Lipid metabolism in riboflavin-deficient rats

1. Effect of dietary lipids on riboflavin status and fatty acid profiles

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1. The increase in activation coefficient (stimulated: basal activity) of erythrocyte NAD(P)H_2:glutathione oxidoreductase (*EC* 1.6.4.2) and reduction in hepatic flavin concentration which occurred in riboflavin-deficient weanling rats were not markedly or consistently affected by differences in the concentration of lipid in the diet nor by differences in the total proportion of saturated or polyunsaturated fatty acids in the dietary lipid.

2. Their gain in body-weight was, however, reduced when the dietary lipid concentration was increased from 30 to 200 g/kg and liver:body-weight and hepatic triglyceride content were correspondingly increased, suggesting a functionally-deleterious effect of high fat intake in the deficient animals. This was especially severe when the diets contained cottonseed oil, which appeared to be toxic for the deficient animals.

3. Comparisons between fatty acid profiles of hepatic phospholipids of deficient, pair-fed and *ad lib.*-fed control animals indicated that the increase in proportion of 18:2 ω6 and the decrease in proportion of 20:4 ω6 observed in deficient animals were due specifically to riboflavin deficiency, whereas certain other changes were probably caused by inanition. The changes in 18:2 ω6 and 20:4 ω6 were observed at both low and high levels of lipid intake and at both low and high levels of dietary lipid polyunsaturation. Similar changes in fatty acid profiles were observed in renal, erythrocyte membrane, and plasma phospholipids, but were not seen in cardiac phospholipids.

4. A consistent increase in proportion of 18:2 ω6 was also observed in the hepatic triglycerides, together with a decrease in proportion of 16:0.

5. It is concluded that acute riboflavin deficiency affects lipid metabolism in a characteristic manner, probably by interfering with β-oxidation of fatty acids, but that diets of high lipid content do not significantly increase the extent of flavin depletion.

Several lines of evidence point towards an important interaction between riboflavin status and tolerance towards, or metabolism of, dietary lipids.

Early studies of riboflavin deficiency in rats indicated that diets rich in lipid increased the requirement of riboflavin for growth and the avoidance of pathological signs of deficiency (Mannering *et al.* 1941; Shaw & Phillips, 1941; Tange, 1941; Mannering *et al.* 1944; Czaczkes & Guggenheim, 1946; Kaunitz *et al.* 1954). Similar conclusions were reached for deficient mice (Wynder & Klein, 1965), guinea-pigs (Hara, 1960) and cats (Gershoff *et al.* 1959). The work of Czaczkes & Guggenheim (1946) and Gershoff *et al.* (1959) in particular, pointed towards a change in intestinal flora being the critical difference between high-fat and high-carbohydrate diets and thus indicated that refection might be an important component. A study by Reiser & Pearson (1949) on chicks indicated that cottonseed-oil-containing diets were more deleterious than those containing a similar amount of lard, suggesting that the polyunsaturated fatty acid content may be relevant.

Many studies have recorded the occurrence of fatty livers in riboflavin-deficient animals (e.g. Wintrobe *et al.* 1944; Gershoff *et al.* 1959; Guggenheim & Diamant, 1959; Kim & Lambooy, 1969). More recently, it has been shown that riboflavin deficiency produces changes in fatty acid composition, both in hepatic triglycerides and in hepatic phospholipids (Taniguchi & Nakamura, 1976; Taniguchi *et al.* 1978): a characteristic feature being a marked increase in proportion of 18:2 ω6 and a decrease in 20:4 ω6.

Finally, recent studies by Hoppel *et al.* (1979) have indicated that hepatic mitochondrial

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fatty acid oxidation is rapidly and severely impaired in riboflavin-deficient rats, and is considerably more sensitive than the oxidation of non-lipid substrates, even though these are also metabolized by flavin-dependent pathways. In support of this, it has also been reported that riboflavin-deficient rats excrete increased amounts of adipic, hexenedioic, suberic, octenedioic, sebacic and decenedioic acids in their urine, indicating inefficient utilization of these fatty acids in their tissues (Goodman, 1981).

The purpose of the present study was to measure biochemical status by the erythrocyte glutathione oxidoreductase (*EC* 1.6.4.2) test and by measurement of hepatic flavin levels, in riboflavin-deficient rats given diets containing different levels and types of lipid, and to investigate changes in fatty acid profiles in the deficient animals, with particular attention to the control of inanition. The accompanying paper (Olpin & Bates, 1982) describes studies of mitochondrial fatty acid oxidation, and of enzymes involved in microsomal fatty acid desaturation, in deficient animals.

MATERIALS AND METHODS

Animals and diets

Female weanling Norwegian hooded rats were housed individually in suspended wire cages. Coprophagy was prevented in all groups of rats by the use of circular collars fitted round the animal's neck. These were cut from 6·5 mm thick sheets of Plastizote; a light semi-rigid plastic foam material obtained from Forest Rubber Co. Ltd, Bishops Stortford, Herts. The diameter of the circle and of the central hole for the neck were adjusted continually as the animals grew: for a 50 g rat the dimensions were 110 mm and 12 mm respectively. During most of the studies described, the material was cut radially and rejoined with staples, but with practice it became possible to push the collar over the animal's head without cutting and rejoining. The collars were fitted when the animals weighed 45–50 g and were generally well-tolerated and were not frequently pulled off.

