Bioavailability of total iron from meat, spinach (*Spinacea oleracea* L.) and meat–spinach mixtures by anaemic and non-anaemic rats*

By D. ZHANG, D. G. HENDRICKS and A. W. MAHONEY

Department of Nutrition and Food Sciences, Utah State University, Logan, Utah 84322-8700, USA

1. Bioavailability of Fe from beef, spinach (*Spinacea oleracea* L.) and their mixtures was studied using anaemic and non-anaemic rats by haemoglobin regeneration efficiency (HRE) and apparent Fe absorption in two trials.

2. The initial haemoglobin levels of severely anaemic, mildly anaemic and non-anaemic rats were 63, 88 and 113 g/l, respectively. The Fe level in diets was about 30 mg/kg. All other nutrients equalled or exceeded the requirement of the growing rat.

3. The spinach Fe was well utilized by the rats with average HRE of 0·41, 0·53 and 0·36, and apparent Fe absorptions averaging 0·48, 0·59 and 0·37 for the severely anaemic, mildly anaemic and non-anaemic animals respectively.

4. Beef Fe was efficiently used by rats as reported by others. Average HRE were 0·42, 0·51 and 0·44, and average apparent Fe absorptions were 0·44, 0·47 and 0·46 for the severely anaemic, mildly anaemic and non-anaemic rats respectively.

5. When the percentage of meat Fe was increased from 0 to 25, 50, 75 or 100 of the dietary Fe, HRE and apparent Fe absorption were not increased significantly. A meat enhancement effect on total Fe absorption, reported by others for non-haem-Fe, did not occur in the present experiment.

6. Negative correlation coefficients between initial haemoglobin and HRE (r −0·79), and initial haemoglobin and apparent Fe absorption (r −0·73) were seen with the rats fed on dietary Fe from $FeSO_4 \cdot 7H_2O$. This was not seen with the rats fed on dietary Fe from beef or spinach.

7. The Fe absorption pattern for the different Fe sources is evidence for a third Fe pool, a pool made up of highly soluble inorganic Fe salt, in addition to haem-Fe and non-haem-Fe complex pools. $FeSO_4 \cdot 7H_2O$ is not in the same gastrointestinal pool as non-haem-Fe complex such as spinach Fe. A suggested mechanism of absorption is discussed.

Fe deficiency is prevalent in the world. This is a result of low Fe absorption from food due to either low Fe intake or low bioavailability. Difference in absorption between haem-Fe in animal products and non-haem-Fe in plant foods has led to the two-pool hypothesis, which suggests the following routes of Fe absorption. Haem is efficiently absorbed intact into the intestinal mucosal cells where it is split to release the Fe (Weintraub *et al.* 1968; Turnbull, 1974). Non-haem-Fe must be solubilized and reduced before absorption and many factors affect this process (Layrisse *et al.* 1968, 1969; Cook & Monsen, 1976; Björn-Rasmussen *et al.* 1973; Gillooly *et al.* 1984; Wollenberg & Rummell, 1987). The released haem-Fe and non-haem-Fe are transferred from the mucosal cells by the same mechanism. Non-haem-Fe absorption is measured by extrinsic labelling (Layrisse *et al.* 1968, 1969; Cook & Monsen, 1976; Björn-Rasmussen *et al.* 1973). Meat enhances non-haem-Fe absorption (Martínez-Torres & Layrisse, 1971; Björn-Rasmussen *et al.* 1973; Björn-Rasmussen & Hallberg, 1979; Layrisse *et al.* 1968, 1969; Cook & Monsen, 1976). However, haem-Fe or total Fe absorptions were not measured in these studies; this information is necessary to determine the true impact of meat on Fe balance. The present study was concerned with the bioavailability of Fe from beef, spinach (*Spinacea oleracea* L.), or proportional mixtures of beef and spinach. Bioavailability in anaemic and non-anaemic rats was determined by haemoglobin regeneration efficiency (HRE) and apparent Fe

* Paper no. 3606 of the Utah Agricultural Experiment Station.

absorption, both of which are measures of total Fe utilization. The rat was used because its mechanism of haem-Fe absorption is similar to that of man (Turnbull, 1974; Thannoun *et al.* 1987).

MATERIALS AND METHODS

Experimental design

Diets with equal amounts of Fe were prepared by mixing lyophilized meat and lyophilized spinach in proportions to vary the added dietary Fe from 100% from meat Fe to 100% from spinach Fe (Table 1). A low-Fe basal diet to which about 20 mg Fe as $FeSO_4 \cdot 7H_2O$/kg were added served as a reference (Table 1). The effect of the Fe status of the rats on Fe utilization was tested in two trials. In trial 1, half the rats were severely anaemic and half were mildly anaemic. In trial 2, half the rats were severely anaemic and half were kept non-anaemic.

Five rats in each group, a total of 120 rats, were used in the experiment. Body-weight gain, haemoglobin gain, haemoglobin-Fe gain and Fe intake were measured and used for calculating HRE. Faecal Fe and Fe intake were measured and used to calculate apparent Fe absorption. These methods of evaluation of Fe bioavailability were analysed and compared.

Food and diet preparation

Ground beef round and spinach were lyophilized for 48 h with the shelf temperature set at 40°. They were then ground in a blender fitted with stainless steel blades. The composition of these products is shown in Table 2.

The diets were formulated to provide 30 mg Fe/kg diet (Table 1). Total protein, fat and fibre were balanced across diets to 180, 100 and 50 g/kg with casein, maize oil and cellulose respectively. Total calcium and phosphorus in the diets were held constant by adjusting the level of inorganic Ca and P added to the diets. Total sodium, potassium and β-carotene were also adjusted among diets according to literature values for these nutrients in meat and spinach (Agricultural Research Service, United States Department of Agriculture, 1963). The dietary ingredients were mixed in a stainless steel bowl and refrigerated in plastic bags until fed.

