

## The effect of riboflavin deficiency in rats on the absorption and distribution of iron

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1. Riboflavin may play a part in the transport of iron across the gastrointestinal mucosa. Fe absorption was measured in the rat by monitoring whole-body retention of a dose of  $^{59}\text{Fe}$  using a small-animal  $\gamma$ -counter.
2. Female Norwegian Hooded rats were fed on a diet deficient in riboflavin ( $B_2^-$ ) from 5 weeks of age. Control animals, fed on a complete diet ( $B_2^+$ ), were weight-matched to rats fed on the  $B_2^-$  diet. After 7 weeks all rats were fed on a test meal extrinsically labelled with  $^{59}\text{Fe}$  and whole-body radioactivity measured for 15 d.
3. Riboflavin deficiency was associated with a reduction in the percentage of the dose absorbed and an increase in the rate of loss of Fe post absorption.
4. A smaller percentage of the absorbed dose was present in the livers of the riboflavin-deficient animals.

There is little doubt that riboflavin plays some part in iron utilization, and this involvement is almost certainly via a flavin-dependent release of Fe from ferritin (Zaman & Verwilghen, 1977; Ulrik & Romslo, 1981; Powers, 1983). The reduction and release of ferritin-bound Fe is important at several stages of Fe metabolism and may be a step in the transport of Fe across the gastrointestinal mucosa.

Studies conducted in The Gambia, among subjects deficient in riboflavin and with relatively poor Fe status, have suggested that riboflavin may be involved in Fe absorption (Powers *et al.* 1983, 1985). Powers (1986) has also demonstrated that riboflavin deficiency in the rat impairs the ability of the proximal gastrointestinal mucosa to mobilize ferritin-Fe *in vitro*. Other workers have also conducted investigations which implicate riboflavin in Fe absorption (Sirivech *et al.* 1977; Adelekan & Thurnham, 1986).

Various problems with experimental design have made the interpretation of some findings difficult and it remains far from clear whether riboflavin is involved in Fe absorption *in vivo*. We have therefore chosen to measure Fe absorption in the rat by monitoring whole-body retention of a dose of  $^{59}\text{Fe}$  using a small-animal  $\gamma$ -counter.

### MATERIALS AND METHODS

Forty 5-week-old female Norwegian Hooded rats were fed on a basal diet containing (g/kg) 30 arachis oil, 700 sucrose, 200 casein supplying 0.52 mg riboflavin/kg diet, and a vitamin and salt mixture (Powers, 1987). Rats were allocated to one of two dietary regimens to receive either the basal diet ( $B_2^-$ ) or the basal diet plus 15 mg riboflavin/kg diet ( $B_2^+$ ). Whereas  $B_2^-$  animals (group 1) were fed *ad lib.*,  $B_2^+$  animals (group 2) were paired by weight with  $B_2^-$  animals and food intakes restricted so that  $B_2^+$  animals weighed the same as the  $B_2^-$  partner. Animals were all trained to meal feed so that by 2 weeks on the experimental diets food intake for 1 d was consumed within a 3 h period during the morning.

After 7 weeks on the experimental diets all animals were given a fixed amount of diet for

3 d (12 g/meal) in order to standardize Fe intakes. Following a 24 h fast each animal was given a test meal of 3 g of a paste of cooked starch and sucrose (equal amounts by uncooked weight) containing 120  $\mu\text{g}$  Fe (as ferrous sulphate in 0.1 M-hydrochloric acid) extrinsically labelled with 1.0  $\mu\text{Ci}$   $^{59}\text{Fe}$  ( $\text{FeCl}_3$ ; Amersham International plc, Amersham, Bucks). Extrinsic-labelling techniques have been validated as a reliable estimate of absorption of endogenous Fe for several foods (Cook *et al.* 1972; Weaver *et al.* 1984). Any rat not consuming at least three-quarters of the test meal within 1 h was excluded from the experiment. At 4 h after receiving the test meal all animals on the control diet were given 12 g food and deficient animals fed *ad lib*. From this point onwards meal feeding was stopped but weight-matching maintained.

