The Effect of Essential Fatty Acid Deficiency on Basal Respiration and Function of Liver Mitochondria in Rats[1]

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ABSTRACT Rats were fed a diet poor (0.05%) in essential fatty acids (EFA) with hydrogenated coconut oil as fat component, or a control diet containing 4% of the total energy intake in the form of linoleic acid. Effects of dietary EFA deficiency were investigated during a period of 2–30 weeks. Growth retardation becomes significant after 4 weeks of deficiency and attains about 25% when the deficiency is maintained for longer than 12 weeks. Respiration, body weight and age of EFA-deficient rats and controls are in a nonlinear relationship. Basal respiration in relation to the body weight is significantly increased by EFA deficiency; it is unchanged when related to total animals under the employed experimental conditions. Oxidative phosphorylation in isolated liver mitochondria is unaffected by EFA deficiency, i.e., the increased metabolic rate of EFA-deficient rats, related to the body weight, cannot be explained from impaired functional integrity of the inner mitochondrial membrane. Respiratory chain enzyme activities in mitochondria from heart and skeletal muscle and specific amounts of mitochondria in these tissues are unchanged by EFA deficiency. J. Nutr. *114*: 255–262, 1984.

INDEXING KEY WORDS essential fatty acids • nutritional deficiency • oxidative phosphorylation • liver mitochondria • rat

The role of essential fatty acids (EFA) in the animal economy has been studied extensively for more than 50 years (see refs. 1–6 for review). Significant growth reduction and intensified basal metabolism, reported as typical symptoms of dietary EFA deficiency, have been related to functional lesions of the mitochondria (3–7). Increased substrate oxidation (8, 9) and partial uncoupling of oxidative phosphorylation (10–16) as measured with liver homogenates and liver mitochondria isolated from rats given lipid-free diets, was attributed to a decreased membrane integrity caused by EFA deficiency (3, 7). Impaired energy coupling was also found in pulsating rat heart cells, when cultured in a lipid-deficient medium (17). Very little notice was given to well-founded reports (18, 19) of unimpaired functional integrity of liver mitochondria from EFA-deficient rats, and symptoms of EFA deficiency are still explained by citing mitochondrial dysfunction in very recent reviews (5, 6) and in general lecturing (7).

It was the purpose of this investigation to scrutinize this question by correlation of physiological and biochemical parameters in EFA-deficient rats and controls. The

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effect of an EFA-deficient regimen on the basal respiration was tested, and functional capacities of liver mitochondria were controlled. Apart from pathophysiological significance under EFA deficiency, possible loose coupling of oxidative phosphorylation in vivo would appear highly interesting with respect to mitochondrial bioenergetics in general.

MATERIALS AND METHODS

Male, weanling, Wistar rats (age 22–25 days, average weight 52 g) were fed a diet poor in EFA with hydrogenated coconut oil as the fat component. Controls were fed the same diet containing linoleic acid in isocaloric quantity (4% of the total energy intake) (table 1). All rats were given tap water ad libitum. They were kept at a constant ambient temperature of 23°C (12-hour light:dark cycle), caged individually in suspended, wire-bottomed cages preventing a possible intake of bacterial EFA by coprophagy (1). After varying periods of EFA-deficiency (2–30 weeks, cf. results) groups of 6–12 rats were investigated and compared with control animals of the same age.

Metabolic O_2 consumption of unanaesthetized rats was measured in a thermostated open system as described by Heldmaier and Steinlechner (20) by means of a two-channel paramagnetic O_2 analyzer (Oxytest S, Hartmann and Braun, Frankfurt, West Germany). After 2 hours of equilibration in the metabolic chamber the basic metabolic O_2 consumption of the rats was measured during a period of 2 hours at an environmental temperature of 28°C.

Rats were killed by a blow on the head and exsanguinated. Liver, heart and combined hing leg skeletal muscles were dissected and immediately worked up for the determination of enzyme activities and the isolation of mitochondria, respectively. Liver mitochondria were isolated as previously described by Wagner and Rafael (21); the mitochondrial fraction was spun down at 7000 × g, and two washing procedures were performed. Mitochondria from heart and skeletal muscle were prepared according to Mela and Seitz (22) and Ernster and Nordenbrand (23), respectively.

