A biochemical evaluation of the erythrocyte glutathione reductase (*EC* 1.6.4.2) test for riboflavin status

2. Dose – response relationships in chronic marginal deficiency

BY A. M. PRENTICE* AND C. J. BATES†

Dunn Nutrition Unit, University of Cambridge and Medical Research Council, Cambridge

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1. Chronic marginal riboflavin deficiency was induced in groups of weanling rats by feeding a deficient diet supplemented with 0, 0·5, 1·0 and 1·5 mg riboflavin/kg diet. *Ad lib.*- and pair-fed controls received 3·0 and 15 mg riboflavin/kg diet respectively.

2. Serial measurement of erythrocyte NAD(P)H_2 glutathione oxidoreductase (glutathione reductase; *EC* 1.6.4.2) and its activation coefficient revealed that after 12 weeks a steady-state of deficiency had been reached following initial fluctuations in status; the animals were then killed, and their tissues analysed.

3. Food intake, growth rate and the appearance of pathological signs were directly proportional to riboflavin content; however relative liver weight was increased above control levels only in the most-severely-deficient group, and anaemia was not detected in any group.

4. The activation coefficient of glutathione reductase in erythrocytes and liver was closely related to dietary riboflavin content; that of skin responded maximally even in the least-severely-depleted animals.

5. Hepatic and renal flavin contents were directly proportional to dietary riboflavin, FAD being conserved at the expense of riboflavin and FMN. ATP:riboflavin 5-phosphotransferase (flavokinase; *EC* 2.7.1.26) activity was reduced, even in the least-severely-deficient animals; ATP:FMN adenylyltransferase (FAD pyrophosphorylase; *EC* 2.7.7.2) was increased in liver, but only in the most-severely-deficient animals.

6. Hepatic succinate:(acceptor) oxidoreductase (succinate dehydrogenase; *EC* 1.3.99.1) activity fell sharply between 1·5 and 0·5 mg riboflavin/kg diet, producing an S-shaped dose-response curve; it showed smaller or less specific changes in other tissues such as brain, skin and intestine. NADH:(acceptor) oxidoreductase (NADH dehydrogenase; *EC* 1.6.99.3) activity declined in liver and intestine, but not in skin or brain.

7. The activation coefficient of glutathione reductase was correlated strongly with nearly all the riboflavin-sensitive variables measured, once equilibrium had been reached in this chronic deficiency model, and it was particularly strongly correlated with hepatic and renal FAD levels. Under equilibrium conditions, therefore, it appears to represent a good index of the extent of riboflavin deficiency, and significant changes in flavin levels and enzymes in the internal organs were detected even under conditions of marginal deficiency, associated with relatively small increases in the activation coefficient.

In the accompanying paper (Prentice & Bates, 1981), it was shown that under the dynamic conditions of acute deficiency the activation coefficient (stimulated:basal activity; AC) of erythrocyte glutathione reductase (*EC* 1.6.4.2; GR) responded more rapidly, and to a greater extent, than many other riboflavin-dependent variables, and was considered, therefore, to over-estimate the extent of deficiency in such a situation. The wide variation in rate of response of these other variables resulted in generally poor correlations with the AC of erythrocyte GR (EGRAC); moreover the interpretation of the results was further complicated by cessation of growth, and in the later stages, by a high mortality rate.

In the investigation reported here, an attempt has been made to simulate the conditions of endemic human riboflavin deficiency more closely, by maintaining rats on a deficient diet, supplemented with riboflavin at several suboptimal levels, until a steady-state of deficiency was reached at each level, as judged by serial measurements of EGRAC. Under these equilibrium conditions, the extent to which EGRAC reflects over-all riboflavin status

* Present address: MRC Laboratory, Keneba, The Gambia, West Africa.
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throughout the body was assessed by measurement of several independent biochemical indices of riboflavin status in several tissues. To our knowledge, no previous studies of EGRAC in experimental riboflavin deficiency have deliberately made use of a chronic, marginal deficiency model in order to study the relationship with other riboflavin-dependent variables. A similar approach has, however, been successfully used in the study of chronic marginal vitamin C deficiency in guinea pigs (Ginter, 1979).

MATERIALS AND METHODS

Animals and diets

Male, weanling Norwegian hooded rats were used throughout. No precautions were taken to prevent refection completely in this study, although the animals were housed individually in suspended wire cages to minimize coprophagy. The distribution of animals to each dietary regimen is shown in Table 1.

The experimental groups received a riboflavin-deficient diet supplemented with 0, 0·5, 1·0 and 1·5 μg riboflavin/g diet. *Ad lib.*- and pair-fed controls received the same diet supplemented with 3 μg (Bro-Rasmussen, 1958) and 15 μg riboflavin/g respectively. The basic riboflavin-deficient diet has been described previously (Prentice & Bates, 1981). Since tail-cups were not employed in the present study, however, the pyridoxine, biotin, tocopherol, menadione and folic acid content of the riboflavin-deficient diet was not increased above that of the *ad. lib.*-fed controls.