The basic riboflavin-deficient diet was similar to that used previously (Prentice & Bates, 1981*a*). The following vitamins were added (mg/kg diet): choline chloride 2000, calcium pantothenate 20, thiamine hydrochloride 4, pyridoxine hydrochloride 9, nicotinamide 25, biotin 1, pteroylglutamic acid 1, cyanocobalamin 0·05, menadione 9, α-tocopherol 250, retinyl acetate 2·1 retinol equivalents and ergocalciferol 0·0075. The riboflavin-deficient diet contained less than 0·3 mg riboflavin/kg, derived from the casein, and to this was added 3 mg riboflavin/kg for the *ad lib.*-fed controls and 15 mg riboflavin/kg for the pair-fed and weight-matched controls, to compensate for their reduced food intake (Prentice & Bates, 1981*a*).

The lipid component of the diet was varied to provide diets with a variety of fatty acid compositions and extents of unsaturation, at two different levels in the diet: 30 g/kg and 200 g/kg, providing approximately 0·071 and 0·388 of the total energy of the diet, by replacing part of the sucrose with an equal weight of lipid. The sources and fatty acid compositions of the dietary lipids used are shown in Table 1.

Pair-fed animals were individually paired with deficient animals, and received the amount of food eaten by the deficient animals on the previous day. In one experiment, a group of animals received 7% less diet each day than the pair-fed group, which maintained their body-weights close to those of the deficient animals (weight-matched controls).

After killing by diethyl ether anaesthesia and exsanguinating by cardiac puncture, the liver, and in some experiments the kidneys and heart were removed, weighed and frozen for further analysis.

Analytical methods

Measurement of riboflavin status. The activation coefficient (AC; stimulated: basal activity) of erythrocyte NAD(P)H_2: glutathione oxidoreductase (*EC* 1.6.4.2; glutathione reductase;

Table 1. *Composition of fatty acids in dietary lipids used*

(Methyl esters of the fatty acids in the dietary lipids used were prepared and separated by gas–liquid chromatography as described in the *Lipid analysis* section; the results are given as proportions by weight of each major fatty acid. Minor components (< 0·005 by weight) are not recorded)

	Lipid				
Fatty acid	Cottonseed oil*‖	Palm oil*	Arachis oil*	Maize oil† (Mazola)	Cod-liver oil‡ (Seven Seas)
16:0	0·23	0·44	0·10	0·11	0·10
16:1	—	0·005	—	—	0·13
18:0	0·025	0·09	0·03	0·07	0·023
18:1	0·21	0·45	0·66	0·24	0·25
18:2 ω 6	0·53	—	0·20	0·61	0·022
20:0	—	—	0·015	—	—
20:1 ω 9	—	—	—	—	0·13
20:5 ω 3	—	—	—	—	0·18
22:5 ω 3	—	—	—	—	0·015
22:6 ω 3	—	—	—	—	0·12

* From Alembic Products Ltd, Sale, Manchester.
† From CPC United Kingdom Ltd, Esher, Surrey.
‡ From British Cod Liver Oils Ltd, Hull.
‖ Contained approximately 20 mg gossypol/kg, by analysis (American Oil Chemists' Society. Tentative Method Ca 13–56).

GR) was measured in washed erythrocyte preparations from blood obtained by cardiac puncture, but otherwise essentially as described previously (Prentice & Bates, 1981*a*). Hepatic FAD, and FMN plus free riboflavin, were measured essentially by the method of Bessey *et al.* (1949) as described previously (Prentice & Bates, 1981*a*).

Measurement of hepatic triglyceride concentration. Weighed portions of liver of approximately 250 mg fresh weight were homogenized in 2·5 ml sodium chloride solution (9 g/l), with an Ultra Turrax homogenizer. Duplicate samples of 0·1 ml homogenate were analysed for triglycerides with a Boehringer-Mannheim test-combination triglyceride kit, 126012.

Lipid analysis. Whole blood was collected into heparinized syringes by cardiac puncture and the plasma separated immediately by centrifugation at 2000 ***g*** for 10 min. Erythrocyte membranes were then prepared and the lipid extracted, as described by Olegard & Svennerholm (1971). Liver, kidney, heart and plasma samples, after storage at −20° for short periods, were extracted with chloroform–methanol (2:1, v/v) as described by Folch *et al.* (1957). Butylated hydroxytoluene (500 mg/l) was added to all solvents. The chloroform layers were evaporated, the lipid extract was redissolved in chloroform, 5 ml, and was dried with a mixture of anhydrous sodium sulphate and sodium bicarbonate (4:1, w/w). The lipid extracts were separated into triglyceride and phospholipid fractions on thin-layer chromatography plates of Kieselgel GF_{254} Type 60 (Merck, Darmstadt, Germany), by a solvent system consisting of light petroleum (b.p. 40–60°) – diethyl ether – acetic acid, (90:10:1, v/v). Lipid fractions were detected by examination under u.v. light, were collected, saponified under reflux for 10 min with methanol containing potassium hydroxide (0·5 mol/l) and methylated under reflux for 15 min with methanol containing ammonium chloride (33·3 g/l) and concentrated sulphuric acid (50 ml/l) (Hartman & Lago, 1973). The fatty acid composition was determined using a Pye Series 204 gas–liquid chromatograph (Pye-Unicam Ltd, Cambridge), operating at 220° with an argon flow of 40 ml/min through a 1·5 m × 4 mm analytical glass column of 50 g Silar 5CP/kg Gas Chrom Q (100–200 mesh)

prepared by Supelco Inc. and fitted with a flame-ionization detector. The proportion of each fatty acid, relative to the total recovered from the column, was determined directly by a computing integrator DP88, (Pye, Unicam Ltd).