TABLE 1

Nutrient composition of diets

Ingredient	EFA poor[1]	Linoleic acid[2]
	g/100 g	
Casein[3]	23.2	23.2
Sucrose	30.4	30.4
Potato starch (steam decomposed)	30.4	30.4
Cellulose	4.0	4.0
Hydrogenated coconut oil[4]	6.0	4.2
Linoleic acid-methyl ester	—	1.8
Mineral mixture[5]	5.0	5.0
Vitamin mixture[6]	1.0	1.0
Energy content, *kJ/100 g*	1647	1647

[1]Linoleic acid content <0.05%. [2]Isocaloric, linoleic acid comprised 4% of energy intake. [3]Enriched with D,L-methionine (0.08 g/100 g diet). [4]Content of linoleic acid 0.2%. [5]Supplied per 100 g of diet: $CaCO_3$, 1.50 g; KH_2PO_4, 1.73 g; NaCl, 1.22 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; iron citrate, 31.00 mg; $MnSO_4 \cdot H_2O$, 9.10 mg; ZnO, 2.50 mg; $CuSO_4 \cdot 5H_2O$, 3.13 mg; NaF, 22.0 μg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 18.0 μg; Na_2SeO_3, 2.2 μg; KI, 26.0 μg. [6]Supplied in milligrams/100 g of diet: *p*-aminobenzoic acid, 10.00; menadione 0.50; thiamin-HCl, 1.00; riboflavin, 1.00; pantothenic acid, Ca-salt, 5.00; niacin, 5.00; pyridoxine-HCl, 1.00; folic acid, 0.20; biotin, 0.04; inositol, 10.00; ascorbic acid, 5.00. In addition to this vitamin mixture the following vitamins were added to 100 g of the diet: vitamin A-acetate, 1.02 mg; vitamin B-12, 3.12 μg; cholecalciferol, 0.25 mg; vitamin E-acetate 9.00 mg; choline chloride, 150 mg.

Protein was determined by a modified biuret method (24).

For enzyme assays the tissues were carefully minced with a pair of scissors and homogenized in ice-cold phosphate buffer (50 mM; pH 7.2), EDTA (1 mM) (30–50 mg tissue per milliliter) by means of a tightly fitting 5 ml glass-glass homogenizer (25–30 strokes at 200 rpm). NADH–cytochrome *c* reductase (EC 1.6.99.3) and succinate–cytochrome *c* reductase (EC 1.3.99.1) were assayed in frozen-and-thawed samples of tissue homogenates and mitochondrial stock suspension at 25°C according to Sottocasa et al. (25). Cytochrome oxidase (EC 1.9.3.1) activity was determined polarographically in freshly prepared unfrozen samples as described by Rafael (26).

Oxidative phosphorylation was measured at 25°C as described by Wagner and Rafael (21). Concentration of respiratory substrates was: α-oxoglutarate 5 mM, succinate 5 mM, glutamate 5 mM, malate 2 mM.

The membrane potential of liver mitochondria was measured fluorometrically with 2-(dimethylaminostyryl)-1-methyl-pyridinium iodide (DSMP$^+$) according to Mewes and Rafael (27). Radioactivity was measured in a Philips LSA counter (Philips GmbH, Kassel, West Germany).

Linoleic acid-methyl ester (*cis-cis*) was obtained from Riedel-de-Haen, Seelze, and hydrogenated coconut oil was from W. Rau, Neuss, West Germany. Lubrol (type WX), cytochrome *c* (type III) and TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) were from Sigma Chemie, Munich, West Germany. $^{32}P_i$ was obtained from Amersham Buchler GmbH, Braunschweig. [^{3}H]DSMP$^+$ was synthesized according to Mewes and Rafael (27). All other reagents were of the highest purity commercially available.

RESULTS

Earlier studies on the metabolic rate of EFA-deficient rats were generally performed with animals kept on fat-free diets (see ref. 1 for review), i.e., under a permanent demand for increased fatty acid synthesis (3). Conditions of dietary deprivation, i.e., duration of deficiency and age of the animals, differ considerably in the available reports. A reproducible model was established by feeding weanling rats, aged 24 days (average body weight 52 g) a diet containing less than 0.05% unsaturated fatty acids; after a defined period of EFA deficiency these rats were compared with controls of the same age, which were fed the same diet supplemented with an isocaloric amount of linoleic acid (for details see methods).

