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Lipid metabolism in riboflavin-deficient rats

2. Mitochondrial fatty acid oxidation and the microsomal desaturation pathway

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1. Oxygen consumption was measured by means of an O_2 electrode in mitochondrial suspensions from riboflavin-deficient and pair-fed control rats, using six different substrates. Whereas consumption of O_2 by glutamate was only slightly depressed in mitochondria from deficient animals, the consumption of O_2 by hexanoate and by palmitoyl-L-carnitine was depressed to approximately half the control value: a highly significant difference. A comparable magnitude of depression was observed for stearoyl-, oleoyl-, and linoleoyl-L-carnitine. There were no major or consistent differences between groups of animals receiving two different types, and two different levels, of fat in their diet.

2. The activity of acyl coenzyme A dehydrogenase (*EC* 1.3.99.3) in hepatic mitochondrial fragments, measured by cytochrome *c* reduction with palmitoyl-coenzyme A as substrate, and expressed as maximum velocity (V_{max}) with respect to phenazine methosulphate, was also reduced to approximately half the control value in deficient animals.

3. In hepatic microsomes, cytochrome b_5 reductase (*EC* 1.6.2.2) activity was unaffected by riboflavin deficiency, although NADPH-cytochrome *c* reductase (*EC* 1.6.2.4) and microsomal flavin content were diminished to approximately half the control values. Acyl CoA (Δ9) desaturase activity (*EC* 1.14.99.5) was virtually identical in deficient, pair-fed, and *ad lib.*-fed control groups.

4. It is concluded that the depression of mitochondrial β-oxidation of fatty acids which is observed in riboflavin-deficient animals is not a secondary result of inanition, and may account for the observed changes in fatty acid profiles of triglycerides and phospholipids. Failure of the microsomal fatty acid desaturation system is less likely to be a major consequence of riboflavin deficiency.

Riboflavin deficiency is associated with a major impairment of lipid economy (Olpin & Bates, 1982). Studies by Hoppel & Tandler (1975) on mice, and by Hoppel *et al.* (1979) on rats have suggested that mitochondrial fatty acid β-oxidation, which involves a sequence of three flavin-dependent enzymes in the initial rate-limiting dehydrogenase reaction, may be especially sensitive to variations in riboflavin status. This could perhaps explain some of the physiological abnormalities, such as hepatic lipid accumulation and the deleterious effects of high-fat diets, which are seen in deficient animals.

On the other hand, some of the characteristics of riboflavin deficiency: notably the accumulation of the fatty acid 18:2 at the expense of 20:4 in liver phospholipids, might be interpreted as evidence for a lesion in fatty acid desaturation: either at the flavin-dependent microsomal NADH cytochrome b_5 reductase (*EC* 1.6.2.2) which channels electrons into fatty acid desaturases, or at some other component of the desaturase systems.

The purpose of the present study was first to examine in greater detail the proposal that mitochondrial fatty acid oxidation is impaired in riboflavin deficiency, and secondly to measure microsomal function in relation to fatty acid desaturation.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]stearic acid, specific activity 56·5 mCi/mmol was obtained from The Radiochemical Centre, Amersham, Bucks. Stearoyl-DL-carnitine chloride, oleoyl-DL-carnitine chloride and

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linoleoyl-DL-carnitine chloride were obtained from PL Biochemicals Inc., Wisconsin. All other biochemicals were obtained from Sigma; the bovine serum albumin (BSA) used was essentially fatty acid-free.

Animals and diets

Female weanling Norwegian hooded rats were used throughout, and were housed and fed as described previously (Olpin & Bates, 1982); neck collars being used to prevent coprophagy. The mean starting body-weights and the duration of each experiment are given in Tables 1–4.

