.021	0.52±0.027†
12 hrs.	0.63±0.058	1.21±0.058‡	1.37±0.157	1.34±0.078	0.43±0.049	0.91±0.031‡
24 hrs.	0.76±0.038	1.54±0.041‡	1.12±0.115	1.46±0.037†	0.62±0.019	1.06±0.037‡
36 hrs.	1.15±0.007	1.67±0.057‡	1.35±0.023	1.37±0.051	0.82±0.029	1.22±0.046‡
72 hrs.	2.26±0.415	2.20±0.127	2.13±0.647	1.19±0.071	1.28±0.073	1.90±0.057†

21 day old rats were injected with 0.75 µCi of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated time intervals. Each value is the mean of 3 or 4 determinations, except for 12 hrs., 24 hrs. and 36 hrs. deprived values, which are the means of 8 determinations.

The significance of the difference between the means of deprived and control values is shown by:

*P<0.05, †P<0.01 and ‡P<0.001.

Table 2A (1).

Percent distribution of radioactivity in fatty acids of whole brain phospholipids from essential fatty acid deprived and control rats following intracerebral administration of [1-¹⁴C] linolenate.

Time	<u>Fatty Acid Percent Distribution ± S.E.M.</u>							
	<u>14:0</u>		<u>16:0</u>		<u>16:1</u>		<u>18:0</u>	
	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>
5 min.	0.8±0.11	0.2±0.09†	2.8±0.19	2.2±0.19*	0.7±0.07	0.2±0.04†	1.4±0.04	0.5±0.06‡
30 min.	0.5±0.15	0.2±0.07	7.4±0.56	4.5±0.80*	0.9±0.29	0.4±0.07	1.5±0.32	0.7±0.00
60 min.	0.7±0.18	0.3±0.11	8.2±0.85	5.5±0.19*	1.1±0.04	0.7±0.32	2.0±0.20	1.2±0.15*
4 hrs.	1.2±0.31	1.3±0.40	11.7±0.41	7.2±0.62‡	1.1±0.11	0.8±0.39	2.5±0.15	1.5±0.26*
12 hrs.	0.6±0.04	0.4±0.04*	13.3±0.76	6.2±0.55‡	0.6±0.08	0.6±0.06	3.7±0.35	2.4±0.11†
24 hrs.	0.5±0.07	0.8±0.32	14.9±0.57	4.6±0.08‡	0.8±0.00	1.1±0.27	5.1±0.15	2.8±0.26‡
36 hrs.	0.8±0.15	0.6±0.16	16.2±0.16	5.6±0.04‡	1.0±0.26	0.8±0.29	5.5±0.15	2.4±0.08‡
72 hrs.	1.6±0.43	0.8±0.07	13.9±1.32	6.4±0.08‡	1.5±0.07	1.0±0.15*	6.8±0.43	3.6±0.12‡

21 day old rats were injected with 0.75 µCi of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. Each value represents the mean of 3 or 4 determinations.

The significance of the difference between the means of deprived and control animals is given by:

*P<0.05, †P<0.01 and ‡P<0.001.

Table 2A(2).

Time	18:1		18:2n-6		18:3n-6 + 20:0		18:3n-3 + 20:1	
	Control	Deprived	Control	Deprived	Control	Deprived	Control	Deprived
5 min.	0.9±0.04	0.3±0.06‡	1.3±0.24	0.2±0.05†	2.9±0.43	2.1±0.29	59.7±0.96	70.1±1.2†
30 min.	1.5±0.39	0.6±0.08	0.7±0.22	0.2±0.04	4.0±0.35	0.1±0.04‡	43.9±5.15	49.0±1.91
60 min.	1.4±0.22	1.3±0.30	0.8±0.17	0.4±0.23	1.9±0.25	2.1±0.59	41.8±1.96	31.6±3.6*
4 hrs.	2.1±0.14	1.6±0.33	1.1±0.04	0.9±0.30	1.2±0.29	3.47±0.29†	24.9±0.68	18.7±1.23†
12 hrs.	2.3±0.11	2.2±0.45	0.7±0.10	0.7±0.25	1.5±0.53	2.0±0.36	11.0±1.17	6.4±0.32*
24 hrs.	4.1±0.15	2.1±0.49*	1.5±0.29	1.3±0.29	1.0±0.23	1.7±0.12*	6.9±0.07	3.0±0.18‡
36 hrs.	5.0±0.21	2.2±0.37‡	1.4±0.17	0.5±0.18†	1.2±0.25	1.5±0.11	4.1±0.50	3.1±0.29
72 hrs.	5.5±0.43	3.4±0.07†	1.4±0.04	0.9±0.04‡	1.4±0.16	1.3±0.07	3.0±0.18	2.1±0.15†

