

Protein utilization, growth and survival in essential-fatty-acid-deficient rats

BY CHRISTIANI JEYAKUMAR HENRY AND AMAL GHUSAIN-CHOUERI

*School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane,
Oxford OX3 0BP*

AND PHILIP R. PAYNE

*Human Nutrition Unit, London School of Hygiene & Tropical Medicine, 2 Taviton Street,
London WC1H 0BT*

(Received 12 December 1994 – Revised 1 May 1995 – Accepted 10 May 1995)

The relationship between essential fatty acids (EFA) deficiency and the utilization of dietary protein, growth rate and survival of offspring was investigated in rats during development and reproduction. EFA deficiency was induced by feeding a 200 g casein/kg-based diet containing 70 g hydrogenated coconut oil (HCO)/kg as the only source of fat. The conversion efficiency of dietary protein was assessed as net protein utilization (NPU), using a 10 d comparative carcass technique. Consumption of the deficient diet during the 10 d assay period induced biochemical changes characteristic of mild EFA deficiency in humans (triene:tetraene 0.27 (SD 0.04) compared with 0.026 (SD 0.004) for non-deficient controls), but there were no significant changes in growth rate or protein utilization. These variables were also unchanged when the deficient diet was fed for an additional 7 d before the assay, although triene:tetraene increased to 0.8 (SD 0.02). Feeding the deficient diet for 63 d before assay produced severe EFA deficiency (triene:tetraene 1.4 (SD 0.3) *v.* 0.036 (SD 0.005) for controls), a fall in growth rate (25% during assay period), and NPU (31.5 (SD 0.63) *v.* 39.0 (SD 0.93) for controls). These severely-EFA-deficient animals had a 30% higher fasting–resting rate of energy metabolism than that of age-matched controls. However, there was no change in the rate of endogenous N loss. Voluntary energy consumption was increased in animals fed on deficient diets, either with 200 g protein/kg, or protein free. The reduced efficiency of protein utilization could be entirely accounted for by the restricted amount of energy available for growth and protein deposition. Consumption of an EFA-deficient diet during pregnancy and lactation resulted in high mortality (11% survival rate at weaning compared with 79% for controls) and retarded growth in the preweaning offspring. It is concluded that animals are particularly sensitive to EFA deficiency during reproduction and pre- and post-natal stages of development. However, after weaning only severe EFA deficiency retarded growth, primarily through changes in energy balance.

Essential fatty acids deficiency: Protein utilization: Metabolic rate: Pregnancy and lactation

Essential fatty acids (EFA) deficiency has been described in human subjects suffering from protein–energy malnutrition (Holman *et al.* 1981). It has been suggested also that EFA deficiency might be the factor responsible for precipitating the acute syndrome of kwashiorkor, aggravating the effects of protein deficiency in malnourished children and contributing to the skin lesions commonly associated with that condition (Naismith, 1973). EFA deficiency has long been associated with poor growth in animals (Burr & Burr, 1930) and in human infants (Hansen *et al.* 1963). Since poor growth (*i.e.* slow weight gain) is almost inevitably accompanied by reduced N balance and changes in appetite, some association between EFA deficiency and altered protein metabolism is to be expected.

However, the direction and nature of the causal mechanisms linking the two have not been clearly established.

Protein deficiency has been reported to decrease the supply of polyenoic acids for normal development and metabolic adaptation in the rat (De Tomas *et al.* 1983). On the other hand, some earlier studies have been interpreted as showing the reverse effect, namely that EFA deficiency has a direct effect on metabolism, leading to impaired protein utilization. Naismith's (1973) studies of the EFA status of Nigerian infants suffering from protein-energy malnutrition (kwashiorkor) were stimulated by his earlier experiments with rats, which showed a 39% fall in N retention in rats maintained on diets devoid of EFA (Naismith, 1962). However, the design of those experiments did not allow the separation of any direct effect of deficiency on protein retention *per se* from that resulting indirectly from the reduced availability of energy. Moreover, there was no objective measure of the degree of EFA deficiency induced.

Other reports have suggested that there may be links between EFA status and energy metabolism that may account for impaired protein retention. Barta-Bedo (1963) attributed a two-fold increase in protein-sparing effect of a supplement of sunflower oil to its EFA content. The sunflower oil was given to protein-depleted rats and compared with rats given lard (animal fat). Others have drawn attention to the effect of EFA deficiency on cell membrane function in general (Robillard & Christon, 1993) and, in particular, to the marked increase in the rate of water transpiration from the skin seen in EFA-deficient animals and human subjects (Sinclair, 1952). Hence, it is possible that a direct effect of EFA deficiency is an increased rate of heat loss. In the absence of a compensatory rise in food consumption, the enhanced rate of heat loss reduces energy balance, and as a consequence N retention.

In addition to the direct, or indirect, effects on protein and energy balance, several animal experiments have demonstrated that maternal EFA deficiency during pregnancy and lactation resulted in growth retardation in the foetuses (Menon *et al.* 1981) and offspring (Alling *et al.* 1974; Menon *et al.* 1981; Parlanti & Orellana, 1985). Arachidonic acid in particular has been shown to have growth-promoting effects mediated through its role in the synthesis of prostaglandins and other eicosanoids (Koletzko & Braun, 1991). The role of EFA deficiency in stunting growth, independent of the nutritional status of the mother, in post-natal animal models has been demonstrated (Stepankova *et al.* 1990; Dvorak & Stepankova, 1992). Despite these numerous studies, the degree of EFA deficiency required to elicit effects in young animals and in the progeny of deficient dams remains controversial.