In agreement with earlier findings (1–5) characteristic visual symptoms of EFA deficiency, like scaling of tail and paws and scrubbiness of the fur, became obvious after 3–4 weeks of EFA deficiency; growth reduction of the rats (28) became statistically significant at the same time. As shown in figure 1, the average body weight of male rats kept for 10 weeks on an EFA-deficient regimen is about 22% lower if compared with controls of the same age. Body weight reduction attains about 25%, when the dietary lack of EFA is maintained. The food intake of EFA-deficient rats and controls was virtually the same during the investigation, indicating a significantly different food efficiency (29).

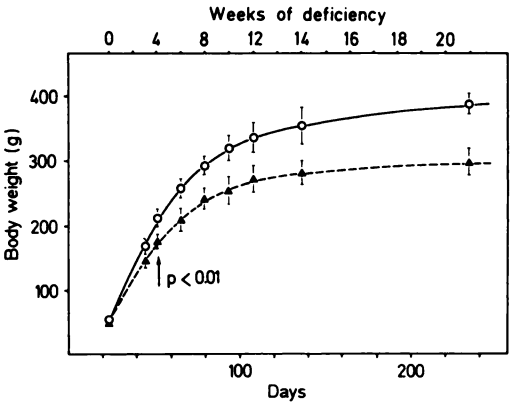

Fig. 1 Retardation of growth in rats induced by EFA deficiency. ▲, EFA-deficient rats; ○, controls. Means ± SE; n = 15 rats.

The relationship between respiratory rates and body weight as measured with controls and EFA-deficient rats after varying times of deficiency is not linear (fig. 2). The basal oxygen uptake is higher in EFA-deficient rats, when compared to controls of the same weight, but it is similar to that of controls of the same age.[2] For example, oxygen consumption of EFA-deficient rats weighing 200 g (aged 65–70 days) is similar to that of controls with a body weight of 250–260 g, i.e., of approximately the same age (fig. 1). This is also demonstrated in table 2 for rats fed an EFA-deficient diet for 10 weeks. The O_2 uptake of the rats related to the body weight (gram^{-1} or gram$^{-0.74}$) is significantly increased by EFA deficiency. The concomitant decrease of the total body weight, however, results in a compensation when the O_2 consumption is determined per animal: no significant difference becomes obvious between the basal respiration of deficient rats and that of controls of the same age.

It is stressed by our experiments that estimation of nutritional effects on the respiratory activity of rats, in general, must respect the age of the animals compared. Earlier statements on increased metabolic oxygen consumption as a symptom of nutritional EFA deficiency (1, 33, 34) have

[2]The basal respiration of control rats as measured in our experiments (fig. 2) is similar to results reported on warm-adapted rats by Jansky et al. (30), Leblanc and Villemaire (31) and Foster et al. (32).

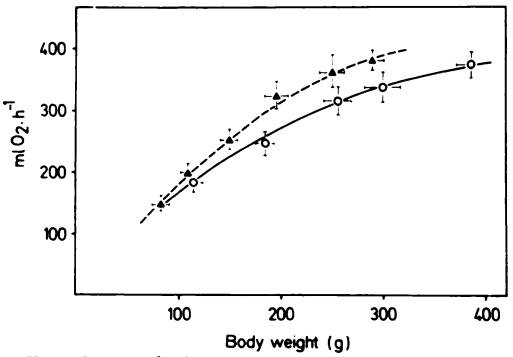

Fig. 2 Basal O_2 consumption (BMR) of EFA-deficient rats and controls. ▲, EFA-deficient rats; ○, controls. Groups of 4–16 rats were investigated. Means ± SE.

to be specified in view of these results: the age-dependent increase of respiratory activity and respiratory capacity of rats is not significantly influenced by EFA deficiency. The age-dependent increase of body weight, however, is considerably depressed by the EFA-deficient regimen, resulting in a significant increase of oxygen uptake per gram body weight.