Animals

Male, weanling (50–60 g body-weight), Sprague-Dawley rats (Simonson Laboratories, Gilroy, CA) were individually housed in stainless steel cages with wire-mesh bottoms and fronts fitted with stainless steel funnels and glass apparatus for separating urine and faeces. Housing was in a temperature-controlled room (22°) with a 12 h light – 12 h dark cycle. Fresh demineralized water was given *ad lib.* in polypropylene bottles fitted with stainless steel lick-spouts and rubber stoppers. Fresh diet was given daily in glass feeders.

The rats were treated to be severely or mildly anaemic in trial 1, and severely anaemic and non-anaemic in trial 2, before the experimental diets were given. Severe Fe depletion was caused by giving the rats a low-Fe basal diet (about 10 mg Fe/kg) for 7 d and bleeding thirty drops of blood from the retro-ocular capillary bed of the rats (Timm, 1979) on days 1 and 4. Heparinized glass capillary tubes were used to penetrate the orbital capillary bed to bleed the rats. The average haemoglobin concentration of these rats at the start of experiment was about 65 (SD 7) g/l in trial 1 and 62 (SE 7) g/l in trial 2. Mild Fe depletion in trial 1 was caused by giving the rats a low-Fe basal diet for 7 d without bleeding, which lowered the haemoglobin level to 88 (SD 8) g/l. Non-anaemic rats in trial 2 were given an $FeSO_4 \cdot 7H_2O$-supplemented diet to produce an average haemoglobin level of 113 (SD 7) g/l at the end of 7 d of feeding.

Table 1. *Formulation of diets containing varying proportions of iron from meat and spinach* (Spinacea oleracea *L.*) (g/kg)

	Proportion of Fe sources in diets					
Meat Fe...	1000	750	500	250	0	FeSO$_4 \cdot$7H$_2$O*
Ingredients Spinach Fe...	0	250	500	750	1000	diet
Meat (lyophilized)	187	140	94	47	—	—
Spinach (lyophilized)	—	23·5	47	70·5	94	—
Casein	38·5	70	101	134	166	198
Maize oil	72·3	78·5	84·6	94·7	97	100
Cellulose	50	48·6	47·2	45·8	44·4	50
NaH$_2$PO$_4$	25·2	25·4	25·5	25·6	25·7	26·9
CaCO$_3$	16·9	16·5	16·1	15·7	15·3	17
Vitamin mixture†	20	20	20	20	20	20
Mineral mixture‡	11·6	11·6	11·6	11·6	11·6	11·6
NaCl	0·8	0·6	0·4	0·2	—	1·8
KCl	4·5	3·4	2·2	1·1	—	9
β-Carotene (mg/kg)	49	36·8	24·5	12·3	—	49
Glucose	573·2	561·9	550·4	537·8	526·0	565·7
Fe (mg/kg)§	27·4	28·0	28·6	28·9	29·6	29·5

* The ferrous sulphate reference diet was made by adding 0·1000 g FeSO$_4 \cdot$7H$_2$O to 1 kg basal diet.

† The vitamin mixture contained (g/kg): α-tocopherol (10 mg/g) 5·0, L-ascorbic acid 45·0, choline chloride 75·0, D-calcium pantothenate 3·0, inositol 5·0, menadione 2·25, niacin 4·5, pyridoxine hydrochloride 1·0, riboflavin 1·0, thiamin hydrochloride 1·0, retinyl acetate 270 mg, calciferol 2·5 mg, biotin 20 mg, folic acid 90 mg, vitamin B$_{12}$ 1·35 mg, and glucose added to make to 1 kg.

‡ The mineral mixture contained (g/kg): KCl 296·7, MgCO$_3$ 121, MnSO$_4$ 12·7, CoCl$_2 \cdot$6H$_2$O 0·7, ZnSO$_4$ ·7H$_2$O 38, CuSO$_4 \cdot$5H$_2$O 1·6, KI 0·8, NaMoO$_4 \cdot$2H$_2$O 0·12, glucose 528·4.

§ Analysed value.

Table 2. *Composition of lean ground beef and spinach* (Spinacea oleracea *L.*)*

	Ground beef			Spinach		
Component	Fresh wt basis	Dry wt basis	Nutrient density (mg/MJ)	Fresh wt basis	Dry wt basis	Nutrient density (mg/MJ)
Iron (mg/kg)	29·3	107·2	5·2	19·8	213·5	18·2
Protein (g/kg)	212	776	—	29	310	—
Fat (g/kg)	40	148	—	—	—	—
Phosphorus (mg/kg)	2000	7500	358	500	5200	454
Calcium (mg/kg)	80	290	14	800	8340	741

* Beef and spinach were freeze-dried before mixing with other diet ingredients. The fresh beef contained 727 g moisture/kg, and the fresh spinach contained 907 g moisture/kg.

On day 8, all rats were weighed, haemoglobin concentration determined, and five rats were allotted to each of the six dietary treatment groups, balancing for haemoglobin concentration and body-weight. This was done by computer which first calculated the average haemoglobin concentrations of the severely anaemic, mildly anaemic or non-anaemic rats (thirty rats per group, two groups per trial). The groups were then subdivided by computer into six test groups allotting rats back and forth across the six diets, beginning by allotting animals with haemoglobin values equal to or above the mean and then allotting

those with values below the mean. Body-weight was balanced by moving the rats with similar haemoglobin levels and different body-weights across the groups. The rats were given 9 g daily of their respective test diets for 10 d.