The aims of the present studies were (1) to investigate the threshold level of EFA deficiency necessary to impair protein utilization during early growth in the rat, (2) to observe the relationship between protein utilization, energy consumption and fasting rate of energy metabolism, (3) to assess the effects of EFA deficiency during pregnancy and lactation on survival and growth of offspring.

MATERIALS AND METHODS

Animals and diets

Weaning (21–23 d old) Sprague–Dawley rats (OLAC Ltd, Shaws Farm, Blackthorn, Oxon.) were used for the protein utilization studies. For the breeding experiments, Sprague–Dawley male and female rats, weighing 343 (SD 5.4) and 217 (SD 6.0) g respectively, were used. One male and one female were caged together, the male was removed after 10 d. Rats were kept in a room maintained between 26 and 28° with a 12 h light–dark cycle. All animals were allowed free access to food and water.

Table 1. *Composition of diets fed to rats in essential fatty acid (EFA) experiments (g/kg)*

Ingredient	Diet...	A	B	C	D
		EFA ⁺ P ⁺	EFA ⁻ P ⁺	EFA ⁺ P ⁻	EFA ⁻ P ⁻
Casein (fat-vitamin-free)		200	200	—	—
Sucrose		580	580	780	780
Sunflower-seed oil*		70		70	
Hydrogenated coconut oil			70		70
Vitamin mix†		100	100	100	100
Salt mix‡		50	50	50	50

P, protein; +, adequate; —, deficient.

* Van den Burghs & Jurgens, Sussex House, Burgess Hill, Sussex RH1 59AW.

† Miller & Bender (1955) mixed with solka floc-cellulose powder.

‡ Miller (1965).

The composition of the experimental diets is given in Table 1. The EFA-deficient diets, containing either 200 or 0 g protein/kg, were constructed using hydrogenated coconut oil (contains no linoleic acid). The control (EFA-containing) diets, both 200 and 0 g protein/kg, contained sunflower-seed oil (53 g linoleic acid/100 g oil). The metabolizable energy (ME) content of the diets was calculated to be 14.6 kJ/g (on an air-dried basis), by applying the conversion factors of 16.7, 37.6 and 16.7 kJ/g respectively for the energy content of protein, fat and carbohydrate.

Measurement of protein retention and energy metabolism. Animals were fed on the experimental diet B (Table 1) for either 0, 7 or 63 d before the net protein utilization (NPU) assay. Rats fed on diet B for 0 d were given BP (Witham, Essex) stock diet. Control animals were fed on diet A, containing sunflower-seed oil. Consumption of the two diets for variable periods before the NPU assay allowed different degrees of EFA deficiency to be induced. At the beginning of the assay period rats were either 30 (for both mild and moderate deficiency) or 83 d old (for the severe deficiency). Animals from each of the two diet treatments were allocated to groups of four, such that the total weights of each group were within a range of 3 g. For each diet treatment some groups of animals were then killed, to provide an initial value for total body N content. During the next 10 d (the duration of NPU assay) some groups from each diet treatment were transferred to protein-free diets C and D (EFA-containing or -deficient respectively). Each group was housed in a cage with wire mesh at the bottom. The spilt feed was collected on a plastic tray covered with filter paper and kept underneath the cage. The powdered diet was dispensed in glass pots. Energy and protein (N) intakes were calculated from the amount of air-dried feed consumed multiplied by the N content of the diets to give the N intakes of the animals, or multiplied by 14.6 to give the ME intakes. On the 10th day of the assay period the animals were killed using chloroform anaesthesia. Liver samples were removed from each rat in the group, pooled and subsequently used for lipid analysis. The water content of the carcasses (including gut contents) were determined by drying to a constant weight.

Breeding experiment. Sixteen female rats were divided randomly into two groups. Group 1 was fed on an EFA-containing diet (diet A, Table 1). Group 2 was fed on an EFA-deficient diet (diet B, Table 1). Gestation period was timed from the introduction of the male. Animals were checked regularly for signs of EFA deficiency.

Analytical methods

Nitrogen content of carcass and feed. The dried carcasses from each group were pooled and macerated. Samples of the finely minced carcass and of dried feed were analysed for N using the Kjeldahl method (Bradstreet, 1965).

Fasting metabolic rate (FMR). FMR was measured during the 10 d NPU assay period in severe-EFA-deficiency experiments at 26–28°. O₂ consumption was measured using an open-circuit system. The groups of rats were fasted for 18 h and placed, two at a time, into a Perspex chamber (400 mm × 200 mm). The flow-rate and temperature of the dried air entering the chamber were recorded at 30 min intervals. The air that passed through the chamber then passed through a paramagnetic O₂ analyser (Taylor Servomex).

The O₂ concentration per unit time was monitored over a period of 55 min using a chart recorder connected to the O₂ analyser. The change in O₂ concentration over the 55 min period was taken to be the area under the curve charted by the pen recorder. For each metabolic study the mean of a 6 h run was taken. The results in Table 4 are calculated on a 10 d basis for comparison with the 10 d intake measurement.

