A proposed intestinal mechanism for the effect of riboflavin deficiency on iron loss in the rat

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The effect of riboflavin deficiency on gastrointestinal Fe distribution and loss was studied in weanling rats. Riboflavin deficiency was associated with a significant increase in crypt depth in the upper and mid small intestine and a twofold increase in the rate of crypt cell production compared with weight-matched and *ad lib.*-fed control rats. The rate of loss of endogenous Fe, measured as faecal ^{59}Fe after intraperitoneally administered ^{59}Fe, was twice that from riboflavin-deficient rats compared with weight-matched controls. We suggest that while there may be a contribution from turnover of enterocytes with an enhanced Fe content, enhanced Fe loss associated with riboflavin deficiency is due predominantly to an accelerated rate of small-intestinal epithelial turnover.

Riboflavin deficiency: Iron: Rat: Cell turnover: Small intestine

Riboflavin deficiency is associated with a disturbance of iron metabolism (Buzina *et al.* 1979; Powers *et al.* 1983; Adelekan & Thurnham, 1986). Studies conducted in rats have indicated that the vitamin deficiency impairs absorption of Fe and increases the gastrointestinal loss (Powers *et al.* 1988, 1991). The increased gastrointestinal loss of Fe, post absorption, in riboflavin-deficient rats may be mediated by an enhanced rate of turnover of the epithelial cells of the mucosa of the small intestine. A recent study has shown that riboflavin deficiency is associated with crypt hypertrophy of the upper small intestine and an enhanced proliferative response of the mucosal epithelium (Powers *et al.* 1991).

It remains to be demonstrated that the apparent effect of riboflavin deficiency on small intestinal mucosal cytokinetics is not merely an effect of the amount of food consumed, and that the enhanced proliferative response of the small intestine leading to an increased rate of turnover of the epithelial cells of the mucosa of the small intestine is responsible for the increased Fe loss.

In these earlier studies of Fe movement in riboflavin-deficient rats it was assumed that the Fe lost through the small intestine was endogenous in origin and was not the result of retention of Fe in the intestinal mucosa with subsequent loss. This remains to be confirmed. Results are presented of a further study into the mechanism of the effect of riboflavin deficiency on the handling of Fe by the rat.

MATERIALS AND METHODS

Two experiments were performed: Expt 1 was designed to determine whether the rate of loss of intraperitoneally administered ^{59}Fe was influenced by riboflavin deficiency; Expt 2

was designed to determine whether riboflavin deficiency was associated with an increased rate of proliferation of the enterocytes of the small intestine.

All the rats were female Norwegian Hooded rats obtained from the breeding colony at the Dunn Nutrition Unit. Rats were weaned at 21–23 d at a body weight of 38·3–67·8 g. Rats were fed on either a basal diet containing (g/kg): arachis oil 30, sucrose 700, casein 200 (supplying 0·52 mg riboflavin/kg diet) and a vitamin and salt mixture (riboflavin-deficient), or the basal diet supplemented with 15 mg riboflavin/kg diet (control diet; Powers, 1987). Diets contained 34 mg Fe/kg diet, supplied as ferric citrate. Wire-bottomed cages were used in order to reduce refection.

Statistical analyses

Analysis of variance and Student's *t* test were used to test the effects of diet on riboflavin status, Fe distribution and loss, and crypt cell proliferation rates.

Expt 1

Nine rats were fed *ad lib.* on the riboflavin-deficient diet; nine rats were paired to the deficient rats and fed on a control diet restricted in amount to maintain the weights equal to that of the deficient partner (weight-matched feeding). The diets were fed for 5 weeks. After 5 weeks, following an overnight fast, ^{59}Fe was administered intraperitoneally in a single dose to all rats as ferrous sulphate in 500 μl isotonic saline (9 g NaCl/l; about 0·136 MBq; Amersham International plc, Amersham, Bucks.). The exact dose to each animal was calculated by weighing the syringe before and after injection.