Erythrocyte GR activity and AC values were measured serially in all rats, following weekly blood collections from the tail vein. After 12 weeks, EGRAC measurements indicated that a steady-state had been achieved, and all animals were then killed for tissue analyses.

Analytical methods

Tissue preparation and enzyme assay techniques have been described previously (Prentice & Bates, 1981). Where P values are quoted, they have been calculated on the basis of the paired t-test, comparing matched pairs of animals in each group described.

RESULTS

Growth and pathology

Table 1 shows the final mean body-weights of rats receiving the four marginally-deficient diets, together with those of their pair-fed and *ad lib.*-fed controls. The levels of riboflavin supplementation chosen produced clear differences in weight gain between the experimental groups. In contrast to acutely-deficient animals in which refection was prevented (Prentice & Bates, 1981), all animals in the present study sustained some growth throughout the experimental period, indicating that even those who were receiving no added riboflavin in their diet must have obtained small amounts through refection (Prentice & Bates, 1980). Food intake was closely correlated with riboflavin content of the diets and with growth, but in contrast to rats suffering from severe acute deficiency, there was no progressive decline in food intake in any group.

There was a clear and direct relationship between the riboflavin content of the diet and the time of onset and subsequent severity of externally-visible pathological lesions (Table 2). The pathological signs in the present study at 12 weeks in the group receiving the unsupplemented diet were more severe than those observed in the acute experiment at 7 weeks, despite the fact that the values for EGRAC were lower and mortality less frequent in the chronically-deficient group. There were marked differences in onset and extent of pathological signs between individuals in each group, which did not correlate with differences in EGRAC within the group.

Table 1. *Final* body-weights (g) of deficient, pair-fed and* ad lib.*-fed control rats receiving different levels of riboflavin in their diet for* 12 *weeks*

(Mean values with their standard errors for four animals except where indicated in parentheses)

Riboflavin content of diet† (μg/g)	Expt 1				Expt 2			
	Deficient		Pair-fed		Deficient		Pair-fed	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	81	7	102	8	90	5	107	12
0·5	118	10 (5)	155	15 (5)	120	11	156	9
1·0	168	9 (5)	216	12 (5)	184	6	231	17
1·5	237	25	297	27	261	27	305	14
3·0‡	435	23 (5)			410	17 (3)		

* Initial body-weight in both experiments was 48 ± 2 g.

† Not including the small contribution (< 0·3 μg/g) from the purified casein component (Prentice & Bates, 1979).

‡*Ad lib.*-fed group.

There was a large increase in relative liver weight ($P < 0·001$) in the group receiving no added riboflavin, when compared with the *ad. lib.*-fed and corresponding pair-fed controls (Fig. 1), but the increase in the other marginally deficient groups was much less marked. Grossly fatty livers, which were frequently observed in acutely-deficient rats, were not evident in any of the chronically-deficient animals in this study.

There was a closer correlation between relative liver weight and EGRAC in the chronic than in the acute experiments, ($r + 0·77$, n 41 for the chronic; $r + 0·60$, n 47 for the acute experiments), and the AC of hepatic GR was significantly correlated with relative liver weight in the chronic, but not the acute, experiments ($r + 0·79$, n 21 for the chronic; $r + 0·09$, n 21 for the acute experiments).

Packed red cell volumes were not significantly altered in any of the chronically-deficient groups.

Erythrocyte GR

Serial measurements on tail-vein blood revealed a complex response to deficiency, in which EGRAC rose sharply to a peak at week 3, decreased again, presumably on the onset of refection, and finally increased once more to reach a plateau from weeks 7–8 onwards (cf. Prentice & Bates, 1979). In the present study, the rats were killed at week 12, by which time the values for EGRAC had remained at a constant level, characteristic of the riboflavin intake, for 4–5 weeks (see Table 3). Interestingly, as the riboflavin content of the diet was increased, the magnitude of the initial fluctuations diminished, until at 1·5 mg/kg diet they were virtually undetectable.

There was a strong inverse correlation between the 12-week values for EGRAC from the deficient groups, and the riboflavin content of the diet (Fig. 2). This correlation was much stronger at 12 weeks than it had been during the earlier stages, where rapid fluctuations in EGRAC were occurring.

Each animal reached a remarkably constant value for EGRAC in the later stages, which was characteristic of the individual animal as well as the riboflavin content of the diet. Within each group receiving the same dietary intake of riboflavin, variations in EGRAC reflected those observed in several other riboflavin-dependent variables.

Both the unstimulated (minus FAD) and stimulated (plus FAD) GR activities showed some reduction in the riboflavin-deficient groups. The reduction in stimulated activity

Table 2. *Appearance of external pathological signs of riboflavin deficiency*
(All animals in Expts 1 and 2 taken together)

Riboflavin supplementation (μg/g diet)	Total no. of animals	Hair lesions*	Foot/tail lesions*	Hunched posture*	Partial paralysis*	Conjunctivitis*	Diarrhoea*	Corneal opacity*	Death*
0	8	8	8	8	5	5	2	2	1
0·5	9	9	9	2	2	2	—	—	—
1·0	9	9	3	1	—	—	—	—	—
1·5	8	8	1	—	—	—	—	—	—
3·0	8	—	—	—	—	—	—	—	1

* No. of animals affected; cumulative record over 12-week period.