Efficiency of protein utilization. This was calculated using the formula for NPU described by Miller & Bender (1955).

$$\text{NPU} = (\text{B} - \text{B}_k) / \text{I}, \quad (1)$$

where B is body N of test-diet-fed group, B_k is body N of non-protein-fed group and I is N intake of test group.

Rate of obligatory N loss. This was calculated as (B₀ - B_k), where B₀ is body N of a weight-matched group killed at the start of the assay period.

Energy-limited value of NPU. The maximum efficiency of protein utilization achievable at a given level of energy intake was calculated from the formula given by Miller & Payne (1961 a):

$$\text{NPU}(\text{max}) \times \text{I} = (\text{C} - \text{C}_b) / \text{E}, \quad (2)$$

where C is energy intake of the test group, C_b is basal metabolic rate of test animals, and E is 36.3 (SD 1.4), the total energy cost associated with the deposition of an amount of N equivalent to 4.18 kJ ME.

Lipid analysis. Lipids from the liver sample were extracted and purified using a modification of the method of Folch *et al.* (1957). The liver lipid extracts were stored at -18° until required for further analysis. The lipid classes of the liver were separated by TLC; the solvent system used for the separation of phospholipids was chloroform-methanol-distilled water (60:30:4, by vol.). Lipid standards choline phosphoacylglycerol (PC) and ethanolamine phosphoacylglycerol (PE; Sigma Ltd, Poole, Dorset) were run alongside the sample to aid in the identification of the lipid fraction.

For visualization, the lipid bands were sprayed with 2 g 2,7-dichlorofluorescein/l methanol, using an aerosol. Whenever identification was difficult, the plates were exposed to fumes of NH₃ and re-examined under u.v. light at 254 μm. The identified PC and PE bands were removed separately by careful scraping and placed into methylation tubes.

Transmethylation was carried out after the addition of 50 ml concentrated H₂SO₄/l to the methylation tubes. The contents were heated in an oven at 70° for 3 h with vigorous shaking every 20 min. Methyl esters were extracted with light petroleum (b.p. 40–60°) and washed with distilled water to remove any traces of H₂SO₄. Samples were analysed by GLC (W. G. Pye and Co., Cambridge). The column used was a 2.0 m glass column, diameter 4 mm, containing 10% diethyl glycol succinate on 80–100 mesh Chromasorb W (Field Instruments Ltd). Samples were run isothermally at 191°, with the flow-rate of carrier gas (N₂) being at 64 ml/min. Peak areas were determined by triangulation.

Once the fatty acid peaks of 20:3n-9 (eicosatrienoic acid) and 20:4n-6 (eicosatetraenoic

or arachidonic acid) were identified on the GLC chart, the peak areas were determined using the formula:

$$\text{peak area} = H \times W_{\frac{1}{2}}Ht \times AF,$$

where H is the ht of peak, $W_{\frac{1}{2}}Ht$ is the width at half the height and AF is the attenuation factor.

The ratio of the two peak areas gave triene:tetraene.

Statistical analysis

Differences between two diet groups were analysed using Student's unpaired t test. When more than two diet groups were compared the differences were analysed using one-way ANOVA. Results were expressed as means and standard deviations.

RESULTS

Effect of mild EFA deficiency

During the 10 d assay period of feeding an EFA-deficient diet no clinical signs of EFA deficiency were observed, and no deaths occurred in the assay groups. Feeding of an EFA-deficient diet for 10 d had no significant effect on growth or food intake compared with the controls (Table 2). As expected, animals on the protein-free diet lost weight and N. However, EFA deficiency did not increase the rate of weight loss, nor did it increase the rate of obligatory loss of body N. The NPU values were also the same, showing that there was no significant reduction of efficiency of protein utilization in mild EFA deficiency. However, the biochemical index of the EFA status, triene:tetraene (20:3:20:4), gave values (Table 2) for deficient animals which were ten times higher than those for the controls, indicating a marked decline in status.

Moderate EFA deficiency

When animals were fed on either the deficient diet B, or the EFA-containing diet A for a 7 d period before the 10 d assay, there was again no significant effect of deficiency on the rate of weight change in animals fed on either the 200 g protein/kg or the protein-free diets (Table 3). No deaths or clinical signs of EFA deficiency were observed in these groups. Calculation of NPU showed no change in efficiency of protein utilization (Table 3). However, triene:tetraene in the deficient animals (Table 3) was twice the value of 0.4 suggested by Holman (1960) as the limit of the normal range. In the protein-free EFA-deficient group there was a significant ($P < 0.05$) decrease in feed intake compared with the group fed on a protein-free EFA-containing diet (Table 3). However, the body N content of these two groups was the same (Table 3), indicating no significant effect of deficiency on the rate of loss of obligatory N. In these animals the rates of daily obligatory N loss were similar to those reported by Miller & Payne (1963; 250 mg N/d per kg body weight (W)^{0.75}) for normal rats of comparable age.

