The effect of riboflavin deficiency on methylenetetrahydrofolate reductase (NADPH) (*EC* 1.5.1.20) and folate metabolism in the rat

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1. Riboflavin deficiency at two levels of severity was produced in weanling rats by feeding deficient diets for 6 weeks and using neck collars to prevent coprophagy. The severity of deficiency was monitored by growth, liver flavin levels and the activation coefficient of erythrocyte glutathione oxidoreductase (NAD(P)H) (*EC* 1.6.4.2). Control groups, receiving the same diet with ample added riboflavin, were fed either *ad lib.*, or were pair-fed with the deficient animals.

2. The hepatic flavoenzyme, methylenetetrahydrofolate reductase (NADPH) (*EC* 1.5.1.20), was very markedly affected by severe riboflavin deficiency and was significantly, but less markedly, affected by the intermediate level of deficiency. This reduction in activity was due primarily to the direct effect of the diminished supply of riboflavin, and occurred to only a small extent as a result of inanition, demonstrated by a moderate reduction in activity in the more severely food-restricted of the two pair-fed groups. Since the enzyme is assayed in the presence of its flavin cofactor, FAD, it clearly cannot be reactivated in vitro, as some other depleted flavoenzymes can. The discriminatory ability in distinguishing between severe and moderate riboflavin deficiency in vivo confers some potential advantages on this oxidoreductase as a possible index of riboflavin status.

3. The hepatic activity of another key folate-metabolizing enzyme, dihydrofolate reductase (*EC* 1.5.1.3), was not diminished by riboflavin deficiency in the present study.

4. The ratio, labelled 5-methyltetrahydrofolic acid:other labelled compounds derived from intraperitoneally injected pteroylglutamic acid in extracts of hepatic tissue was significantly reduced in the riboflavin-deficient groups, indicating the possibility of an effect of riboflavin deficiency on folate metabolism in vivo.

In view of the widespread occurrence of riboflavin deficiency in developing countries, which is especially severe in the later stages of pregnancy (Bates *et al.* 1984), where it may have undesirable consequences for fetal development and homeostasis, it is important to understand the nature and extent of riboflavin involvement in vulnerable metabolic pathways. Hitherto, attention has been mainly focussed on flavoprotein components of the electron transport chain because of their implication in the release of energy, and on the erythrocyte flavoenzyme, glutathione reductase (NAD(P)H) (*EC* 1.6.4.2), because of its practical usefulness as an indicator of riboflavin status in man. The effects of riboflavin deficiency on other flavoenzyme-dependent pathways have, however, been little studied. Since it is necessary to consider not only the occurrence of flavoenzymes in each metabolic pathway, but also their relative sensitivity to the effects of dietary riboflavin-deficiency, which affects particular flavoenzymes and flavin-dependent pathways rather selectively, riboflavin-depletion studies in animals are needed to complement our growing repository of knowledge about flavoenzyme structure and kinetics.

The reaction catalysed by methylenetetrahydrofolate reductase (*EC* 1.5.1.20; MTHF reductase) is an important gateway between the partially oxidized, folate-activated, one-carbon units which are used in DNA synthesis, and the reactions involving serine, glycine, histidine and formate on the one hand, and on the other, the fully reduced methyl group, whose most important role quantitatively is the formation of methionine from homocysteine. Fine tuning of this reaction to ensure appropriate proportions of oxidized and reduced one-carbon units in different metabolic states is achieved by feedback inhibition by the effector, S-adenosyl methionine (Kutzbach & Stokstad, 1972). The possibility that MTHF reductase may be sensitive to variations in nutritional status has, however, received less attention, and a single report of its sensitivity to riboflavin deficiency (Narisawa *et al.*

1968) does not appear to have been confirmed or re-investigated. In addition, conflicting reports exist about the effects of riboflavin deficiency on certain folate-metabolizing enzymes and folate-sensitive processes, suggesting that a new investigation would be timely.

