Essential Fatty Acid Malnutrition and Brain Development

An experimental study on rats from foetal to adult age of the effects of low dietary levels of essential fatty acids on brain phosphoglycerides in relation to extra-neural organs.

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GÖTEBORG 1974

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CONTENTS

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I. Changes in lipid concentrations and fatty acid compositions in rat cerebrum during maturation.
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Together with I. Karlsson.

II. Effect of maternal essential fatty acid supply on fatty acid composition of brain, liver, muscle and serum in 21-day-old rats.
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Together with Å. Bruce, I. Karlsson, O. Sapia and L. Svennerholm

III. The effect of different dietary levels of essential fatty acids on growth of the rat.
Nutr. Metabol. 16: 38-50 (1974)
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IV. The effects of different dietary levels of essential fatty acids on body composition of the rat.
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The papers will be referred to by the above Roman figures.

INTRODUCTION

ESSENTIAL FATTY ACIDS

Essential fatty acids (EFA) is a term originally used to designate fatty acids which cured symptoms occurring in experimental animals fed a fat-free diet (Burr and Burr, 1930). These acids cannot be synthetized in the mammalian body and must be provided in the diet. Linoleic acid, 18:2 (n-6), linolenic acid, 18:3 (n-3) and arachidonic acid, 20:4 (n-6) are traditionally regarded as the major essential fatty acids. Linoleic acid, 18:2 (n-6) and linolenic acid, 18:3 (n-3) undergo a series of desaturations and elongations to form higher polyunsaturated fatty acids. Linoleic acid has the first double bond after the sixth carbon atom, the methyl group being counted as number 1 (figure 1). All derivatives of linoleic acid have the same terminal structure, denoted (n-6), and are called the linoleic acid series. Linolenic acid has the first double bond after the third carbon atom, denoted (n-3), and gives rise to the linolenic acid series (figure 1). There are no conversions of acids of one of the series into acids of the other, and there is no endogenous synthesis de novo of the higher polyunsaturated members of either series.

Absence of EFA in the diet of rats has been reported to give rise to a number of conditions such as diminished growth, dermatitis, caudal necrosis, impaired reproduction and impaired water balance (for review see Holman, 1968). Fatty acids of the linolenic acid series cannot prevent all the symptoms of EFA deficiency to the same extent as those of the linoleic acid series. Neither the degeneration of the testes nor the infertillity of the females is prevented by fatty acids of the linolenic acid series, and their ability to cure increased permeability of the skin is only one tenth of that of linoleic acid (Houtsmuller, 1972). Tinoco et al., (1971) claim on the basis of analyses of the fatty acid composition of total lipids of whole head, liver and heart of rats fed a linolenic acid-free diet through two generations, that linolenic acid is not essential to the rat. According to Crawford and Sinclair (1972a), however, the experiments by Tinoco et al., (1971) were not properly designed to show whether linolenic acid is essential or not.

In EFA deficiency polyunsaturated fatty acids derived from oleic acid, 18:1 (n-9) occur in tissue lipids (figure 1). Fulco and Mead (1959) showed that the fatty

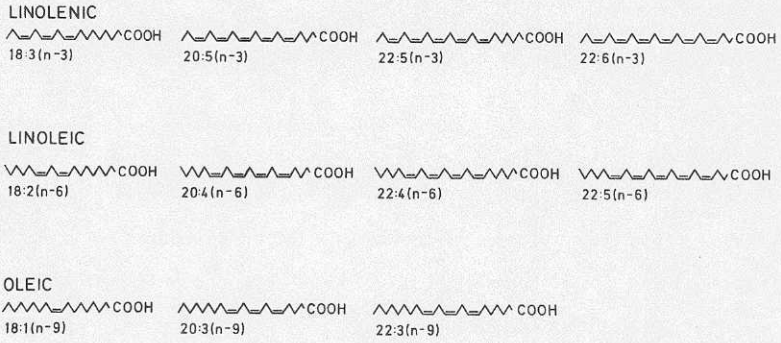

Figure 1. Major polyunsaturated fatty acids of the linolenic, linoleic and oleic acid series.

acid with three double bonds that increased in EFA deficiency was derived from oleic acid. Mohrhauer and Holman (1965), who used radioactive tracers, demonstrated that not only 20:3 (n-9) but also the fatty acid, 22:3 (n-9), was formed in the liver in EFA deficient rats.

LIPID CONCENTRATIONS AND PHOSPHOGLYCERIDE FATTY ACID PATTERNS IN BRAIN DURING DEVELOPMENT

A comparison between species

Biochemical research on the influence of specific environmental factors on the human brain during development is limited for several reasons and comprises only the final outcome of the effect of the total environment, measured by analysis of autopsy material. The use of large animals with a development similar to man would be preferable, but is virtually impracticable. One must, therefore, resort to small mammals, particularly the rat. However, the anatomical elements and the general course of development of the mammalian brain do not appear to vary substantially with species, (Davison and Dobbing, 1968; Dobbing, 1970; 1973). In comparisons between man and rat, however, there are two important differences which must be considered viz the difference in the duration of the maturation processes, and the times of events in relation to birth. Dobbing (1973) has stressed the importance of comparing stages, rather

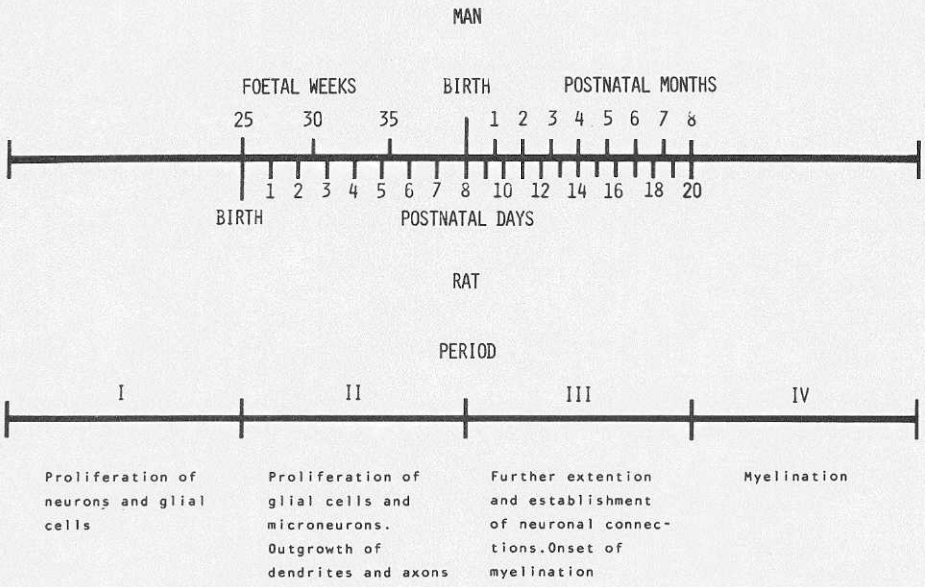

Figure 2. Major stages in brain development.