Severe EFA deficiency

These animals were fed on EFA-deficient or EFA-containing diets for 63 d (9 weeks) before the 10 d assay. The growth curves for the two groups began to diverge at week 5, and by week 6 there was a marked change in growth rate compared with the controls. By week 9 they showed scaliness over the whole length of the tail, some superficial haemorrhage of the tail, roughened coat and scaly feet. During the subsequent assay period, weight gain was significantly ($P < 0.05$) reduced in EFA-deficient animals fed on the 200 g protein/kg diet. Calculations of NPU values showed a highly significant ($P < 0.01$) decrease in the efficiency

Table 2. *Nitrogen metabolism and triene:tetraene in mild essential fatty acid (EFA) deficiency in rats*

(Mean values and standard deviations for six groups of four rats over a 10 d assay period)

Dietary treatment†...	A EFA ⁺ P ⁺		B EFA ⁻ P ⁺		C EFA ⁺ P ⁻		D EFA ⁻ P ⁻	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wt gain (g)‡	87.0 ^a	12.5	79.3 ^a	7.9	-70.5 ^b	7.4	-71.4 ^b	8.6
Energy intake (MJ)	6.70 ^a	0.38	6.57 ^a	0.22	3.39 ^b	0.10	4.00 ^b	0.15
N intake (g)	11.36 ^a	0.65	11.15 ^a	0.37				
N retained (g)	4.75 ^a	0.39	4.59 ^a	0.13	-1.91 ^b	0.19	-1.95 ^b	0.25
NPU	41.8 ^a	1.60	41.2 ^a	1.31				
Obligatory N loss (mg/d per kg W ^{0.75})§					321 ^b	21.9	327 ^b	29.1
Triene:tetraene	0.026	0.004	0.269**	0.04				

^{a, b} Mean values in the same horizontal row with different superscript letters were significantly different by ANOVA ($P < 0.001$).

P, protein; +, adequate; -, deficient; NPU, net protein utilization; W, body weight.

Mean values were significantly different from those for the controls (Student's *t* test): ** $P < 0.01$.

† For details of diets, see Table 1 and pp. 238-239.

‡ Average starting weight approximately 80 g.

§ Mean values for individual rats (one rat taken from each of the six groups).

Table 3. *Nitrogen metabolism and triene:tetraene in moderate essential fatty acid (EFA) deficiency in rats*

(Mean values and standard deviations for four groups of four rats over a 10 d assay period)

Dietary treatment†...	A EFA ⁺ P ⁺		B EFA ⁻ P ⁺		C EFA ⁺ P ⁻		D EFA ⁻ P ⁻	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wt gain (g)‡	52.2 ^a	2.2	52.0 ^a	3.9	-38.2 ^b	2.6	-39.0 ^b	2.2
Energy intake (MJ)	4.37 ^a	0.17	4.40 ^a	0.23	2.94 ^b	0.08	2.38 ^{c*}	0.23
N intake (g)	7.4 ^a	0.32	7.48 ^a	0.36				
N retained (g)	2.91 ^a	0.15	2.96 ^a	0.17	-1.08 ^b	0.08	-1.10 ^b	0.08
NPU	39.3 ^a	1.55	39.5 ^a	0.40				
Obligatory N loss (mg/d per kg W ^{0.75})§					230 ^b	15.2	237 ^b	14.1
Triene:tetraene	0.025	0.004	0.800***	0.020				

^{a, b, c} Mean values in the same horizontal row with different superscript letters were significantly different by ANOVA ($P < 0.001$).

P, protein; +, adequate; -, deficient; NPU, net protein utilization; W, body weight.

Mean values were significantly different from those for the controls (Student's *t* test): * $P < 0.05$, *** $P < 0.001$.

† For details of diets, see Table 1 and pp. 238-239.

‡ Average starting weight approximately 60 g.

§ Mean values for individual rats (one rat taken from each of four groups).

of protein retention (21%) in these severely-deficient animals (Table 4). The feed intake of deficient animals fed on 200 g protein/kg was significantly ($P < 0.05$) increased compared with those of the EFA-fed group (approximately 15%). There was also a slight, but not significant, increase (approximately 4%) in feed consumption for the EFA-deficient

Table 4. Nitrogen metabolism and triene:tetraene in severe essential fatty acid (EFA) deficiency in rats

(Mean values and standard deviations for two groups of four rats over a 10 d assay period)

Dietary treatment†...	A EFA ⁺ P ⁺		B EFA ⁻ P ⁺		C EFA ⁺ P ⁻		D EFA ⁻ P ⁻	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wt gain (g)	100.5 ^a	6.4	75.0 ^{b*}	2.8	-72.0 ^c	2.8	-77.0 ^c	2.8
Energy intake (MJ)	7.52 ^a	0.23	8.63 ^{b*}	0.19	5.40 ^c	0.18	5.60 ^c	0.18
N intake (g)	12.1	0.49	14.0*	0.23				
N retained (g)	4.72 ^a	0.08	4.41 ^a	0.01	-2.10 ^b	0.14	-2.22 ^b	0.09
NPU	39.0	0.93	31.5*	0.63				
Metabolic rate (MJ/10 d)	4.64	0.18	6.02*	0.12				
NPU(max)	40.0	2.0	30.3*	2.0				
Obligatory N loss (mg/d per kg W ^{0.75})§					211 ^b	1.8	221 ^b	1.2
Triene:tetraene	0.036	0.005	1.406***	0.300				

^{a, b, c} Mean values in the same horizontal row with different superscript letters were significantly different by ANOVA ($P < 0.001$).

P, protein; +, adequate; -, deficient; NPU, net protein utilization; W, body weight.

Mean values were significantly different from those for the controls (Student's *t* test): * $P < 0.05$, *** $P < 0.001$.

† For details of diets, see Table 1 and pp. 238-239.

‡ Average starting weight approximately 155 g.