A small sample of blood was collected from the tail vein for the measurement of riboflavin status using the erythrocyte glutathione reductase (*EC* 1.6.4.2) activation coefficient (EGRAC) test. The erythrocyte cells were washed in isotonic saline and frozen in 3 vol. distilled water. The EGRAC measurement was made using a u.v. spectrophotometer with the same incubation conditions as have been described previously (Powers *et al.* 1983).

After the ^{59}Fe dose the diets were given *ad lib.* to all the animals and faeces were collected from each animal each morning for 14 d. ^{59}Fe in the faeces was determined and expressed as a percentage of the dose given to each animal, taking into account radioactive decay. The loss of ^{59}Fe over 14 d was calculated for each animal. The percentage of the dose retained each day was also calculated and a regression analysis performed of $\log_{10}$ percentage dose retained *v.* time for days 1–7. This allowed calculation of the daily rate of loss of ^{59}Fe. Animals were killed on day 14 by cervical dislocation. Cardiac blood was collected into lithium heparin for the measurement of EGRAC.

The small intestine was removed, washed with saline and divided into duodenum (upper 10%), jejunum (mid 40%) and ileum (lower 50%). Tissues were weighed, ^{59}Fe of each section measured and expressed as percentage dose retained by each animal at the time of kill. Tissues were freeze-dried and reweighed. The three gut sections from each animal were pooled and total Fe was measured by atomic absorption spectrometry.

Tissue Fe was measured by atomic absorption spectrometry on a Perkin Elmer 3030 atomic absorption spectrometer with u.v. background correction. Freeze-dried tissue (600–800 mg) was ground into a fine powder and ashed in silica crucibles at 550° for 24 h. Ashed samples were dissolved in concentrated HCl, filtered through Whatman no. 542 hardened ashless filter paper and stored at 4° until required for measurement of Fe.

Expt 2

Forty-five female Hooded Norwegian rats were allocated to one of three dietary groups at between 21 and 23 d of age. Fifteen rats received a diet deficient in riboflavin which was fed

ad lib.; fifteen rats were weight-matched to the deficient rats and fed on a complete diet; fifteen rats were fed a complete diet *ad lib.*

The feeding regimens were continued for 5 weeks, after which time crypt morphometry and crypt cell production rate (CCPR) were measured in the rats after an overnight fast.

Experiments were performed in the mornings of three consecutive days. Animals were given an intraperitoneal injection of vincristine (1 mg/kg) as 1 mg/5 ml isotonic saline at time zero. Animals were killed by cervical dislocation 0, 10, 30, 60 and 90 min thereafter. Three animals from each group were killed at each time-point. The small intestine from the pylorus to the ileo-caecal valve was removed, laid on the bench under its own weight and its length was measured. Segments 10 mm long were removed at a distance of 5–10% and 50–55% along the length of the small intestine, mounted on card and fixed in Clarke's solution (ethanol–acetic acid (750:250, v/v)) for 24 h. The fixed tissue was stored in ethanol (750 ml/l) before microdissection.

On the day of microdissection the tissue was bulk stained with Schiff reagent by the Feulgen reaction (Wright & Irwin, 1982). The serosal and muscle layers were removed and individual crypts dissected on a microscope slide in acetic acid (450 ml/l). The depths and widths of ten well-orientated crypts per animal were measured under $\times 250$ magnification using an eyepiece micrometer. The tissue was then gently squashed under a coverslip and the number of metaphase arrest figures per crypt was measured. CCPR was calculated from the slope of the plot of the mean number of metaphase arrest figures per animal *v.* time after vincristine injection (30, 60 and 90 min) (Wright & Irwin, 1982).

At the time of killing, cardiac blood was collected into heparinized tubes and the erythrocytes stored at $-20°$ for the measurement of EGRAC.