Student's *t* test was used for all tests of significance, except where otherwise stated, since the distribution of values was approximately Gaussian in all instances.

RESULTS

Tables 2 and 3 summarize the results of two experiments which were designed to examine the effects of riboflavin deficiency in comparison with control groups receiving adequate riboflavin, and of different levels and types of dietary lipid fed to the riboflavin-deficient animals.

Growth

Although the *ad lib.*-fed control animals grew much more rapidly than either the pair-fed or the deficient animals (Table 2), the pair-fed groups also had a significantly higher body-weight at 25 d than the deficient group at both levels and with both types of lipid used ($P < 0{\cdot}001$). In one experiment, therefore, weight-matched controls were also included, to demonstrate unequivocally that the biochemical differences observed between deficient animals and pair-fed controls were not attributable to inanition.

Deficient animals given cottonseed oil at the 200 g/kg level grew consistently less well than those given the same lipid at the 30 g/kg level (Tables 2 and 3); they also had a slightly lower energy intake. Addition of palm oil did not have a comparable growth-depressing effect, at least during the first 25 d. Animals given cottonseed oil in their diets grew less rapidly, or lost weight more rapidly than animals which received maize oil in similar concentrations (Table 3), while cod-liver oil-based diets resulted in an intermediate performance (Table 3). However, in every instance there was significantly better growth ($P < 0{\cdot}001$) at the lower than at the higher level of dietary lipid. Growth on a diet with 200 g arachis oil/kg was similar to that with 200 g maize oil/kg, and for most of the variables discussed later, the animals given arachis oil resembled those given maize oil.

The second experiment (Table 3) was continued for longer than the first (Table 2) and during the final 11 d of the second experiment (Table 3) it was noted that most groups were tending to lose weight gradually, those with the higher lipid intakes still faring generally worse than those with lower lipid intakes.

Hepatic flavin levels and AC of erythrocyte GR

Hepatic FAD levels were approximately fivefold lower in deficient than in control animals, while hepatic FMN plus free riboflavin levels were ten- to twenty-fold lower (Table 2). However, there was no clear indication that different amounts or types of dietary lipid affected the extent of flavin depletion. The same was true for the AC of erythrocyte GR, where there was a highly-significant difference between control and deficient groups, but no detectable difference between groups receiving different amounts or levels of dietary lipid. A similar conclusion was reached for groups of deficient animals given 30 g maize-oil, 30 g arachis-oil and 200 g arachis-oil/kg diets: mean ($\pm$SE) values for the AC of erythrocyte GR being $2{\cdot}23 \pm 0{\cdot}35$ (n 7), $2{\cdot}25 \pm 0{\cdot}23$ (n 7) and $2{\cdot}20 \pm 0{\cdot}25$ (n 7) respectively.

The experiment described in Table 3 confirmed the conclusion that different levels of dietary lipid have no significant effect on the hepatic FAD levels attained in deficient animals, but suggested that there may in some instances be different responses to different types of lipid: thus analysis of variance revealed a significant difference between treatments ($P < 0{\cdot}001$) and the animals receiving cod-liver-oil diets had hepatic FAD levels which were significantly higher than those achieved with cottonseed-oil diets ($P < 0{\cdot}001$) for both levels of lipid used. An increase in liver: body-weight is usually associated with a decrease in flavin

Table 2. *Body-weight change, hepatic flavin and triglyceride concentrations and EGRAC for rats given different levels and types of dietary lipid*

(Mean values with their standard errors; no. of animals in parentheses. The animals were killed between days 25 and 30 of the experiment. The biochemical analyses were not carried out on every animal in all the groups, but the means are considered representative of the whole group where some results are missing)

Dietary lipid (g/kg)	Group	Starting wt (g)		Body-wt change§ (g)		Hepatic FAD (μg/g)‡		Hepatic FMN† (μg/g)‡		Hepatic triglyceride (mg/g)‡		EGRAC	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cottonseed oil: 30	Deficient	49·5	1·6 (6)	+4·7	0·9 (6)	4·5	0·2 (5)	0·7	0·1(5)	20·9	1·6 (6)	2·25	0·14 (6)
	Pair-fed control	47·8	2·0 (6)	+11·0***	1·2 (6)	20·9***	1·1 (5)	9·2***	0·8 (5)	12·0***	1·7 (6)	1·26***	0·02 (6)
Cottonseed oil: 200	Deficient	51·1	3·8 (7)	−4·0	1·1 (7)	4·5	0·7 (3)	0·5	0·1 (3)	31·1	4·3 (4)	2·08	0·14 (4)
	Pair-fed control	53·2	2·2 (7)	+4·2***	1·3 (7)	24·4***	1·2(3)	10·7***	1·2 (3)	10·2**	1·5 (4)	1·36***	0·06 (4)
	Ad-lib.-fed control	51·3	3·3 (6)	+32·0***	5·1 (6)	23·4	1·2 (6)	4·5**	0·6 (6)	13·9	1·7 (6)	1·46	0·07 (6)
Cottonseed oil: 30	Deficient	50·2	3·5 (6)	+5·7	0·7 (6)	4·7	0·5 (5)	0·3	0·1 (5)	34·4	2·4 (6)	2·44	0·39 (5)
	Pair-fed control	49·3	3·9 (6)	+17·0***	1·6 (6)	22·8***	1·3 (5)	8·0***	0·6 (5)	12·7***	1·1 (6)	1·23***	0·05 (5)
plus palm oil: 17	*Ad lib.*-fed control	49·2	2·6 (6)	+43·3***	0·4 (6)	21·7	1·0 (6)	5·7	0·8 (6)	21·2**	2·2 (6)	1·37	0·11 (5)

EGRAC, activation coefficient of erythrocyte glutathione reductase.