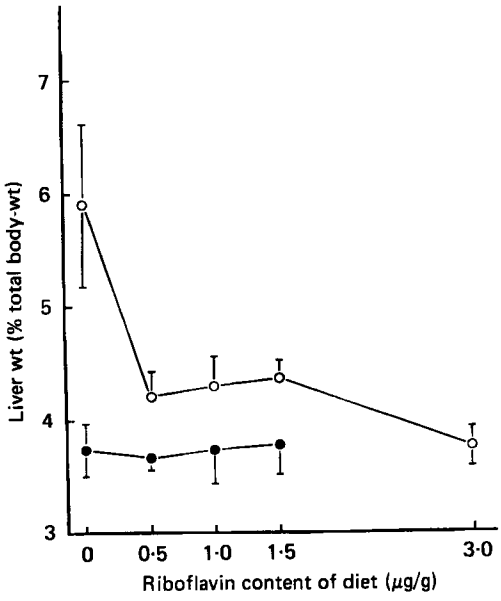

Fig. 1. Relative liver weight of riboflavin-deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 8 or 9; for details, see Table 1).

Table 3. *Serial measurements of the activation coefficient of erythrocyte glutathione reductase* (EC 1.6.4.2) *for the last six weeks of Expt.* 1

(Values are means with their standard errors)

Riboflavin supplementation (μg/g diet)	Week														
	6		7		8		9		10		11		12		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*n*
0	3·04	0·10	2·88	0·21	3·83	0·21	3·62	0·17	3·67	0·12	3·63	0·15	3·76	0·15	4
0·5	2·63	0·17	2·90	0·31	2·88	0·20	3·10	0·09	2·92	0·21	2·92	0·19	2·90	0·15	5
1·0	2·21	0·17	2·35	0·12	2·54	0·44	2·46	0·08	2·46	0·25	2·57	0·15	2·38	0·11	5
1·5	2·21	0·04	2·27	0·15	2·25	0·06	2·29	0·04	2·31	0·16	2·12	0·19	2·19	0·07	4
3·0	1·28	0·06	1·29	0·08	1·28	0·04	1·30	0·10	1·29	0·06	1·28	0·10	1·28	0·07	5

appeared to be related entirely to inanition since, when corrected for differences in the efficiency of food utilization, the decreases for each deficient group were equal to those seen in the corresponding pair-fed groups. The decrease in stimulated activity in the pair-fed groups was inversely proportional to food intake.

Hepatic and skin GR

As noted in the acute deficiency experiments (Prentice & Bates, 1981), both liver and skin respond to riboflavin deficiency by an increase in the AC of GR. In the chronic situation, AC values for hepatic GR (HGRAC) were linearly correlated with the riboflavin content

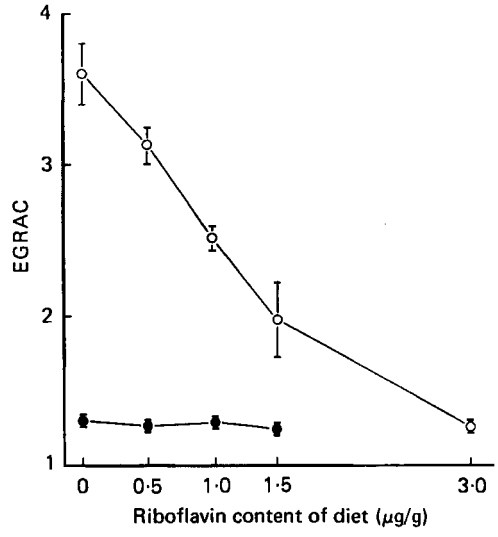

Fig. 2. Erythrocyte glutathione reductase (*EC* 1.6.4.2; GR) activation coefficient (stimulated:basal activity; AC) (EGRAC) from riboflavin-deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 8 or 9; for details, see Table 1).