§ Mean values for individual rats.

protein-free group compared with their controls. The rate of obligatory N loss was still not significantly increased by severe EFA deficiency in the protein-free diet. FMR was measured in the protein-fed, severely-EFA-deficient animals and the controls during the NPU assay period. The results (Table 4) show that metabolic rate was increased by 30% in the EFA-deficient animals in comparison with the controls. Triene:tetraene for the EFA-deficient animals was forty times that of the controls (Table 4).

Reproductive performance

Pregnant rats fed on the EFA-deficient diet B showed clinical signs of EFA deficiency by week 3 of pregnancy. A scaly tail tip was seen in half the animals. However, no scaliness of the feet was apparent, nor did scaliness spread to the whole length of the tail in any animal. The reproductive performance in deficient (fed on diet B) and control (fed on diet A) rats is shown in Table 5. The two deaths of EFA-deficient rats occurred on the day of parturition and on the following day. Both were due to excessive haemorrhaging, a problem associated with EFA deficiency in rats (Quakenbush *et al.* 1942).

Although 'gestation time' was slightly increased and litter size decreased in EFA-deficient rats, the differences were not statistically significant. However, the reduction in total litter weight and the mean birth weight observed in neonates born to EFA-deficient animals were significant ($P < 0.05$; Table 5). The difference in mean weight of EFA-deficient pups increased ($P < 0.001$) during lactation, but since mortality was higher in EFA-deficient animals the total litter weight was progressively reduced relative to controls throughout lactation (Fig. 1). The survival rate for the controls and EFA-deficient pups is illustrated in Fig. 2. By day 7 after birth, only 22% of the pups were still alive in comparison with 80% of the controls. At day 21 only eight pups (11%) survived in the

Table 5. *Reproductive performance in essential fatty acid (EFA)-deficient female rats*
(Mean values and standard deviations)

Dietary treatment†	n	Reproductive performance							
		Gestation time		Maternal deaths	No. of live litter	Litter size		Birth wt (g/pup)	
		Mean	SD			Mean	SD	Mean	SD
EFA ⁺ P ⁺	8	22.4	0.92	—	8	10.9	1.46	5.8	0.11
EFA ⁻ P ⁺	8	23.5	1.69	2	7	10.0	2.58	5.27*	0.07

P, protein; +, adequate; -, deficient.

Mean values were significantly different from those for the control values (Student's *t* test): **P* < 0.05.

† For details of diets, see Table 1 and pp. 238-239.

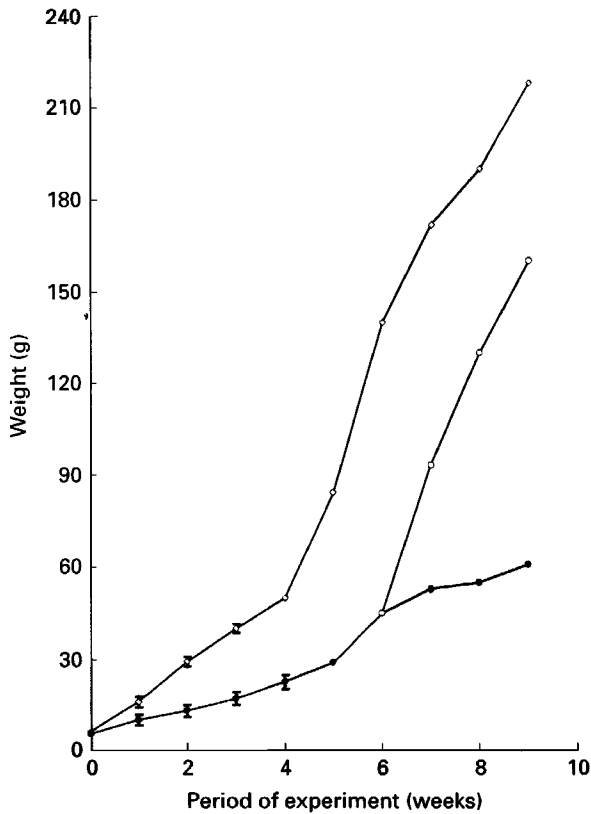


Fig. 1. Growth curves for essential fatty acid-deficient (●), rehabilitated (○) and control rats (◇). For details of dietary treatments, see Table 1 and pp. 238-239. Points are means and standard deviations, represented by vertical bars.

EFA-deficient group compared with sixty-nine (79%) of the controls. Survival to weaning amongst EFA-deficient pups averaged only one animal per litter. There was no correlation in either dietary groups between survival to day 21 (expressed either as a percentage of animals born, or as an absolute number of pups) and litter size. In both EFA-deficient

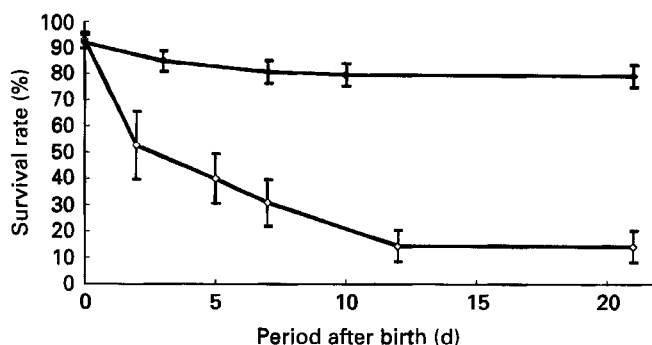


Fig. 2. Survival rate for pups born to essential fatty acid-deficient (◇) and control (◆) dams. For details of dietary treatments, see Table 1 and pp. 238–239. Points are means and standard deviations, represented by vertical bars.

animals and controls, most deaths occurred in the first week after birth. In both dietary groups, those animals which survived to day 10 almost always reached weaning.

