

Refection in rats fed on a sucrose-based, riboflavin-deficient diet

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1. Refection, resulting in an increased supply of riboflavin to riboflavin-deficient rats through coprophagy, was demonstrated on a sucrose-based diet when sensitive biochemical tests of riboflavin status were employed: these included measurements of NAD(P) H_2 :glutathione oxidoreductase (*EC* 1.6.4.2); succinate:(acceptor) oxidoreductase (*EC* 1.3.99.1) and NADH:(acceptor) oxidoreductase (*EC* 1.6.99.3).

2. The use of tail-cups to eliminate coprophagy, and hence refection, resulted in a more rapid and reproducible progress into severe deficiency.

3. The occurrence of refection on a sucrose-based diet may account for hitherto unexplained differences between previous publications on the biochemical effects of riboflavin deficiency.

It is difficult to achieve an uninterrupted progression towards severe B vitamin deficiency in animals receiving a raw starch-based diet. Changes in intestinal flora described by Fridericia *et al.* (1928) as 'refection', and probably accompanied by coprophagy (Roscoe, 1928) result in the ability of some animals to thrive without an external source of B vitamins. Refection, which occurs several weeks after the introduction of a vitamin-deficient diet and is characterized by an increase in the daily weight gain, has since been observed in many laboratories when raw starch-based diets have been used. It has often been stated that the contribution of intestinal synthesis to the over-all riboflavin economy of the rat is greatly reduced when sucrose replaces raw starch as the main energy source of the diet (Guerrant *et al.* 1935; Mannering *et al.* 1944).

As a result of these reports many laboratories now use sucrose-based diets to induce riboflavin deficiency. However, with the introduction of sensitive techniques for assessing riboflavin status, in particular the glutathione reductase (NAD(P) H_2 :glutathione oxidoreductase, *EC* 1.6.4.2) test, some inconsistencies have appeared in a number of publications relating to the biochemical effects of riboflavin deficiency (Tillotson & Sauberlich, 1971; Bamji & Sharada, 1972; Prentice, 1977), even though the conditions used to induce deficiency have been broadly similar and have involved sucrose-based diets in each case.

This publication describes a partial refection observed when rats were fed on a sucrose-based, riboflavin-deficient diet. The occurrence of some extent of refection may help to explain differences in previously reported biochemical responses of the rat to experimental riboflavin deficiency.

MATERIALS AND METHODS

Male, weanling Norwegian hooded rats were used throughout, and were housed individually in suspended wire cages. Where appropriate, tail-cups (Barnes *et al.* 1963) were fitted in order to prevent coprophagy.

The riboflavin-deficient diet contained (g/kg):sucrose 811, acid-washed casein (Glaxo Ltd) 105, salt mixture (Greenfield *et al.* 1969) 50, cotton-seed oil 30, choline chloride 2 and cystine 1.5. Microbiological analysis of the riboflavin content of acid-washed casein using *Lactobacillus casei* (Barton-Wright, 1961) indicated a level of less than 3 $\mu\text{g/g}$. The following vitamins were added (mg/kg diet):calcium pantothenate 20, thiamine hydrochloride 3, pyridoxine hydrochloride 3, nicotinamide 25, biotin 0.1, cyanocobalamin 0.05. When

tail-cups were fitted the following were also added to the diet (mg/kg): pyridoxine hydrochloride 3, biotin 0.9 and pteroylglutamic acid 1, as recommended by Clarke *et al.* (1977), in order to compensate for the elimination of the faecal source of these vitamins. Each rat received a weekly dose of 300 μg retinol, 5 μg ergocalciferol, 2 mg α -tocopherol and 0.05 mg menaphthone. When tail-cups were fitted, an additional 8 mg α -tocopherol and 0.25 mg menaphthone were given weekly.

On killing, blood was collected from the tail vein in heparinized capillary tubes, and liver samples were immediately cooled to 4°. Haemolysates were prepared according to the method of Thurnham *et al.* (1970).