The liver from each animal was removed, washed in isotonic saline and frozen at $-20°$. Liver flavins were measured using a fluorimetric method (Bessey *et al.* 1949), with a Kontron SFM25 programmable fluorimeter.

Total liver Fe was measured by atomic absorption spectrometry. Portions (500 mg) were freeze-dried overnight and stored in a desiccator until ashing, which took place within 1 week.

RESULTS

Expt 1

Riboflavin status is conventionally described in terms of an activation coefficient for a flavin-dependent enzyme, glutathione reductase (NAD(P)H) (*EC* 1.6.4.2). Riboflavin depletion leads to an elevation of the activation coefficient (Sauberlich *et al.* 1974). Rats fed on the riboflavin-depleted diet showed a significantly higher activation coefficient than their weight-matched controls at the time of the ^{59}Fe dose; this difference was still evident 14 d later (two-way analysis of variance; Table 1). An indeterminate amount of dose was lost during administration to one animal in each group. These animals are, therefore, not included in the ^{59}Fe calculations.

At the time of the ^{59}Fe dose all animals were still gaining weight and mean body weights were 111·1 (SE 4·57) g and 117·0 (SE 2·70) g for deficient and weight-matched groups respectively. At the time of death, after 14 d *ad lib.* feeding, mean body weights were 133·5 (SE 4·97) g and 163·8 (SE 1·89) g for deficient and weight-matched groups respectively. Riboflavin deficiency was associated with a twofold increase in the faecal loss of intraperitoneally administered ^{59}Fe (Table 2).

Faecal ^{59}Fe in the 7 d following the dose accounted for about 20% of the dose administered to the riboflavin-deficient rats compared with about 10% from the weight-matched controls. In the subsequent 7 d a further 6% of the dose was lost from the

Table 1. *Expt 1. Riboflavin status, measured at the time of* ^{59}*Fe dosing (day 0) and 14 d later in rats fed on a riboflavin-deficient diet (RD) and their weight-matched controls (WM)**

(Mean values with their standard errors for nine rats)

	EGRAC			
	Day 0		Day 14	
Treatment group	Mean	SEM	Mean	SEM
RD	1·94	0·151	2·13	0·099
WM	1·29	0·037	1·24	0·039
Statistical significance of difference (Student's *t* test):	$P < 0·001$		$P < 0·001$	

EGRAC, erythrocyte glutathione reductase (*EC* 1.6.4.2) activation coefficient.
* For details of treatments and procedures, see pp. 553–554.

Table 2. *Expt 1. Faecal loss of intraperitoneally administered* ^{59}*Fe over 14 d post-dose in rats fed on a riboflavin-deficient diet (RD) and their weight-matched controls (WM)**

(Mean values with their standard errors for eight rats)

	Faecal ^{59}Fe (% dose)				Statistical significance of effect of †:	
	Days 1–7		Days 8–14			
Treatment group	Mean	SEM	Mean	SEM	Diet	Time
RD	20·44	2·512	5·99	0·248	$P < 0·001$	$P < 0·001$
WM	10·39	0·702	3·69	0·416		

* For details of treatments and procedures, see pp. 553–554.
† Analysis was by two-way analysis of variance.

riboflavin-deficient rats compared with only 3% from the controls. These differences between groups were significant (two-way analysis of variance).

The rate of loss of ^{59}Fe over the period of most rapid loss was calculated from a regression of $\log_{10}$ percentage dose retained *v.* time (d). The results are shown in Fig. 1. Riboflavin-deficient rats lost ^{59}Fe at the rate of 1·72 (SE 0·20) %/d compared with a rate of only 0·83 (SE 0·06) %/d in the control rats. These values are significantly different ($P < 0·001$; Student's *t* test).

The distribution of the ^{59}Fe in the tissues of the small intestine at the time of killing the animals is shown in Table 3. Analysis of variance did not reveal any differences between the two dietary treatments. Riboflavin deficiency had no effect on either the concentration of Fe in the small intestine or the specific activity of ^{59}Fe (Student's *t* test; Table 4).