Immediately after consuming the test meal each rat was placed in a small-animal  $\gamma$ -counter (NE 8112, Nuclear Enterprises, Edinburgh) and whole-body radioactivity monitored, using an external  $^{59}\text{Fe}$  standard. The NE 8112 small-animal  $\gamma$ -counter has a detector made of plastic scintillant in the shape of a well, with a counting chamber 360 mm long by 170 mm diameter. Rats were placed in a ventilated Perspex box, 240 by 120 mm square, with the longitudinal axis of the box coincident with that of the counting chamber. Variation in counts from an isotope point-source at any given position within the counting volume was not more than 1% of the overall mean, and reproducibility was better than 99%. Animals were counted for 0.5 min, with a counting efficiency of approximately 50%. Radioactivity was monitored every day for 14 d. On the 14th day after receiving the test meal animals were killed by exsanguination under anaesthesia, and liver, spleen, femur, biceps femoris muscle, 250 mm of washed proximal small intestine and blood were collected for measurement of the distribution of absorbed  $^{59}\text{Fe}$ . Measurements were made in a Philips PW4580 automatic  $\gamma$ -counter and counted using a 75  $\times$  75 mm sodium iodide crystal, centre 1180 keV, width 440 keV, with a counting efficiency of 14%. Riboflavin status was determined at the time of receiving the test meal, in blood collected from the tail vein, and at the time of death, by measuring blood glutathione reductase (NAD(P)H) (*EC* 1.6.4.2) activation coefficient (BGRAC) (Powers, 1986). Packed cell volumes were measured in blood samples at the time of death of the animals.

## RESULTS

The mean weights of rats at the time of receiving the test meal were 170 (SE 3) g in group 1 and 175 (SE 2) g in group 2. These were not significantly different. One rat in group 2 failed to consume three-quarters of the test meal within 1 h and therefore has been excluded from the experiment. The riboflavin status of the rats, both at the time of receiving the test meal and at the time of killing are shown in Table 1, expressed as BGRAC; a BGRAC of > 1.30 is considered to indicate a biochemical deficiency of riboflavin. Rats in group 1 had a significantly higher BGRAC than those of group 2 at both times of measurement, reflecting a depletion of riboflavin.

The mean packed cell volume of rats at the time of killing was 0.414 (SE 0.005) in group 1 and 0.415 (SE 0.007) in group 2. Tissue weights at the time of killing are shown in Table 2. The fresh and dry weights of the proximal 250 mm of the small intestine were significantly lower in the weight-matched controls than in the riboflavin-deficient rats. No differences were observed for any other tissues measured.

The proportion of  $^{59}\text{Fe}$  retained for each day after receiving the test meal is plotted in Fig. 1. The initial rapid fall in whole-body  $^{59}\text{Fe}$  activity was due mainly to excretion of unabsorbed  $^{59}\text{Fe}$ . The daily rate of loss stabilized at day 8 and it is assumed that the subsequent low rate of fall of whole-body  $^{59}\text{Fe}$  reflected basal losses. The rate of loss of whole-body  $^{59}\text{Fe}$  was calculated for 8–11 and 8–14 d after the test meal for each rat, from

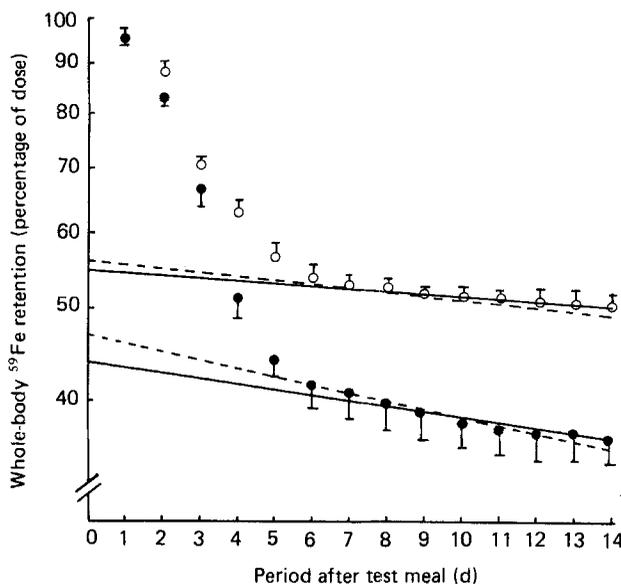


Fig. 1. Whole-body retention of <sup>59</sup>Fe from 0 to 14 d after a <sup>59</sup>Fe-labelled test meal in control (○) and riboflavin-deficient (●) rats (for details of dietary regimens, see p. 381). Points are means, with their standard errors represented by vertical bars. Log linear regression lines are shown for the periods 8–11 d (---) and 8–14 d (—) after the <sup>59</sup>Fe-labelled test meal.

Table 1. Riboflavin status of rats, measured on the day of receiving a <sup>59</sup>Fe-labelled test meal after 7 weeks on experimental diets, and at the time of killing, 14 d later (Mean values with their standard errors)

Group	No. of rats	BGRAC			
		Day of test meal		14 d after test meal	
		Mean	SE	Mean	SE
B <sub>2</sub> -	20	1.64*	0.06	1.56*	0.04
B <sub>2</sub> +	19	1.28	0.02	1.18	0.03

B<sub>2</sub>- , rats fed on a riboflavin-deficient diet; B<sub>2</sub>+, rats fed on a complete diet (control); BGRAC, blood glutathione reductase (EC 1.6.4.2) activation coefficient.