than ages, in investigations of development of the brain. Koch and Koch (1913) had already divided development of the rat brain into stages, to facilitate the discussion of lipid changes. This division has since been used in comparisons of species (McIlwain, 1966; Brante, 1949; Vanier et al., 1971).

The most extensive investigations on the concentrations of major lipids during development of the brain and the developmental patterns of the fatty acid composition of the phosphoglycerides in man, are those by Svennerholm and Vanier (1972, 1973a). In order to estimate to what extent it was justified to compare rats with man in investigations of the effects of EFA deficiency on brain phosphoglycerides, we analysed rat cerebrum at frequent intervals from birth to 45 days of age (paper I). Myelin in rat cerebrum does not appear until 10 days of age (Friede, 1966), and between 10 and 20 days of age, myelin constitutes only a minor part of cerebrum compared to whole brain. The results obtained in rat cerebrum up to 20 days of age, were therefore compared with those obtained in human grey matter (Svennerholm and Vanier, 1972, 1973a) and studied for any intercorrelation with the methods suggested by McIlwain (1966) and Vanier et al., (1971). A detailed comparison was not possible since it is not known exactly how the time scales correspond to each other. The material did not allow any definite intercorrelations for the end of periods I and III and

Table 1. Quantitative lipid values (μmol/g) in rat cerebrum and human cerebral cortex during development.

Age			
Rat	Man	Rat cerebrum	Human cerebrum
0–8 days	25th foetal week to term		
		Cholesterol	
		9–> 14	10–> 20
		Phospholipids	
		22–> 34	20–> 30
		Gangliosides	
		1.0–> 1.5	1.0–> 1.7
		Rat cerebrum	Human cerebral cortex
8–20 days	Birth to 8 months postnatally		
		Cholesterol	
		14–> 25	20–> 25
		Phospholipids	
		34–> 52	30–> 40
		Gangliosides	
		1.5–> 2.5	1.7–> 2.5

Table 2. Fatty acid composition of ethanolamine phosphoglycerides in rat cerebrum and human cerebral cortex during development.

Age		Rat cerebrum	Human cerebrum
Rat	Man		
0–8 days	25th foetal week to term	**Saturated acids**	
		16:0 decreased, 18:0 increased, and together constituted 36–43%.	16:0 decreased, 18:0 increased, and together constituted 30–40%.
		Monoenoic acids	
		Decreased from 12 to 8%.	Decreased from 15 to 10%.
		Linoleic acid series	
		Increased from 25 to 28% from the 1st to the 4th day.	Constant around 38%.
		Linolenic acid series	
		Increased from the 1st to the 3rd day, 22 to 25% and decreased from the 3rd to the 8th day, 25 to 21%.	Increased from 10 to 20%.
8–20 days	Birth until 8 months postnatally	Rat cerebrum	Human cerebral cortex
		Saturated acids	
		Constant around 38%.	Constant around 35%.
		Monoenoic acids	
		Increased from 8 to 12%.	Constant around 10%.
		Linoleic acid series	
		Increased from 26 to 30% and reached a maximal plateau of 30% until 16 days after which it slowly decreased.	Increased shortly after birth and reached a maximal plateau of 45% between the 4th and the 12th month.
		Linolenic acid series	
		Decreased to 16 days after which it increased. Lowest value 20%.	Constant. Range 15–20%.

therefore the divisions at these stages are those suggested by Vanier et al., (1971). In the present comparison (Fig. 2) the day of birth of man was said to correspond to the age of 8 days in the rat, because at these ages the fatty acid patterns of the two species agree better than when the rats are 10 days old. In McIlwain's (1966) compilation the day of birth of man was compared to 10 days in the rat, but the age of 5-7 days has recently been suggested (Dobbing, 1973).

The comparison (Tables 1 and 2) revealed that the quantitative lipid values were approximately the same in rats as in man at corresponding stages of development. The major difference was the increase in cholesterol, which was less pronounced in human cerebral cortex than in rat. The increase in cholesterol is due to an increasing amount of cell membrane and myelination. It is quite possible that the accumulation of myelin in rat cerebrum before 20 days of age had already caused this difference between man and rat. After 20 days of age the concentrations of cholesterol and cerebrosides in rat cerebrum reached such a level, due to myelination, that comparison with human cerebral cortex was no longer justified. The comparison of the fatty acid patterns of ethanolamine phosphoglycerides revealed that the principle changes were the same, and occurred at corresponding stages.

PRESENT EVIDENCE FOR A DISTURBED BIOCHEMICAL COMPOSITION OF BRAIN DUE TO MALNUTRITION

Malnutrition means a pathological state resulting from a relative or absolute deficiency or excess of one or more essential nutrients, sufficient to produce disease. Disease may be clinically manifest or detectable only by biochemical or physiological tests. A specific deficiency refers to the pathological state, resulting from relative or absolute lack of individual nutrients. Undernutrition is the pathological state resulting from the consumption of an inadequate quantity of food over an extended period of time (Scrimshaw et al., 1968). The term "protein-calorie malnutrition" (PCM) was introduced by Jeliffe in 1959 (Jeliffe, 1959) and covers a range of pathological conditions arising from coincident lack of protein and calories in varying proportions. A synonymous term is "protein-calorie deficiency", which was proposed by the joint FAO/WHO Expert Committee on Nutrition in its sixth report (1962).

PCM is more frequently used in the literature than "protein-calorie deficiency".