Tissue preparation

Isolation of mitochondria for fatty acid oxidation and acyl CoA dehydrogenase measurements. Animals were anaesthetized with diethyl ether and exsanguinated. Liver samples (5 g) were then homogenized, in a Potter-Elvehjem homogenizer, in 20 ml of a buffer (pH 7·4) containing sucrose 0·25 mol/l, Tris (hydroxymethyl methylamine) chloride 0·05 mol/l, ethyleneglycol bis (β-aminoethyl ether) *N,N'*tetraacetic acid (EGTA) 10^{-3} mol/l, at 0°. The homogenates were diluted to a tissue concentration of 100 g/l and centrifuged at 700 ***g*** for 10 min to remove nuclei, unbroken cells, and cell debris. Mitochondria were then isolated from the supernatant layers by centrifugation at 6000 ***g*** for 10 min, the pellets were washed by resuspension in a further 20 ml buffer, centrifugation, and resuspension at approximately 10 mg protein/ml.

Isolation of microsomes for the assay of NADH cytochrome b_5 *reductase* (EC *1.6.2.2*) *and NADPH cytochrome* c *reductase* (EC *1.6.2.4*). The postmitochondrial supernatant fractions were centrifuged at 105000 ***g*** for 30 min, the resulting pellets were washed by resuspension in phosphate-buffered saline (9 g sodium chloride/l), pH 7·4, recentrifuged, and suspended in approximately 1 ml phosphate-buffered saline. They were diluted (1:50, v/v) in a solution containing 20 ml Triton X-100 and 0·1 mol sodium phosphate/l, pH 7·7, to yield a soluble enzyme preparation.

Isolation of microsomes for the assay of acyl CoA ($\Delta 9$) *desaturase* (EC *1.14.99.5*). Liver samples were homogenized in a Potter-Elvehjem homogenizer in a buffer, pH 7·4, at 0° containing 0·3 mol sucrose/l and 0·2 mol sodium phosphate/l (2 ml/g fresh liver). These homogenates were centrifuged at 15000 ***g*** for 30 min and the resulting supernatant fractions were further centrifuged at 100000 ***g*** for 60 min. The microsomal pellets thus obtained were resuspended in the same buffer (0·5 ml/g original liver weight).

O_2 consumption by mitochondria

A Clarke-type O_2 electrode was used (Rank Bros, Bottisham, Cambridge). The incubation medium, pH 7·0, contained/l: potassium chloride 0·08 mol, morpholino propane sulphonic acid (MOPS) 0·05 mol, sodium phosphate 5×10^{-3} mol, EGTA 10^{-3} mol, bovine serum albumin 1 g. The operating temperature was 30° and the volume 1·5 ml.

Endogenous mitochondrial substrates were first depleted by addition of small amounts of ADP until no further O_2 uptake was observed. ADP (2·3 μmol) was then added and state-3 respiration (Chance & Williams, 1955) was initiated by addition of substrate.

Final substrate concentrations were (mol/l): L-glutamate 10^{-2}, hexanoate 4×10^{-4} plus L-malate 2×10^{-3}, palmitoyl-L-carnitine 4×10^{-5} plus L-malate 2×10^{-3}, stearoyl-L-carnitine 4×10^{-5} plus L-malate 2×10^{-3}, oleoyl-L-carnitine 4×10^{-5} plus L-malate 2×10^{-3} linoleoyl-L-carnitine 4×10^{-5} plus L-malate 2×10^{-3}. The concentration of mitochondrial protein in the incubating medium was 1–2 mg/ml.

Enzyme assays

Acyl CoA dehydrogenase (EC *1.3.99.3*) *in mitochondrial fragments.* Mitochondrial fragments were prepared by addition of cholic acid, 1 g/l sodium phosphate buffer (0·05 mol/l) pH 7·4. Acyl CoA dehydrogenase activity was then determined essentially by the methods