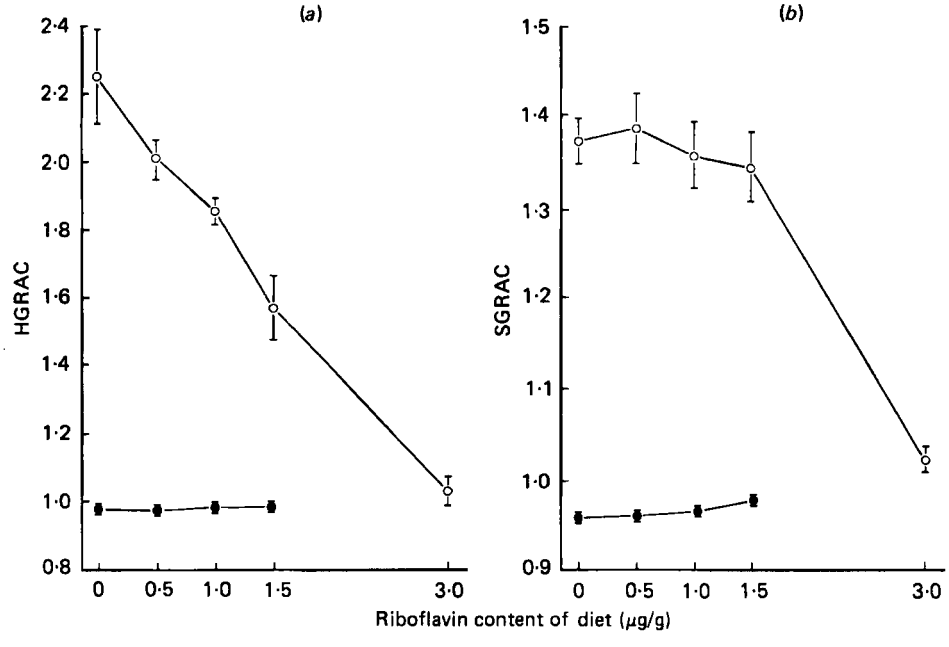

Fig. 3. Glutathione reductase (*EC* 1.6.4.2) activation coefficient in (*a*) liver (HGRAC) and (*b*) skin (SGRAC) from deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 4).

of the diet (Fig. 3). Since there was also a pronounced, inanition-related decrease in absolute GR activity in the liver, the extent of deficiency as measured by AC values appeared to be greater than the true increase in amount of unsaturated apoenzyme, which was calculated by subtracting unstimulated from stimulated activity. The correlation between EGRAC and free hepatic apoenzyme was significant, though weaker than that between EGRAC and HGRAC (Table 4).

The AC for skin GR (SGRAC) showed a small, but highly significant, increase in response to mild deficiency (Fig. 3) (all deficient *v.* all pair-fed controls: $P < 0{\cdot}001$), but somewhat surprisingly, this response did not increase as the severity of deficiency increased. The total amount of glutathione reductase per unit (wet) weight of skin in the *ad lib.*-fed controls was threefold higher than that in the weanling animals. Inanition partially counteracted this increase in pair-fed controls, and owing to the lower efficiency of food utilization in the deficient animals, the growth-related increase was completely eliminated in the most-severely-deficient group.

Flavin-metabolizing enzymes

Flavokinase (*EC* 2.7.1.26) showed a similar response in both the liver and kidney of deficient animals (Fig. 4), the activity being reduced in all the deficient groups, and reaching approximately 60% of the pair-fed value in the most-severely-deficient group.

FAD pyrophosphorylase (*EC* 2.7.7.2) activity in liver was unaffected at riboflavin intakes at and above 0·5 μg/g diet, but in the most-severely-deficient group it increased to nearly double the activity observed in the pair-fed controls (Fig. 4). Renal FAD pyrophosphylase activity was unaffected by riboflavin deficiency.

As observed in the acute experiments (Prentice & Bates, 1981), there was no change in FMN phosphatase activity in response either to inanition or to chronic riboflavin deficiency.

Flavin coenzyme levels

Liver was considerably more sensitive than kidney, with respect to reduction in the level of acid-extractable flavin coenzymes in the deficient animals. In the group receiving 1·5 μg riboflavin/g diet, hepatic riboflavin, FMN and FAD concentrations were all reduced to approximately 70% of the level in the *ad lib.*-fed controls (Fig. 5). In the more-severely-deficient groups there was a differential drain on the flavin coenzymes, and this was particularly apparent in the unsupplemented group (FAD *v.* riboflavin, $P < 0{\cdot}02$; riboflavin *v.* FMN, $P < 0{\cdot}01$). As a result, FAD was conserved at the expense of FMN, to the extent that FAD levels were similar in all three groups receiving the lowest riboflavin intakes (0, 0·5 and 1·0 μg/g diet). This effect is presumably due to the elevation of FAD pyrophosphorylase activity, which helps to maintain FAD levels in severely-deficient animals (Fass & Rivlin, 1969).

In the absence of any increase in FAD pyrophosphorylase activity in the kidney, free riboflavin levels decreased to a greater extent than FMN levels (Fig. 5) in each of the two groups receiving the lowest riboflavin intakes ($P < 0{\cdot}05$). Similarly, in these two groups FAD showed smaller deficiency-related changes than the other two flavins. (FAD *v.* FMN, $P < 0{\cdot}05$; FAD *v.* riboflavin, $P < 0{\cdot}01$).

In all instances the pair-fed controls had flavin levels which did not differ significantly from those of the *ad lib.*-fed controls, which justifies the choice of dietary riboflavin levels in these groups, and indicates that their tissues were probably saturated.

Succinate dehydrogenase (EC 1.3.99.1; *SDH*) *and NADH dehydrogenase* (EC 1.6.99.3)
Preliminary experiments showed that both SDH and NADH dehydrogenase from kidney and heart were unresponsive during acute deficiency (Prentice & Bates, 1981); therefore these tissues were not included in the present study.