Values for the deficient group were statistically significantly different from the pair-fed controls, and values for the pair-fed controls were statistically significantly different from the *ad lib.*-fed controls: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

† Includes free riboflavin.

‡ Concentration per g wet liver.

§ Body wt. change refers to the first 25 d of the experiment.

Table 3. *Body-weight change, liver:body-weight value, hepatic FAD and triglyceride concentration of riboflavin-deficient rats fed diets containing several types of lipid at two concentrations*

(Mean values with their standard errors; no. of animals in parentheses; animals were killed between days 37 and 44 of the experiment)

	Starting wt (g)		Body-wt change (g)				Liver: body-wt		Hepatic triglyceride (mg/g)†		Hepatic FAD (μg/g)†	
			1–25 d		26–37 d							
Dietary lipid (g/kg)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cod-liveroil: 30	53·5	1·31 (6)	+17·5	1·4 (6)	−3·4	2·3 (6)	0·060	0·003 (6)	10·3	1·3 (6)	7·9	0·7 (5)
Cod-liver oil: 200	55·8	4·16 (6)	+5·3***	0·8 (6)	−5·8	0·3 (6)	0·070*	0·002 (6)	16·0**	1·1 (5)	7·3	0·5 (5)
Maize oil: 30	52·0	3·14 (6)	+20·5	0·7 (6)	+0·8	0·6 (5)	0·059	0·002 (5)	15·1	2·0 (5)	6·7	0·5 (6)
Maize oil: 200	58·8	4·5 (6)	+14·0***	1·2 (6)	−4·7***	0·8 (6)	0·076***	0·002 (6)	18·4	2·6 (5)	5·3	0·5 (5)
Cottonseed oil: 30	56·8	3·84 (6)	+11·8	2·2 (6)	−4·2	0·4 (6)	0·079	0·004 (6)	19·1	2·7 (6)	4·5	0·6 (6)
Cottonseed oil: 200	56·3	3·51 (6)	−2·2***	0·7 (6)	−6·7	2·3 (3)	0·100*	0·008 (3)	43·8***	5·2 (6)	4·3	0·4 (5)
Cottonseed oil: 30 plus palm oil: 170	57·0	3·22 (6)	+10·3	2·1 (6)	−8·2***	0·9 (6)	0·104*	0·008 (6)	36·1*	6·8 (6)	5·2	0·5 (6)

Values for the high lipid intake group were statistically significantly different from the corresponding low lipid intake group: $*P < 0·05$, $**P < 0·01$, $***P < 0·001$.
† Concentration per g of wet liver.

concentration, but the differences in FAD concentration between groups cannot be explained entirely in terms of dilution with new tissue components which contained little or no flavin.

Liver: body-weight and hepatic triglyceride concentrations

Liver weight as a proportion of body-weight was approximately 0·04 for all control animals, and was 0·0407 ± 0·0014 (mean ± SE) for a group of pair-fed controls given the 200 g cottonseed oil/kg diet. All the deficient groups showed increased values (mean ± SE): varying from 0·0587 ± 0·0011 for the 30 g maize oil/kg diet to 0·1042 ± 0·0083 for the 30 g cottonseed oil plus 170 g palm oil/kg diet (Table 3), which were all significantly different from the pair-fed control value ($P < 0·005$). The 200 g lipid/kg diets yielded higher values than the 30 g lipid/kg diets, and the cottonseed-oil-containing diets yielded higher values than the other diets.

Likewise, hepatic triglyceride concentrations were significantly higher in the deficient groups than in either pair-fed or *ad lib.*-fed controls (Table 2); the pair-fed controls had the lowest hepatic triglyceride levels and the *ad lib.*-fed controls were intermediate. There were some marked differences between the various deficient groups (Tables 2 and 3): the highest accumulations occurred in groups which received cottonseed oil, and greater amounts occurred in those which received 200 g than in those which received 30 g dietary lipid. However, although the increases in hepatic triglyceride generally paralleled the increases in liver: body-weight, they only accounted for a small proportion of the increased value, since triglyceride contents never exceeded 50 g/kg of the wet weight of the liver. The deficient animals did not accumulate lipid to any visible extent in subcutaneous or mesenteric depots.

Pathological signs of deficiency

In the deficient animals, the pathological signs included those observed in a previous study (Prentice & Bates, 1981*a*), together with patchy alopecia, and a yellowish sticky exudate on the eyelids. The onset was earlier and severity greater in the group given 200 g cottonseed oil/kg than in any other group. There was no relationship between individual values of the AC of erythrocyte GR and the severity of pathological signs, within groups.

Fatty acid profiles

Hepatic phospholipids. The values obtained for deficient, pair-fed and *ad lib.*-fed control animals receiving three diets with different levels and types of lipid are recorded in Table 4. Weight-matched control animals yielded values indistinguishable from those of the pair-fed controls.

The most striking differences between the deficient animals and their pair-fed controls were the increase in the fatty acid 18:2 ω6 ($P < 0·01$–$0·001$) and decrease in 20:4 ω6 ($P < 0·001$) in the deficient groups, for all three dietary lipid variants. In the animals given 170 g palm oil plus 30 g cottonseed oil/kg there was also a highly-significant increase in 18:1 ω9 ($P < 0·001$), and in both groups given 200 g lipid/kg, 18:0 was decreased ($P < 0·01$–$0·001$).