Evaluation methods

HRE. All diet food given and that refused or spilled were weighed to determine total diet intake. Fresh diet was given to the rats every day. The initial and final body-weights and haemoglobin concentrations were determined to calculate HRE (Mahoney & Hendricks, 1982). The formulas used were as follows:

$$\text{haemoglobin-Fe (mg)} = \text{body-weight (g)}$$
$$\times \text{ml blood/g body-weight (assumed to be } 0.067 \text{ ml)}$$
$$\times \text{g haemoglobin/ml blood}$$
$$\times \text{mg Fe/g haemoglobin (assumed to be } 3.35 \text{ mg)}.$$

$$\text{HRE} = \frac{\text{mg haemoglobin-Fe (final)} - \text{mg haemoglobin-Fe (initial)}}{\text{mg Fe consumed}}.$$

Apparent Fe balance. Apparent Fe absorption was calculated for each rat as follows:

$$\text{apparent Fe absorption} = \frac{\text{Fe intake (mg)} - \text{faecal Fe (mg)}}{\text{Fe intake (mg)}}.$$

The Fe in urine, perspiration and sloughed skin was considered negligible (Beutler, 1980). No urinary Fe was detected in the five rats sampled, so urine was not collected nor used in the formula.

Chemical analysis

The protein content of meat and spinach was measured by the Kjeldahl method with an automatic nitrogen analyser (Tecator Kjeltec Auto 1030 Analyser). The fat in the meat was measured by the Mojonnier method (Atherton & Newlander, 1982). Samples for Fe measurement were wet ashed using sulphuric and nitric acids, and diluted to volume with demineralized water. Fe^{3+} in the sample was reduced to Fe^{2+} by hydroxylamine hydrochloride. α,α-Dipyridyl was used as a colour reagent to form a pink Fe complex. The absorbance was measured with a Beckman DB-GT grating spectrophotometer at 510 nm (Association of Official Analytical Chemists, 1980). Analysed values for National Bureau of Standards bovine liver (NBS 1577_a) and wheat flour (NBS 1567) reference samples were 177 (SD 7) mg Fe/kg (91 % of the certified value of 194 (SD 20) mg Fe/kg) and 16 (SD 0·9) mg Fe/kg (90 % of the certified value of 18·3 (SD 1·0) mg Fe/kg) respectively. P was measured by colorimetry (Fiske & Subbarow, 1925). Samples for Ca were ashed in a muffle furnace at 550° for 48 h or until completely ashed. The ash was solubilized with 5 ml 6 M-hydrochloric acid and diluted to volume with lanthanum oxide solution (50 g/l) and deionized water (final sample solution containing 10 g lanthanum/l). Ca was measured with an atomic absorption spectrophotometer (model no. 457; Instrumentation Laboratories) using nitric oxide flame at 422·7 nm.

Statistical analysis

Results were analysed statistically by factorial analysis of variance (Dowdy & Wearden, 1983). Two factors, Fe status of animals and dietary Fe source, were tested for their effect on Fe bioavailability. When F was significant ($P < 0.05$), means were compared by least significant difference (LSD) values. The LSD test is suitable for multiple comparisons of

mean differences (Carma & Swanson, 1973). Correlation analysis was used to test if there was a linear relation between Fe status and Fe bioavailability indicators in varied dietary Fe sources. The significance of the correlation was tested by the one-tailed t test (Dowdy & Wearden, 1983).

<div align="center">RESULTS</div>

<div align="center">*Food composition*</div>

The protein, fat, total Fe, P and Ca contents of beef and spinach are summarized in Table 2. Spinach contained two-thirds as much Fe as beef on a fresh weight basis. However, on a dry weight or energy basis, spinach contained two to three times more Fe than beef. The beef and spinach used in the present study had similar amounts of P. However, beef contained little Ca, about 0·02 of the Ca contained in spinach.

<div align="center">*Body-weight of rats*</div>

The anaemic rats initially weighed less than the non-anaemic rats ($P < 0.001$), possibly due to the stress of blood lost and anaemia caused by the two phlebotomies during the 7-d depletion period. Rats in trial 1 fed on the low-Fe diet and not bled (mildly anaemic rats) initially weighed more than all other groups in both trials. The anaemic rats in trial 2 gained more weight than non-anaemic rats during the 10-d repletion period ($P < 0.001$). Severely and mildly anaemic rats in trial 1 had similar body-weight gains. There were no significant differences in body-weight gains ($P > 0.05$) among groups of rats fed on the different diets. Differences in body-weight gain and Fe intake do not affect HRE (Mahoney *et al.* 1974; Mahoney & Hendricks, 1976) if metabolizable Fe intake does not exceed Fe need.

<div align="center">*HRE*</div>

HRE values and the values used to calculate them are listed in Table 3. Dietary Fe sources affected the haemoglobin gain ($P < 0.001$). After 10 d of Fe repletion, the haemoglobin concentration of rats receiving $FeSO_4 \cdot 7H_2O$ increased the most. Rats fed on meat, meat–spinach or spinach diets gained similar amounts of haemoglobin, with the exception of the non-anaemic rats fed on the spinach diet which gained less haemoglobin. Anaemic rats gained more haemoglobin than non-anaemic rats ($P < 0.001$). This was apparent when $FeSO_4 \cdot 7H_2O$ and spinach Fe were the dietary Fe sources.

Fe status of the rats affected ($P < 0.001$) haemoglobin-Fe gain (Table 3). There was a tendency for mildly anaemic rats (initial haemoglobin 88 g/l) to gain the most Fe as haemoglobin when dietary Fe was from spinach and meat. This was different from the rats fed on dietary Fe from $FeSO_4 \cdot 7H_2O$, which showed an inverse relation between haemoglobin-Fe gain and initial Fe status. Haemoglobin-Fe gain was similar for the severely anaemic and non-anaemic rats when they were fed on diets with Fe from spinach and meat. The significant effect of dietary Fe source on haemoglobin-Fe gain ($P < 0.001$) was mainly the result of $FeSO_4 \cdot 7H_2O$ which caused the greatest haemoglobin-Fe gain. However, rats fed on the meat, meat–spinach and spinach diets gained similar amounts of haemoglobin-Fe.