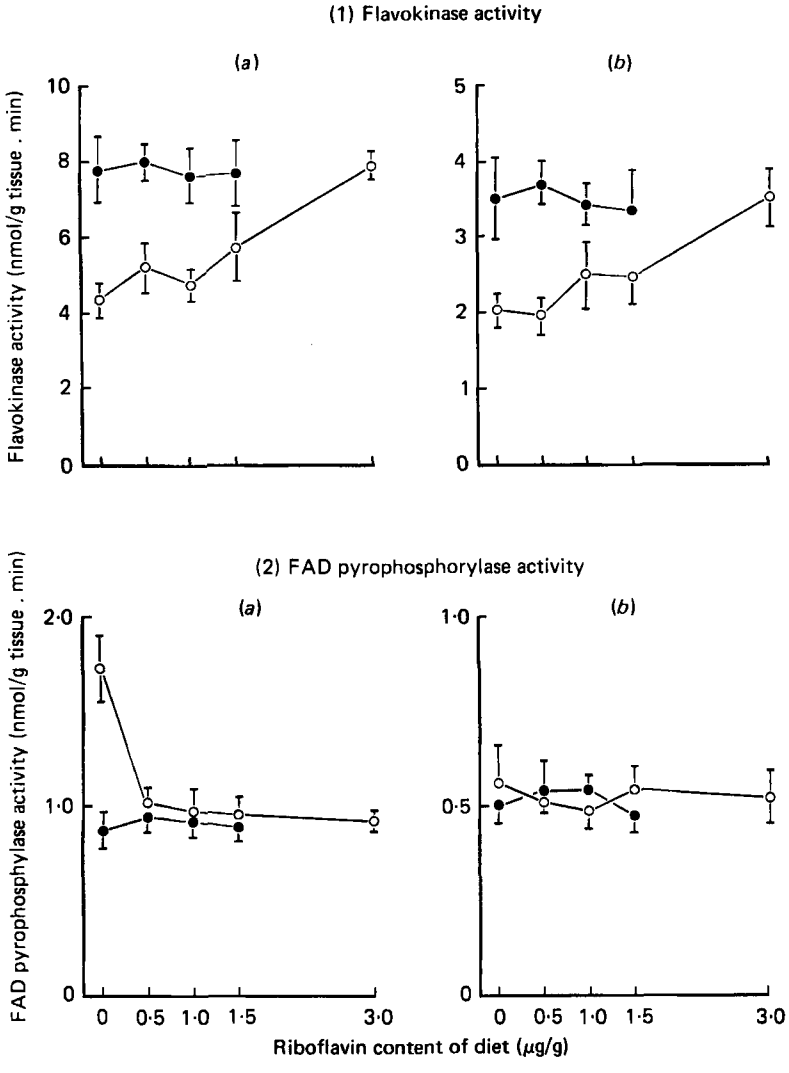

Fig. 4. (1) Flavokinase (*EC* 2.7.1.26) activity and (2) FAD pyrophosphorylase (*EC* 2.7.7.2) activity in (*a*) liver and (*b*) kidney from deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 4).

Hepatic SDH activity was severely reduced, especially at the lowest levels of riboflavin intake (Fig. 6). In the group receiving 0·5 μg riboflavin/g diet, the activity was only 30% of that of the pair-fed and *ad lib.*-fed controls; no further reduction was observed in the completely unsupplemented group, possibly because of the compensating increase in FAD pyrophosphorylase activity and therefore of FAD availability.

Brain SDH activity was reduced by 70% in the unsupplemented deficient group, and skin SDH activity was reduced by 50%, in comparison with *ad lib.*-fed controls (Fig. 6). In the brain, there was a slight increase in activity in the pair-fed animals in response to inanition (pair-fed *v. ad lib.*-fed controls, $P < 0.05$).

Intestinal SDH activity showed a response which was markedly different from the other