Enzyme assays

In riboflavin-deficient animals the erythrocyte glutathione reductase (EGR) apoenzyme is not fully saturated with its cofactor, FAD, and can be resaturated *in vitro*. The activation coefficient (AC) is the ratio, activity measured with added FAD: activity measured without added FAD, and provides an index of riboflavin status (Bamji, 1969; Beutler, 1969; Glatzle *et al.* 1970). EGR and its AC were measured by an automated technique (Prentice, 1977) in which the final concentrations (mol/l) of reagents were: phosphate buffer (pH 7.4) 0.1, EDTA 3.3×10^{-3} FAD (where added), 8×10^{-6} for the erythrocyte enzyme and 4×10^{-6} for the hepatic enzyme, oxidized glutathione 9×10^{-4} , NADPH 7.5×10^{-5} . The reagents were added to the haemolysate in the order shown and the change in extinction at 340 nm was followed continuously for 4 min at 37°.

Succinate:(acceptor) oxidoreductase (succinate dehydrogenase *EC* 1.3.99.1) is also a flavoprotein, and when measured by a suitably-specific assay it reflects riboflavin status. Hepatic succinate dehydrogenase activity was assayed by a semi-automated modification of the method of Arrigoni & Singer (1962). Final reagent concentrations (mol/l) were: phosphate buffer (pH 7.4) 5×10^{-2} , sodium succinate 2×10^{-2} , cysteine sulphinate 9×10^{-3} , potassium cyanide 1×10^{-3} , calcium chloride 7.5×10^{-4} , phenolindo-2,6-dichlorophenol 6×10^{-5} and phenazine methosulphate at three concentrations: 0.18, 0.54 and 1.09×10^{-3} . Following activation of the enzyme by pre-incubation with succinate for 20 min at 30° the reaction was initiated by the addition of the dyes and the change in extinction at 600 nm was recorded for 2 min. Results are expressed as V_{\max} values.

NADH:(acceptor) oxidoreductase, (NADH dehydrogenase, *EC* 1.6.99.3) requires flavin mononucleotide as its cofactor and may also reflect riboflavin status (Burch *et al.* 1960). Ferricyanide was found to be the most suitable electron acceptor and the assay therefore had to be performed on mitochondrial fragments to ensure specificity. These were prepared by the method of Kopaczyk (1967). The following reagent concentrations (mol/l) were used: triethanolamine buffer (pH 7.8) 4×10^{-2} , reduced nicotinamide adenine dinucleotide (NADH) 2.7×10^{-4} and potassium ferricyanide at two concentrations: 3.5 and 8.0×10^{-4} . The change in extinction at 420 nm was followed for 1 min at 30° and results are expressed as V_{\max} values.

RESULTS

Expt 1

In Expt 1, thirty weanling rats were fed on the sucrose-based, riboflavin-deficient diet without tail-cups, and groups of three animals were killed at weekly intervals for analysis of glutathione reductase and its AC, succinate dehydrogenase and NADH dehydrogenase in several tissues. During the first 4 weeks on the deficient diet these biochemical indices showed a steady and continuous progress towards deficiency, but by the sixth week a sudden, though transient, reversal had occurred. This reversal was demonstrated by all the indices of riboflavin status which were investigated.

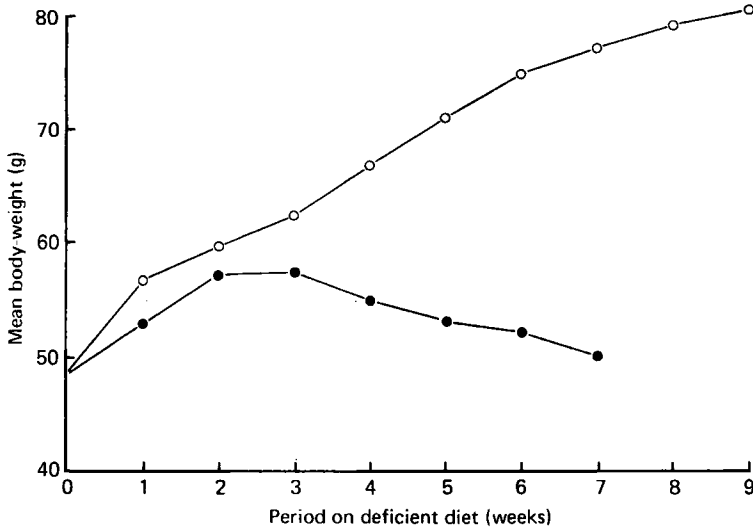


Fig. 1. Growth curves of rats without tail-cups (○, Expt 1 (for details of diet and experiments, see p. 171)) and with tail-cups (●, Expt 2) fed on a sucrose-based diet. Each point represents the mean weight of five rats which survived for the duration of each experiment. Other animals, which were culled at earlier periods for biochemical measurements, grew at a similar rate in each experiment.