A great number of reports on morphological and functional alterations of liver mitochondria from EFA-deficient rats and mice (8–16, 35–37) suggest impaired energy coupling in the organelles in connection with changes of the membrane lipid composition. To investigate this explanation respiration and phosphorylation were measured in liver mitochondria isolated from rats fed an EFA-deficient diet for 5 to 25 weeks. Data measured after 12 weeks of EFA deficiency (table 3) indicate virtually identical P:O ratios in mitochondria of EFA-deficient rats and controls; no uncoupling of oxidative phosphorylation is indicated. This holds for succinate as respiratory substrate and also for substrates oxidized via NAD^+, as α-oxoglutarate and glutamate; thus uncoupling of phosphorylation site 1, suggested in earlier reports, is excluded. Data in table 3 are representative of results obtained during the whole investigation period. In agreement with results in table 3 no difference was found between the mitochondrial membrane potential of liver mitochondria from EFA-deficient rats and from controls when measured fluorometrically or from the distribution of radioactive labeled $DSMP^+$ under state 4 conditions (table 4).

These results indicate that the membrane integrity of EFA-deficient mitochondria is not impaired by the altered fatty acid profile of membrane lipids (18) as far as oxidative phosphorylation is concerned. Results by Stancliff et al. (18) and Williams et al. (19), indicating intact energy coupling in EFA-deficient rat liver mitochondria are confirmed. Extensive swelling and structural damage have been reported of these mitochondria (12, 13). It appears likely that functional impairment of the organelles in vitro is mainly due to insufficient isolation procedures. Swelling properties of liver mitochondria have been introduced as a criterion in an EFA bioassay (35). We found no difference of the matrix volume in EFA-deficient mitochondria and controls during succinate respiration in the employed incubation medium (table 4). Again, differences may be explained from the increased sensitivity of EFA-deficient mitochondria to the isolation process.

As shown in table 3, respiration of liver mitochondria from EFA-deficient rats is about 25–30% higher if compared with controls. The uptake of $^{32}P_i$ is equivalently increased. Specific activities of respiratory chain enzymes as measured in rat liver and in liver mitochondria after 12 weeks of EFA deficiency do not reflect an increase of the mitochondrial functional capacity in EFA-deficient rat liver (table 5). In agreement with results on liver mitochondria, heart and skeletal muscle mitochondria reveal unchanged succinate-cytochrome *c* reductase and cytochrome oxidase activity when isolated from EFA-deficient rats (table 5). No increase of respiratory activity was found with these mitochondria in preliminary experiments (not shown here).

Specific amounts of mitochondrial protein were calculated from specific activities of mitochondrial marker enzymes in tissues and tissue mitochondria, respectively. It turns out that the specific amount of mitochondria in liver, heart and skeletal muscle (table 6) is unchanged by EFA deficiency. Since the weight of most of the inner organs

TABLE 2

Basal metabolic rate (BMR) of 90- to 95-day-old EFA-deficient rats and controls

Measure	Controls	EFA-deficient rats[1]	P-value[2]
Body weight, *g*	302 ± 13.6	249 ± 17.2	0.001
BMR, *ml O_2/hr*			
Total	332 ± 43.1	358 ± 29.0	NS
Per gram	1.10 ± 0.148	1.44 ± 0.095	0.001
Per gram$^{0.74}$	4.85 ± 0.632	6.04 ± 0.512	0.001

[1]Duration of EFA deficiency 10 weeks. Experimental details as described in methods section. Means ± SE are given from 12 rats in each group. [2]Significance of differences was evaluated by Student's double-tailed *t*-test. NS, not significant.

in rats is reduced by EFA deficiency (36), this finding might indicate a decrease of total mitochondria in EFA-deficient rats concomitant with the general growth reduction. This appears interesting with respect to the increased energy turnover per gram body weight (cf. discussion section).

DISCUSSION

Increased basal metabolism associated with EFA deficiency in rats was originally described by Wesson and Burr in 1931 (33) and was subsequently confirmed by several workers (see ref. 1 for review). Though little study has been given to this phenomenon since 1957 (34), the increased metabolic rate has been accepted as a fundamental EFA deficiency symptom, and its explanation became subject to numerous speculations (1–7).

In this investigation respiratory rates of EFA-deficient rats and controls have been defined in correlation to the body weight and the age of the animals, respectively. This became necessary since interrelations are not linear and may change under varying dietary conditions (fig. 2); this nonlinearity cannot be eliminated simply by relating metabolic rates to the body weight of the animals. As demonstrated here, relations may become even more complicated when these conditions are changed. In order to avoid substantial errors, nutritional effects on the respiratory activity of rats should be quantitated in relation to controls of the same age, irrespective of whether the O_2 consumption is related to the total animal or to its body weight.