The present study was designed primarily to re-investigate the claim that MTHF reductase is sensitive to riboflavin deficiency in vivo. This prediction has been tested at two levels of riboflavin depletion, and with particular attention to the potentially confounding effects of inanition. Certain other facets of folate economy have also been examined, in an attempt to clarify the wider question of riboflavin–folate interactions in vivo.

ANIMALS AND METHODS

Female weanling Norwegian hooded rats reared on Purina chow diets were housed individually in suspended wire cages; mean body-weight was 60 g at the beginning of the experiment. Coprophagy was prevented in the deficient and pair-fed control animals by the use of Plastizote collars as described previously (Olpin & Bates, 1982). Because of the difficulty in adjusting the overall size of the collar and of its central hole for rapidly growing animals, collars were not applied to the ad lib.-fed controls.

The basic riboflavin-deficient diet was the same as that described previously (Olpin & Bates, 1982; Duerden & Bates, 1985), the lipid component being arachis oil (30 g/kg), and the casein (100 g/kg) was 'low in vitamins' (BDH, Poole, Dorset), providing about 0·20 mg riboflavin/kg diet (Duerden & Bates, 1985).

Five matched groups of eight animals were used: group D1 (severely deficient), with no added riboflavin in their diet; group P1, receiving the same diet containing added riboflavin (15 mg/kg diet) and individually pair-fed to the animals in group D1; group D2 (moderately deficient), with added riboflavin (0·5 mg/kg diet); group P2, with added riboflavin (15 mg/kg diet) and individually pair-fed to the animals in group D2; and finally group A, receiving ad. lib. the diet with riboflavin added at 15 mg/kg. A riboflavin content of 15 mg/kg has previously been shown to be adequate for optimal response of several riboflavin-sensitive factors even in the presence of reduced total food intake of pair-fed control animals (Prentice & Bates, 1981 b). Animal weights and food intakes were measured every day, livers were removed, and blood was anticoagulated with heparin. At 24 h before each animal was killed, it received an intraperitoneally injected dose of [3′,5′,7,9-^{3}H]-pteroylglutamic acid (Amersham International plc, Amersham, Bucks; 500 mCi/mmol, 50 μCi/kg body-weight). The animals were killed during the 7th week after starting the special diets; two animals from each group on each successive day. Blood samples, anticoagulated with heparin, were used to prepare washed erythrocytes, and liver samples were removed for enzyme assays and for folate separation by high pressure liquid chromatography (HPLC) as described later. Liver flavin levels were measured by the method of Bessey et al. (1949) as described previously (Prentice & Bates, 1981 a). The activation coefficient (stimulated:basal activity) of erythrocyte glutathione reductase (EGRAC) was measured in washed erythrocyte preparations by a previously-described method (Prentice & Bates, 1981 a), modified for use with a Roche Cobas Bioanalyser (Powers et al. 1983). Preincubation was for 30 min with and without 6 μM-FAD, the assay reaction time was 5 min, following addition of NADPH as start reagent.

Enzyme assays

Samples of fresh liver were homogenized in 10 vol. buffer, pH 6·3, containing potassium phosphate (0·05 mol/l) and EDTA (1 mmol/l), and were centrifuged for 30 min at 105 000 g to obtain a particle-free supernatant fraction.

MTHF reductase activity was measured by a method based on studies by Donaldson & Keresztesy (1962). Portions (0·1 ml) of the liver supernatant were mixed with 0·65 ml of a solution, pH 6·3, containing potassium phosphate (0·05 mol/l), EDTA (1 mmol/l), menadione (0·04 mmol/l), FAD (0·1 mmol/l) and [^{14}C]methyltetrahydrofolic acid, barium salt (Amersham International plc; 0·2 μCi/mol; 0·58 mmol/l). After incubation for 60 min at 37°, 50 μl formaldehyde (0·4 mol/l) were added, followed by 0·47 ml potassium acetate, pH 4·5 (1·0 mol/l), and 0·47 ml of a solution of dimedone (0·43 mol/litre ethanol). The mixture was heated for 5 min at 95°, cooled, extracted by shaking with 5·0 ml toluene, and 4·5 ml toluene extract was mixed with 6·0 ml scintillant containing 2,5-diphenyloxazole (10·8 g/l) and 1,4-di-(2-(5-phenyl oxazolyl)) benzene (0·27 g/l) in toluene, for liquid-scintillation counting. A blank correction was introduced by using buffer in place of the enzyme extract. Proportionality of the reaction to the amount of enzyme added was verified.