The following survey is confined to PCM and lipid malnutrition. The effects of vitamin deficiencies on the brain in experimental animals have recently been summarized by Dreyfus and Geel (1972). Deficiencies of minerals in man, including their effects on the nervous system have been reviewed by Manocha (1972).

Protein-calorie malnutrition (PCM)

Studies on PCM are more abundant than those on other types of malnutrition. This is obviously because the frequent occurrence of PCM in man and its clinical manifestations in early life are well documented (Béhar, 1968; Guzmán, 1968). Experimental studies in animals have shown that PCM induces absolute and relative alterations in the amounts of chemical constituents of the developing brain.

The total brain DNA content is reduced in rats with experimental neonatal and/or early postnatal PCM (Winick and Noble, 1966; Winick, 1970). The total brain DNA is a measure of total cell number and gives no information about the influence on different kinds of cells, or about their shape, size and other qualities. PCM has been reported to have a stronger effect on weight and cell number of cerebellum than of cerebrum in the suckling period in rats (Culley and Lineberger, 1968; Chase et al., 1969; Dobbing et al., 1971). This might be due to the fact that the cerebellum grows more rapidly than the cerebrum during the suckling period (Dickerson and Dobbing, 1966). Winick (1970), however, found that the cell number of the cerebellum in 16-day-old rat foetuses was reduced much more than that of the cerebrum, when the maternal protein supply was restricted.

Of the biochemical parameters related to the quality of the brain, cholesterol, phospholipids and cerebrosides have been studied most often. In severely undernourished 1-year-old pigs weighing only 3% of age-matched controls the concentration of cholesterol was reduced by 21%; that of cerebrosides by 36%, and that of phospholipids by 13% (Dickerson et al., 1967; Dickerson and Dobbing, 1967). Even moderate undernutrition during the early postnatal development reduces the concentration of cholesterol in the ages between 6 and 60 days in rats (Dobbing, 1964; Culley and Mertz, 1965; Dobbing and

Widdowson, 1965; Benton et al., 1966; Culley and Lineberger, 1968; Gieson and Waisman, 1970; Ghittoni and Raveglia, 1972). When undernutrition was continued until older ages (Dobbing, 1964; Benton et al., 1966; Dickerson and Walmsley, 1967; Dobbing and Widdowson, 1965; Guthrie and Brown, 1968) the reduction either diminished or disappeared. Cholesterol is a component of all membranes, but cerebrosides and sulfatides are confined to myelin, and their concentrations can be taken as a measure of the amount of myelin. In studies in which both cholesterol and cerebrosides have been quantified the concentration of cerebrosides was reduced more than that of cholesterol (Dickerson and Dobbing, 1967; Culley and Mertz, 1965; Benton et al., 1966; Culley and Lineberg, 1968; Geison and Waisman, 1970; Ghittoni and Raveglia, 1972). A diminished synthesis of sulfatides due to a reduction of the galactocerebroside sulfokinase has also been found (Chase et al., 1967). Fishman et al., (1971) reported that the amount of isolated myelin from 53-day-old rats, undernourished from birth, was only 71% of normals, while the brain weight was 78% of normals. The only consistent change in the composition of that myelin was a reduction in ethanolamine plasmalogen.

Changes in the patterns of soluble protein (Lee, 1969), the proteins of the optic nerve (Wood, 1973) and in the activity of some enzymes (Serini et al., 1966; Kumar and Sanger, 1970; Swaiman et al., 1970; Adlard and Dobbing, 1971) have been reported, but the biological significances of these changes are difficult to assess.

Studies on human brains deserve special attention. Such investigations are few and have been based on relatively few subjects. The interpretation of the findings offers several problems. The difficulty in collecting a normal control material for the early postnatal period has been discussed (Svennerholm and Vanier, 1972). The exact chronological age of children living in developing countries is often difficult to determine. Brown (1965) reported that the brain weight of 96 malnourished Ugandan children, aged 0-5 years, was lower than in non-malnourished children. It is questionable, however, whether a low brain weight for age has any relevance at all to brain function, (Dobbing, 1970; 1973). Moreover, the brain/body weight ratio was higher in the malnourished children. Winick and Rosso (1969) analysed 9 brains from

Chilean children, who had suffered from malnutrition. They found a proportional reduction in weight, protein, DNA and RNA content. Three of these infants weighed less than 2,000 grams at birth, and might, therefore, have been prematures. A low content of cerebral lipids in 4 infants who had suffered from malnutrition and aged 2, 4, 12 and 22 months has been reported by Fishman et al., (1969). For only 2 of these children was age-matched control material analysed. They found that the concentrations of glycolipids and plasmalogens were lower in malnourished brains. Myelin was isolated from 3 of these brains (Fox et al., 1972). No significant difference in composition of the myelin from the brains was found between the malnourished and the control children of comparable ages, and it was concluded that malnutrition did not affect the lipid composition of myelin, but decreased its deposition. Rosso et al., (1970) measured the concentrations of cholesterol and phospholipids in 9 malnourished children and found that during the 2nd year of life the DNA was reduced less than the lipids. They also concluded that myelination was affected more than brain growth or cell number. Winick et al., (1970) reported that cerebrum and cerebellum were affected approximately equally with regard to DNA, RNA and protein content in 16 malnourished children up to 2 years of age. An unexplainably wide range of variation of the ratio between the protein and dry weight limits the value of that study.

Lipid malnutrition

A low amount of lipids in the diet involves both the problem of an insufficient amount of calories derived from fat and the problem of a deficiency of EFA.

A diet poor in fat calories and rich in calories derived from other nutrients gives rise to profound changes in the metabolism in the liver with increased activity of the liver fatty acid synthetase (Volpe and Vagelos, 1973). Recently the content, synthesis and degradation of fatty acid synthetase in the brain has been studied in various nutritional states and during development (Volpe et al., 1973). Neither feeding a fat-free diet nor fasting the rats induced any changes in synthetase activity in the brain at 32 days of age, in contrast to dramatic alterations in hepatic activity, which was measured simultaneously.