21 day old rats were injected with 0.75 μCi of $[1-^{14}\text{C}]$ linolenate intracerebrally and were sacrificed at the indicated times. Each value represents the mean of 3 or 4 determinations.

The significance of the difference between the means of deprived and control animals is given by:
* $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$.

Table 2A(3).

<u>Time</u>	<u>20:3n-3</u> <u>+20:4n-6</u>		<u>20:4n-3+20:5n-3</u> <u>+22:3n-9</u>		<u>22:4n-6</u>		<u>22:5n-6</u>	
	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>	<u>Control</u>	<u>Deprived</u>
5 min.	5.1±0.59	4.2±0.46	9.4±1.06	11.9±0.76	1.1±0.13	0.4±0.08†	1.0±0.04	0.5±0.07
30 min.	7.3±0.93	7.0±0.58	13.7±1.64	25.2±0.92†	1.0±0.14	0.8±0.14	1.0±0.26	1.1±0.06
60 min.	9.3±0.78	8.4±2.0	13.4±0.45	19.0±3.4	1.1±0.04	2.0±0.39*	1.2±0.04	1.6±0.50
4 hrs.	13.8±5.1	9.8±0.63†	13.2±0.36	19.9±0.76‡	1.7±0.05	1.1±0.29*	1.5±0.12	2.0±0.33
12 hrs.	18.4±2.35	9.1±1.04†	13.0±0.95	18.8±0.35†	1.9±0.04	1.3±0.10†	1.7±0.18	2.4±0.08†
24 hrs.	14.1±1.57	9.1±0.70*	9.2±0.27	14.8±0.08‡	1.5±0.29	1.6±0.26	1.7±0.00	2.3±0.24
36 hrs.	8.4±0.15	6.9±0.23†	8.4±0.49	14.0±1.10†	1.6±0.04	1.3±0.19	1.6±0.16	2.4±0.15†
72 hrs.	5.2±0.57	3.7±0.19*	8.1±1.11	7.6±0.28	2.1±0.29	1.4±0.12*	1.8±0.33	1.7±0.11

21 day old rats were injected with 0.75 μ ci of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. Each value represents the mean of 3 or 4 determinations.

The significance of the difference between the means of deprived and control animals is given by:

*P<0.05, †P<0.01 and ‡P<0.001.

Table 2A(4).

Time	22:5n-3		22:6n-3	
	Control	Deprived	Control	Deprived
5 min.	1.8±0.15	1.7±0.11	1.0±0.23	0.7±0.04
30 min.	4.3±0.39	8.4±0.11‡	1.9±0.25	2.0±0.11
60 min.	5.9±0.40	10.4±1.7*	2.4±0.19	3.7±0.41*
4 hrs.	10.7±0.50	16.8±2.30*	5.1±0.25	8.1±0.53†
12 hrs.	18.1±1.24	27.2±0.46†	10.1±0.55	15.5±0.39‡
24 hrs.	16.8±0.39	22.0±0.90‡	15.2±0.48	27.3±1.92†
36 hrs.	19.7±0.93	26.9±1.21†	20.6±0.99	27.4±1.40†
72 hrs.	13.8±0.43	21.2±1.15†	24.9±2.35	38.9±0.64‡

21 day old rats were injected with 0.75 μ Ci of [1-¹⁴C] linolenate intracerebrally and were sacrificed at the indicated times. Each value represents the mean of 3 or 4 determinations.