Expt 2

At the time of killing all animals were still gaining weight, the mean values for the deficient, weight-matched and *ad lib.*-fed groups respectively being 112·7 (SE 2·19) g, 106·0 (SE 2·25) g and 150·5 (SE 4·19) g. Riboflavin status was determined both by the EGRAC test and by

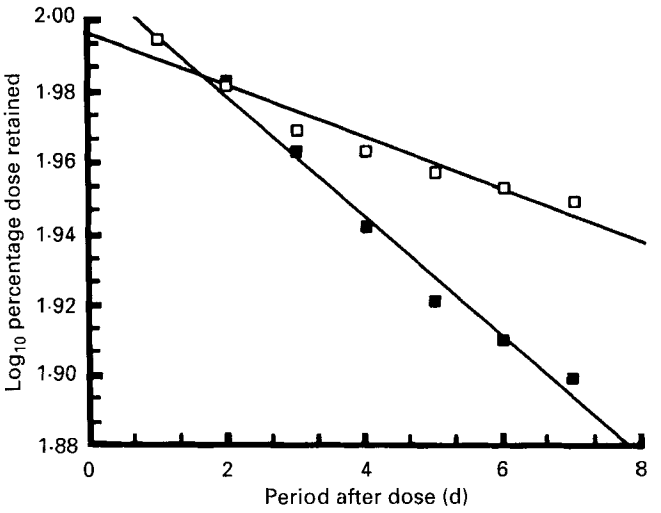

Fig. 1. Rate of faecal loss of ^{59}Fe from eight riboflavin-deficient rats (■) and eight weight-matched control rats (□) over 8 d after intraperitoneal administration. For details of treatments and procedures, see pp. 553–554.

Table 3. *Expt 1. Distribution of intraperitoneally administered ^{59}Fe 14 d post-dose in rats fed on a riboflavin-deficient diet (RD) and their weight-matched controls (WM)**

(Mean values with their standard errors for eight rats)

	^{59}Fe (% of the retained dose at 14 d)					
	Duodenum		Jejunum		Ileum	
Treatment group	Mean	SEM	Mean	SEM	Mean	SEM
RD	0·60	0·088	1·10	0·156	1·30	0·245
WM	0·44	0·078	0·99	0·235	1·08	0·264
Statistical analysis: *t* value (Student's *t* test)	1·339		0·394		0·590	

* For details of treatments and procedures, see pp. 553–554.

measuring the concentration of flavins in the liver of each rat at the time of killing (Table 5). Analysis of variance revealed a highly significant effect of diet on EGRAC and liver flavin concentrations. Feeding the riboflavin-deficient diet produced an increase in EGRAC and a reduction in liver flavin concentrations compared with the control groups. Differences between the control groups were not significant (Student's *t* test). The concentration of Fe in the liver was significantly different between the groups (analysis of variance; Table 6). Riboflavin depletion was associated with a significant reduction in the concentration of Fe in the liver compared with the weight-matched control group despite the fact that the latter consumed about 30% less diet daily than the deficient group. The *ad lib.*-fed group, which ate about two to three times more diet than the other two groups, had a significantly higher liver Fe concentration than either group (Student's *t* test).

One-way analysis of variance revealed a highly significant effect of diet on crypt depth and crypt width in the upper and mid small intestine (Table 7). Detailed comparisons

Table 4. *Expt 1. Iron concentration and specific activity of* ^{59}Fe *in the small intestine 14 d post-dose in rats fed on a riboflavin-deficient diet (RD) and their weight-matched controls (WM)**

(Mean values with their standard errors)

Treatment group	Fe (μg/g dry tissue)			Specific activity (Bq/μg dry tissue)		
	n	Mean	SEM	*n*	Mean	SEM
RD	8	75·00	3·807	8	33·75	4·144
WM	9	73·67	2·321	9	29·67	5·911
Statistical analysis: *t* value (Student's *t* test)		0·307			0·552	

* For details of treatments and procedures, see pp. 553–554.