In contrast with the limited research on the effect of a low fat diet, the nutritional role played by linoleate and linolenate has received more attention

in studies on the effect of dietary lipids on the central nervous system. This is natural since these fatty acids cannot be synthetised by mammals and must be supplied in the diet. Investigations of EFA deficiency during gestation and early postnatal development are faced with a number of methodological problems from a nutritional point of view. These circumstances are discussed in detail in the next chapter. Mohrhauer and Holman (1963) and Biran et al., (1964) included brain in their analyses of different organs for the effect of post-weaning dietary EFA deficiency on the fatty acid composition of phospholipids. They found the brain to be affected much less than other organs. The fatty acid composition was only slightly changed and then mainly by an increase in eicosatrienoic acid. The administration of graded doses of purified linoleate, linolenate or arachidonate to rat weanlings on an EFA-free diet for a period of 100 days (Mohrhauer and Holman, 1963c) gave no further information on the effects on the brain, because of a number of unknown factors in that experiment. Rathbone (1965) maintained adult rats (6-8 weeks) for 34 weeks on an EFA-free diet. In contrast with the findings in serum only very minute changes were demonstrable in the fatty acid pattern of total lipids. Eicosatrienoic acid was not measured.

Until 1968 no studies were available on attempts to expose the brain to EFA deficiency during the period of most rapid growth. A study by Steinberg et al., (1968) on newborn offspring from rats fed an EFA free diet was non-conclusive owing to lack of the necessary precision of the determinations of the fatty acid patterns. A series of publications on the effects of EFA deficiency on brain have since 1970 been published by Paoletti and coworkers (Galli et al., 1970; White Jr. et al., 1971; Galli et al., 1971a; Galli et al., 1971b; Galli et al., 1971c; Paoletti and Galli, 1972). All the experiments were similar in design i.e. an EFA free diet was fed to pregnant rats from about five days before delivery and to their offspring after weaning up to one year of age. The effect on brain weight, quantitative lipid values and the fatty acid pattern of ethanolamine phosphoglycerides were compared with a control group fed a diet with a different nutrient composition and a different ratio between linoleic and linolenic acid. For reasons discussed later, this design made it difficult or impossible to interpret the results. Some of their results are incontestable. After one years continuous feeding of the EFA free diet the fatty acid pattern

of ethanolamine phosphoglycerides in whole brain differed from that in the controls in the following way; polyunsaturated fatty acids of both the linoleic and linolenic acid series were decreased and at the same time 20:3 (n-9) and 22:3 (n-9) were increased to a corresponding extent. The sum of these two last mentioned fatty acids (15%) was larger than that hitherto reported. Recently Sinclair and Crawford (1973) analysed brains of newborn rats for the effects of a low-EFA diet of the mothers (0.2 calorie-%). The newborns were offspring of the second generation of rats fed this low EFA diet. They found the intrauterine influence to be substansial and reported a 10% decrease in highly polyunsaturated EFA in ethanolamine phosphoglycerides, with a corresponding increase in 20:3 (n-9) and 18:1.

THE AIM OF THE PRESENT STUDY

Increasing attention is being paid on the eventual ill effects of malnutrition on brain during development. The research in this field has been concerned mainly with PCM and before the beginning of the present investigation the paucity of information about the effect of EFA deficiency during brain growth was striking. This is remarkable because the growing brain requires substantial amounts of EFA (Svennerholm, 1968; Sinclair and Crawford, 1972; Svennerholm and Vanier, 1973a) and because there is good evidence that when protein is lacking in the food there is also a deficiency of EFA, (Sinclair and Crawford, 1972). The development of the gas-chromatographic technique made it possible to perform detailed studies on the fatty acid patterns of brain lipids. For these reasons it was decided to analyse the effects on brain during development of three different levels of EFA in a diet that was adequate in all other respects. In the course of this study also other research groups have been working on the relation between dietary EFA and the fatty acid pattern of brain lipids (Galli et al., 1970; White et al., 1971; Galli et al., 1970b; Paoletti and Galli, 1972; Sinclair and Crawford, 1973), but with different aims and different methods.

METHODOLOGICAL CONSIDERATIONS

NUTRITIONAL DESIGN

The caloric composition of the diets, the designation of the diets and of the groups of rats used in papers III - VI are given in table III. Rats fed the HP 3.0 and HP 0.75 diets were bred for two generations or more on these diets before animals were taken for analyses. In the beginning of the experiments, the temperature and humidity of the animals' room were not controlled. The mortality of young rats from dams fed the HP 0.07 (10:1) diet was high (Svennerholm, et al., 1972). Therefore, rats from the HP 0.75 group were transferred to the HP 0.07 (10:1) diet 45 days before delivery and their offspring were taken for analyses; the HP 0.07A group. With controlled temperature (23°C) and humidity (60%) it was possible to breed rats on the HP 0.07 (10:1) and HP 0.07 (4:1) diets with a very low mortality. These rats were designated the HP 0.07B and HP 0.07C groups, respectively.

Essential fatty acid depots

The composition of the fatty acids in a diet fed to a rat determines the fatty acid composition of whole body triglycerides (Hilditch and Williams, 1964). The concentration of EFA in whole body total lipids was also related to the level of EFA of the diet (paper IV). The concentration of EFA in milk produced by the rat was directly related with the dam's dietary intake of EFA (paper III). The fatty acid compositions of the triglycerides in the maternal diet and in the triglycerides of adipose tissue, liver and serum in her 21-day-old offspring, were also strongly correlated with each other (paper II). These relations are not established as soon as a certain level of EFA is fed to a rat. Sinclair and Collins (1968 and 1970) found that in the rat the change from a normal EFA state to a deficient state was slow, while the reverse change was relatively rapid. In preliminary experiments (Alling and Svennerholm, 1970) we found that a reduction of dietary EFA was only slowly reflected in the fatty acid composition of the organs. The difference in fatty acid composition between rats fed a low EFA diet and those fed a high EFA diet increased from one litter to the next. Only the second and later generations fed a low EFA diet had an approximately constant phosphoglyceride fatty acid pattern in their brain. This importance of the EFA depots in the dams for the fatty acid composition of the brain in the offspring was described in more detail by the difference between the HP 0.07A and HP 0.07B group (paper V). Although the dams of both the

HP 0.07A and HP 0.07B groups were fed the same diet after mating, the difference in EFA depots between them was reflected in the differences in fatty acid composition in the brains of their offspring.