The Fe status of rats affected HRE ($P < 0.001$). When the dietary Fe source was spinach, the mildly anaemic rats had the highest average HRE. Rats fed on the diet with Fe from meat had similar average HRE, irrespective of Fe status. However, an inverse relation between initial Fe status and HRE was found in rats fed on the $FeSO_4 \cdot 7H_2O$ diet. The significant effect of dietary Fe source on HRE ($P < 0.001$) was mainly the effect of $FeSO_4 \cdot 7H_2O$ which caused higher HRE than all the other dietary groups. HRE values for anaemic rats fed on $FeSO_4 \cdot 7H_2O$ were similar to those reported by Jansuittivechakul *et al.*

Table 3. *Body-weight, haemoglobin concentration, iron intake and haemoglobin regeneration efficiency (HRE) for diets with varying amounts of Fe from meat or spinach* (Spinacea oleracea *L.*) *given to anaemic and non-anaemic rats*

(Each value is the mean of five rats)

	Severely anaemic rats*						
Proportion of meat Fe...	1000	750	500	250	0	$FeSO_4 \cdot 7H_2O$	
Proportion of spinach Fe...	0	250	500	750	1000	diet	
Body-wt (g)							
Initial							
Trial 1	88	90	88	78	87	92	
Trial 2	84	87	84	86	86	90	
Gain							
Trial 1	31	36	28	40	32	30	
Trial 2	30	31	26	33	29	28	
Haemoglobin (g/l)							
Initial							
Trial 1	65·0	64·5	64·8	64·6	65·0	65·6	
Trial 2	61·6	59·5	61·8	61·8	61·5	63·1	
Gain							
Trial 1	23·1	25·8	26·6	26·8	27·4	57·8	
Trial 2	26·3	26·7	28·8	25·7	22·6	57·1	
Haemoglobin Fe gain (mg)							
Trial 1	1·07	1·25	1·12	1·29	1·20	2·01	
Trial 2	1·10	1·10	1·05	1·14	0·98	1·91	
Fe intake (mg)							
Trial 1	2·46	2·52	2·56	2·55	2·66	2·66	
Trial 2	2·46	2·51	2·55	2·60	2·67	2·68	
HRE							
Trial 1	0·44	0·50	0·44	0·51	0·45	0·76	
Trial 2	0·45	0·44	0·41	0·44	0·37	0·71	
	Mildly anaemic or non-anaemic rats*						
Proportion of meat Fe...	1000	750	500	250	0	$FeSO_4 \cdot 7H_2O$	LSD†
Proportion of spinach Fe...	0	250	500	750	1000	diet	$\alpha = 0.05$
Body-wt (g)							
Initial							
Trial 1	96	101	96	97	97	98	
Trial 2	91	91	91	94	91	90	
Gain							
Trial 1	34	32	31	32	30	33	8
Trial 2	24	26	27	24	27	24	7
Haemoglobin (g/l)							
Initial							
Trial 1	87·5	87·9	89·0	88·6	87·8	86·8	
Trial 2	112	111	112	113	113	113	
Gain							
Trial 1	19·1	22·1	23·8	23·5	26·7	38·2	1·04
Trial 2	21·7	17·6	15·7	22·2	10·7	26·1	1·05

Table 3 continued.

	Mildly anaemic or non-anaemic rats*						
Proportion of meat Fe...	1000	750	500	250	0	$FeSO_4 \cdot 7H_2O$	LSD†
Proportion of spinach Fe...	0	250	500	750	1000	diet	$\alpha = 0.05$
Haemoglobin Fe gain (mg)							
Trial 1	1·25	1·28	1·30	1·32	1·38	1·75	0·27
Trial 2	1·16	1·11	1·12	1·19	0·97	1·29	0·20
Fe intake (mg)							
Trial 1	2·46	2·52	2·57	2·60	2·63	2·64	—
Trial 2	2·46	2·51	2·57	2·56	2·66	2·68	—
HRE							
Trial 1	0·51	0·51	0·50	0·51	0·53	0·66	0·10
Trial 2	0·47	0·45	0·43	0·46	0·36	0·48	0·08

* Mildly anaemic rats were used in trial 1 and non-anaemic rats were used in trial 2; for details see p. 335.
† LSD, least significant difference. Mean differences must be equal or exceed the LSD values to be statistically significant ($P < 0.05$).

Table 4. *Correlation coefficients of initial haemoglobin (Hb) v. Hb gain, Hb-Fe gain, Hb regeneration efficiency (HRE) and apparent Fe absorption for rats fed on the $FeSO_4 \cdot 7H_2O$, meat or spinach (Spinecea oleracea L.) diets*

	r†	t‡	
Initial Hb *v.* Hb gain			
$FeSO_4 \cdot 7H_2O$	−0·85	−6·69	S
Meat	−0·21	−0·92	NS
Spinach	−0·47	−2·25	S
Initial Hb *v.* Hb-Fe gain			
$FeSO_4 \cdot 7H_2O$	−0·80	−5·70	S
Meat	0·24	1·03	NS
Spinach	−0·09	−0·38	NS
Initial Hb *v.* HRE			
$FeSO_4 \cdot 7H_2O$	−0·79	−5·48	S
Meat	0·22	0·95	NS
Spinach	0·07	0·29	NS
Initial Hb *v.* apparent Fe absorption			
$FeSO_4 \cdot 7H_2O$	−0·73	−4·51	S
Meat	0·10	0·42	NS
Spinach	−0·32	−1·40	NS

* S, significant; NS, not significant. For details, see p. 338.
† Each correlation coefficient was calculated from twenty pairs of experimental units.
‡ t0·05, 18 = 1·73. If $t > 1.73$ or $t < -1.73$, the correlation coefficient was significant.