Dihydrofolate reductase (*EC* 1.5.1.3) activity was measured by the method of Mathews *et al.* (1963), adapted to a Roche Cobas bioanalyser. Portions (12·5 μl) of the liver supernatant were first mixed with 300 μl buffer, pH 7·5, containing potassium phosphate (0·05 mol/l), mercaptoethanol (0·01 mol/l) and dihydrofolic acid (Sigma, Poole, Dorset; 0·05 mmol/l) and the reaction was started with 10 μl reduced NADP (1 mmol/l). The change in optical density at 340 nm was followed for 5 min at 37°, and a blank correction was made for the small amount of oxidation occurring in the absence of dihydrofolic acid substrate.

The protein content of the enzyme preparation was measured by the biuret method (Gornall *et al.* 1949).

Hepatic folate separation

Immediately after killing, samples of liver (1 g) were extracted with sodium ascorbate pH 6·0 (100 g/l; 2 ml) by homogenization using a Potter–Elvehjem homogenizer. They were then mixed with 0·3 ml rat plasma to provide additional folate conjugase (pteroyl polyglutamyl hydrolase), and were incubated for 3 h at 37° to liberate the folate monoglutamates. The extract was then heated for 5 min at 100° and centrifuged to remove protein by precipitation, and folate separation was performed by the HPLC method of McMartin *et al.* (1981). A column, 250 mm × 3·9 mm of μ-Bondapak C$_{18}$ resin was eluted with methanol, (350 ml/litre water) containing PIC-A (*N*-tetra-*n*-butylammonium phosphate, supplied by Waters, Millford, Massachusetts, 5 mmol/l) at a flow rate of 1·0 ml/min. Extract samples were mixed with unlabelled 5-methyltetrahydrofolic acid marker, which was then detected by its optical density at 254 nm. (The peak of 5-methyltetrahydrofolic acid, which appeared after 14 min, was well separated from pteroylglutamic acid, 5-formyl tetrahydrofolate and tetrahydrofolic acid when run as external markers.) Fractions (0·5 ml) of the eluate were collected and were mixed with a scintillant containing 2,5-diphenyl oxazole (0·6 g/l), 1,4-di(2-(5-phenyl oxazolyl)) benzene (60 mg/l), Triton X-100 (200 ml/l) and toluene (800 ml/l) for liquid-scintillation counting. There was usually a clear peak of radioactivity which coincided with the marker 5-methyltetrahydrofolic acid peak and several peaks in the region occupied by more oxidized folate derivatives. The proportion of the total recovered radioactivity which coincided with the 5-methyltetrahydrofolate peak could thus be calculated.