Mohrhauer and Holman (1963c) fed varying amounts of ethyl linoleate, linolenate, and arachidonate, and a mixture of linoleate and linolenate for a period of 100 days to weanling rats on a fat-free diet. The diet of their dams was not described. Linoleic acid 18:2 (n-6) was fed in amounts ranging from 0.009 to 4.5 and linolenic acid from 0.009 to 9.4 cal-%. The fatty acid 22:6 (n-3) belonging to the linolenic acid series was not influenced, while 20:4 (n-6) and 22:5 (n-6) of the linoleic acid series were. The weanlings obviously had depots of these fatty acids which were utilized to an extent unknown to the investigators. The effect of the graded levels of EFA on the brain fatty acid composition obtained in that study is therefore not easy to interpret.

Paoletti and coworkers (White Jr. et al., 1971; Galli et al., 1971a; Galli et al., 1971b; Galli et al., 1972) have analysed the fatty acid composition of brain ethanolamine phosphoglycerides of young offspring of dams fed an EFA deficient diet from about five days before delivery. That experimental design led to a continuous decrease in the EFA depots of the dam and consequently a decreasing supply of EFA to the sucklings. The increasing biochemical signs of EFA deficiency that were found with time were, therefore, not a function of time, but of a continuously reduced supply.

As early as 1969 Berg-Hansen and Clausen (1969) commented on the pre-experimental EFA depots of the dams. Pregnant rats were fed an EFA deficient diet from 1-2 weeks before delivery. At 14 or 28 days of age the fatty acid composition in the offspring was not found to differ from that in control rats. The authors pointed out that when this experimental approach was used the sucklings did not become EFA deficient as long as they were feeding at the breast.

This apparent importance of the EFA depots prompted us to analyse the size of the fat depots and their EFA-concentration around the time of breeding, in rats fed the three different levels of EFA for two generations or more before the actual experiment (paper IV). A reduction of EFA from 3.0 to 0.75 cal-% resulted in an increase in the concentration of the total body fat, but a further

decrease to 0.07 cal-% led to a fat concentration lower than that in rats fed 0.75 cal-%, but higher than that in those fed 3.0 cal-%. These findings indicate two effects of EFA on the total body fat:

1. Increasing amounts of dietary EFA leads to a decreasing amount of total body fat.
2. A severe deficiency of EFA during early postnatal life leads to a diminished number of adipocytes.

The combined effect of these two mechanisms explains the results obtained. Analyses of the fatty acid composition of the total lipids of the rats revealed that the concentration of linoleic acid (18:2 (n-6)) was six to eight times as high in rats fed 3.0 cal-% EFA as in those fed 0.07 cal-%. Arachidonic acid (20:4 (n-6)) was twice as high in rats fed 3.0 cal-% EFA as in those fed 0.07 cal-%.

Dietary composition

Contribution of nutrients. Throughout this study we endeavoured to use a cautious experimental approach to the dietary aspects of the problem, in order to obtain results that were both more informative and easier to interpret. A critical evaluation of nutritional methods used in metabolic research on rats has recently been published (Greenfield and Briggs, 1971). Improvements in such methods, especially in the dietary composition, were suggested in that review. Most of these suggestions were considered at the beginning of the present investigation.

We used the following criteria for the composition of the experimental and control diets:

1. The diets contained adequate amounts of all the essential nutrients except EFA.
2. The experimental and control diets were identical, except for the amount of EFA.
3. The carbohydrates were supplied mainly by starch.
4. The diets were not fat-free.

The reasons for these criteria were presented in papers II and III and their relevance to the results has been discussed in papers II - VI. The adequacy

of the amounts of the essential nutrients in the present diets has been commented on in paper II. The importance of the same nutrient composition in control and experimental diets in studies on EFA deficiency has been pointed out by Sinclair and Collins (1968).

If one, or more, of these criteria is ignored in investigations on the effects of EFA deficiency on growth, the effects on growth cannot be ascribed to the EFA deficiency. In most of the studies on growth in EFA deficiency the experimental diets have been fat-free, and fat has, as a rule, been replaced by sucrose on a weight basis. (Burr and Burr, 1929; Burr and Burr, 1930; Greenberg et al., 1950; Greenberg et al., 1951; Pudelkewiez et al., 1968; Aas-Jörgensen and Hölmer, 1969; Galli et al.,1970; White Jr et al., 1971; Sun, 1972). From a metabolic point of view, a withdrawal of fat from the diet leads to a change in the fatty acid metabolism (Volpe and Vagelos, 1973) and, from the caloric point of view, to an increased percentage of calories derived from proteins. In rats a high-sucrose diet resulted in a slower weight gain, higher body and liver fat concentration, and higher serum triglyceride concentration, than did an equivalent amount of starch (Laube et al., 1973).

In studies on the fatty acid composition of brain lipids in EFA deficiency the use of control diets containing a different proportion of calories from fat than the experimental diet, were probably less deleterious than in studies on body growth. However, such control diets introduce unknown factors. The use of a commercial stock diet as a control diet must, under all circumstances, be avoided in studies on the effect of EFA deficiency on brain. There are generally several differences between the commercial diet and the experimental diet which can influence the results. A commercial diet has nevertheless been used in some studies (Biran et al., 1964; Rathbone, 1965; Steinberg et al., 1968; Sun, 1972).

Linoleic and linolenic acids. The effects of three different dietary levels of EFA - a very low, a suboptimal, and an optimal level - were investigated. Previous studies have, as a rule, applied the all-or-none approach in the investigation of the relation between dietary EFA and the fatty acid composition of organs. Only Mohrhauer and Holman (1963a, 1963b and 1963c) have previously analysed the effects of graded levels of EFA, but they did not include

the intra-uterine and early postnatal life in their studies. The use of graded levels creates a situation that is surely more likely to occur in man than the total absence of EFA.