(1986). The HREs of rats fed on the diets formulated with meat, meat–spinach or spinach did not differ significantly. The only significant difference was that the HRE of non-anaemic rats in trial 2 fed on spinach Fe was lower than that of rats fed on meat or meat–spinach diets.

For rats fed on the $FeSO_4 \cdot 7H_2O$ diet, there was an inverse relation between initial Fe

Table 5. *Liver, iron, faecal Fe and apparent Fe absorption for diets with varying amounts of meat and spinach* (Spinacea oleracea *L.*) *Fe fed to severely and mildly anaemic and non-anaemic rats*

(Each value is the mean of five rats)

	Severely anaemic rats						
Proportion of meat Fe...	1000	750	500	250	0	FeSO$_4$·7H$_2$O	
Proportion of spinach Fe...	0	250	500	750	1000	diet	
Liver wt (g)							
Trial 1	3·03	3·14	2·87	3·30	3·52	3·27	
Trial 2	2·97	3·09	3·03	3·31	3·20	3·07	
Liver Fe (μg/g)							
Trial 1	26	23	24	29	25	35	
Trial 2	26	30	30	37	32	32	
Fe in faeces (mg)							
Trial 1	1·12	1·10	1·11	1·02	1·06	0·49	
Trial 2	0·94	0·91	0·94	0·97	1·02	0·43	
Apparent Fe absorption							
Trial 1	0·43	0·46	0·46	0·49	0·50	0·77	
Trial 2	0·45	0·48	0·48	0·46	0·46	0·79	
	Mildly anaemic or non-anaemic rats*						
Proportion of meat Fe...	1000	750	500	250	0	FeSO$_4$·7H$_2$O	LSD†
Proportion of spinach Fe...	0	250	500	750	1000	diet	$\alpha = 0.05$
Liver wt (g)							
Trial 1	3·22	3·32	3·04	3·06	4·03	3·75	0·42
Trial 2	3·04	3·18	3·05	3·33	3·25	3·04	0·39
Liver Fe (μg/g)							
Trial 1	25	30	34	26	31	36	10
Trial 2	37	32	34	35	37	61	12
Fe in faeces (mg)							
Trial 1	1·04	1·10	1·01	1·11	0·85	0·56	0·18
Trial 2	0·93	1·03	0·97	0·95	1·16	0·88	0·18
Apparent Fe absorption							
Trial 1	0·47	0·46	0·51	0·47	0·59	0·73	0·09
Trial 2	0·46	0·41	0·47	0·46	0·37	0·54	0·10

* Mildly anaemic rats in trial 1 and non-anaemic rats in trial 2; for details, see p. 339.

† LSD, least significant difference. Mean differences must be equal or exceed the LSD values to be statistically significant ($P < 0.05$).

status and haemoglobin gain, haemoglobin-Fe gain or HRE, as shown in Table 4. The correlation coefficients between initial haemoglobin and these three variables were −0·85, −0·80 and −0·79, respectively (Table 4). However, the correlation coefficients between initial haemoglobin and these variables were not statistically significant for rats fed on diets containing Fe from meat or spinach, with the only exception of initial haemoglobin *v.* haemoglobin gain in rats fed on the spinach diet (Table 4).

Table 6. *Relative haemoglobin regeneration efficiency (rHRE) and relative absorption of Fe (rAbs) from meat or spinach* (Spinacea oleracea L.) *diets compared with the FeSO$_4$ diet**

Proportion of meat Fe...	1000	750	500	250	0	FeSO$_4 \cdot$7H$_2$O
Proportion of spinach Fe...	0	250	500	750	1000	diet
Severely anaemic rats						
Trial 1						
rHRE	58	66	58	67	59	100
rAbs	56	60	60	64	65	100
Trial 2						
rHRE	63	62	58	62	52	100
rAbs	57	61	61	58	58	100
Mildly anaemic rats						
Trial 1						
rHRE	77	77	76	77	80	100
rAbs	64	63	70	64	81	100
Non-anaemic rats						
Trial 2						
rHRE	98	94	90	96	75	100
rAbs	85	76	87	85	69	100

* For details, see p. 340.

Liver Fe

Liver Fe values are presented in Table 5. Dietary Fe source was the factor affecting liver weight ($P < 0.001$). The livers from rats fed on the spinach diet weighed more than those from rats fed on most of the other diets (LSD 0·22 g). Liver Fe concentration was affected by both Fe status and dietary Fe source ($P < 0.001$). The concentration of liver Fe was highest in rats fed on the FeSO$_4 \cdot$7H$_2$O diet, and was particularly high in the non-anaemic rats (trial 2). There were no differences in the concentration of liver Fe among the groups of rats fed on meat, meat–spinach or spinach. Non-anaemic rats had higher liver Fe concentrations (LSD 4·3 μg/g) at the end of the experiment than severely and mildly anaemic rats. The concentration was especially higher in rats fed on the FeSO$_4 \cdot$7H$_2$O diet. However, there was no significant difference in liver Fe concentration between severely and mildly anaemic rats in any other diet groups.