Mean values were significantly different from those of control animals (Student's *t* test): \* *P* < 0.001.

a regression of log<sub>10</sub> percentage dose <sup>59</sup>Fe retained *v.* period (d) after dose. The results are given in Table 3, and show that the daily rate of loss was significantly higher (*P* < 0.001) in the riboflavin-deficient group than in the controls when calculated over either period. The percentage of dose retained at day 0 was determined by extrapolating the individual regression lines back to day 0. The values are given in Table 3 and show that predicted retention at day 0 (percentage absorption) was significantly lower in the riboflavin-deficient group, when calculated over either period.

The tissue distribution of absorbed <sup>59</sup>Fe is shown in Table 4. It is evident that the liver of the deficient animals contained a smaller percentage of the absorbed <sup>59</sup>Fe than that of the controls, whereas the reverse was true for a 0.5 ml sample of whole blood. The observed difference between the two groups for 250 mm of proximal small intestine disappeared

Table 2. *Wet weights of tissues from riboflavin-deficient and control rats at the time of killing*  
(Mean values with their standard errors)

	Riboflavin-deficient		Weight-matched controls	
	Mean	SE	Mean	SE
Liver	5.292	0.142	5.405	0.143
Spleen	0.381	0.012	0.386	0.009
Femur	0.907	0.014	0.882	0.014
Proximal SI	1.584***	0.044	1.163	0.042
Proximal SI (dry wt)	0.356***	0.009	0.279	0.008

SI, small intestine.

Mean values were significantly different from those of control animals (Student's *t* test): \*\*\*  $P < 0.001$ .

Table 3. *Percentage of  $^{59}\text{Fe}$  retained and daily percentage rate of loss of  $^{59}\text{Fe}$  retained by rats after an oral dose of [ $^{59}\text{Fe}$ ]ferrous sulphate*  
(Mean values with their standard errors)

Period after test meal (d)...		8-11		8-14	
Group	No. of rats	Mean	SE	Mean	SE
				Percentage absorption†	
B <sub>2</sub> -	20	47.51*	3.11	44.29**	2.75
B <sub>2</sub> +	19	55.37	1.29	54.81	1.21
				Daily percentage rate of loss‡	
B <sub>2</sub> -	20	2.17***	0.35	1.41***	0.20
B <sub>2</sub> +	19	0.76	0.11	0.57	0.06

B<sub>2</sub>-, rats fed on a riboflavin-deficient diet; B<sub>2</sub>+, rats fed on a complete diet (control).

Mean values were significantly different from those of control animals (Student's *t* test): \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

† Calculated as the percentage dose retained at day 0, from log linear regression *v.* period (d) after a  $^{59}\text{Fe}$ -labelled test meal as shown.

‡ Calculated from the log linear regression of percentage dose retained *v.* period (d) after a  $^{59}\text{Fe}$ -labelled test meal as shown.

Table 4. *Tissue distribution of absorbed  $^{59}\text{Fe}$  (percentage of whole body count) 14 d after an oral dose of  $^{59}\text{Fe}$*   
(Mean values with their standard errors)

	Riboflavin-deficient ( <i>n</i> 20)		Weight-matched controls ( <i>n</i> 19)	
	Mean	SE	Mean	SE
Liver	30.2**	2.1	37.4	1.1
Blood (0.5 ml)	2.98**	0.11	2.60	0.07
Spleen	1.34	0.07	1.31	0.05
Femur	0.579	0.022	0.616	0.027
Muscle (per g)	0.072	0.003	0.078	0.003
Proximal SI	0.362*	0.017	0.306	0.019
(per g fresh wt)	0.232	0.013	0.271	0.020
(per g dry wt)	1.025	0.049	1.099	0.073

SI, small intestine.

Mean values were significantly different from those of control animals (Student's *t* test): \*  $P < 0.05$ , \*\*  $P < 0.01$ .

when the  $^{59}\text{Fe}$  content was expressed on a weight basis, that is  $^{59}\text{Fe}$  (% of total)/g fresh or dry tissue.

#### DISCUSSION

In attempting to clarify whether or not riboflavin deficiency impairs Fe absorption, great care was taken in controlling for factors now known to influence the absorption of  $^{59}\text{Fe}$  from a test meal. Thus all rats received the same concentration of Fe in the diet, and ate their 24 h intake as a single meal at the same time daily. It has been clearly demonstrated that dietary Fe intake during the 3 d before a test meal influences the amount of  $^{59}\text{Fe}$  retained (Fairweather-Tait *et al.* 1985). Therefore, all rats were given similar levels of Fe for 3 d before receiving the test meal.