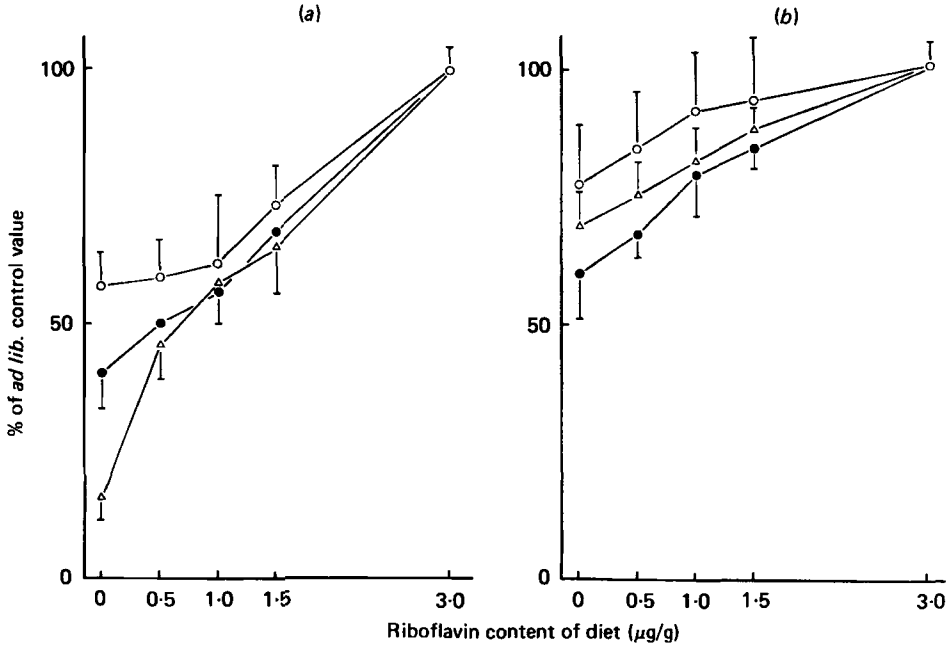

Fig. 5. FAD(○), FMN(△) and free riboflavin (●) concentrations in (*a*) liver and (*b*) kidney from riboflavin-deficient rats expressed as a percentage of the concentration in rats receiving the diet supplemented with 3·0 μg riboflavin/g, which were (mean ± SE; μg/g): hepatic FAD 26·3 ± 3·1; hepatic FMN 4·7 ± 0·5; hepatic riboflavin 1·6 ± 0·1; renal FAD 23·0 ± 2·7; renal FMN 12·4 ± 0·8; renal riboflavin 1·8 ± 0·2. Points are mean values with their standard errors represented by vertical bars (*n* 4).

tissues (Fig. 6): there was a pronounced decrease in the pair-fed groups which, when corrected for efficiency of food utilization, accounted for a large proportion of the decrease seen in the most-severely-deficient groups. However, in the groups receiving 1·0 and 1·5 μg riboflavin/g diet, food intake was voluntarily restricted to only a minor extent, yet SDH values were reduced to 40% of the *ad lib.*-fed control values in the deficient groups. It appears, therefore, that intestinal SDH may show a dual response to both inanition and riboflavin deficiency.

NADH dehydrogenase from brain and skin failed to respond to riboflavin deficiency in the present study. The activity of the hepatic enzyme was reduced only in the group receiving the diet with no added riboflavin (Fig. 7); it was in this group that the reduction in FMN levels was particularly severe (Fig. 5). Intestinal NADH dehydrogenase activity was reduced at all levels of deficiency (Fig. 7), and like intestinal SDH, there appeared to be a dual response to both inanition and riboflavin deficiency.

Correlations with EGRAC

Correlation coefficients from log–log plots of EGRAC *v.* each of the other potential indices of riboflavin status are presented in Table 4. Correlations were invariably higher in these chronic deficiency experiments than in the corresponding ones on acutely-deficient animals (Prentice & Bates, 1981). This was particularly evident for those indices which responded slowly, where correlations often failed to achieve significance in the acute experiments and yet were highly significant in the chronic ones.