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of Hauge (1956) and Hoskins (1969) in a 1·0 ml reaction mixture, pH 7·2 at 30°, containing (mol/l): phosphate buffer 0·034, potassium cyanide $1·5 \times 10^{-3}$, cytochrome *c* $1·5 \times 10^{-4}$, rotenone $3·75 \times 10^{-6}$ and phenazine methosulphate (PMS) at three different concentrations (0·05, 0·1 and 0·2 g/l). The enzyme preparation consisted of approximately 40 μg mitochondrial protein, and the substrates, added last, were palmitoyl CoA or stearoyl CoA (5×10^{-5} mol/l). The rate of increase of optical density was followed continuously for 3 min at 550 nm in a Pye Unicam SP 1800 recording spectrophotometer. The difference between the molar extinction coefficients of cytochrome *c* in the oxidized and reduced states was used to calculate the rate of reaction for each concentration of PMS, which was then used to construct a double reciprocal plot and to obtain the maximum velocity with respect to PMS and the Michaelis constant (PMS) for each preparation. This procedure was used in order to overcome the problem of contamination of enzyme with electron-transferring flavoprotein (ETF), which diverts some of the electrons away from PMS, especially at low concentrations of the latter.

NADPH-cytochrome c *reductase* (EC *1.6.2.4*) *in microsomal extracts.* The incubation medium, pH 7·7 at 25° contained (mol/l): potassium phosphate buffer 0·3, NADPH 10^{-4} and cytochrome *c* 4×10^{-5} in a final volume of 1·0 ml (Phillips & Langdon, 1962). The reaction was started by addition of 40 μl Triton-treated microsomal extract, and the change in optical density was followed for 2–5 min at 550 nm.

NADH-cytochrome b_5 *reductase* (EC *1.6.2.2*) *in microsomal extracts.* The incubation medium, pH 7·7 at 25° contained (mol/l): potassium phosphate buffer 0·08, potassium ferricyanide 10^{-3}, in a final volume of 1·0 ml (Mihara & Sato, 1978). The reaction was started by addition of 20 or 40 μl Triton-treated microsomal extract and the change in optical density was followed for 5 min at 420 nm.

Acyl CoA (Δ*9*) *desaturase* (EC *1.14.99.5*) *in intact microsomes.* [1-^{14}C]stearic acid bound to BSA was prepared by mixing 177 nmol (10 μCi) [1-^{14}C]stearic acid with 355 nmol unlabelled stearic acid in 100 μl methanol containing potassium hydroxide (0·05 mol/l). A further 500 μl of the methanolic potassium hydroxide was added, the methanol was removed in a stream of nitrogen, and 250 μl of an aqueous solution of BSA (4·2 g/l) was added.

A solution, pH 7·0, containing (mol/l): potassium phosphate 1, magnesium chloride 0·08, EDTA 0·01, was mixed with NADH (final concentration $3·0 \times 10^{-3}$ mol/l) and CoA (final concentration $1·5 \times 10^{-3}$ mol/l). The following: 0·1 ml of this solution, 0·1 ml adenosine triphosphate (0·1 mol/l), 0·1 ml buffer pH 7·0 containing citric acid, (0·05 mol/l), potassium phosphate (0·1 mol/l in phosphate), 0·6 ml BSA (4·2 g/l) and 0·05 ml stearic acid (2·0 μCi; $1·06 \times 10^{-4}$ M final concentration) bound to BSA were mixed together and were preincubated for 5 min at 37° (R. Jeffcoat, personal communication). The reaction was initiated by addition of microsomal preparation containing approximately 1·5 mg protein, and was terminated after 6 min at 37° by addition of 2·0 ml potassium hydroxide (0·5 mol/l).

Methyl esters of the fatty acids were prepared as described elsewhere (Olpin & Bates, 1982). These were then separated and the distribution of radioactivity between fatty acids 18:0 and 18:1 measured on a Pye series 204 gas–liquid chromatograph, operating at 150° with a carrier gas of carbon dioxide in argon (50 ml/l) through a 1·5 m × 4 mm column of 100 g EGSS-X/kg Gas Chrom Q (100–120 mesh) (prepared by Pye Unicam Ltd) with a stream splitter, one side of which was connected to a Panax Radiogas detector system and the other to a flame-ionization detector.