The pair-fed controls also differed in several respects from the *ad lib.* controls, and in many ways were intermediate between them and the deficient animals. However, for all three dietary lipid contents, the proportion of 18:2 was very similar in the pair-fed and *ad lib.*-fed controls.

Phospholipids in other tissues. Plasma, erythrocyte membrane, and renal phospholipid fatty acid profiles for animals receiving the 30 g cottonseed oil/kg diet are shown in Table 5. For plasma, erythrocyte membrane and renal phospholipids, the patterns are very similar to those found for the hepatic phospholipids: the major changes attributable to riboflavin

Table 4. *Hepatic phospholipid fatty acid composition from riboflavin-deficient and control rats fed different levels and types of dietary lipid*

(Mean proportions by weight with their standard errors; no. of animals in parentheses. The groups of animals were the same as those described in Tables 2 and 6)

Dietary lipid (g/kg)	Fatty Acid	Group: Deficient Mean	Deficient SE	Pair-fed control Mean	Pair-fed control SE	*Ad lib.*-fed control Mean	*Ad lib.*-fed control SE
Cottonseed oil: 30	16:0/1†	0·241	0·005 (6)	0·247	0·011 (6)	0·166***	0·005 (6)
	18:0	0·186	0·005 (6)	0·195	0·011 (6)	0·284***	0·007 (6)
	18:1	0·124	0·005 (6)	0·126	0·008 (6)	0·070***	0·004 (6)
	18:2	0·172	0·007 (6)	0·108***	0·006 (6)	0·092	0·006 (6)
	20:3/4‡	0·172	0·005 (6)	0·224***	0·010 (6)	0·274***	0·005 (6)
	22:4/5§	0·027	0·005 (6)	0·031	0·004 (6)	0·054**	0·005 (6)
	22:6	0·064	0·002 (6)	0·061	0·005 (6)	0·052	0·003 (6)
Cottonseed oil: 200	16:0/1†	0·214	0·015 (5)	0·207	0·008 (5)	0·174*	0·008 (6)
	18:0	0·168	0·005 (5)	0·218***	0·004 (5)	0·252***	0·002 (6)
	18:1	0·065	0·003 (5)	0·054	0·002 (5)	0·040***	0·008 (6)
	18:2	0·279	0·005 (5)	0·194***	0·006 (5)	0·164**	0·004 (6)
	20:3/4‡	0·134	0·006 (5)	0·217***	0·007 (5)	0·253**	0·006 (6)
	22:4/5‖	0·039	0·006 (5)	0·040	0·003 (5)	0·075***	0·006 (6)
	22:6	0·061	0·003 (5)	0·063	0·009 (5)	0·032**	0·003 (6)
Cottonseed oil: 30 plus palm oil: 170	16:0/1†	0·250	0·012 (6)	0·223	0·004 (6)	0·158***	0·009 (6)
	18:0	0·151	0·006 (6)	0·185***	0·005 (6)	0·239***	0·011 (6)
	18:1	0·204	0·006 (6)	0·144***	0·006 (6)	0·136	0·006 (6)
	18:2	0·159	0·004 (6)	0·121**	0·008 (6)	0·120	0·003 (6)
	20:3/4‡	0·158	0·005 (6)	0·224***	0·008 (6)	0·257**	0·004 (6)
	22:4/5‖	0·021	0·002 (6)	0·043***	0·004 (6)	0·047	0·002 (6)
	22:6	0·046	0·002 (6)	0·054	0·003 (6)	0·037***	0·001 (6)

Values for the deficient group were statistically significantly different from the pair-fed controls and values for the pair-fed controls were statistically significantly different from the *ad lib.*-fed controls: $*P < 0·05$, $**P < 0·01$, $***P < 0·001$.

† Mainly 16:0; (< 0·004 16:1).

‡ Mainly 20:4 ω6; (< 0·001 20:3).

‖ Consists of 22:4 ω6 and 22:5 ω3.

deficiency being an increase in 18:2 and a decrease in 20:4. The kidneys from animals receiving the 200 g lipid/kg diets were also analysed, and these too, showed the same trends. However, in another experiment, cardiac phospholipids in the deficient groups showed no evidence of the increase in 18:2 and a decrease in 20:4 seen in liver, kidney, plasma and erythrocyte membranes.

Although the mean values of 20:4:18:2 in phospholipids from several of the tissues examined varied by a factor of six between different groups of animals, the total number of double bonds per unit weight of fatty acid varied by a factor of only 1·2.

Hepatic and renal triglycerides. The hepatic triglyceride fatty acid profiles are recorded in Table 6. Clearly there were some very pronounced effects of riboflavin deficiency, especially with respect to 18:2. In contrast to the phospholipid fatty acid profiles, however, the patterns for the *ad lib.*-fed controls were closer to those of the deficient groups than to those of the pair-fed controls, especially for the 200 g cottonseed oil/kg diet.

In deficient animals, the proportion of 16:0 was consistently decreased, and that of an unidentified fatty acid, moving approximately in the position of 20:1, was increased.

For the renal triglycerides, differences between deficient and pair-fed control groups were smaller than those for the hepatic triglycerides, and the increase in proportion of 18:2 was significant ($P < 0·01$) only for the deficient group receiving the 30 g cottonseed oil/kg diet.