The deficiency-induced increase of the specific metabolic rate, as indicated by the increased basal respiration related to the body weight, appears plausible in view of the substantial loss of water through the skin observed in EFA-deficient rats (1, 3), which causes an adequate loss of heat. In agreement with earlier reports, the water uptake of EFA-deficient rats in our laboratory was approximately doubled if compared with controls of the same age and no significant increase of renal water elimination was found. It may be speculated that the relative increase of the specific basal O_2 consumption is sufficient to compensate the heat loss

TABLE 3

Respiration and phosphorylation of liver mitochondria from EFA-deficient rats and controls[1,2]

Rats	Respiratory substrate[3]	State 4 respiration	State 3 respiration	$^{32}P_i$ uptake	P:O[4]
		nmol O/(min • mg protein)		*nmol P/ (min • mg protein)*	
Control	α-Oxoglutarate	9.7 ± 0.42	40.2 ± 2.02	107.2 ± 5.14	2.67
	Glutamate/malate	11.0 ± 0.34	51.9 ± 1.75	138.8 ± 5.05	2.68
	Succinate	20.4 ± 0.91	82.0 ± 3.35	137.3 ± 5.96	1.67
EFA-deficient	α-Oxoglutarate	13.2 ± 0.73**	51.1 ± 2,20*	137.4 ± 6.02**	2.68
	Glutamate/malate	13.4 ± 0.42*	59.4 ± 1.65*	159.2 ± 4.36*	2.69
	Succinate	27.9 ± 1.19**	104.3 ± 2.27**	171.9 ± 7.54*	1.65

[1]Age of rats was 14–15 weeks; EFA deficiency was for 12 weeks. Means ± SEM; n = 8–9 rats in each group. [2]Differences from controls were evaluated by Student's double-tailed *t*-test: $^*P < 0.01$; $^{**}P < 0.001$. [3]Succinate respiration in the presence of 8 μM rotenone. [4]P:O ratios were determined from $^{32}P_i$ uptake and state 3 respiration (25°C) as described by Wagner and Rafael (21).

TABLE 4

Membrane potential and matrix volume of liver mitochondria from EFA-deficient rats and controls[1]

Rats	Respiratory substrate	State 4 membrane potential	State 4 matrix volume
		mV	*μl/mg protein*
Control	α-Oxoglutarate	186 ± 1.4	—
	Succinate[2]	186 ± 5.1	1.18 ± 0.112
EFA-deficient	α-Oxoglutarate	184 ± 4.7	—
	Succinate[2]	187 ± 2.6	1.22 ± 0.136

[1]Age of rats was 14–15 weeks. EFA deficiency for 12 weeks. Means ± SE for 7 experiments. [2]Plus 8 μM rotenone.

of deficient rats kept under the experimental conditions we employed (23°C).

There is little doubt that the absence of EFA in the phospholipids of membranes alters their functional properties and many physiological and histological alterations due to EFA deficiency can be attributed to impaired membrane integrity in connection with changed membrane fatty acid profiles (1–5). Altered membrane properties are also very likely a major reason for the increased energy turnover in EFA-deficient rats (cf. below), but it is clearly shown that they do not affect the fundamental functional integrity of the inner mitochondrial membrane, as still suggested by many workers in this field (see refs. 5 and 6 for review). As demonstrated in this study partial uncoupling of oxidative phosphorylation (8–16) can be excluded as a reason for the increased oxidative metabolism induced by EFA deficiency (1, 5–7), and earlier reports manifesting functional integrity of EFA-deficient rat liver mitochondria (18, 19) are confirmed. Increased fragility and sensitivity of EFA-deficient mitochondria to isolation procedures have been described (11–13), and structural damage of the organelles during the isolation process yields a most plausible explanation of the impaired functional activity reported. In fact, careful preparation methods employed in this investigation were originally developed to isolate extremely sensitive chloramphenicol-induced megamitochondria from mouse liver (21).