Table 5. *Expt 2. Riboflavin status of the rats after 5 weeks on a riboflavin-deficient diet for deficient rats and for weight-matched controls and* ad lib.*-fed controls**

(Mean values with their standard errors for fifteen rats)

Treatment group...	Riboflavin-deficient		Weight-matched		*ad lib.*		Statistical significance of difference†: $P <$
	Mean	SEM	Mean	SEM	Mean	SEM	
Liver FAD (μg/g wet wt)	9·74	0·567	23·30	1·499	20·91	1·247	0·001
Liver FMN + B_2 (μg/g wet wt)	2·17	0·201	6·55	0·421	7·17	0·458	0·001
EGRAC	1·93	0·072	1·35	0·031	1·38	0·083	0·001

FAD, flavin adenine dinucleotide; FMN: flavin mononucleotide; B_2; free riboflavin; EGRAC, erythrocyte glutathione reductase (*EC* 1.6.4.2) activation coefficient.

* For details of treatments and procedures, see pp. 554–555.

† Analysis was by one-way analysis of variance.

Table 6. *Expt 2. Total liver iron concentrations of rats fed on a riboflavin-deficient diet for deficient rats and for weight-matched controls and* ad lib.*-fed controls**

(Mean values with their standard errors for fifteen rats)

Treatment group...	Riboflavin-deficient		Weight-matched		*ad lib.*		Statistical significance of difference†: $P <$
	Mean	SEM	Mean	SEM	Mean	SEM	
Liver Fe (μg/g dry wt)	285	17·8	485	27·6	877	96·0	0·001

* For details of treatments and procedures, see pp. 554–555.

† Analysis was by one-way analysis of variance.

between the groups (Student's *t* test) showed a significant increase in crypt depth ($P <$ 0·001) in the upper and mid small intestine and in crypt width ($P < 0·01$) in the mid small intestine compared with *ad lib.* and weight-matched controls. Differences between the two control groups were not significant. CCPR (no. of cells produced by the crypt/h) was

Table 7. *Expt 2. Crypt morphometry and crypt cell production rate of rats fed on a riboflavin-deficient diet for deficient rats and for weight-matched controls and* ad lib.*-fed controls**

Treatment group...	Riboflavin-deficient		Weight-matched		*ad lib.*		Statistical significance of difference†: *P*
	Mean	SEM	Mean	SEM	Mean	SEM	
Upper SI‡							
Crypt depth	253	6·4	196	6·0	188	9·1	< 0·001
Crypt width	63	3·1	55	2·2	59	2·3	0·003
CCPR	20	2·3	11	2·8	8	0·7	< 0·001
Mid SI§							
Crypt depth	204	5·7	174	5·1	149	4·0	< 0·001
Crypt width	60	1·2	51	1·7	51	1·1	< 0·001
CCPR	16	2·1	7	0·6	9	3·0	< 0·001

SI, small intestine; CCPR, crypt cell production rate.
* For details of treatments and procedures, see pp. 554–555.
† One-way analysis of variance was conducted on crypt depths and widths.
‡ The upper 5–10% of the SI.
§ The mid 50–55% of the SI.

approximately doubled in the riboflavin-deficient rats compared with controls at both sites ($P < 0·001$; Student's t test). Differences between the control groups were not significant.