Those pups born to EFA-deficient dams which survived to weaning were smaller relative to the controls, and grew very slowly when weaned onto an EFA-deficient diet (Fig. 1). When some of these deficient animals were fed on the EFA-containing diet A their growth rate improved to the same rate as the controls but they did not show catch-up; their growth curve was parallel to that of the control animals.

DISCUSSION

Feeding weaning rats on hydrogenated coconut oil (EFA-deficient) diet for a relatively short period of time (10–17 d) markedly altered triene:tetraene in liver phospholipids. Similar changes in triene:tetraene in plasma and liver phospholipids after a short period (1–2 weeks) of dietary EFA deficiency have been reported previously, both in rats (Trugnan *et al.* 1985) and in human infants (Lee *et al.* 1993). The threshold value for triene:tetraene originally proposed to denote deficiency was 0.4 (Holman, 1960). Half that value (0.2) was later recommended as the lower limit of the normal range in humans by Holman (1978) and by a Food and Agriculture Organization/World Health Organization Expert Committee (1977).

In the present study the mild to moderate degrees of deficiency did not induce visible clinical signs of deficiency, or affect voluntary feed intake, growth rate or the rate of obligatory N loss. Furthermore, they did not reduce the efficiency of protein utilization. Thus, the biochemical indicator of EFA deficiency can reach and exceed the currently accepted threshold value well before any clinical or metabolic changes are detectable.

With longer exposure to EFA-deficient diets, and in agreement with other studies (Holman, 1968; Rafael *et al.* 1988; Yazbeck *et al.* 1989), significant growth retardation was visible by week 5 of feeding a deficient diet. By week 9 other classical clinical signs of EFA deficiency were visible, at which point triene:tetraene of the animals was very high (1.4). Even this severe degree of deficiency did not result in any increase in the rate of obligatory N loss, in contrast to the results of Barta-Bedo (1963) who stated that the level of EFA in the diet influenced the rate of obligatory N loss.

The efficiency of retention of protein, however, was considerably reduced in severe EFA deficiency (NPU 21% lower than controls). As can be seen from Table 4, the smaller proportion of N intake which was retained by these animals was partly due to the smaller absolute amount of N retained (the numerator), but also because of increased N intake (the denominator) associated with the increased feed consumption. Reduced N retention could

be the result of some effect of EFA deficiency on protein metabolism *per se*, but could also be due to a restriction of the amount of energy available. It is well established that energy restriction can impose an upper limit on the rate of positive N balance (Calloway & Spector, 1955; Miller & Payne, 1961*b*).

The deficient animals were found to have a substantially (30%) increased metabolic rate. This has also been reported both at a reduced environmental temperature of 23° (Rafael *et al.* 1984), and at thermal neutrality 28–29° (Rafael *et al.* 1988; Yazbeck *et al.* 1989). The higher metabolic demand is thought to compensate for enhanced evaporative heat loss. This is associated with the observed increase (up to 88%) in transdermal water loss in EFA deficiency (Panos *et al.* 1958; Hansen & Jensen, 1985). Phinney *et al.* (1993) have shown that these losses can be suppressed by keeping the deficient animals in air at a high relative humidity (RH; 90% at 30°). Moreover, EFA-deficient animals housed under these conditions suffered less growth retardation than when kept at 40% RH and 20°.

The elevated FMR might be an important factor in limiting the amount of energy available for protein deposition if the increased feed intake is only partially effective in compensating for the increased rate of heat loss. A similar situation has been observed during cold exposure. Payne & Jacob (1965) maintained rats at a reduced air temperature (15°, compared with 27°) and observed a substantial increase in FMR and voluntary feed intake, accompanied by a 40% reduction in the NPU of a 250 g casein/kg diet. These authors showed that the reduction in efficiency could be accounted for by the fact that the increase in energy intake was insufficient to compensate fully for the rise in FMR, so that the N retention was limited by the available energy rather than by the quantity and quality of protein intake. To demonstrate this they made use of the equation proposed by Miller & Payne (1963) (equation 2), which predicts the maximum value of NPU which is possible at any given level of energy intake. Substituting the value for FMR, measured at the lower temperature, for the BMR (C_b in equation 2, p. 240) gave predictions of the maximum energy-limited values of NPU which were in good agreement with those observed. It seems reasonable to suppose that the increased evaporative heat loss in severe EFA deficiency could have a similar effect on both the FMR and on feed intake, as a lowered air temperature does in normal rats, leading in turn to a quantitatively predictable reduction in NPU.

The calculated values of NPU(max) in Table 4 are the result of replacing C_b in equation 2 (see p. 240) with the value for FMR observed in severely-deficient rats. This demonstrates that the NPU values actually observed were the maximum which could have been expected, given the actual levels of energy intake and expenditure.

In contrast to the young growing rats, feeding the EFA-deficient diet during pregnancy resulted in the onset of typical skin lesions as early as day 16. No cannibalism was observed, contrary to earlier reports about rats (Sinclair & Crawford, 1973) or mice (Rivers & Crawford, 1974). However, in those studies the animals were fed on EFA-deficient diets for at least 12 weeks before mating.