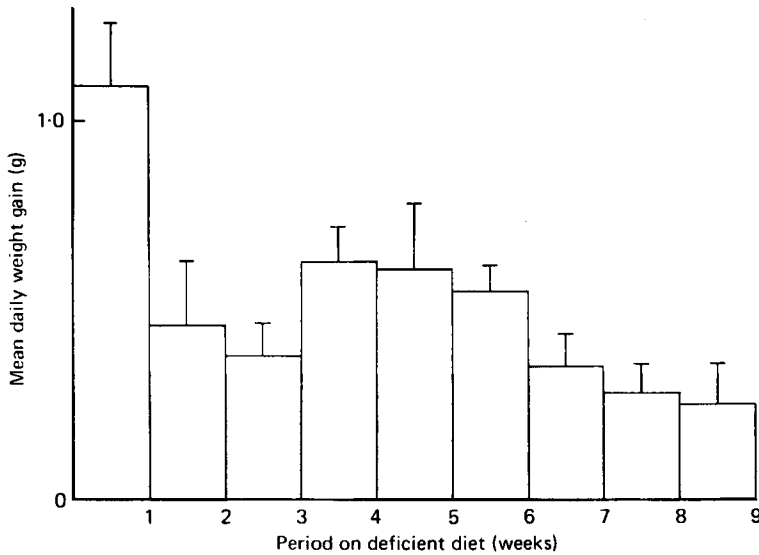


Fig. 2. Daily weight gain (g) in rats fed on a sucrose-based diet and showing refection (for details of diet and experiment, see p. 171). The weight changes illustrated are from five rats which survived the duration of the experiment. Vertical bars represent the standard error of the mean.

The growth curve of the rats in Expt 1 is shown as part of Fig. 1. Although the animals in this experiment grew faster than non-refected animals, the onset of refection only became apparent when weight changes were plotted in terms of the daily weight gain (Fig. 2). Food consumption also showed a marked increase at this time.

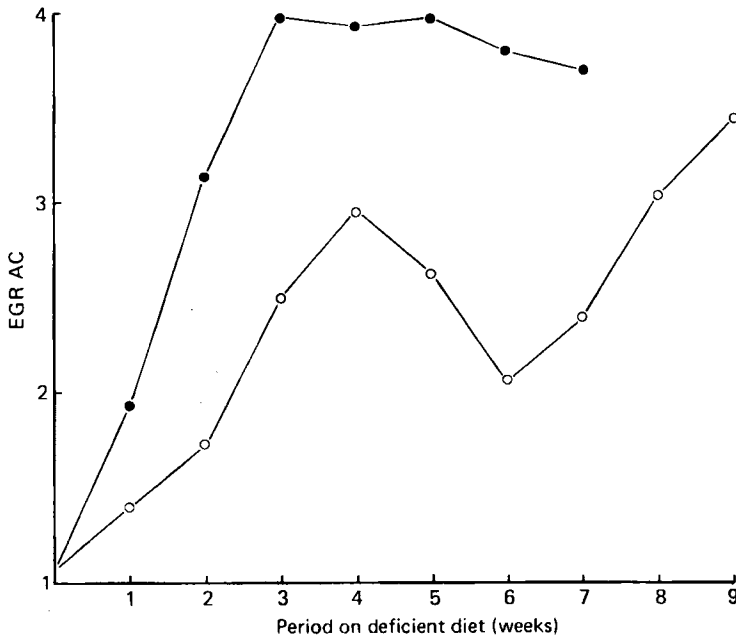


Fig. 3. Activation coefficients (activity measured with added FAD:activity measured without added FAD; AC) for erythrocyte glutathione reductase (NAD(P)H₂: glutathione oxidoreductase, EC 1.6.4.2; EGR) from rats with tail-cups (●) and those without tail-cups (○) fed on a sucrose-based diet (for details of diet and experiment, see p. 171). Each point represents the mean value from three rats.