DISCUSSION

The feeding of a diet deficient in riboflavin to weanling rats resulted in an increased crypt width in the mid small intestine and crypt depth in the upper and mid small intestine. Riboflavin deficiency also resulted in an accelerated rate of production of the crypt cells of the small intestine. The rate of gastrointestinal loss of endogenous Fe occurred at twice the rate from riboflavin-deficient rats as from weight-matched controls. In our experience, and in that of others (Duerden & Bates, 1985; Powers, 1986; Powers *et al.* 1991), rats fed on a complete diet but in restricted amounts sometimes have EGRAC values above 1·30, a commonly used threshold for normality. This was particularly evident in Expt 2 and, therefore, confirmation of the riboflavin status of the rats was sought by measuring the actual flavin concentrations in the liver. There was a twofold difference in the concentration of flavin adenine dinucleotide and a threefold difference in the concentration of flavin mononucleotide and free riboflavin in the livers of the rats fed on the riboflavin-deficient or complete diets. All the rats receiving the riboflavin-deficient diet were clearly riboflavin depleted, while those in both control groups had adequate riboflavin status.

We have previously demonstrated that the post-absorption loss of orally administered Fe from rats is totally accounted for by faecal Fe loss (Powers *et al.* 1991). We showed that the enhanced loss of orally administered Fe in riboflavin deficiency was associated with a preferential accumulation of the radio-Fe by the tissues of the small intestine. We also showed that riboflavin deficiency was associated with an increased mitotic index in the crypt cells of the mucosal epithelium of the upper small intestine. We suggested that either or both of these observations could explain the accelerated rate of post-absorption Fe loss in riboflavin deficiency.

To demonstrate conclusively that the post-absorption faecal ^{59}Fe loss was entirely endogenous in origin, the ^{59}Fe was administered intraperitoneally in the first experiment described here. We demonstrated that intraperitoneally administered ^{59}Fe was lost more

rapidly from riboflavin-deficient rats than weight-matched controls. There were no significant differences in the specific activity of ^{59}Fe in the small intestine between the two groups at 14 d and tissues of the small intestine from the riboflavin-deficient animals did not account for significantly more of the ^{59}Fe than their controls. There was a trend towards higher values in the riboflavin-deficient group, and we cannot rule out the possibility that this contributed to the enhanced Fe loss in these animals.

We suggest that the enhanced rate of Fe loss in riboflavin deficiency is due predominantly to an accelerated rate of villus enterocyte turnover in the small intestine. The significantly increased concentration of orally administered ^{59}Fe seen in the tissues of the small intestine of riboflavin-deficient rats in a previous study (Powers *et al.* 1991) may have been due to a retention of ^{59}Fe in a non-epithelial part of the small intestine before absorption, and not exclusively to preferential uptake of absorbed Fe, as was believed to be the case. It may be prudent also to consider the possibility that the observed difference in the rate of Fe loss between the riboflavin-deficient and control animals may be due, at least in part, to increased blood loss in riboflavin deficiency. This possibility needs to be addressed using erythrocyte kinetic studies, or by the measurement of occult blood.

Riboflavin-deficient rats have a significantly increased crypt depth in the upper and mid small intestine, and crypt width in the mid small intestine, compared with weight-matched and *ad lib.*-fed controls. CCPR is also significantly increased in riboflavin deficiency. Hypotrophy of the small intestine is a feature of restricted food intake (Powers, 1986) which could have explained the difference in crypt morphometry between the deficient and weight-matched groups. However, the *ad lib.*-fed group exhibited the same crypt morphometry as the weight-matched group, indicating that the relative hypertrophy of the riboflavin-deficient group was an effect of the riboflavin deficiency *per se*.

The effect of the riboflavin deficiency was not simply to increase the size of the crypts, but to increase the rate at which the crypt cells were produced. The stathmokinetic technique is a robust and well-validated method for the measurement of CCPR (Wright & Irwin, 1982). The small intestinal mucosa may adapt to a wide variety of physiological and pathological stimuli, and the methods described here have been used to measure proliferative changes in many of these (Goodlad *et al.* 1988; Weaver & Carrick, 1989). It is suggested that an increased rate of crypt cell proliferation in riboflavin deficiency leads to an accelerated rate of Fe loss via a change in the rate of cellular sloughing from the villus tip. An effect of this nature would be expected to increase the rate at which Fe was lost from the body. It is also possible that an accelerated enterocyte production and an influx of immature enterocytes into the villus from the crypts disturb Fe absorption. This requires further investigation.