Protein determination. The protein content of mitochondrial preparations was estimated by the method of Lowry *et al.* (1951) after precipitation with trichloroacetic acid (final concentration 50 g/l). Protein in the microsomal preparations was estimated by the Biuret procedure (Gornall *et al.* 1949) on samples dissolved in Triton X-100, with a blank correction for turbidity.

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RESULTS

O_2 utilization by mitochondria

The effects of riboflavin deficiency on rates of hepatic mitochondrial O_2 consumption for groups of rats given three diets of different lipid composition, are shown in Table 1. For all three groups, with hexanoate as substrate, there were highly-significant reductions in the rate of O_2 consumption in the mitochondria from deficient animals, to approximately 50% of the values observed for the pair-fed controls. The picture was similar with palmitoyl-L-carnitine as substrate, where the mean deficient values were approximately 44% of the mean control values, and the extent of reduction was similar, or slightly greater, with stearoyl-L-carnitine (39% of control values); oleoyl-L-carnitine (37% of control values) and linoleoyl-L-carnitine (32% of control values). The effect of riboflavin deficiency on glutamate oxidation was relatively small, the deficient mitochondria attaining 79% of the control activities over all, and falling significantly below the controls only in the group given the maize-oil diet. There were no consistent differences in O_2 consumption rates attributable to the different lipid contents of the diets, for either the deficient or the control sets.

Acyl CoA dehydrogenase (EC *1.3.99.3*)

Riboflavin deficiency resulted in a significant reduction in V_{max}(PMS) for this enzyme for all three diets of different lipid contents (Table 2), and the magnitude of the reduction was similar to the reduction in O_2 utilization by intact mitochondria, with palmitoyl carnitine as substrate. The apparent K_m(PMS) was also reduced in the mictochondria from the deficient animals: this attained significance for those receiving the 200 g arachis-oil/kg diet ($P < 0{\cdot}05$) and the 30 g maize-oil/kg diet ($P < 0{\cdot}001$).

Microsomal enzyme activities

Despite a highly-significant reduction in NADPH-cytochrome *c* reductase (*EC* 1.6.2.4) activity in the microsomes of riboflavin-deficient animals (Table 3) to 55% of the mean pair-fed control value, and a similar reduction in total flavin concentration in the microsomal fraction, there was no detectable change in the NADH cytochrome b_5 reductase (*EC* 1.6.2.2) activity (Table 3). Likewise, acyl CoA (Δ9) desaturase (*EC* 1.14.99.5) activity in the microsomes was apparently unaffected by riboflavin deficiency (Table 4).

DISCUSSION

A reduction of 50–70% in rates of oxidation of fatty acid substrates and of V_{max} for fatty acyl CoA dehydrogenase activity in the deficient animals is consistent with the observations of Hoppel *et al.* (1979), and confirms that these effects of riboflavin deficiency are still evident when the control animals receive diets identical with those fed to the deficient groups, apart from their riboflavin content, and are pair-fed to control the effects of inanition. Likewise, it is clear that the oxidation of fatty acids is impaired to a greater extent than that of glutamate. The apparent change in K_m with respect to phenazine methosulphate in the dehydrogenase assay is puzzling; it suggests that the affinity for the electron acceptor is increased in the deficient animals. However, it is not known whether a similar result would be obtained with the natural electron acceptor, nor whether this observation has any physiological significance.

The reduction in oxidation of linoleoyl carnitine was at least as great, and possibly greater, than that of the other fatty acid substrates, which is consistent with the observed accumulation of fatty acid 18:2 in tissue phospholipids and triglycerides (Olpin & Bates, 1982).