RESULTS

Fig. 1 depicts the growth curves of the five groups of rats, and Fig. 2 depicts their food intakes. The *ad lib.* control group gained five times as much weight as the most severely
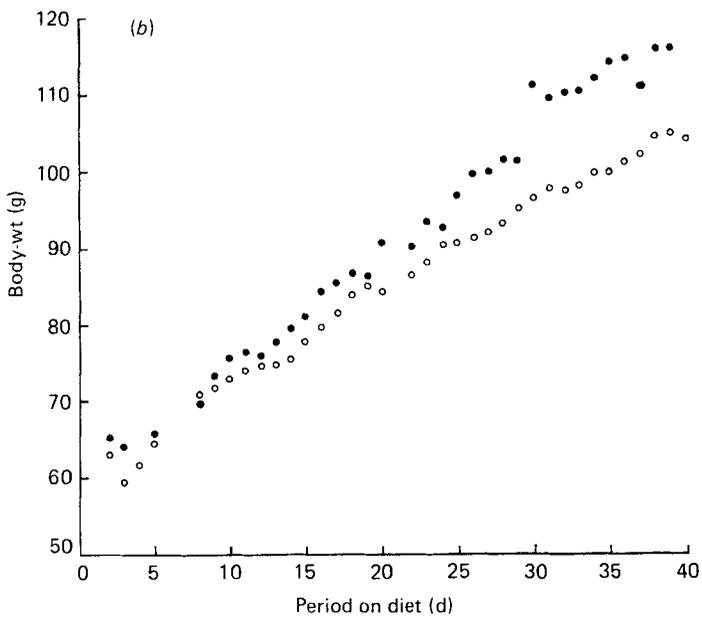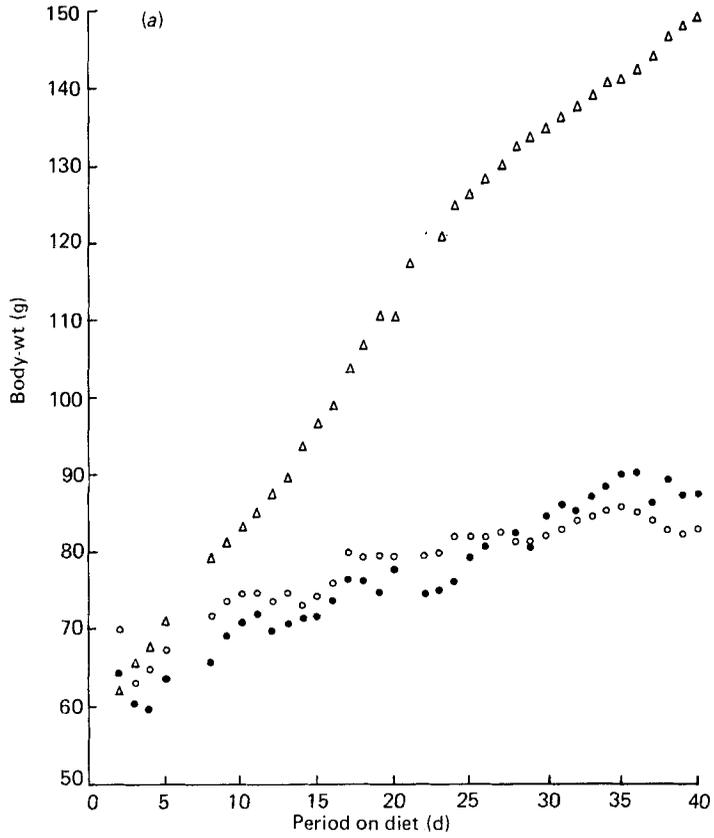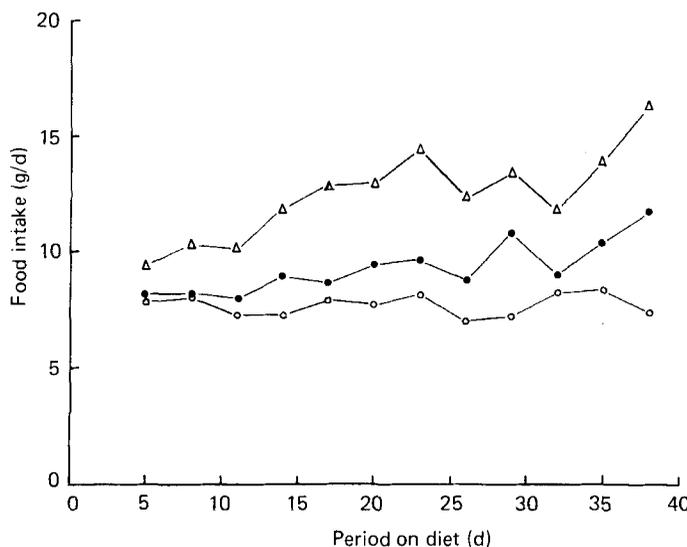
Fig. 2. Daily food intakes. Mean values for successive 3 d intervals: group D1 (no added riboflavin) (○); group D2 (0·5 mg riboflavin/kg) (●); group A (15 mg riboflavin/kg fed *ad lib.*) (△).
    Mean coefficients of variation for each group over the entire 40 d period were: group D1 20·4, group D2 17·6, group A 19·0.