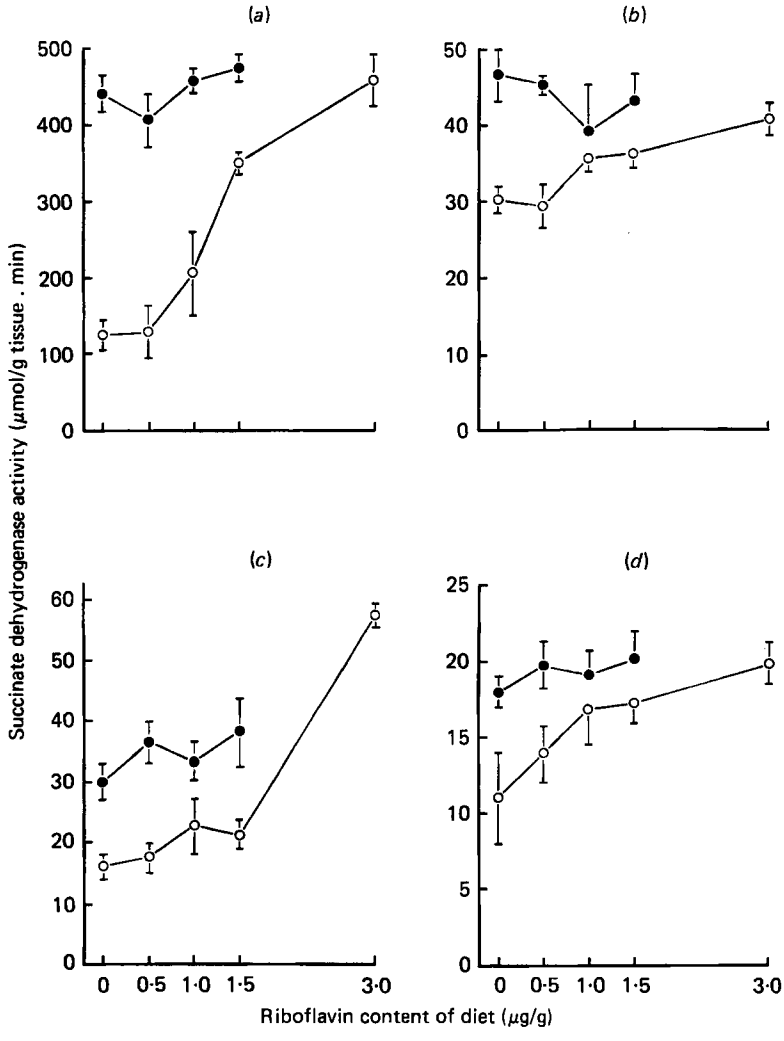

Fig. 6. Succinate dehydrogenase (*EC* 1.3.99.1) activity in four tissues (*a*) liver (*b*) brain (*c*) intestine and (*d*) skin from deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 4).

DISCUSSION

By prolonging the equilibration period to 12 weeks, the initial fluctuations in riboflavin status which are probably due to refection (Prentice & Bates, 1980) can be overcome, and a reproducible and progressive dose-response relationship to riboflavin intake can be achieved. This permits tail-cups to be eliminated, which minimizes stress to the animals. The various extents of mild chronic deficiency induced by this technique were associated with corresponding increases in EGRAC and reductions in weight gain. There were also significant and related changes in several other riboflavin-dependent variables in the internal organs. Individual animals, even within each experimental group, showed consistent and reproducible differences in status, as measured both by EGRAC and by other sensitive variables such as HGRAC. These differences may be due to individual variations related

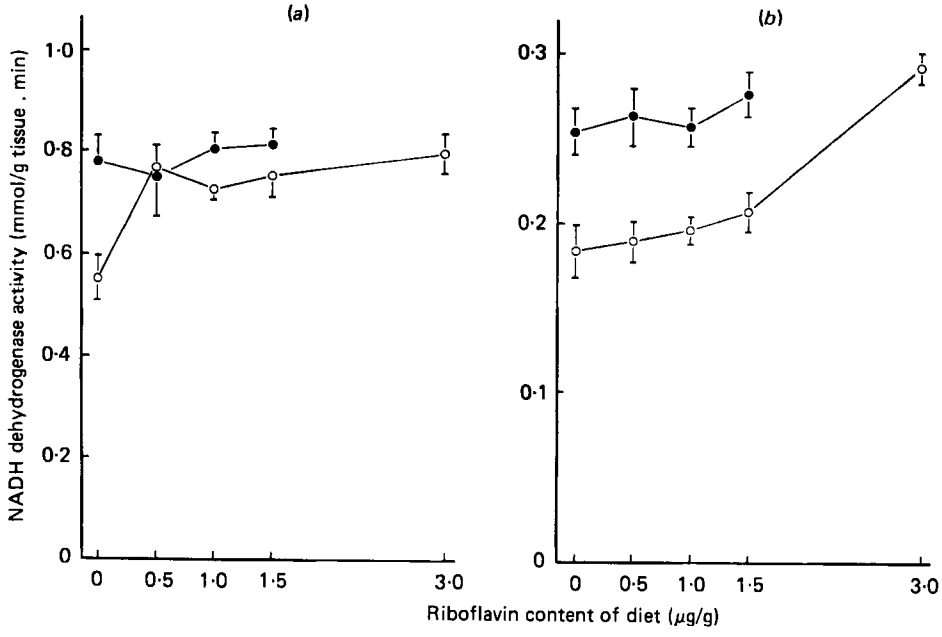

Fig. 7. NADH dehydrogenase (*EC* 1.6.99.3) activity in (*a*) liver and (*b*) intestine from deficient (○) and pair-fed (●) control rats. Points are mean values with their standard errors represented by vertical bars (*n* 4).

to the efficiency of riboflavin retention or the extent of refection in individual animals. Similar consistent differences between individuals have been noted in human subjects receiving controlled riboflavin-deficient diets (Tillotson & Baker, 1972).

Although there was a wide inter-tissue variation, the following approximate order of sensitivity was observed for different riboflavin-sensitive variables in the present study: (1) most sensitive: growth, food intake, tissue flavin levels, flavokinase (particularly in liver), AC of GR in several tissues; (2) intermediate sensitivity: hepatic SDH activity; (3) least sensitive: relative liver weight, hepatic NADH dehydrogenase activity and FAD pyrophosphorylase activity.

The relative sensitivity of different tissues is more difficult to summarize, because some enzymes appear to be affected to a greater extent in some tissues than in others, and the picture is complicated by inanition-related changes, changes in apparent K_m and differences in rates of change between different tissues. Of the tissues studied, erythrocytes and liver were undoubtedly among the most sensitive; kidney and brain were generally less sensitive, and skin showed paradoxical effects: some variables being virtually unaffected whereas others (such as the AC of GR) responded significantly even to a very mild deficiency. To some extent, factors such as inter-tissue variation in riboflavin transport and flavokinase activity, possibly hormonally controlled, must be important, but other as yet undetermined factors evidently affect the distribution of flavin coenzymes between flavoproteins. That this is not simply related to over-all tissue cofactor concentration is demonstrated, for instance, by the relative insensitivity of hepatic NADH dehydrogenase, despite the large reduction observed in its cofactor, FMN. The response of hepatic SDH is more closely related to that of its cofactor FAD (cf. Figs. 5 and 6), but the relationship is still not a simple one. The response of FAD pyrophosphorylase (Fig. 4) is probably partly responsible for the maintenance of floor levels of hepatic FAD and SDH.