Apparent Fe absorption

Both Fe status and dietary Fe source affected faecal Fe excretion and apparent Fe absorption ($P < 0.001$). Severely or mildly anaemic rats, when fed on the FeSO$_4 \cdot$7H$_2$O diet, excreted less Fe in the faeces and had a higher apparent absorption than those fed on diets with food Fe sources (Table 5). However, in non-anaemic rats, faecal Fe loss and apparent absorption were similar for the rats fed on the FeSO$_4 \cdot$7H$_2$O diet and food Fe diets. In rats fed on the FeSO$_4 \cdot$7H$_2$O diet, the initial haemoglobin level was positively related to faecal Fe excretion, and negatively related to apparent absorption (Table 4). However, these relations were not apparent in rats fed on diets with Fe from meat or spinach. Of the non-anaemic rats, those fed on spinach had the lowest apparent Fe absorption. However, all the Fe-deficient rats absorbed more Fe from spinach than from the other sources of food Fe. The pattern of HRE (Table 3) for all these animals was similar to that of apparent Fe absorption (r 0·87). Both methods are affected by Fe status and both distinguish differences in the bioavailability of Fe.

Relative HRE and relative Fe absorption

The relative HRE and relative Fe absorption of rats fed on meat, spinach or meat–spinach mixtures are shown in Table 6. The HRE of anaemic rats fed on meat or meat–spinach mixtures was about 60 % of that of rats fed on the $FeSO_4 \cdot 7H_2O$ diet. This finding is similar to that observed for meat by Jansuittivechakul *et al.* (1985, 1986) and Shah *et al.* (1983). On meat, meat–spinach and spinach diets, the mildly anaemic and non-anaemic rats had higher relative HREs than the severely anaemic rats. This is because the HRE for rats on the $FeSO_4 \cdot 7H_2O$ diet was inversely associated (r -0.79) with initial haemoglobin concentration (Table 4), but there was little or no association between initial haemoglobin concentration and HRE for rats on the meat, meat–spinach or spinach diets. Therefore, relative HRE of mildly anaemic and non-anaemic rats fed on the meat diet increased to 77 and 98 % respectively. The relative HRE values of the mildly anaemic and non-anaemic rats fed on the spinach diet increased to 80 and 75 %, respectively. The pattern of relative Fe absorption among severely and mildly anaemic and non-anaemic rats was similar to that of relative HRE values.

DISCUSSION

The effect of Fe status on Fe bioavailability

On the $FeSO_4 \cdot 7H_2O$ diet, rats with the lowest initial haemoglobin had the highest HRE and apparent Fe absorption; on the spinach diet, they were highest in the mildly anaemic rats; and on the meat diet, they were unaffected by haemoglobin level. It is possible that the ability of the severely Fe-deficient rats to absorb haem-Fe (beef) or non-haem-Fe complex (spinach) in foods may have been damaged somehow without affecting the ability to absorb simple inorganic Fe ($FeSO_4 \cdot 7H_2O$). It is also possible that both the anaemic and non-anaemic rats utilized all the metabolizable Fe available in diets containing food Fe, and that the metabolizable Fe available in the $FeSO_4 \cdot 7H_2O$ diet exceeded the requirements of the non-anaemic rats, which then absorbed (Table 5) and utilized (Table 3) less Fe than Fe-deficient rats. But this cannot explain why mildly anaemic rats had higher HRE and apparent Fe absorption than severely anaemic rats fed on the spinach diet; rather, this may be due to random experimental variations between trials 1 and 2.

Utilization of Fe from spinach and beef

The results of the present study were similar to those of Pye & MacLeod (1946), who found that anaemic rats retained 26 % of the Fe from spinach and 32 % from beef muscle. The absorption of spinach Fe relative to meat Fe in the present study was similar to that in a study involving human subjects by MacMillan & Johnston (1951). They found that subjects on a spinach diet absorbed 11·4 % of the dietary Fe, and subjects on a spinach–beef diet absorbed 9·5 % of the dietary Fe; the difference was not statistically significant. The differences in percentage Fe absorption between the animal and human studies are probably due to differences in Fe status, maturity and Fe intake relative to requirement (Thannoun *et al.* 1988). The high quantity and utilization of Fe in spinach (Pye & MacLeod, 1946; Ruegamer *et al.* 1946; McMillan & Johnston, 1951; Van Campen & Welch, 1980) means that spinach should be a good Fe source.

Effect of meat on Fe absorption

Meat is a high-quality Fe source (Cardon *et al.* 1980; Jansuittivechakul *et al.* 1985, 1986; Thannoun *et al.* 1987) that enhances the absorption of non-haem-Fe from other components of a meal (Layrisse *et al.* 1968, 1984; Cook & Monsen, 1976). Most of the
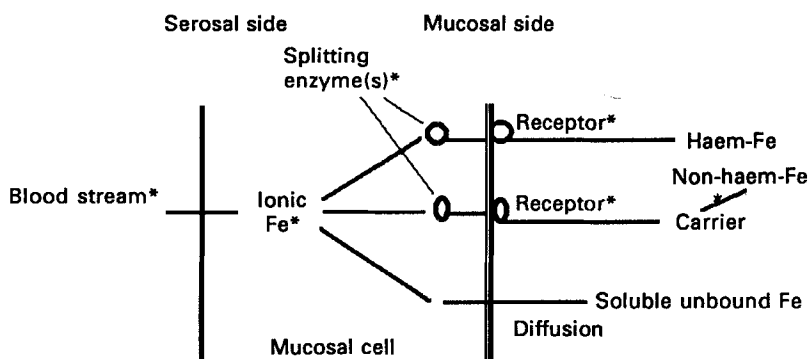
Fig. 1. Suggested mechanism of iron absorption (three Fe pool hypothesis). * Control points for Fe absorption.

experiments showing this enhancement involved measuring absorption of extrinsic radio-Fe labels. This method measures only the absorption of non-haem-Fe, not the total Fe in food, and involves one acute dose over a very short period of time. This technique would not indicate whether the absorption of haem-Fe in meat was reduced when the absorption of non-haem-Fe was enhanced. Martínez-Torres & Layrisse (1971) used a dual radioisotope technique and found that non-haem-Fe absorption from maize (*Zea mays*) and black bean (*Phaseolus nigra*) increased by 87 and 121 % respectively when meat was added to the meal. However, the absorption of Fe from meat was reduced. Total dietary Fe absorption was slightly lower when the meal contained maize and meat or black beans and meat.