deficient (D1) group and about twice as much as the intermediate (D2) group. Some of the difference in weight gain between the *ad lib.* and deficient groups may be attributable to the stress of wearing collars; therefore the most useful comparisons are between the deficient groups and their corresponding pair-fed controls. Both pair-fed groups gained more weight than the corresponding deficient groups: thus group D1 gained 0·056 (SD 0·032) g body-weight/g food eaten compared with 0·082 (SD 0·024) g body-weight/g food eaten in the P1 group ($0·05 < P \leqslant 0·1$ by paired t test). Group D2 gained 0·125 (SD 0·022) g body-weight/g food eaten compared with 0·164 (SD 0·018) g body-weight/g food eaten in the P2 group ($P < 0·005$ by paired t test). These comparisons illustrate the difference in efficiency of food conversion to new tissue between deficient animals and the corresponding controls.

Table 1 shows the effect of riboflavin deficiency on hepatic flavin levels, and on EGRAC. The picture in the D1 group is of fairly severe biochemical deficiency and in the D2 group of an intermediate level of deficiency. The three control groups were all very similar to each other and were within normal limits with respect to the biochemical indices of riboflavin status.

Table 2 shows the effect of riboflavin deficiency on the two hepatic enzymes, MTHF reductase and dihydrofolate reductase. MTHF reductase was clearly extremely sensitive to riboflavin deficiency, in both the D1 and the D2 groups, and its specific activity was considerably below that of the corresponding control group. In addition, there was a highly significant difference between the two deficient groups ($P < 0·001$), which was not observed in the case of the primary indices of riboflavin status: hepatic FAD levels and EGRAC (Table 1). This point is further illustrated in Fig. 3, which depicts the relations of individual

Fig. 1. Growth curves. (*a*) Mean values for: group D1 (no added riboflavin) (○); group P1 (15 mg riboflavin/kg, pair-fed to group D1) (●); group A (15 mg riboflavin/kg, fed *ad lib.* (△). (*b*) Mean values for: group D2 (0·5 mg riboflavin added/kg) (○); group P2 (15 mg riboflavin/kg, pair-fed to group D2) (●).
    Mean coefficients of variation for each group over the entire 40 d period were: group D1 7·9, group D2 5·9, group P1 8·4, group P2 7·1, group A 12·7.

Table 1. *Hepatic flavin concentrations and erythrocyte glutathione reductase NAD(P)H*
(EC 1.6.4.2) activation coefficients (EGRAC)

(Mean values with their standard errors for eight animals per group)

Group of animals	Riboflavin added to diet (mg/kg)	Hepatic flavin concentrations					
		Free riboflavin + FMN (μg/g)		FAD (μg/g)		EGRAC	
		Mean	SE	Mean	SE	Mean	SE
D1	0	2·0***	0·33	9·0***	0·66	2·03***	0·105
P1	15	10·1	3·58	22·6	1·34	1.31	0·030
D2	0·5	3·2***†	0·18	11·0***	1·10	1·81***	0·062
P2	15	8·6	0·33	23·2	1·26	1·27	0·018
A	15	9·3	0·41	19·1	0·82	1·25	0·018

P1, pair-fed to animals in group D1; P2, pair-fed to animals in group D2; A, fed *ad lib.*
*** Significantly different from the corresponding values for the pair-fed control group (paired *t* test): $P < 0.001$.
† Significantly different from group D1 (by Student's *t* test): $P < 0.05$.