Table 4. *Correlations between the activation coefficient of erythrocyte glutathione reductase (EC 1.6.4.2; EGRAC) and other riboflavin-dependent variables*

(Animals in Expts 1 and 2. The calculation included all deficient animals and the *ad lib.*-fed controls, which were considered the 'top' end of the deficiency scale, but did not include pair-fed controls. All the correlation coefficients were calculated from logarithmically transformed values, to allow for deviations from Gaussian distribution in some of the factors measured)

Independent variable		No. of animals	*r*
Liver:	AC* of glutathione reductase	21	+0·96
	Glutathione reductase, unsaturated apoenzyme†	21	+0·63
	Succinate dehydrogenase (*EC* 1.3.99.1) activity	21	−0·88
	NADH dehydrogenase (*EC* 1.6.99.3) activity	22	−0·61
	Flavokinase (*EC* 2.7.1.26) activity	20	−0·87
	FAD pyrophosphorylase (*EC* 2.7.7.2) activity	20	+0·68
	FMN phosphatase activity	20	+0·01
	Riboflavin concentration	21	−0·84
	FMN concentration	20	−0·88
	FAD concentration	20	−0·95
	Weight (% body-wt)	41	+0·77
Kidney:	Flavokinase activity	20	−0·83
	FAD pyrophosphorylase activity	20	+0·02
	FMN phosphatase activity	20	−0·04
	Riboflavin concentration	20	−0·78
	FMN concentration	20	−0·73
	FAD concentration	20	−0·88
Brain:	Succinate dehydrogenase activity	21	−0·88
	NADH dehydrogenase activity	21	+0·04
Skin:	AC of glutathione reductase	21	+0·90
	Succinate dehydrogenase activity	21	−0·81
	NADH dehydrogenase activity	21	+0·05
Intestine:	Succinate dehydrogenase activity	21	−0·93
	NADH dehydrogenase activity	21	−0·90

* AC activation coefficient (stimulated:basal activity).
† Difference between FAD-stimulated activity and unstimulated activity.

In acute deficiency, abrupt cessation of growth appears to represent an adaptation which, in the short-term at least, mitigates some of the worst effects of specific nutrient deficiencies in growing rats, and this is well illustrated in the acute deficiency model (Prentice & Bates, 1981). However, pathological signs do eventually appear, and accumulate, even in animals which are still growing under conditions of mild chronic deficiency, which demonstrates that reduction of growth is only partly effective as a protective response in the long-term. This emphasizes the importance of the time factor in the aetiology of pathological changes: animals surviving for relatively long periods on chronically-deficient diets frequently had more obvious pathological signs than those which were approaching death through acute deficiency, yet the biochemical indices were often less markedly affected in the chronically-deficient animals.

The poor correlation between EGRAC and clinical signs of riboflavin deficiency in cross-sectional studies of human populations (Thurnham *et al.* 1971; Buzina *et al.* 1973; Bamji, 1975; Bamji *et al.* 1979) may also be due to a time-lag between the response of these variables. Many of the biochemical changes in specific flavoproteins probably represent loss of spare capacity, which can be tolerated for moderate periods in the absence of additional stress, without significant impairment of over-all metabolic pathways and of tissue function. However, much remains to be learned about the nature of those critical

biochemical lesions which determine the onset and course of observable pathological changes.

The particularly close correlation between EGRAC and the alternative indices of riboflavin status in rats suffering from chronic deficiency supports the use of the erythrocyte GR test as a primary index of riboflavin status, and demonstrates that even marginal changes in EGRAC are accompanied by significant changes in riboflavin-sensitive processes elsewhere in the body. In these experiments, the sensitivity of the erythrocyte GR test was sufficient to detect small differences in riboflavin status between individual rats within the same experimental group, and the reproducibility was sufficient to reveal a consistent rank-order of individuals once equilibrium had been achieved.

The relatively rapid response during acute deficiency, in comparison with other sensitive variables (Prentice & Bates, 1981), might produce misleading results under conditions of rapidly fluctuating riboflavin intake, but it seems unlikely that any other practicable index of status would avoid this problem, while retaining the specificity and sensitivity of EGRAC.

Furthermore, except possibly when precipitated by the stress of infection or by the increased requirements of pregnancy, naturally-occurring riboflavin deficiency in man is usually a chronic condition caused by long-term suboptimal intakes of riboflavin. Under such conditions (and in the absence of specific genetic or clinical disturbances which are known to interfere with the test), we conclude that EGRAC is likely to give an accurate indication of the over-all riboflavin status of the body.

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