The observed effect of riboflavin status on hepatic Fe is a reflection of the disturbance that riboflavin deficiency makes to Fe economy. The weanling rat has relatively small Fe stores, and in the period after weaning the stores increase in size until they reach a value similar to that of the adult (Powers, 1986). A failure to accumulate liver Fe is the result of a limited surplus after the demands of erythropoiesis have been met and may result from increased Fe turnover, reduced absorption or a combination of both. We have already demonstrated that riboflavin deficiency in the rat impairs Fe absorption and increases Fe loss.

Conclusions

Riboflavin deficiency increases the rate at which endogenous Fe is lost from the body through the intestine. This could be explained by an increased rate of turnover of small intestinal epithelial cells. The mechanism for the increased crypt cell proliferation is not known but is independent of the amount of food passing into the small intestine. The

increased crypt cell proliferation may also impair Fe absorption via an effect on the functional maturity of enterocytes in the villi. The effect of riboflavin deficiency in reducing available Fe (by increasing loss and reducing absorption) is reflected in a failure to accumulate hepatic Fe after weaning.

REFERENCES

Adelekan, D. A. & Thurnham, D. I. (1986). A longitudinal study of the effect of riboflavin status on aspects of iron storage in the liver of growing rats. *British Journal of Nutrition* **56**, 171–179.

Bessey, V. A., Lowry, V. H. & Love, R. H. (1949). The fluorimetric measurement of the nucleotides of riboflavin and their concentration in tissues. *Journal of Biological Chemistry* **180**, 755–769.

Buzina, R., Jusic, M., Milanovic, N., Sapunar, J. & Brubacher, G. (1979). The effects of riboflavin administration on iron metabolism parameters in a school-going population. *International Journal of Vitamin and Nutrition Research* **49**, 136–143.

Duerden, J. M. & Bates, C. J. (1985). Effect of riboflavin deficiency on reproductive performance and on biochemical indices of riboflavin status in the rat. *British Journal of Nutrition* **53**, 97–105.

Goodlad, R. A., Plumb, J. A. & Wright, N. A. (1988). Epithelial cell proliferation and intestinal absorptive function during starvation and refeeding in the rat. *Clinical Science* **25**, 301–306.

Powers, H. J. (1986). Investigation into the relative effects of riboflavin deficiency in the weanling rat and the adult. *Annals of Nutrition and Metabolism* **30**, 308–315.

Powers, H. J. (1987). A study of maternofetal iron transfer in the riboflavin deficient rat. *Journal of Nutrition* **117**, 852–856.

Powers, H. J., Bates, C. J. & Duerden, J. M. (1983). Effects of riboflavin deficiency in rats on some aspects of iron metabolism. *International Journal of Vitamin and Nutrition Research* **43**, 371–376.

Powers, H. J., Weaver, L. T., Austin, S., Wright, A. J. A. & Fairweather-Tait, S. J. (1991). Riboflavin deficiency in the rat: effects on iron utilization and loss. *British Journal of Nutrition* **65**, 487–496.

Powers, H. J., Wright, A. J. A. & Fairweather-Tait, S. J. (1988). The effect of riboflavin deficiency on the absorption and distribution of iron. *British Journal of Nutrition* **59**, 381–387.

Sauberlich, H. E., Skala, J. H. & Dowdy, R. P. (1974). *Laboratory Tests for the Assessment of Nutritional Status*. Cleveland, Ohio: CRC Press, Inc.

Weaver, L. T. & Carrick, B. M. (1989). Changes in upper intestinal morphology and kinetics in the growing guinea pig. *Paediatric Research* **26**, 31–33.

Wright, N. A. & Irwin, M. (1982). The kinetics of villus cell populations in the mouse small intestine. *Cell and Tissue Kinetics* **15**, 595–609.

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