Table 5. *Phospholipid fatty acid composition from three tissues of riboflavin-deficient and control rats fed a diet containing 30 g cottonseed oil/kg*

(Mean proportions by weight with their standard errors, for six rats/group for plasma phospholipids and means of two values† for erythrocyte phospholipids and renal phospholipids. Mean starting weight was 51·0 g and animals were killed between days 25 and 31 of the experiment)

	Group					
	Fatty acid-deficient		Pair-fed control		*Ad lib.*-fed control	
	Mean	SE	Mean	SE	Mean	SE
			Plasma phospholipids			
16:0/1‖	0·351	0·017	0·308	0·008	0·199***	0·012
18:0	0·144	0·008	0·176	0·012	0·270***	0·009
18:1	0·109	0·004	0·117	0·006	0·077***	0·003
18:2	0·200	0·007	0·154**	0·010	0·138	0·006
20:3/4‡	0·118	0·005	0·169**	0·015	0·236**	0·009
22:4/5§	0·015	0·003	0·017	0·003	0·036**	0·003
22:6	0·029	0·002	0·028	0·004	0·031	0·004
			Erythrocyte phospholipids			
16:0/1‖	0·334	—	0·312	—	0·286	—
18:0	0·132	—	0·138	—	0·174	—
18:1	0·103	—	0·118	—	0·102	—
18:2	0·109	—	0·079	—	0·069	—
20:3/4‡	0·237	—	0·268	—	0·281	—
22:4/5§	0·015	—	0·019	—	0·035	—
22:6	0·056	—	0·055	—	0·045	—
			Renal phospholipids			
16:0/1‖	0·221	—	0·233	—	0·244	—
18:0	0·189	—	0·177	—	0·173	—
18:1	0·122	—	0·136	—	0·126	—
18:2	0·140	—	0·105	—	0·091	—
20:3/4‡	0·279	—	0·308	—	0·311	—
22:4/5§	0·013	—	0·012	—	0·019	—
22:6	0·025	—	0·020	—	0·021	—

Values for deficient groups were statistically significantly different from the pair-fed controls, and values for the pair-fed controls were statistically significantly different from the *ad lib.*-fed controls: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

† Each value represents a pooled sample from three rats.

‡ Mainly 20:4 ω6; (< 0·001 20:3).

‖ Mainly 16:0; (< 0·004 16:1).

§ Consists of 22:4 ω6 and 22:5 ω3.

DISCUSSION

Prevention of coprophagy, and the effects of dietary lipids on growth and severity of deficiency

The use of semi-rigid plastic foam collars proved an effective and relatively simple means of preventing coprophagy; they were simpler to apply and to adjust in size than tail cups, (Prentice & Bates, 1980), were well tolerated, and could be used with either male or female rats. Females were used for the present study because they are reported to respond more uniformly to a riboflavin-deficient diet (Lambooy, 1975). The virtual cessation of weight gain between days 26 and 37 in the deficient animals in Expt. 2 (Table 3) is an indication of the efficacy of the technique. Although the magnitude of the changes in flavin levels and of the AC of erythrocyte GR were somewhat different from those of a previous study of acute deficiency (Prentice & Bates, 1981*a*) in which male rats were used, the severity of the deficiency was probably similar. The smaller weight gain observed in the *ad lib.* controls

Table 6. *Hepatic triglyceride fatty acid composition from riboflavin-deficient and control rats fed different levels and types of dietary lipid*

(Mean proportions by weight with their standard errors; no. of animals in parentheses. The groups of animals were the same as those described in Tables 2 and 4)

Dietary lipid (g/kg)	Fatty Acid	Deficient		Pair-fed control		*Ad lib.*-fed control	
		Mean	SE	Mean	SE	Mean	SE
Cottonseed oil: 30	16:0/1†	0·317	0·010 (6)	0·479***	0·015 (6)	0·446	0·009 (6)
	18:0	0·063	0·003 (6)	0·056	0·005 (6)	0·034**	0·002 (6)
	18:1	0·351	0·009 (6)	0·411**	0·017 (6)	0·423	0·007 (6)
	18:2	0·189	0·009 (6)	0·033***	0·004 (6)	0·069**	0·009 (6)
	UN‡	0·063	0·006 (6)	0·016***	0·005 (6)	0·005	0·002 (6)
	20:3/4	0·011	0·002 (6)	0·011	0·003 (6)	0·003	0·001 (6)
Cottonseed oil: 200	16:0/1†	0·124	0·013 (5)	0·405***	0·020 (5)	0·297*	0·014 (6)
	18:0	0·054	0·001 (5)	0·081**	0·008 (5)	0·044*	0·095 (6)
	18:1	0·148	0·006 (5)	0·171	0·019 (5)	0·170	0·004 (6)
	18:2	0·493	0·008 (5)	0·201***	0·029 (5)	0·402**	0·038 (6)
	UN‡	0·135	0·020 (5)	0·066*	0·010 (5)	0·024**	0·004 (6)
	20:3/4	0·044	0·008 (5)	0·077	0·013 (5)	0·063	0·014 (6)
Cottonseed oil: 30 plus palm oil: 170	16:0/1†	0·277	0·017 (6)	0·376**	0·013 (6)	0·353	0·016 (6)
	18:0	0·054	0·003 (6)	0·053	0·009 (6)	0·031*	0·002 (6)
	18:1	0·455	0·004 (6)	0·410	0·036 (6)	0·499*	0·004 (6)
	18:2	0·137	0·009 (6)	0·089***	0·004 (6)	0·097	0·005 (6)
	UN‡	0·069	0·003 (6)	0·033**	0·008 (6)	0·011	0·001 (6)
	20:3/4	0·008	0·001 (6)	0·045	0·020 (6)	0·008	0·001 (6)

Values for the deficient group were statistically significantly different from the pair-fed controls, and values for the pair-fed controls were statistically significantly different from the *ad lib.*-fed controls: $*P < 0·05$, $**P < 0·01$, $***P < 0·001$.