Table 2. *Hepatic activity of methylenetetrahydrofolate reductase (NADPH)*
(EC 1.5.1.20) and of dihydrofolate reductase (EC 1.5.1.3)

(Mean values with their standard errors for eight animals per group)

Group of animals	Riboflavin added to diet (mg/kg)	Methylenetetrahydrofolate reductase (nmol/mg protein per h)		Dihydrofolate reductase (nmol/mg protein per h)	
		Mean	SE	Mean	SE
D1	0	0·44***	0·10	250	13
P1	15	6·45††	0·22	186	10
D2	0·5	2·04***‡‡‡	0·19	257	16
P2	15	8·56	0·66	221	8
A	15	8·96	0·58	218	16

P1, pair-fed to animals in group D1; P2, pair-fed to animals in group D2; A, fed *ad lib.*
*** Significantly different from the corresponding values for the pair-fed control group (paired *t* test): $P < 0.001$.
†† Significantly different from the value for the *ad lib.*-fed control group (A) (Student's *t* test): $P < 0.005$.
‡‡‡ Significantly different from the value for group D1 (Student's *t* test): $P < 0.001$.

values of MTHF reductase with those of FAD and of EGRAC. The two deficient groups were clearly distinguishable from each other by their different MTHF reductase activities but were not adequately distinguishable by the other two indices. Although MTHF reductase levels were slightly reduced in group P1 compared with group A, the inanition effect on this enzyme was clearly much smaller than the direct effect of riboflavin supply.

Unlike MTHF reductase, hepatic dihydrofolate reductase was not significantly reduced by riboflavin deficiency; indeed the mean activity in the two deficient groups was slightly above that of the controls (Table 2) and significantly so in comparison with group P1, the most severely food-restricted of the three control groups. In a separate experiment it was shown that folic acid reductase activity (with pteroylglutamic acid as substrate) was also unaffected by severe riboflavin deficiency.
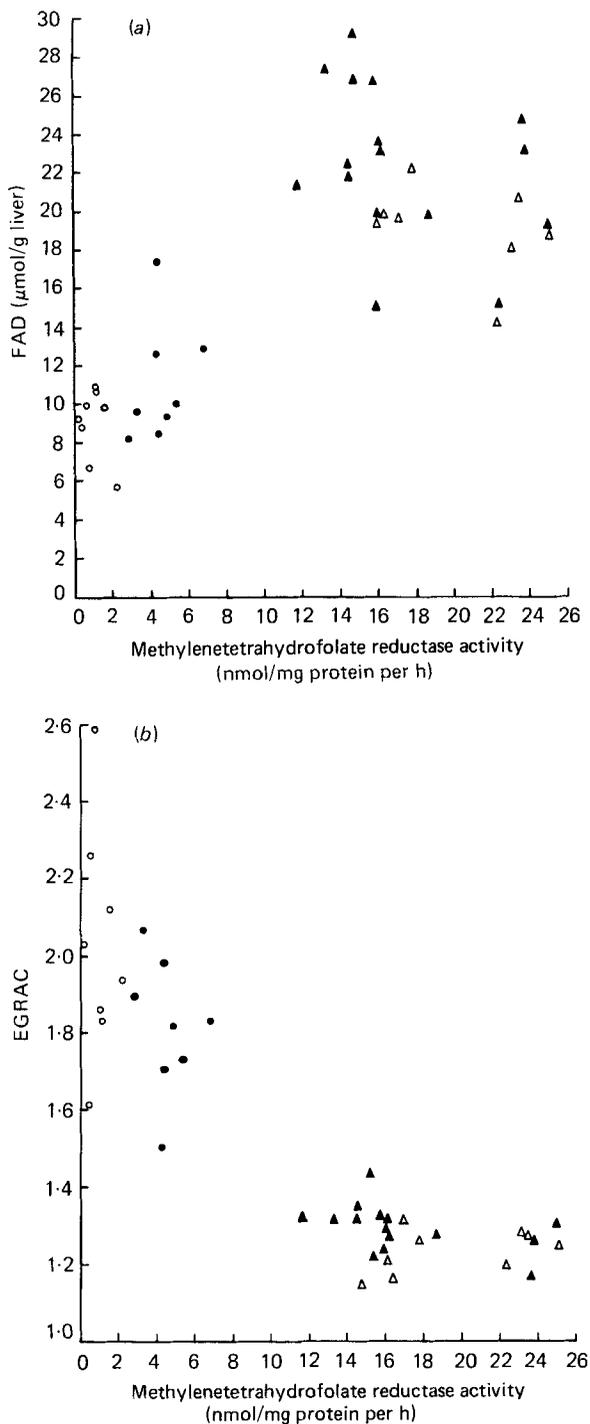
Fig. 3. Relation of hepatic methylenetetrahydrofolate reductase (*EC* 1.5.1.20) with indices of riboflavin status. (*a*), Relation to hepatic FAD levels; (*b*), relation to the activation coefficient of erythrocyte glutathione reductase (NAD(P)H) (*EC* 1.6.4.2; EGRAC). Group D1 (no added riboflavin) (○); group D2 (15 mg riboflavin/kg, pair-fed to group D1) (●); groups P1 and P2 (15 mg riboflavin/kg, pair-fed to groups D1 and D2 respectively) (▲); group A (15 mg riboflavin/kg, fed *ad lib.*) (△).