The significance of the difference between the means of deprived and control animals is given by:

*P<0.05, †P<0.01 and ‡P<0.001.

Table 3A.

Ratio of specific activities of individual phosphatides
derived from the microsome fraction of whole rat brain.

	<u>Ratio ± S.E.M.</u>		
<u>Time</u>	<u>PE/PC</u>	<u>PE/PS+PI</u>	<u>PS+PI/PC</u>
5 min.	0.25±0.023	0.42±0.001	0.59±0.051
30 min.	0.42±0.100	0.75±0.207	0.56±0.019
60 min.	0.38±0.070	0.72±0.210	0.54±0.060
4 hrs.	0.68±0.194	1.64±0.530	0.42±0.016
12 hrs.	0.74±0.036	1.36±0.325	0.57±0.163
24 hrs.	0.82±0.019	1.29±0.071	0.75±0.055
36 hrs.	0.88±0.089	1.29±0.085	0.78±0.022
48 hrs.	1.32±0.120	1.34±0.071	0.99±0.024
72 hrs.	1.06±0.088	1.40±0.039	0.74±0.046
96 hrs.	1.10±0.165	1.23±0.014	1.05±0.200

21 day old rats were injected with 1.0 μ Ci of [1-¹⁴C] linolenate intracerebrally, and were sacrificed at the indicated times. Each value represents the mean of 2 or 3 determinations. Each determination is derived from three rat brains which were pooled for the subcellular fractionation.

Table 4A.

Ratio of specific activities of individual phosphatides derived from the synaptosome fraction of whole rat brain.

<u>Ratio ± S.E.M.</u>			
<u>Time</u>	<u>PE/PC</u>	<u>PE/PS+PI</u>	<u>PS+PI/PC</u>
5 min.	0.44±0.039	0.46±0.057	0.95±0.032
30 min.	0.50±0.177	0.66	0.57
60 min.	0.41±0.012	0.72±0.086	0.47±0.067
4 hrs.	0.48±0.028	1.28	0.39
12 hrs.	0.59±0.042	1.26±0.156	0.47±0.025
24 hrs.	0.85±0.100	1.20±0.256	0.74±0.077
36 hrs.	0.85±0.135	1.41±0.361	0.61±0.057
48 hrs.	1.10±0.461	1.30±0.240	0.78±0.204
72 hrs.	0.74±0.026	1.20±0.177	0.61±0.110
96 hrs.	0.95±0.006	1.00±0.468	1.02±0.457

21 day old rats were injected with 1.0 μ Ci of [1-¹⁴C] linolenate, intracerebrally, and were sacrificed at the indicated times. Three rat brains were pooled for each determination, and each value represents the mean of 2 or 3 determinations, except where no value for S.E.M. is shown, which are single determinations.

Table 5A.

Ratio of specific activities of individual phosphatides derived from the myelin fraction of whole rat brain.

<u>Time</u>	<u>Ratio \pm S.E.M.</u>		
	<u>PE/PC</u>	<u>RE/PS + PI</u>	<u>PS + PI/PC</u>
5 min.	0.25 \pm 0.088	0.44 \pm 0.300	0.64 \pm 0.240
30 min.	0.69	2.11	0.33
60 min.	0.32 \pm 0.037	2.17 \pm 1.31	0.17 \pm 0.086
4 hrs.	0.81 \pm 0.060	1.57 \pm 0.205	0.52 \pm 0.030
12 hrs.	0.88 \pm 0.339	1.61 \pm 0.297	0.54 \pm 0.110
24 hrs.	---	---	---
36 hrs.	1.62 \pm 0.007	1.67 \pm 0.672	1.06 \pm 0.430
48 hrs.	1.88 \pm 0.170	1.45 \pm 0.304	1.32 \pm 0.163
72 hrs.	1.98 \pm 0.170	1.65 \pm 0.573	1.30 \pm 0.559
96 hrs.	1.36 \pm 0.113	1.07 \pm 0.014	1.80 \pm 0.085

21 day old rats were injected with 1.0 μ Ci of [1-¹⁴C] linolenate intracerebrally, and were sacrificed at the indicated times. Three rat brains were pooled for each determination, and each value represents the mean of 2 or 3 determinations, except where no value for S.E.M. is shown, which are single determinations.