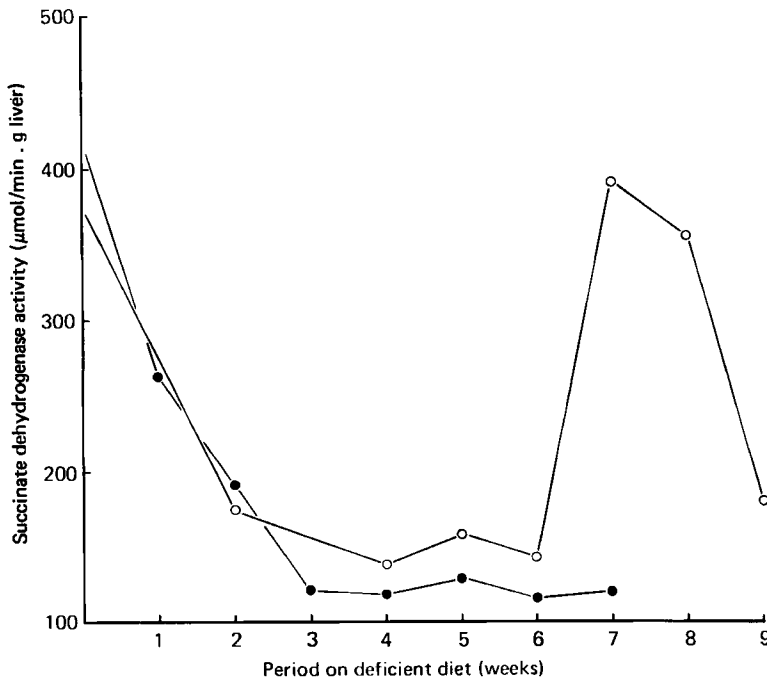


Fig. 4. Hepatic succinate dehydrogenase activity ($\mu\text{mol}/\text{min}$ per g liver) from rats with tail-cups (●) and those without tail-cups (○) fed on a sucrose-based diet (for details of diet and experiment, see p. 171). Each point represents the mean value from 3 rats.

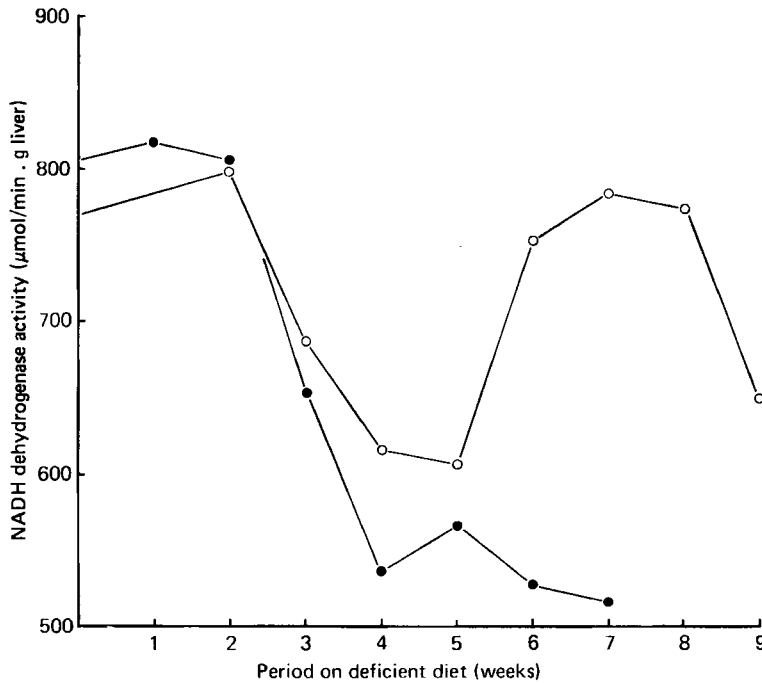


Fig. 5. Hepatic NADH dehydrogenase activity ($\mu\text{mol}/\text{min}$ per g liver) from rats with tail-cups (●) and those without tail-cups (○) fed on a sucrose-based diet (for details of diet and experiment, see p. 171). Each point represents the mean value from three rats.

For EGR the value for AC rose to 2.9 by week 4 of Expt 1 and then decreased sharply until week 6, after which it started to rise again (Fig. 3). Changes in basal activity mirrored those of the AC, and the activity of glutathione reductase from liver and skin showed similar changes, though somewhat later than those in the erythrocytes.