The fatty acid composition of the phosphoglycerides in the brain depends to a large extent on the ratio between the linoleic and the linolenic acid in the diet. Walker (1967) fed pregnant rats either a diet containing linoleic and linolenic acid with a ratio of 45:1 or 8:1. The former contained 10% fat; the latter, 3%. Fatty acid determinations of the total lipids in the brain of the offspring of these dams showed that the high ratio resulted in lower levels of 22:6 (n-3) and higher levels of 22:5 (n-6) than the diet with the lower ratio, but no appreciable change in the sum of the two fatty acids. Similar results were obtained at 0, 12 and 24 days of age. Galli et al., (1971c) supplemented the diet of one group of rats with fish oil, containing fatty acids (5%) of the linoleic acid series and fatty acids (33%) of the linolenic acid series, and safflower oil, containing 80% linoleic acid, to another group. Both oils were fed in an amount corresponding to 1% of the consumed diet. The level of linoleic acid in the fish oil and that of linolenic acid in the safflower oil were extremely low and the diets were obviously deficient in either linoleic or linolenic acid. The fatty acids of the linolenic acid series in the fish oil were mainly 20:5 (n-3) and 22:6 (n-3). The ethanolamine phosphoglyceride of brains of rats fed fish oil had a high level of 22:6 (n-3) and a low level of 22:5 (n-6). In the rats fed safflower oil the concentrations were the reverse. In contrast with Walker (1967), Galli et al., (1971c) found that the sum of 22:6 (n-3) and 22:5 (n-6) was not equal in the groups, but lower in rats fed the safflower oil.

The effect of two different ratios between linoleic and linolenic acid in the same amount of dietary fat has been analysed in our laboratory (Svennerholm et al., 1972, paper II). The level of dietary fat was 21 cal-% and the linoleic/linolenic acid ratios were 85:15 and 98:2, respectively. The effect on the fatty acid pattern of ethanolamine phosphoglycerides in cerebrum was analysed at the same dietary level of EFA. Two experiments were performed; one at 1cal-% EFA and another at 5 cal-% EFA. Walker's experimental design (1967) differed from ours in that he did not feed the same amount of fat or the same amount of EFA to the two groups of rats that received different linoleic/linolenic acid ratios. In both Walker's experiment and in ours the dams were fed the diet for

a long time before they were mated. We found that the concentration of 22:6 (n-3) and 22:5 (n-6) in ethanolamine phosphoglyceride of rat cerebrum was dependent only on the linoleic/linolenic acid ratio and not on the amount of linoleic or linolenic acid in the diet. Furthermore, like Walker, (1967) we found that the sum of 22:5 (n-6) and 22:6 (n-3) was equal in both groups. One exception was found; the rats fed the ratio 98:2 and 1 cal-% had less 22:6 (n-3) than those fed the same ratio but 5 cal-% EFA. Further studies in our laboratory have indicated that a drastic reduction of the cal-% of linolenic acid (below 0.20 cal-%) decreases the concentration of 22:6 (n-3) in ethanolamine phosphoglyceride in rat cerebrum, more than one should expect from the linoleic/linolenic acid ratio (paper V).

The realization of the importance of the dietary linoleic/linolenic acid ratio for the fatty acid pattern of brain phosphoglycerides has improved our knowledge of the factors determining these fatty acid patterns. Although Walker's results were published in 1967, some subsequent studies on the effect of EFA deficiency on the fatty acid composition of brain lipids, have not considered the importance of the linoleic/linolenic acid ratio in the composition of the diets (Steinberg et al., 1968; Berg-Hansen and Clausen, 1968; Galli et al., 1970; White Jr et al., 1971; Galli et al., 1971b; Paoletti and Galli, 1972; Galli et al., 1972). The value of these investigations has, therefore, been limited.

Growth

The effect of EFA deficiency upon growth was the first symptom of this deficiency that was noticed and analysed. Burr and Burr (1929) rigidly excluded fat from the diet of growing rats and found that the growth curves reached a plateau 20 - 30% below that for the controls. The level of dietary linoleic acid necessary for maximum growth has been measured by several investigators in rats between 21 and 150 days of age. A variety of values have been reported from 10 to 100 mg per day, per rat, (Holman, 1970). The value of previous investigations on the growth-promoting activity of dietary linoleic acid or the growth retarding effect of the deficiency of linoleic acid is limited. Firstly, the experiments have started on weanlings and the results were therefore influenced by the dietary history of the dam (see previous chapter "EFA depots"). Secondly, the experimental diets were, as a rule, made EFA deficient by excluding fat from the diet, which may by itself influence growth (see previous

chapter "Dietary composition").

The experimental design used in this laboratory, permitted analysis of the effect of varying dietary levels of EFA on growth during the early postnatal period, from 5 days of age, up to 120 days of age (paper III). The growth-retarding effect of 0.75 or 0.07 cal-% EFA compared with that of 3.0 cal-% EFA was small but significant and was demonstrable at such an early age as 5 days. Paoletti and coworkers (White Jr et al., 1971; Galli et al., 1971b; Paoletti and Galli, 1972) have reported reduced body weight of young offspring from dams fed an EFA deficient diet from 5 days before delivery. We do not consider that the design of their study was such as to give appropriate information on growth during EFA deficiency and for the following reasons: Only very few animals were analysed; no acceptable growth curve could be drawn for the control group, in which the females diminished in weight with increasing age; the control diet and the experimental diet differed from each other in fat content.

Also the effect of two levels of protein combined with different levels of EFA in otherwise identical diets was analysed in this laboratory (paper III). It was found that the effect of a low EFA diet on growth was superimposed on the effect of a low protein diet. The opposite has been claimed by Holman (1968) in that if a fat-free diet is deficient also in other nutrients and thereby retains growth, the development of symptoms of EFA deficiency will be delayed or prevented. The kind of EFA deficiency symptoms in question were however not described.

ANALYTICAL

Identification and quantification of fatty acids

The methods used for identifying fatty acids and the accuracy or precision for quantification are, as a rule, not properly described in studies on EFA deficiency. This is especially unfortunate in studies on the brain, because the differences obtained are small, and their demonstration requires the use of methods with a high degree of precision. In the present investigation fatty acids were analysed with gas-liquid chromatographic methods elaborated in this laboratory in the early 1960s and since used extensively on human brain material. The identification and quantification of the fatty acids are briefly summarized below:

Identification.