Similar to our findings with rats, McMillan & Johnston (1951) concluded that meat did not affect absorption of spinach Fe by young women. They found that Fe absorption increased in the first week when meat was given with spinach, but then decreased to a level lower than that from the spinach diet during the following 3 weeks. Thus, short-term experiments, especially those involving a single dose, may not accurately reflect actual Fe absorption.

Hypothesis of a third Fe pool

In our experiment, the pattern of Fe absorption from the different Fe sources indicates that the commonly accepted non-haem-Fe pool may be separated into two: a simple inorganic non-haem-Fe salt pool and an organically complexed non-haem-Fe pool (Fig. 1). Gitlin & Cruchaud (1962) suggested that two different mechanisms of Fe absorption might operate simultaneously in the gastrointestinal lumen: (1) a process in which absorption is limited by the amount of carrier, and (2) a first-order process governed by the amount of absorbable Fe in the gastrointestinal lumen. They also suggested that diffusion, a first-order process, may be one of the mechanisms of control. Geisser & Müller (1987) reported that ferrous sulphate and ferric-hydroxide polymaltose (ferripolymaltose) appear to be governed by totally different Fe absorption or distribution mechanisms, or both; the absorption of ferrous sulphate may depend on passive diffusion, which is only limited by the membrane surface area and the Fe concentration gradient, while the absorption of ferripolymaltose may depend on active transport, which is energy dependent.

Non-haem-Fe complexes (such as Fe in spinach or ferripolymaltose) need to be bound to a carrier protein which may limit Fe absorption either by differences in binding of the carrier with the Fe complex, or by differences in movement of the Fe-complex-loaded carrier across the mucosal cell membrane. Haem-Fe is absorbed intact by the mucosal cells

via a carrier system. In severe Fe deficiency, the function of the carrier system may be reduced by pathological factors, such as reduced Fe enzyme activity, reduced energy supply or low O_2 supply. Thus, the absorption of both non-haem- and haem-Fe complexes may be affected by the carrier system that allows Fe to pass through the membrane into mucosal cells. However, highly soluble, simple inorganic Fe salts (such as $FeSO_4 \cdot 7H_2O$) diffuse into mucosal cells and are not affected by the carrier system. Once in the mucosal cells, all three Fe pools seem to enter the bloodstream via the same pathway, which is regulated by Fe requirements. Fe in excess of the body requirement is trapped as ferritin and discarded when the mucosal cells exfoliate. The portion of Fe needed by the body is bound to transferrin, which is released into the blood.

Relative Fe bioavailability (RIBA)

RIBA is the Fe bioavailability of a test source expressed as a percentage of the Fe bioavailability of a reference material, usually ferrous sulphate (100%). This makes it possible to control for extraneous dietary and environmental factors that may affect Fe bioavailability and to rate Fe sources. This approach assumes that factors affecting the absorption of Fe in food will also have the same effect on the absorption of reference Fe. However, this may not always be true. In our experiments, severe Fe depletion increased the amount of Fe that rats absorbed from a diet containing $FeSO_4 \cdot 7H_2O$, but did not increase absorption of Fe from food sources (Table 5). The higher RIBA in the non-anaemic rats was due to a decreased absorption of Fe from $FeSO_4 \cdot 7H_2O$, not an increased absorption of Fe from foods. The HRE of non-anaemic rats was similar regardless of whether diets contained Fe from $FeSO_4 \cdot 7H_2O$ or from foods. However, the HRE of anaemic rats was much higher when diets contained $FeSO_4 \cdot 7H_2O$. If, as we hypothesize, $FeSO_4 \cdot 7H_2O$-Fe is not in the same gastrointestinal pool as non-haem-Fe complex, calculation of RIBA would give confusing results. Thus, an Fe complex may be a better reference material for determining RIBA of foods than $FeSO_4 \cdot 7H_2O$.

REFERENCES

Agricultural Research Service, United States Department of Agriculture (1963). *Composition of Foods*. Agriculture Handbook no. 8. Washington DC: US Government Printing Office.

Association of Official Analytical Chemists (1980). *Official Methods of Analysis*, 13th ed. Washington DC: Association of Official Analytical Chemists.

Atherton, H. V. & Newlander, J. A. (1982). *Chemistry and Testing of Dairy Products*, 4th ed., pp. 105–116. Westport, Connecticut: AVI Publishing Company Inc.

Beutler, E. (1980). Iron. In *Modern Nutrition in Health and Disease*, 6th ed., p. 136 [R. S. Goodhart and M. E. Shils, editors]. Philadelphia: Lea & Febiger.

Björn-Rasmussen, E. & Hallberg, L. (1979). Effect of animal proteins on the absorption of food iron in man. *Annals of Nutrition and Metabolism* 23, 192–202.

Björn-Rasmussen, E., Hallberg, L. & Walker, R. B. (1973). Food iron absorption in man II: isotopic exchange of iron between labelled foods and between a food and an iron salt. *American Journal of Clinical Nutrition* 26, 1311–1319.

Cardon, K. M., Anthony, R. J., Hendricks, D. G. & Mahoney, A. W. (1980). Effect of atmospheric oxidation on bioavailability of meat iron and liver weights in rats. *Journal of Nutrition* 110, 567–574.

Carma, S. G. & Swanson, M. R. (1973). An evaluation of ten pairwise multiple comparison procedures by Monte Carlo methods. *Journal of the American Statistical Association* 68, 66–74.