† Mainly 16:0 (< 0·008 16:1).

‡ Unidentified peak moving approximately in the position of 20:1.

here than in the *ad. lib.* controls of a previous study (Prentice & Bates, 1981*a*) may be due partly to the use of collars on the control animals here, and partly to the use of females instead of males.

In some early studies of the effect of high-fat diets on riboflavin requirements (Mannering & Lipton, 1941; Shaw & Phillips, 1941; Tange, 1941; Mannering *et al.* 1944; Czaczkes & Guggenheim, 1946; Kaunitz *et al.* 1954), no precautions were taken to prevent coprophagy, and high-fat diets were generally compared with high-starch diets, the latter tending to favour the supply of B-vitamins when refection is permitted. Any metabolic effect of the high lipid content of the diet would, presumably, have been masked by the effect of refection under these conditions (Prentice & Bates, 1980). Nevertheless, our present observations suggest that a high lipid intake will usually result in some further reduction in weight gain in deficient animals, even when coprophagy is effectively prevented. This did not occur in every instance, however; for instance the animals given 30 g cottonseed oil plus 170 g palm oil/kg grew as well as those given 30 g cottonseed oil alone. The lower weight gains in animals fed the high-fat diets may be partly attributable to a small decrease in energy intake in these animals.

Cottonseed oil seemed to be particularly deleterious at high concentrations, with respect both to weight gain and to the appearance of pathological signs of deficiency. This cannot, apparently, be explained simply in terms of its high content of polyunsaturated fatty acids (cf. Reiser & Pearson, 1949), since maize oil and arachis oil were much better tolerated at the 200 g/kg level. In our experience there was little clear-cut evidence of a correlation

between polyunsaturated fatty acid content and the severity of riboflavin deficiency produced by the different dietary lipids, although it remains possible that the toxic constituents in some of the lipids, particularly cottonseed oil, may have obscured any trends which were due to the level of polyunsaturation. The cottonseed oil used was found to contain 20 mg gossypol/kg, which is unlikely to be enough to produce toxic effects in normal animals (Berardi & Goldblatt, 1969), and it is also known to contain cyclopropene fatty acids which also have some potentially toxic effects (Gurr & James, 1975). It is conceivable that these are exacerbated in riboflavin-deficient animals. In addition it is conceivable that relatively high levels of vitamins A and D in the 200 g/kg cod-liver-oil diet may have exerted a slight toxic effect, which could partly have obscured the effects of the fatty acid composition of this diet.

Neither the hepatic flavin levels nor the values of the AC of erythrocyte GR provided any clear evidence that high lipid intakes exacerbated the extent of flavin depletion in the deficient animals, although in order to confirm this beyond doubt, it would be necessary to measure the time-course of depletion or to use a chronic deficiency model (Prentice & Bates, 1981*b*). Intriguingly, there was some evidence that the cod-liver-oil-containing diets failed to deplete liver flavin levels to the same extent as some of the other deficient diets.

Hepatic enlargement and triglyceride accumulation were more pronounced for the 200 g lipid/kg diets than for those containing 30 g/kg lipid, and both were exacerbated by cottonseed oil. These changes would be compatible with reduced mobilization and turnover of hepatic lipid. The increase in 18:2:20:4 in hepatic phospholipids did not correlate closely with the extent of hepatic triglyceride accumulation, suggesting a somewhat different control mechanism for these two responses.

Fatty acid profiles

The relative accumulation of 18:2 and depletion of 20:4 in hepatic phospholipids of deficient animals is in accord with the observations of Taniguchi *et al.* (1976, 1978). Although the latter authors did not control inanition, in our experience pair-feeding proved to be an essential precaution to eliminate the interfering effects of inanition. The additional refinement of weight-matching did not, however, appear to be necessary. Similar fatty acid changes were observed in plasma phospholipids in the present study, in agreement with Koyanagi & Oikawa (1965) and were also seen in renal and erythrocyte membrane phospholipids, but not in cardiac phospholipids. These observations are further evidence that the phospholipid fatty acid changes are not a consequence of hepatic lipid accumulation, and the accompanying changes in its triglyceride fatty acid profile.

An increase in proportion of 18:2 and a decrease in 20:4 might arise as a direct result of over-all reduction in fatty acid metabolism (Hoppel *et al.* 1979); 18:2 was present in all of the dietary lipids used and cannot be synthesized *de novo*, while 20:4 was absent from all the dietary lipids and would only arise by elongation and desaturation of 18:2. Thus, when fatty acid metabolism is generally depressed, the proportion of 18:2 would presumably rise, that of 20:4 would presumably fall, but the proportion of the saturated and monounsaturated fatty acids might be less affected, since a reduction in their metabolism could be offset by a corresponding reduction in their *de novo* synthesis. When the dietary lipid had a high content of 18:1 (as in palm oil), this fatty acid did accumulate in the phospholipids of deficient animals.

The minimal extent of variation observed in total double bond concentration per unit weight of fatty acids may be a reflexion of the need to maintain membrane fluidity, although wide variations in amounts and relative proportions of individual fatty acids can apparently be tolerated.

Taniguchi *et al.* (1978) have found that the time-course of changes in phospholipid fatty

acid profiles did not closely parallel changes in mitochondrial morphological structure, suggesting that the morphological change is not a major cause of the fatty acid changes.