In agreement with the observation of intact oxidative phosphorylation, formation of the membrane electrochemical potential is not affected by the altered membrane fatty acid pattern (18). It has apparently no or only selective influence on the permeability of the mitochondrial membrane, since no change of the matrix volume was found with EFA-deficient liver mitochondria suspended in 250 mM sucrose. This agrees with results by Stancliff et al. (18) who report that ion transport-induced mitochondrial swelling is unaltered by EFA deficiency. It may be suggested that altered swelling properties of EFA-deficient mitochondria, introduced as a bioassay for EFA deficiency (35), are at least partly due to impairment

TABLE 5

Activity of respiratory chain enzymes in tissues and tissue mitochondria from EFA-deficient rats and controls[1]

Tissue	Substrate[2]	Control rats		EFA-deficient rats	
		Tissue	Mitochondria	Tissue	Mitochondria
		nkat/g	*nkat/mg protein*	*nkat/g*	*nkat/mg protein*
Liver	SCR	127 ± 10.9	2.33 ± 0.194	123 ± 4.1	2.33 ± 0.156
	COX	4033 ± 223.8	93.7 ± 6.76	4430 ± 237.1	97.7 ± 8.32
	NCR	—	5.00 ± 0.313	—	5.00 ± 0.335
Heart	SCR	172 ± 8.2	2.33 ± 0.144	168 ± 10.1	2.17 ± 0.077
	COX	9767 ± 615.6	130.7 ± 9.57	9623 ± 291.7	121.7 ± 6.90
Skeletal muscle	SCR	37 ± 4.5	1.83 ± 0.298	35 ± 3.5	2.33 ± 0.335
	COX	1797 ± 285.4	115.2 ± 14.04	1797 ± 218.6	114.7 ± 11.99

[1]Rats were 100–105 days old; duration of EFA deficiency was 12 weeks. Means ± SEM are given from 5–9 experiments where 1 nkat = 1 nmol electrons/sec. [2]SCR, succinate-cytochrome *c* reductase; COX, cytochrome oxidase; NCR, NADH-cytochrome *c* reductase.

of the organelles during the isolation procedure.

Loose coupled oxidative phosphorylation does not appear to be necessary to explain the physiological phenomena of EFA deficiency. We have not investigated energy coupling of other than hepatic mitochondria. However, if oxidative phosphorylation in general remains coupled during EFA deficiency, increased oxygen consumption would also indicate an increased ATP production per gram body weight and thus an intensified ATP requirement. Altered membrane properties during EFA deficiency may provide several reasons of an increased ATP requirement. Impairment of membrane integrity was shown to cause many pathological symptoms in EFA deficiency. The highly increased dermal loss of water in EFA-deficient rats (cf. above), for instance, may well require increased ATP production.

The weight of most organs and tissue of EFA-deficient rats, including skeletal muscle, liver and heart, is less than in controls (36). Since the specific amount of mitochondria in liver, heart and skeletal muscle is unchanged, the total mass of mitochondria in the animal appears reduced due to EFA deficiency. The functional capacity of the remaining mitochondria, however, should suffice to comply with the relative increase of the ATP requirement. We do not know if the small increase of oxidative phosphorylation capacity observed in isolated liver mitochondria from EFA-deficient rats is of any compensatory significance in this respect. We observed no concomitant functional increase in muscle and heart mitochondria so far. Another question we cannot explain is why the basal respiration per animal is similar to levels measured in controls during EFA deficiency for up to 7 months. It appears possible that the agreement of O_2 consumption and thus of ATP-consuming processes in EFA-deficient rats and controls might cease when the environmental temperature is changed.

Evidence of unimpaired oxidative phosphorylation in EFA-deficient rat liver mitochondria should help to direct further efforts on metabolic effects of EFA deficiency out of an impass (5–7). It should be further stressed that reasons for the growth reduction induced by EFA deficiency remain unclear; it appears questionable if this phenomenon can be attributed to a diminished nutritional efficiency (5–7) or rather to effects of EFA deficiency on the endocrine system (1–4).

TABLE 6

Specific amounts of mitochondrial protein in tissues from EFA-deficient rats and controls[1]

Tissue	Mitochondrial protein	
	Control rats	EFA-deficient rats
	mg/g tissue	
Liver	49.5 ± 7.22	49.9 ± 4.22
Heart	75.8 ± 15.39	80.3 ± 19.03
Skeletal muscle	16.9 ± 5.78	16.3 ± 5.38

[1]Data were calculated from activities of mitochondrial (mitochl) marker enzymes in tissues and mitochondria, respectively (table 5):

$$\frac{\text{nkat/g tissue}}{\text{nkat/mg mitochl protein}} = \text{mg mitochl protein/g tissue}$$

Means ± SE for 5–9 experiments.

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