Cook, J. D. & Monsen, E. R. (1976). Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. *American Journal of Clinical Nutrition* 29, 859–867.

Dowdy, S. & Wearden, S. (1983). *Statistics for Research*. New York: John Wiley & Sons, Inc.

Fiske, C. H. & Subbarow, Y. (1925). The colorimetric determination of phosphorus. *Journal of Biological Chemistry* **66**, 375–400.

Geisser, P. & Müller, A. (1987). Pharmacokinetics of iron salts and ferric hydroxide carbohydrate complexes. *Arzneim.-Forsch./Drug Research* **37**, 100–104.

Gillooly, M., Torrance, J. D., Bothwell, T. H., Macphail, A. P., Derman, D., Mills, W. & Mayet, F. (1984). The relative effect of ascorbic acid on iron absorption from soy-based and milk-based infant formulas. *American Journal of Clinical Nutrition* **40**, 522–527.

Gitlin, D. & Cruchaud, A. (1962). On the kinetics of iron absorption in mice. *Journal of Clinical Investigation* **41**, 344–350.

Jansuittivechakul, O., Mahoney, A. W., Cornforth, D. P., Hendricks, D. G. & Kangsadalampai, K. (1985). Effect of heat treatment on bioavailability of meat and hemoglobin iron fed to anemic rats. *Journal of Food Science* **50**, 407–409.

Jansuittivechakul, O., Mahoney, A. W., Cornforth, D. P., Hendricks, D. G. & Sisson, D. V. (1986). Effect of heat treatment on meat enhancement of dietary iron bioavailability of meat/ferrous sulfate and meat/hemoglobin mixtures fed to anemic rats. *Journal of Food Science* **51**, 263–267.

Latunde-Dada, G. O. & Neale, R. J. (1986). Effect of soya-bean protein on meat iron solubility and absorption in rats. *British Journal of Nutrition* **55**, 419–426.

Layrisse, M., Cook, J. D., Martínez-Torres, C., Roche, M., Kuhn, I. N., Walker, R. B. & Finch, C. A. (1969). Food iron absorption: a comparison of vegetable and animal foods. *Blood* **33**, 430–443.

Layrisse, M., Martínez-Torres, C., Leets, I., Taylor, P. & Ramírez, J. (1984). Effect of histidine, cysteine, glutathione or beef on iron absorption in humans. *Journal of Nutrition* **114**, 217–223.

Layrisse, M., Martínez-Torres, C. & Roche, M. (1968). Effects of interaction of various foods on iron absorption. *American Journal of Clinical Nutrition* **21**, 1175–1183.

McMillan, T. J. & Johnston, F. A. (1951). The absorption of iron from spinach by six young women, and the effect of beef upon the absorption. *Journal of Nutrition* **44**, 383–398.

Mahoney, A. W. & Hendricks, D. G. (1976). Effect of dietary iron level on efficiency of converting food iron into hemoglobin by the anemic rat. *Nutrition Metabolism* **20**, 222–227.

Mahoney, A. W. & Hendricks, D. G. (1982). Efficiency of hemoglobin regeneration as a method of assessing iron bioavailability in food products. In *Nutritional Bioavailability of Iron*, pp. 1–11 [C. Kries, editor]. Washington DC: American Chemical Society.

Mahoney, A. W., Van Orden, C. C. & Hendricks, D. G. (1974). Efficiency of converting food iron into hemoglobin by the anemic rat. *Nutrition Metabolism* **17**, 223–230.

Martínez-Torres, C. & Layrisse, M. (1971). Iron absorption from veal muscle. *American Journal of Clinical Nutrition* **24**, 531–540.

Miller, J. (1982). Factors affecting evaluation of relative biological value of dietary iron for rats. *Journal of Nutrition* **12**, 2381–2385.

Pye, O. F. & MacLeod, G. (1946). The utilization of iron from different foods by normal young rats. *Journal of Nutrition* **32**, 677–687.

Ruegamer, W. R., Michaud, L., Hart, E. B. & Elvehjem, C. A. (1946). The use of the dog for studies on iron availability. *Journal of Nutrition* **32**, 101–111.

Shah, B. G., Giroux, A. & Belonje, B. (1983). Bioavailability of iron in ground beef and plant protein concentrates. *Nutrition Research* **3**, 547–555.

Thannoun, A. M., Mahoney, A. W., Hendricks, D. G. & Zhang, D. (1987). Effect of meat–bread mixtures on bioavailability of dietary iron for anemic rats. *Cereal Chemistry* **64**, 399–403.

Thannoun, A. M., Mahoney, A. W., Sisson, D. V. & Hendricks, D. G. (1988). Heme and nonheme iron absorption from meat and meat loaf by anemic and healthy rats. *Nutrition Reports International* **37**, 487–497.

Timm, K. (1979). Orbital venous anatomy of the rat. *Laboratory Animal Science* **29**, 636–638.

Turnbull, A. (1974). Iron absorption. In *Iron in Biochemistry and Medicine*, pp. 369–403 [A. Jacobs and M. Worwood, editors]. Orlando, FL: Academic Press.

Van Campen, D. & Welch, R. M. (1980). Availability to rats of iron from spinach: effects of oxalic acid. *Journal of Nutrition* **110**, 1618–1621.

Weintraub, L. R., Weinstein, M. B., Huser, H. & Rafal, S. (1968). Absorption of hemoglobin iron: the role of a heme-splitting substance in the intestinal mucosa. *Journal of Clinical Investigation* **47**, 531–539.

Wollenberg, P. & Rummel, W. (1987). Dependence of intestinal iron absorption on the valency state of iron. *Naunyn-Schmiedeberg's Archives of Pharmacology* **336**, 578–582.