Table 3. *Proportion of radioactivity in 5-methyltetrahydrofolate in hepatic extracts after injection of [³H] pteroylglutamic acid*

(Mean values with their standard errors)

Group of animals	Riboflavin added to diet (mg/kg)	Proportion of total radioactivity which coincided with the marker 5-methyltetrahydrofolic acid		
		Mean	SE	n†
D1	0	0·22**	0·010	5
P1	15	0·41	0·049	5
D2	0·5	0·32*	0·026	6
P2	15	0·44	0·044	4
A	15	0·42	0·015	7

P1, pair-fed to animals in group D1; P2, pair-fed to animals in group D2; A, fed *ad lib.*

Significantly different from the corresponding values for the pair-fed control groups (Student's *t* test): * $P < 0.05$, ** $P < 0.005$.

† Although all the animals received the injection of labelled pteroylglutamic acid, only a proportion yielded sufficient labelling of hepatic folates for the chromatographic analysis to be successful.

The radioactive tracer experiment showed a smaller percentage of labelled 5-methyl tetrahydrofolic acid in the two deficient groups than in the controls, and very similar values between each of the three control groups (Table 3).

DISCUSSION

The animals in the most severely riboflavin-deficient group (D1) grew somewhat better than those in similarly-treated groups in previous studies (Prentice & Bates, 1981*a*; Olpin & Bates, 1982), possibly because their body-weights at the outset were slightly greater. Nevertheless it is clear that group D1 was quite severely deficient when biochemical indices were compared with those from each of the control groups. The intermediately deficient (D2) group clearly was less severely affected than group D1, particularly in terms of growth, which is in agreement with previous experience of the levels of riboflavin intake (Prentice & Bates, 1981*b*). The picture of riboflavin-restriction and inanition responses for the conventional indices of riboflavin status: liver flavin levels and EGRAC is consistent with previous studies (Prentice & Bates, 1981*a*, *b*; Olpin & Bates, 1982).

The results presented in Table 2 and in Fig. 3 show clearly that riboflavin deficiency has a major effect on the hepatic enzyme, MTHF reductase, consistent with the role of FAD as an essential cofactor (Donaldson & Keresztesy, 1962). Since different flavoenzymes within the same tissue vary greatly in their relative response to riboflavin deprivation in vivo (Burch *et al.* 1956; Hoppel *et al.* 1979; Prentice & Bates, 1981*a*, *b*; Olpin & Bates, 1982), it is important to be able to assess their relative sensitivity, and in this respect MTHF reductase clearly falls into the most highly sensitive group of FAD-dependent enzymes.

Compared with group A, group D2 showed a bigger reduction in MTHF reductase than the severely food-restricted control group P1, despite the fact that group P1 was eating less and growing much more slowly than group D2. Thus the effect of inanition on this enzyme is clearly much smaller than that of riboflavin deficiency. The fact that activity is not restored by re-addition of FAD in vitro suggests that once the cofactor is lost in vivo, the apoprotein probably undergoes irreversible inactivation.

The observations of the present study with respect to the response of MTHF reductase to riboflavin deficiency confirm and extend those of Narisawa *et al.* (1968). It was considered

especially important to verify these observations using control groups which were specifically designed to eliminate the confounding effects of inanition, which is a vital consideration in all studies of experimental riboflavin deficiency in animals (Prentice & Bates, 1981 *a*).