Foetal growth in weight is known to be significantly decreased by EFA deficiency. However, the association between EFA deficiency and neonatal mortality is less clearly described in the literature. A high post-natal mortality was observed in the pups born to dams fed on an EFA-deficient diet in the present experiments. Similar rates of pup mortality were reported in litters born to first- (Parlanti & Orellana, 1985) and second-generation EFA-deficient rats (Sinclair & Crawford, 1973; Alling *et al.* 1974). In our study, triene:tetraene for liver phospholipids in expired pups had a mean value of 0.7, a value not exceptionally high when compared with those observed in deficient animals in the post-weaning experiments. The high mortality, therefore, may not be directly due to EFA deficiency but may, for example, reflect a reduction in maternal milk output, with mortality due to semi-starvation. This in turn may have been exacerbated by a rise in heat loss, as

is found in the post-weaning experiment, especially when the pups' high surface area:body weight is considered.

The surviving EFA-deficient pups failed to have a catch-up growth period when weaned onto an EFA-adequate diet. This suggests that an EFA deficiency experienced in infancy (prenatal and/or early post-natal) is irreversible, even when the deficiency is corrected later on.

This is an interesting parallel with the recent recognition that children who suffer growth retardation from a combination of maternal or concurrent malnutrition and infectious disease within the first 2 years of life do not subsequently fully recover their potential for catch-up growth (Beaton, 1989), even when given prolonged and intensive supplementary feeding (Lutter *et al.* 1990).

In conclusion, these studies have shown that the progressive development of EFA deficiency in the rat can be monitored using triene:tetraene. The sensitivity of this measurement is such that the threshold level suggested by the Food and Agriculture Organization/World Health Organization Expert Committee (1977) as indicative of a state of deficiency can be reached and greatly exceeded before any of the expected clinical signs or physiological symptoms become patent. EFA deficiency, especially relating to the energy requirement for growth during the neonatal and weaning periods, has serious functional consequences which may contribute to a permanent stunted growth. This finding is of importance for the nutritional management of human pregnancy and weaning in developing countries (Robillard & Christon, 1993). Moreover, the study as a whole highlights the danger of relying only on clinical symptoms to identify the risk of EFA deficiency in humans. The more obvious physiological effects of moderate-to-severe deficiency are an increase in the level of energy exchange and higher fasting expenditure rates coupled with an increased food consumption. The raised energy intake, although substantial, was not sufficient to sustain normal rates of growth and protein deposition. In contrast to many previous reports, which have implied that EFA deficiency has a direct effect on protein metabolism, the present study showed no effect on the rate of endogenous N loss. Moreover, the reduction in the efficiency of protein utilization was entirely consistent with the reduced level of energy available to support protein deposition.

REFERENCES

- Alling, C., Bruce, A., Karlsson, I. & Svennerholm, L. (1974). The effect of different dietary levels of essential fatty acids on growth in the rat. *Nutrition and Metabolism* **16**, 38–50.
- Barta-Bedo, M. (1963). The effect of different fats upon the protein sparing mechanism. *Proceedings of the VIth International Congress of Nutrition (Edinburgh)*, p. 495. Edinburgh: E. Livingstone.
- Beaton, G. H. (1989). Small but healthy? Are we asking the right question? *Human Organisation* **48**, 31–37.
- Bradstreet, R. (1965). *The Kjeldahl Method for Organic Nitrogen*. New York and London: Academic Press.
- Burr, G. O. & Burr, M. M. (1930). On the nature and role of the fatty acids essential in nutrition. *Journal of Biological Chemistry* **86**, 587–621.
- Calloway, D. H. & Spector, H. (1955). Nitrogen utilisation during caloric restriction. The effect of dietary fat content. *Journal of Nutrition* **56**, 533–541.
- De Tomas, M. E., Mercuri, O. & Serres, C. (1983). Effect of cross-fostering rats at birth on the normal supply of essential fatty acids during protein deficiency. *Journal of Nutrition* **113**, 314–319.
- Dvorak, B. & Stepankova, R. (1992). Effects of dietary essential fatty acid deficiency on development of the rat thymus and immune system. *Prostaglandins Leukotrienes and Essential Fatty Acids* **46**, 183–190.
- Food and Agriculture Organization/World Health Organization Expert Committee (1977). *The role of the dietary fats and oils in human nutrition. FAO Technical Paper Series Report no 3*. Rome: FAO.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry* **226**, 497–509.
- Hansen, A. E., Wiese, H. F., Boelsche, A. N., Haggard, M. E., Adam, D. J. D. & Davis, H. (1963). Role of linoleic acid in infant nutrition. *Paediatrics* **31**, 171–192.
- Hansen, H. S. & Jensen, B. (1985). Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinatate and alpha-linolenate. *Biochimica et Biophysica Acta* **834**, 357–363.