An accumulation of 18:2 relative to 20:4 has also been observed in animals deprived of protein (Rogers, 1971), of vitamin B_{12} (Peifer & Lewis, 1979), of vitamin B_6 (Dussault & Lepage, 1975), and as a result of intoxication with ethanol (French *et al.* 1970, 1971) or with ethionine (Lyman *et al.* 1968). It may therefore represent a fairly non-specific response of a vulnerable pathway to metabolic stress. One of the suggested mechanisms for the fatty acid changes in all the previously-mentioned instances has been an impairment in the conversion of 18:2 to 20:4, and this is also a possibility in riboflavin deficiency.

The accumulation of triglyceride in the liver frequently occurs as a result of impaired β-lipoprotein synthesis, resulting in reduced transport of triglyceride out of the liver. There is no evidence currently available to indicate whether this mechanism contributes to the fatty liver of riboflavin deficiency. Increased synthesis of lipid appears unlikely, because fatty acids derived entirely from the diet such as 18:2 tend to accumulate, in preference to those which can be synthesized *de novo*.

In the accompanying paper, some enzyme activities and metabolic functions of hepatic mitochondria and microsomes, which may be relevant to the utilization and interconversions of the fatty acids in riboflavin-deficient animals, are examined.

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REFERENCES

American Oil Chemists Society. Tentative Method Ca 13–56.
Berardi, L. C. & Goldblatt, L. A. (1969). In *Toxic Constituents of Plant Foodstuffs*, p. 211 [I. E. Liener, editor]. New York and London: Academic Press.
Bessey, O. A., Lowry, O. H. & Love, R. H. (1949). *J. biol. Chem.* **180**, 755.
Czaczkes, J. W. & Guggenheim, K. (1946). *J. biol. Chem.* **162**, 267.
Dussault, P. E. & Lepage, M. (1975). *J. Nutr.* **105**, 1371.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
French, S. W., Ihrig, T. J. & Morin, R. J. (1970). *Q. Jl Stud. Alc.* **31**, 801.
French, S. W., Ihrig, T. J., Shaw, G. P., Tanaka, T. T. & Norum, M. L. (1971). *Res. Commun. Chem. Path. Pharmac.* **2**, 567.
Gershoff, S. N., Andrus, S. B. & Hegsted, D. M. (1959). *J. Nutr.* **68**, 75.
Goodman, S. I. (1981). *Amer. J. clin. Nutr.* **34**, 2434.
Guggenheim, K. & Diamant, E. J. (1959). *Br. J. Nutr.* **13**, 61.
Gurr, M. I. & James, A. T. (1975). *Lipid Biochemistry. An Introduction*, 2nd ed. London: Chapman and Hall.
Hara, H. (1960). *J. Vitam.* **6**, 24.
Hartman, L. & Lago, R. C. A. (1973). *Lab. Pract.* **22**, 475.
Hoppel, C., DiMarco, J. P. & Tandler, B. (1979). *J. biol. Chem.* **254**, 4164.
Kaunitz, H., Wiesinger, H., Blodi, C. F., Johnson, R. E. & Slanetz, C. A. (1954). *J. Nutr.* **52**, 467.
Kim, Y. S. & Lambooy, J. P. (1969). *J. Nutr.* **98**, 467.
Koyanagi, T. & Oikawa, K. (1965). *Tohoku J. exp. Med.* **86**, 19.
Lambooy, J. P. (1975). In *Riboflavin*, p. 304 [R. S. Rivlin, editor]. New York and London: Plenum Press.
Lyman, R. L., Rosmire, M. A., Giotas, C. & Miljanish, P. (1968). *J. Nutr.* **94**, 74.
Mannering, G. J., Lipton, M. A. & Elvehjem, C. A. (1941). *Proc. Soc. exp. Biol. Med.* **46**, 100.
Mannering, G. J., Orsini, D. & Elvehjem, C. A. (1944). *J. Nutr.* **28**, 141.
Olegard, R. & Svennerholm, L. (1971). *Acta Paediat., Scand.* **60**, 505.
Olpin, S. E. & Bates, C. J. (1982). *Br. J. Nutr.* **47**, 589.
Peifer, J. J. & Lewis, R. D. (1979). *J. Nutr.* **109**, 2160.
Prentice, A. M. & Bates, C. J. (1980). *Br. J. Nutr.* **43**, 171.
Prentice, A. M. & Bates, C. J. (1981*a*). *Br. J. Nutr.* **45**, 37.
Prentice, A. M. & Bates, C. J. (1981*b*). *Br. J. Nutr.* **45**, 53.
Reiser, R. & Pearson, P. B. (1949). *J. Nutr.* **38**, 247.
Rogers, C. G. (1971). *J. Nutr.* **101**, 1547.
Shaw, J. H. & Phillips, P. H. (1941). *J. Nutr.* **22**, 345.
Tange, U. (1941). *J. agric. Chem. Soc. Japan* **17**, 1050.
Taniguchi, M. & Nakamura, M. (1976). *J. Nutr. Sci. Vitam.* **22**, 135.
Taniguchi, M., Yamamoto, T. & Nakamura, M. (1978). *J. Nutr. Sci. Vitam.* **24**, 363.
Wintrobe, M. M., Buschke, W., Follis, R. H. & Humphreys, S. (1944). *Bull. Johns Hopkins Hosp.* **75**, 102.
Wynder, E. L. & Klein, U. E. (1965). *Cancer* **18**, 167.

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