The experiment described here shows very clearly that riboflavin deficiency altered both the absorption of Fe and the rate of loss of the Fe absorbed. The difference in rates of post-absorption loss was more marked when calculated over the period 8–11 d after the test meal. This was due to a greater individual variation in daily loss of Fe during days 11–14 after the test meal.

Sirivech *et al.* (1977) have suggested that riboflavin deficiency may affect Fe absorption via a mucosal oxidoreductase but as they failed to control the marked inanition effect of riboflavin deficiency, interpretation of their findings is difficult. Powers (1985, 1986) and Adekan & Thurnham (1986) have demonstrated that young riboflavin-deficient rats fail to build up liver Fe stores as effectively as controls, both groups of workers suggesting that the effect might be due to an impaired Fe absorption. Powers (1986) has also demonstrated that riboflavin deficiency in young and adult rats is associated with a reduction in the *in vitro* Fe-mobilizing activity of gastrointestinal mucosa, which suggests an effect on Fe absorption. The monitoring of  $^{59}\text{Fe}$  retention using a whole-animal  $\gamma$ -counter is a sensitive, non-invasive means of measurement of Fe absorption. We believe that the present study constitutes the first rigorously controlled *in vivo* demonstration of an impaired absorption of Fe in riboflavin deficiency.

The elevated post-absorption loss of  $^{59}\text{Fe}$  in riboflavin deficiency was entirely unexpected, but was highly significant. The most likely route of this Fe, assuming no gastrointestinal blood loss, is a serosal to mucosal transfer into the small intestine. There is evidence in the literature that such a transfer might take place. Refsum & Schreiner (1980) observed Fe in goblet cells of the intestinal epithelium and macrophages of the lamina propria of human subjects; they suggested that excretion of Fe via the goblet cell is a normal feature of Fe balance. Bjorn-Rasmussen *et al.* (1980) demonstrated a loss of radioiron from human subjects for up to 1 month after an oral dose, which must represent Fe lost from a non-epithelial part of the intestinal mucosa. In their studies of Fe movement in the rat, Guy & Schachter (1975) showed in both *in vitro* and *in vivo* experiments, that Fe could be transferred from the serosal to the mucosal surface of the small intestine. Although this is a subject that has received very little attention it does seem as though Fe excretion may play a more significant part in Fe balance than has been hitherto acknowledged. Further studies are required to confirm the route of loss of Fe in riboflavin deficiency and to clarify the mechanism involved.

The lower percentage of the absorbed Fe in the livers of the deficient animals is compatible with the reduced accumulation of hepatic ferritin-Fe observed in riboflavin deficiency. This must be a reflection of the need to maintain supply to key tissues, hence limiting the amount of Fe available for hepatic storage. The erythroid marrow has a high requirement for Fe which is met in preference to other tissues, which would account for the higher percentage of the total absorbed  $^{59}\text{Fe}$  being present in 0.5 ml whole blood from the deficient animals.

Of the tissues weighed, only the proximal small intestine was affected by the different

dietary regimens. One explanation for the heavier tissue in the riboflavin-deficient group is that the difference was due to different food intakes in the two groups. The weight-matched control animals consumed about 10% less of the complete diet than the deficient animals. The fine structure of the small intestine is apparently influenced not only by the quality of the diet consumed but also by the actual passage of food down the intestinal tract (Brunser *et al.* 1968; Williamson, 1978). If, for example, a rat is fed intravenously, even when kept in positive nitrogen balance, it loses gut mass (Levine *et al.* 1974). It is possible that even this relatively small difference in food intakes by the two groups of rats in the current experiment was responsible for the reduced mass of small intestine in the control group. It is worth noting that if this were to affect Fe absorption at all one would expect it to reduce rather than to enhance absorption in the control group. Alternatively it has been reported that riboflavin deficiency in the mouse is associated with an increased cell turnover rate in the mucous epithelial cells of the small intestine (Miyaji & Hala, 1965). This may be a mechanism to compensate for an impaired absorption of Fe in riboflavin deficiency.

It is evident that a moderate riboflavin deficiency in rats receiving a normal supply of dietary Fe was associated with both a reduced absorption of Fe and an elevated post-absorption loss. The rats were able to maintain a normal packed cell volume, although in our experience liver stores are seriously depleted. If a similar situation prevails in humans then there are important implications. Population groups consuming diets low in riboflavin invariably have low Fe intakes also. It is not uncommon for riboflavin deficiency and poor Fe status to occur simultaneously, and we have demonstrated that in such a situation riboflavin supplements can enhance the haematological response to Fe (Powers *et al.* 1983).

It is intended that we clarify the effect that low riboflavin intakes have on Fe absorption in man using a stable isotope of Fe. It is likely that such studies will provide information relevant to preventive and therapeutic approaches to reducing the incidence of Fe-deficiency anaemia in vulnerable groups.

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