- Holman, R. T. (1960). The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. *Journal of Nutrition* **70**, 405-410.
- Holman, R. T. (1968). Essential fatty acid deficiency. In *Progress in the Chemistry of Fats and other Lipids*, pp. 279-348. New York: Pergamon Press.
- Holman, R. T. (1978). Essential fatty acid deficiency in humans. *CRC Handbook Series in Nutrition and Food* **3**, 335-353.
- Holman, R. T., Johnson, S. B., Mercuri, C., Itarte, H. J., Rodrigo, M. A. & De Tomas, M. E. (1981). Essential fatty acid deficiency in malnourished children. *American Journal of Clinical Nutrition* **34**, 1534-1539.
- Koletzko, B. & Braun, M. (1991). Arachidonic acid and early human growth: is there a relation? *Annals of Nutrition and Metabolism* **35**, 128-131.
- Lee, E. J., Simmer, K. & Gibson, R. A. (1993). Essential fatty acid deficiency in parenterally fed preterm infants. *Journal of Paediatrics and Child Health* **29**, 51-55.
- Lutter, C. K., Mora, J. O., Habicht, J. P., Rasmussen, K. M., Robson, D. S. & Herrera, M. G. (1990). Age specific responsiveness of weight and length to nutritional supplementation. *American Journal of Clinical Nutrition* **51**, 359-364.
- Menon, N. K., Moore, C. & Dhopeswarkar, G. A. (1981). Effect of essential fatty acid deficiency on maternal, placental, and fetal rat tissue. *Journal of Nutrition* **111**, 1602-1609.
- Miller, D. S. (1965). A procedure for determination of NPU using rats body N technique. *Evaluation of Protein Quality*. NRC Publication no. 1100. Washington, DC: National Research Council/National Academy of Science.
- Miller, D. S. & Bender, A. E. (1955). The determination of the net utilisation of proteins by a shortened method. *British Journal of Nutrition* **9**, 382-388.
- Miller, D. S. & Payne, P. R. (1961*a*). Problems in the prediction of protein values of diets. *British Journal of Nutrition* **15**, 11-19.
- Miller, D. S. & Payne, P. R. (1961*b*). Problems in the prediction of protein values of diets: caloric restriction. *Journal of Nutrition* **75**, 225-230.
- Miller, D. S. & Payne, P. R. (1963). A theory of protein metabolism. *Journal of Theoretical Biology* **5**, 1-14.
- Naismith, D. J. (1962). The importance of dietary fat for the utilisation of protein in the very young. *Proceedings of the Nutrition Society* **21**, 8.
- Naismith, D. J. (1973). Kwashiorkor in western Nigeria. A study of traditional weaning foods, with particular reference to energy and linoleic acid. *British Journal of Nutrition* **30**, 567-576.
- Panos, T. C., Finerty, J. C., Klein, G. F. & Wall, R. L. (1958). Metabolic studies in fat deficiency. In *Essential Fatty Acids*, p. 205 [H. M. Sinclair, editor]. London: Butterworths.
- Parlanti, I. A. & Orellana, L. C. (1985). The influence of an essential fatty acid deficient-diet on the reproductive performance of female rats. *Reproductive Nutrition and Development* **25**, 851-860.
- Payne, P. R. & Jacob, M. (1965). Effect of environmental temperature on utilisation of dietary protein by the growing rat. *Journal of Nutrition* **87**, 221-227.
- Phinney, S. D., Clarke, S. D., Odin, R. S., Moldawer, L. L., Blackburn, G. L. & Bistrian, B. R. (1993). Thermogenesis secondary to transdermal water loss causes growth retardation in essential fatty acid-deficient rats. *Metabolism* **42**, 1022-1026.
- Quakenbush, F. W., Kummerow, F. A. & Steenbock, H. (1942). The effectiveness of linoleic, arachidonic and linolenic acids in reproduction and lactation. *Journal of Nutrition* **24**, 213-224.
- Rafael, J., Patzelt, J. & Elmadfa, I. (1988). Effect of dietary linoleic acid and essential fatty acid deficiency on resting metabolism, nonshivering thermogenesis and brown adipose tissue in the rat. *Journal of Nutrition* **118**, 627-632.
- Rafael, J., Patzelt, J., Schafer, H. & Elmadfa, I. (1984). The effect of essential fatty acid deficiency on basal respiration and function of liver mitochondria in rats. *Journal of Nutrition* **114**, 255-262.
- Rivers, J. P. W. & Crawford, M. A. (1974). Maternal nutrition and the sex ratio at birth. *Nature* **252**, 297-298.
- Robillard, P. Y. & Christon, R. (1993). Lipid intake during pregnancy in developing countries: possible effects of essential fatty acid deficiency on fetal growth. *Prostaglandins Leukotrienes and Essential Fatty Acids* **48**, 139-142.
- Sinclair, A. J. & Crawford, M. A. (1973). The effect of a low-fat maternal diet on neonatal rats. *British Journal of Nutrition* **29**, 127-137.
- Sinclair, H. M. (1952). *Essential Fatty Acids and their Relation to Pyridoxine*. Cambridge: Cambridge University Press.
- Stepankova, R., Dvorak, B., Sterzl, J. & Trebichavsky, I. (1990). Effects of essential fatty acids deficiency in milk diets on the development of germ-free and conventional rats. *Physiologica Bohemoslovakia* **39**, 135-146.
- Trugnan, G., Thomas-Benhamou, G., Cardot, P., Rayssiguier, Y. & Bereziat, G. (1985). Short term essential fatty acid deficiency in rats. Influence of dietary carbohydrate. *Lipids* **20**, 862-868.
- Yazbeck, J., Gubern, M., Senault, C., Chapey, M. F. & Portet, R. (1989). The effects of essential fatty acid deficiency on brown adipose tissue activity in rats maintained at thermal neutrality. *Comparative Biochemistry and Physiology* **94A**, 273-276.