1. Comparison of the retention times of saturated and monounsaturated fatty acids with those of synthetic compounds, (Ställberg-Stenhagen and Svennerholm, 1965), and the relative retention times of polyunsaturated fatty acids compared with those reported at the same colomn temperature (Ackman, 1963). Addition of a known standard to a sample containing a tentatively identified fatty acid revealed only one peak.

2. Plotting of the logarithm of retention time versum carbon number and comparing the equivalent chain length with those reported (Hofstetter et al., 1965).

3. Group separation of the fatty acid methyl esters according to degree of unsaturation on silver nitrate impregnated thin-layer plates prior to gas-liquid chromatography (Morris, 1966).

4. Analysis of the same methyl ester on two stationary phases with different separation characteristics (e.g. a non-polar phase (Apiezon L, OV-1) and a polar phase (DEGS, EGSS-X)).

5. Mass spectrometry (Svennerholm, 1968).

Accuracy.

1. Results obtained with National Institute of Health fatty acid mixtures agreed with the stated composition with an error of less than 5% for major components (>10% of the mixtures) and less than 10% for minor components (<10% of the mixture).

2. Bull testes stored at -20°C were regularly extracted and methyl esters of their total lipids were included in the analyses. The determinations of this fatty acid mixture has the same error as commercial standards in the longitudinal control.

3. All solvents were freshly distilled. As large samples as possible were used in order to minimize the effect of unspecific peaks that originate from the solvents and had the same retention times as some of the major fatty acids (Svennerholm and Vanier, 1973b).

Precision. The coefficients of variation ($\frac{SD \times 100}{M}$) were calculated and are given in paper V.

Evaluation of differences. The number of animals must necessarily be limited when the effects of different diets on brain lipid fatty acid patterns are to be

analysed in animals of many ages. In order to increase the validity of the results we based the evaluation of the differences on the following measures:

1. We used an inbred strain of rats (more than 75 generations);
2. We used animals of representative body weight;
3. We used a known coefficient of variation which included the interindividual difference within a litter and the experimental error of the analyses.

EFFECTS OF VARIOUS LEVELS OF DIETARY ESSENTIAL FATTY ACIDS

The optimum dietary level of EFA during early development was not known before the present investigation. A pilot study was therefore undertaken. Diets containing 1, 5 and 10 calorie-% EFA (linoleic/linolenic acid ratio 98:2) were fed to pregnant rats and their offspring. The fatty acid composition of total phosphoglycerides and ethanolamine phosphoglycerides in the cerebrum were analysed at eight different ages, from term up to 360 days of age. No differences between the groups were found at any age. The fatty acid composition at one year of age is given in Table 3. In further investigations, 3 cal-% was considered an optimal level, sufficient for requirements during pregnancy and all stages of development.

Table 3. Fatty acid composition of ethanolamine phosphoglyceride in cerebrum of one year old rats fed 1, 5 and 10 cal-% EFA.

	1 cal-%	5 cal-%	10 cal-%
16:0	6.1	5.6	5.7
16:1	0.3	0.3	0.3
18:0	22.0	21.9	21.6
18:1	16.3	17.0	16.1
18:3 (n-3)	2.8	2.9	3.3
20:3 (n-9)	0.3	0.2	0.4
20:4 (n-6)	17.0	16.1	16.5
22:4 (n-6)	7.2	7.7	8.4
22:5 (n-6)	8.4	4.3	6.1
22:5 (n-3)	0.1	0.2	0.1
22:6 (n-3)	18.2	22.3	20.0
18 - 22 (n-6)	33.9	29.5	32.4
18 - 22 (n-3)	18.3	22.6	20.2
(n-6) + (n-3)	52.2	52.1	52.6

EFFECTS AT DIFFERENT DEVELOPMENTAL STAGES

The results of our investigations on the effects of low dietary levels of EFA on brain, are given in papers II and V. The quantitative lipid data are discussed in these papers and the following survey is mainly confined to the influence on the fatty acid composition of phosphoglycerides during intrauterine life and postnatal development.

The intrauterine period. The possibility of inducing changes in the fatty acid composition of rat brain lipids during gestation was first described by Walker (1967). He studied the effects of two different linoleic/linolenic acid ratios. The influence was appreciable during this period. The concentration of the fatty acid 22:6 (n-3) was higher, and that of 22:5 (n-6) was lower, in the group that received a low linoleic/linolenic acid ratio than in the group that received a high ratio. But the sum of these two fatty acids was always constant. Sinclair and Crawford (1973) fed rats through two generations on a low fat diet and examined the brains of the third generation of newborns. The aim of that study was to compare the effect of a low (0.3 cal-%) and a high (6 cal-%) level of EFA. In their study the linoleic/linolenic acid ratio was the same in the low-fat group as in the control group. The brains from the animals fed a low level had decreased concentrations of 20:4 (n-6), 22:4 (n-6) and 22:6 (n-3) and increased concentrations of 18:1 and 20:3 (n-9). In paper V the vulnerability of the brain to a low EFA supply during intrauterine life was compared with that of other periods of development. The value of 22:6 (n-3) was lower and those of 20:3 (n-9) and 22:3 (n-9) were higher in the HP 0.07 group and in the HP 0.75 group than in the HP 3.0 group. In contrast with Sinclair and Crawford (1973), we found a constant level of 20:4 (n-6). One can conclude from these studies that the fatty acid composition of brain lipids can vary considerably during intrauterine life as a result of maternal EFA deficiency. Moreover, the effects were larger than during the suckling period, provided that the dams had the same dietary supply during both periods. It is possible that the relatively low degree of cellular differentiation of the brain at this age can tolerate a large variation in the fatty acid pattern without any injurious effect.