In view of its discriminatory power at moderate to severe levels of deficiency (Fig. 3), which clearly exceeds that of EGRAC and liver FAD levels, measurement of the activity of MTHF reductase is a potentially useful candidate for the assay of riboflavin status in experimental animals and, indeed, in any situation where hepatic tissue is available for analysis. It was found to remain active for long periods in frozen liver samples. Set against these advantages, however, are the facts that it cannot, apparently, be assayed in blood (in the present study, an attempt to detect it in erythrocytes was unsuccessful) and that inanition effects do occur to a moderate extent.

Another hepatic enzyme of the folate reduction pathway which, while not being a flavoprotein, is nevertheless sensitive to a wide variety of intervention stresses, including those of nutritional origin (Morgan & Winick, 1978), is dihydrofolate reductase which is responsible for the reduction of dihydrofolate to tetrahydrofolate. A claim that the activity of this enzyme is reduced by riboflavin deficiency in rats (Bovina *et al.* 1969) was not, however, substantiated in the present study. Likewise, the reduction of pteroylglutamic acid to di- and tetrahydrofolates was unaffected by riboflavin deficiency in the present study, a conclusion which accords with that of Narisawa *et al.* (1968), who also measured 'folate reductase' activity, but did not include the dihydrofolate reductase assay. Of the eight folate-metabolizing enzymes which the latter authors measured, the only one, apart from MTHF reductase, which was affected by riboflavin deficiency, albeit to a much lesser extent, was 5-methyltetrahydrofolate transferase which is responsible for the transfer of methyl groups to homocysteine in the synthesis of methionine.

Reports in the literature concerning the possible effects of riboflavin deficiency on folate metabolism beyond the level of enzyme activities in vitro are conflicting and difficult to assess. Two studies have claimed evidence for a riboflavin–folate interaction in animals (Foy *et al.* (1966) in baboons; Dako & Hill (1980) in rats), but the nature of such an effect, if it exists, is not clear. Three other studies have reported a reduction in the proportion or amount of oxidized folates in riboflavin-deficient rats, as measured by the growth of *Streptococcus faecalis* or of *Pediococcus cerevisiae* on tissue extracts (Miller *et al.* 1962; Bovina *et al.* 1969; Tamburro *et al.* 1971) but, in contrast, a study by Honda (1968) in association with studies by Narisawa *et al.* (1968, 1969) reported a diminished conversion of folic acid to 5-methyltetrahydrofolate. In the present study, the experiment reported in Table 3 seems to be consistent with the latter conclusion, suggesting that the conversion of labelled pteroylglutamic acid to 5-methyltetrahydrofolate may be attenuated in the deficient animals. This, in turn, is consistent with the observed reduction in hepatic MTHF reductase activity. In view of the complexity of dynamic processes and control mechanisms in determining the throughput and steady-state levels of these cofactors, it would be premature to suggest that the reduction in the proportion of 5-methyltetrahydrofolate is attributable entirely to the reduction in MTHF reductase activity, and further metabolic studies are clearly needed to elucidate the implications for overall folate economy.

Two groups have reported on the effects of riboflavin deficiency on formiminoglutamic acid production from histidine, as an index of the integrity of folate-dependent pathways in vivo (Narisawa *et al.* 1968; Pasquali *et al.* 1969). Experience with this approach in the present study, however, suggested that the results of formiminoglutamic acid excretion trials in riboflavin-deficient animals are greatly influenced by confounding factors such as different patterns of meal-eating, which make its interpretation very difficult.

In conclusion, the observations from the present study have shown that riboflavin deficiency in rats does have an important effect on the pathways of folate metabolism and,

in particular, on the flavoenzyme, MTHF reductase. The contention that this enzyme lesion may have consequences for folate metabolism and economy is supported by the evidence from pteroylglutamic acid conversion to hepatic 5-methyltetrahydrofolate, but further studies are needed to explore and delineate the full implications and consequences of the metabolic interaction between the two B vitamins. Such an interaction could have important practical consequences, especially during fetal development in riboflavin-deficient communities.

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