The microsomal studies have demonstrated that despite a considerable reduction in NADPH-cytochrome *c* reductase activity in deficient animals, which is consistent with the

Table 1. *Effect of riboflavin deficiency on the rate of mitochondrial oxygen consumption† for six substrates*

(Mean values with their standard errors; no. of animals in parentheses. Mean starting weights were 41·5 g, and the animals were killed between days 20 and 26 of the experiment. Mean (±SE) for the activation coefficient of erythrocyte glutathione reductase (EGRAC) for the deficient animals in this experiment were: for the 30 g arachis oil/kg diet 2·25±0·23 (*n* 7), for the 200 g arachis oil/kg diet 2·20±0·26 (*n* 7) and for the 30 g maize oil/kg diet 2·23±0·35 (*n* 7). The pair-fed controls (*n* 21) had an aggregated EGRAC value of 1·29±0·09)

Dietary lipid (g/kg)	Group	Substrate											
		Glutamate		Hexanoate		Palmitoyl-L-carnitine		Stearoyl-L-carnitine		Oleoyl-L-carnitine		Linoleoyl-L-carnitine	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Arachis oil: 30	Deficient	55·2	5·0 (7)	27·2	2·0*** (7)	29·0	3·1*** (7)	22·6	2·3 (2)	15·2	— (1)	14·5	— (1)
	Pair-fed control	68·2	4·3 (7)	57·9	3·4 (7)	55·8	1·6 (7)	52·9	13·6 (2)	45·3	2·5 (2)	52·5	4·1 (2)
Arachis oil: 200	Deficient	73·7	5·2 (6)	41·2	7·0* (5)	23·3	2·2*** (3)	20·9	2·0* (3)	19·6	2·5 (3)	21·2	2·8 (3)
	Pair-fed control	75·0	2·6 (6)	64·3	3·1 (5)	70·4	3·1 (3)	52·5	8·9 (3)	60·7	4·0 (2)	72·7	3·1 (2)
Maize oil: 30	Deficient	49·7	2·6** (6)	25·6	1·3*** (6)	28·7	2·9*** (6)	19·8	1·4*** (3)	20·1	1·4*** (3)	19·5	1·0 (3)
	Pair-fed control	77·4	7·1 (6)	59·5	5·1 (6)	61·4	5·3 (6)	55·5	2·9 (3)	44·5	1·3 (3)	50·4	1·5 (2)

Values were significantly different from the corresponding pair-fed controls: * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

† O_2 consumption is expressed as no. atoms O_2 consumed/mg mitochondrial protein per min.

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Table 2. *Effects of riboflavin deficiency on the activity of acyl-CoA dehydrogenases* (EC *1.3.99.3*)

(Mean values with their standard errors; no. of animals in parentheses. Mean starting weights were 46·0 g, and the animals were killed between days 27 and 34 of the experiment. V_{max} [phenazine methosulphate] is the activity extrapolated to infinite concentration of phenazine methosulphate: see MATERIALS AND METHODS, using palmitoyl-CoA as substrate)

Dietary lipid (g/kg)	Group	V_{max}[phenazine methosulphate]	
		Mean	SE
Arachis oil:	Deficient	54·4	8·1 (4)
30	Pair-fed control	122·8	19·2* (4)
Arachis oil:	Deficient	78·3	8·3 (3)
200	Pair-fed control	130·6	8·8** (3)
Corn oil:	Deficient	68·4	2·4 (3)
30	Pair-fed control	158·5	15·6*** (3)

Values were significantly different from the corresponding pair-fed controls: $^{*}P < 0·02$, $^{**}P < 0·01$, $^{***}P < 0·001$.

A similar reduction in V_{max} [phenazine methosulphate] in deficient animals as compared to pair-fed controls was obtained using stearoyl-CoA as substrate, under otherwise identical conditions.

observations of Shargel & Mazel (1973), of Patel & Pawar (1974) and of Taniguchi (1980) in rats and of Yang (1974) in mice, together with a corresponding reduction in total microsomal-flavin content, there was nevertheless no detectable reduction in NADH-cytochrome b_5 reductase activity, (a flavoprotein which channels electrons into the fatty acid desaturase pathway; Holloway, 1971; Shimakata *et al.* 1972), as measured by reduction of ferricyanide (Mihara & Sato, 1978). Likewise, the activity of acyl CoA Δ9 desaturase was not diminished in the deficient animals. These conclusions differ from those of Okayasu *et al.* (1977), but it is difficult to make meaningful comparisons since their experimental conditions were very different, and no statistical analyses were recorded.