The suckling period. The triglyceride concentration of rat milk is high,

approximately 200 mmol/l (approx. 16 g%), and fat constitutes about 70% of the calories (Czajka et al., 1968). When rats were fed through several generations on their respective dietary EFA levels, we found that the concentration of linoleic acid in rat milk triglycerides was directly linearly correlated with the dietary EFA level (paper III). This enabled us to study the effect of three different dietary EFA levels on brain via the milk during the suckling period. The design of previous studies has not resulted in controlled EFA concentrations of the dam's milk. Galli et al., (1971a) analysed the fatty acid composition of the stomach contents obtained from the suckling rats and reported a considerable dimunition of linoleic acid during the lactation period when the dams were fed the EFA deficient diet from 5 days before delivery. In our study (paper III), however, we found that at a given dietary EFA level the concentration of linoleic acid in the rat milk was the same on the 4th as on the 14th day of lactation. This means that the experimental design by Paoletti and coworkers resulted in an increasing deficiency of EFA to the sucklings with time and that their conclusions about the effects of EFA deficiency during the various stages of brain development are not valid. Berg-Hansen and Clausen (1969) fed pregnant rats an EFA-free diet from 1-2 weeks before delivery and found no difference in the brains of 14 and 28 day old offsprings, compared to controls. They concluded that the sucklings were not EFA-deficient as long as they received breast milk.

The only study on the effects of different dietary levels of EFA on brain during development, as analysed at the end of the suckling period, i.e. 21 days of age is that presented in paper II. Because rats gradually start to eat the food of adults some days earlier the 18th day of life was chosen for analyses in the later study (paper V). During the suckling period the cerebrum has passed through the phase of fastest EFA deposition, which occurrs between 10 and 18 days of age. In spite of this large requirement of EFA (a tenfold increase per cerebrum) and in spite of the large differences in the EFA supply (a tenfold difference in the concentration of linoleic acid in the milk triglycerides of rats fed 0.07 cal-% and 3.0 cal-%), only minor differences were found. The induced changes were, however, larger during the suckling period than in later life.

The post-suckling period. After 24 days of age the differences between the

groups in our study (paper V) became smaller with but one exception. The accretion of 22:6 (n-3) ceased in rats fed the lowest EFA levels. Though no explanation can be offered for this several might be imagined.

1. An increased exploitation of 18:3 (n-3) by other organs at the expense of brain.
2. The supply of 18:3 (n-3) in terms of cal-% might decrease when the animals are weaned.
3. The brain can tolerate wide variation of the level of 22:6 (n-3) thanks to compensation by 22:5 (n-6).

In this connection it should be mentioned that also Galli et al., (1970) found a decrease in 22:6 (n-3) in the EFA deficient group in spite of a linoleic/linolenic acid ratio of 3.5:1 compared with 38:1 in the control groups.

EFFECTS IN RELATION TO EXTRA-NEURAL ORGANS

The fatty acid composition of ethanolamine phosphoglycerides was analysed in the cerebrum, and that of lecithin (choline phosphoglycerides) in serum and in the liver. This was done for the following reasons. In the cerebrum lecithin has a much lower concentration of polyunsaturated fatty acids than ethanolamine phosphoglycerides (paper I) and is, therefore, less suitable for the study of the effect of EFA deficiency. Ethanolamine phosphoglycerides in serum, on the other hand, are such a small fraction of the phosphoglycerides that they cannot be studied from a practical point of view. Lecithin and the total phosphoglyceride fraction of cerebrum was analysed for effects of low dietary EFA levels (papers II and V), but showed less pronounced changes than ethanolamine phosphoglycerides.

The effect of a low EFA-diet was much larger in serum and in the liver, than in the cerebrum. In lecithin from serum and the liver (papers II and VI) the concentrations of the fatty acids 18:2 (n-6), 20:4 (n-6) and in particular that of 22:6 (n-3) were strongly reduced. These reductions were compensated for by an increase in 18:1 and 20:3 (n-9), but in contrast with the cerebrum, not by 22:3 (n-9). The concentration of 20:4 (n-6) of ethanolamine phosphoglycerides was extremely constant in the cerebrum but not at all in serum or liver lecithin. The largest alterations in serum and liver were found at birth and at the end of the suckling period, i.e. at the same ages as in the cerebrum.

SUMMARY

Previous studies on the effects of malnutrition during development on the biochemical composition of brain, have been concerned mainly with protein-calorie malnutrition. In experimental animals this form of malnutrition has been shown to induce a reduction in the amount of DNA, protein and myelin lipids. However, the paucity of information does not permit any conclusions about the situation in man.

This study has been devoted to another kind of malnutrition - essential fatty acid deficiency -. Objections are raised against previous studies, viz their inadequacy with respect to the pre-experimental diet, the representativity of the animals and the dietary composition. Nutritional methods were, therefore, designed, which enabled the study of the effects of low dietary levels of essentail fatty acids in the rat from foetal to adult age. It was found important, first, to feed the rats a certain low dietary level of essential fatty acids through more than two generations, before rats were taken for analyses and, second, not to feed a fat-free diet but the same fat level and the same linoleic/linolenic acid ratio in all diets.

The effects on growth, body composition, the lipid concentrations and the fatty acid compositions of phosphoglycerides of cerebrum, and lecithin in serum and liver were analysed. A significant dimunition was found in growth, not only in rats fed a very small amount of essential fatty acids (0.07 cal-%), but also for those fed only moderately reduced amounts. The latter animals became obese, particularly the females. When the dietary level of essential fatty acids was reduced at weaning to very low levels, the fattening became worse. In brain the changes in the lipid concentrations were small. Larger changes were found in the fatty acid composition of ethanolamine phospho-glycerides. The concentrations of cholesterol and cerebrosides were slightly lower between 18 and 45 days of age in rats fed 0.07 calorie-% essential fatty acids than in rats fed 3.0 calorie-%. The accretion of the fatty acids of the linolenic acid series was strongly reduced in rats fed 0.07 calorie-% and, though to a lesser extent, in those fed 0.75 calorie-%. In the fatty acid pattern of ethanolamine phosphoglycerides, essential fatty acid deficiency led to a reduction of fatty acids of the linolenic acid series. This decrease

was compensated for by the appearance of polyunsaturated fatty acids of the oleic acid series, and by an increase of the fatty acid 22:5 (n-6). The arachidonic acid, 20:4 (n-6) was remarkably constant between the groups. In serum and liver, low dietary levels of essential fatty acids induced large reductions of the fatty acids of the linoleic and of the linolenic acid series in lecithin. In brain, serum and liver the largest changes occurred simultaniously i.e. at birth and in the late suckling and early postsuckling periods.

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