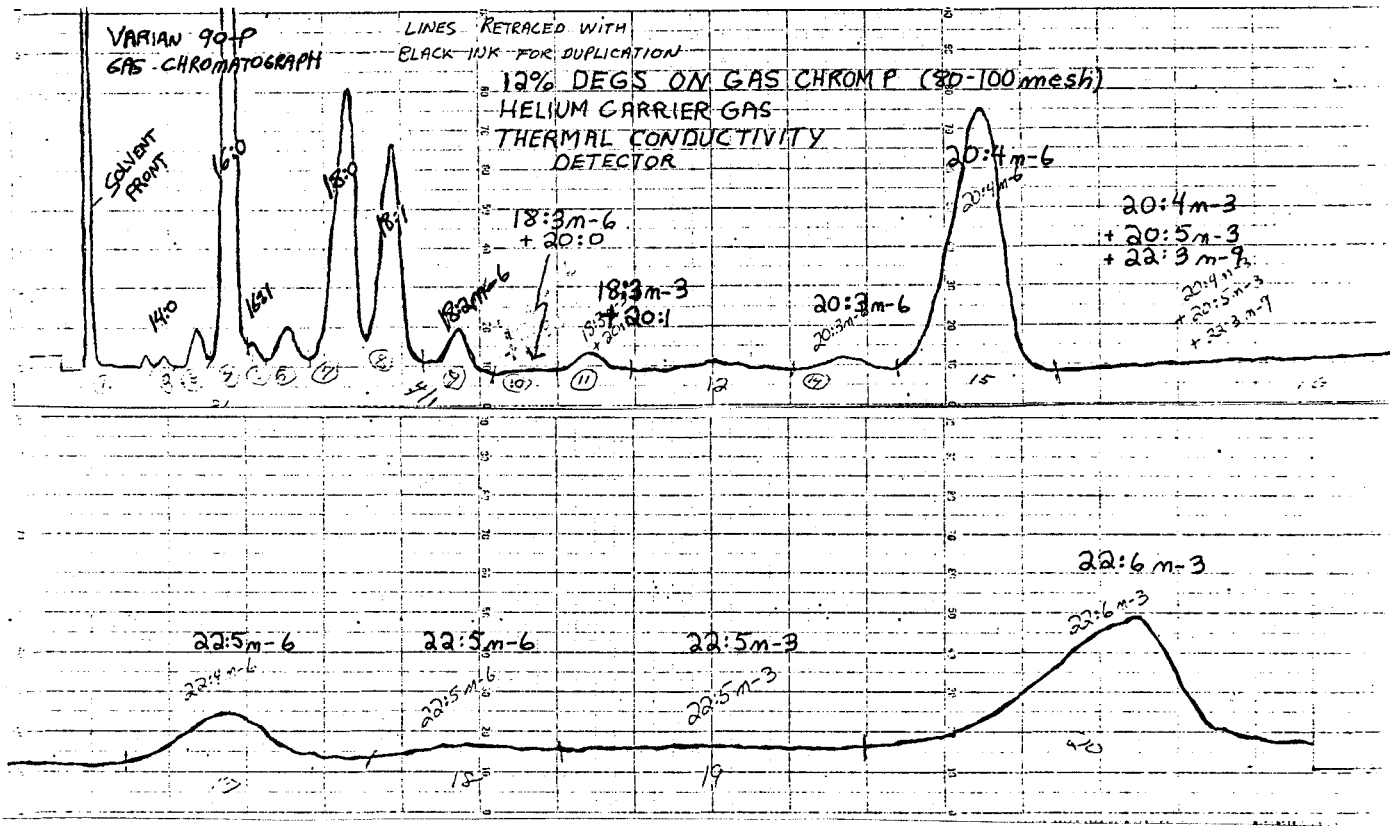


FIG 11: Fatty Acid Methyl Ester Chart From Varian 90-P Gas Chromatography

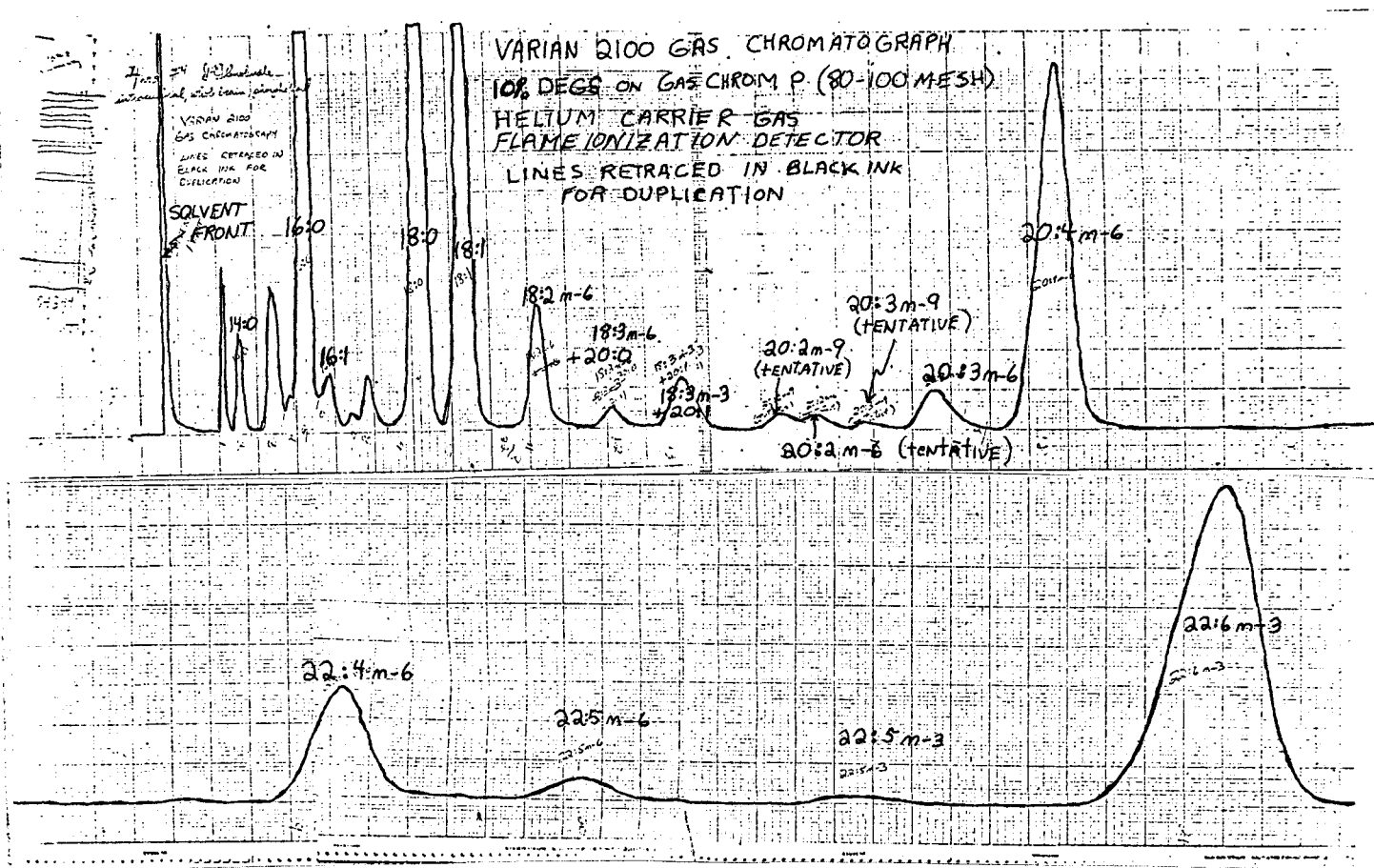


FIG 12: Fatty Acid Methyl Ester Chart From Varian 2100 Gas Chromatography

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

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