The lack of an effect on the desaturase is consistent with the fact that no flavin-dependent process beyond cytochrome b_5 reductase has yet been identified in the desaturase pathway. Some preliminary attempts to study Δ6 desaturase activity have not yet yielded conclusive results however, and it remains possible that the conversion of 18:2 to 20:4 may be specifically impaired. An impairment in this conversion was not, however, detected by Williams *et al.* (1967) during recovery from combined riboflavin and essential fatty acid deficiency in the rat, and at present, the simplest explanation for the relative accumulation of fatty acid 18:2 and reduction in 20:4 levels in tissues of deficient animals would appear to be an over-all reduction in β-oxidation, resulting in the accumulation of those fatty acids which are present in dietary lipids, but which are not synthesized *de novo* in the body.

Whether these effects of severe riboflavin deficiency on fatty acid oxidation in rats are relevant to the pathology of riboflavin deficiency in human populations is not yet known. Suckling rats derive a higher proportion of their energy from lipids than do foetal or adult animals (Drahota *et al.* 1965–6; Yeh & Zee, 1979), and it is thus possible that human babies may be especially vulnerable to this metabolic lesion during their first few months of life.

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Table 3. *Effects of riboflavin deficiency on microsomal flavin concentration, NADPH cytochrome* c *reductase* (EC *1.6.2.4*) *activity and NADH cytochrome* b_5 *reductase* (EC *1.6.2.2*) *activity*

(Mean values with their standard errors; no. of animals in parentheses. Mean starting weights were 36·6 g and the animals were killed between days 20 and 29 of the experiment. As no differences in either cytochrome *c* or b_5 reductase activity could be detected between groups of deficient rats given each of the three deficient diets, (arachis oil, 30 g/kg; arachis oil, 200 g/kg; and maize oil, 30 g/kg), values for all deficient animals were combined. Similarly, combined values are shown for pair-fed control animals given the three corresponding control diets)

	Activation coefficient of erythrocyte glutathione reductase		Microsomal flavin concentration†		NADPH cytochrome *c* reductase‡		NADH cytochrome b_5 reductase‖	
Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Deficient	2·26	0·02*** (5)	0·143	0·004*** (13)	0·105	0·006*** (10)	4·70	0·15 (12)
Pair-fed controls	1·16	0·08 (5)	0·271	0·007 (13)	0·198	0·008 (10)	4·75	0·16 (12)

Values were significantly different from the corresponding pair-fed controls; ***$P < 0.001$.

† Microsomal flavin concentration is expressed as μg total flavin/mg microsomal protein.

‡ Cytochrome *c* reductase activity is expressed as μmol cytochrome *c* reduced/mg microsomal protein per min.

‖ Cytochrome b_5 reductase activity (NADH ferricyanide reductase activity) is expressed as μmol ferricyanide reduced/mg microsomal protein per min.

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Table 4. *Effect of riboflavin deficiency on acyl CoA* ($\Delta 9$) *desaturase* (EC *1.14.99.5*) *activity*

(Mean values with their standard errors; no. of assays in parentheses. The diets contained maize oil (30 g/kg) and the animals (whose mean starting weight was 50·2 g) were killed between days 28 and 33. Each assay was carried out on the microsomes obtained from two rats after combining equal weights of liver from each animal. The severity of the riboflavin deficiency, and responses of the controls, were similar to those of corresponding groups of animals described in Table 2, and in the accompanying paper (Olpin & Bates, 1982))

	Δ9 desaturase activity*	
Group	Mean	SE
Deficient	1·8	0·15 (6)
Pair-fed controls	1·82	0·21 (6)
Ad lib.-fed controls	1·64	0·14 (5)

* nmol 18:1 produced/mg protein per min.

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