Hepatic succinate dehydrogenase activity decreased until week 4 and then showed a sharp rise at week 6 of Expt 1 (Fig. 4); the activity decreased again thereafter. Succinate dehydrogenase activity from brain and intestine responded in a similar manner; the increase in activity in the brain occurred after week 4. Fig. 5 shows the similar response of hepatic NADH dehydrogenase activity.

Expt 2

A second experiment exactly replicated Expt 1 except that tail-cups were fitted to the rats. The tail-cups were inspected and emptied daily to ensure complete elimination of the faecal source of riboflavin. The growth curve in Expt 2 is shown in Fig. 1 and clearly demonstrates the greater severity of deficiency than that seen in Expt 1. The AC values for EGR increased smoothly to reach a plateau after 4 weeks on the deficient diet (Fig. 3). Similarly the activities of the dehydrogenases decreased smoothly to reach a steady basal level after 3–4 weeks (Figs. 4 and 5).

Pathological signs of riboflavin deficiency occurred earlier and with greater severity in the rats to which tail-cups were fitted. In addition, Expt 2 could only be continued for 7 weeks because 30% of the animals had died by this stage whereas in Expt 1 there was no mortality even after 9 weeks.

DISCUSSION

Each of the indices of riboflavin status investigated in Expt 1 suggested that the animals had received an increase in their riboflavin supply approximately 4 weeks after the start of the experiment. The possibility of a technical error in the administration of the diets was excluded by the fact that a similar AC response for EGR was observed in several subsequent experiments in which tail-cups were not used. The apparent increase in riboflavin supply must therefore be attributable to partial refection despite the use of a sucrose-based diet. The AC results for EGR obtained by Bamji & Sharada (1972) suggest that refection may have occurred in their experiments, and Hassan and D. I. Thurnham (personal communication) have also observed remission from deficiency with a sucrose-based diet. Faulkner & Lambooy (1961) have calculated that as much as 3 μg riboflavin/d may be synthesized in the gastrointestinal tract of rats which are receiving a sucrose-based, riboflavin deficient diet.

Although early investigations into the aetiology of refection have concentrated on the type of dietary carbohydrate employed, this does not appear to be the sole determinant of the extent of refection. High levels of dietary fat (Mannering *et al.* 1941; Shaw & Phillips, 1941; Tange, 1941; Czaczkes & Guggenheim, 1946) and, less conclusively, high levels of dietary protein (Sarett *et al.* 1942; Czaczkes & Guggenheim, 1946) have been reported to increase the riboflavin requirement of rats, and may therefore inhibit refection even when a raw-starch based diet is employed (cf. Glatzle *et al.* 1973).

The extent of refection on the sucrose-based diet in the present study was marginal in terms of weight gain when compared to that observed elsewhere in rats fed raw-starch based diets. It appears that the additional riboflavin made available only partly counteracted the effects of the riboflavin-deficient diet. The relapse from the refected state, which has not been recorded in the instance of raw-starch-based refection, may indicate a delicate balance of factors which determine improvement or deterioration. In subsequent experiments in which AC values for EGR were measured longitudinally in individual rats, the AC characteristically increased to a peak after 3 weeks of deficiency, decreased when refection occurred and increased again to a plateau which was always lower than the initial peak, suggesting a continuous supply of riboflavin. In experiments in which rats were fed graded, suboptimal amounts of riboflavin it was found that above 1.5 mg/kg diet no evidence of refection could be detected, despite the presence of marginal deficiency as measured both by reduced weight gain and by increased AC values.

With the introduction of sensitive biochemical methods of assessing vitamin status, such as those based on enzyme activation by a vitamin-derived cofactor *in vitro*, even marginal refection may become important and could explain the fluctuations in AC values for EGR which were observed in the present study, and which are also apparent in the results presented by Bamji & Sharada (1972). The results of Expt 1 demonstrate that the use of suspended wire cages with wide-meshed bases is not effective in preventing refection. The use of tail-cups as described by Barnes *et al.* (1963) was found to be effective and gave reproducible results in this study. Its main disadvantage was that it was time-consuming since tail-cups required daily checks and regular reapplication. Tail-cups could not be fitted to female rats and when fitted to male rats they prevented the testes from descending normally and, although testicular development was not obviously impaired, this was not studied in detail. Preliminary studies in this laboratory (S. Olpin, unpublished results) have suggested that neck collars made of soft expanded plastic foam are an effective alternative, and are easier to apply.

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