# The influence of previous iron intake on the estimation of bioavailability of Fe from a test meal given to rats

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1. Iron retention and its subsequent distribution from 3 g $^{59}$Fe extrinsically-labelled wholewheat flour in rats given a low (8 μg Fe/g)- or high (566 μg Fe/g)-Fe diet during the previous 3 d was measured (Expt 1). The mean (with SE) proportion of Fe retained from wholewheat flour was 0·46 (0·04) in the group given the low-Fe diet and 0·15 (0·01) in the group given the high-Fe diet ($P < 0·001$). There was no difference in distribution of absorbed $^{59}$Fe in the tissues examined.

2. The procedure was repeated in rats given diets containing a range of Fe concentrations, groups 1–6 respectively: 8, 77, 136, 334, 566, 1270 μg Fe/g (Expt 2). The mean (with SE) proportions of Fe retained in groups 1–6 respectively were 0·60 (0·02), 0·34 (0·02), 0·30 (0·02), 0·20 (0·20), 0·17 (0·02), 0·09 (0·01). Regression analysis showed that Fe retention was a function of the logarithm of the Fe concentration of the diet consumed before the test meal ($R -0·997$, $P < 0·0001$) where Fe retained (μg) $= 95 - 28 \log_{10}$ Fe concentration of diet.

3. Rats were given a low-, medium- or high-Fe diet (8, 136 or 1270 μg Fe/g respectively) for 1 or 2 d instead of 3 d before measuring Fe retention from 3 g wholewheat flour (Expt 3). The mean (with SE) proportions of Fe retained in rats given the low-, medium- or high-Fe diets for 1 d were 0·45 (0·02), 0·25 (0·02) and 0·13 (0·01) ($P < 0·001$) and for 2 d 0·47 (0·03), 0·31 (0·03) and 0·18 (0·02) ($P < 0·001$). There were no significant differences in Fe retention from animals given similar diets for 1 or 2 d before the test meal. As in Expt 2 regression analysis showed a highly-significant relationship between Fe retention and the logarithm of the previous dietary Fe concentration in both sets of groups.

4. It was concluded that the amount of Fe retained by rats from a 3 g meal of wholewheat flour depended on the previous dietary Fe intake. Only one meal in the previous 24 h was required to influence the 'mucosal setting' since there was no difference in response between rats preloaded for 1 or 2 d before the test meal.

One of the most accurate and widely used techniques to estimate bioavailability of iron in foods involves measuring absorption from the food (labelled with an Fe isotope) and comparing it with absorption from a reference salt such as ferrous sulphate. In this way each subject acts as his/her own control and if the results are expressed as the ratio, food Fe:ferrous sulphate, inter-individual differences in absorptive capacity are theoretically removed. It is usual practice to administer the test substances to subjects on a self-selected, uncontrolled diet after an overnight fast and, if given within a few days of each other, it is assumed that the subject's capacity to absorb Fe has not significantly altered. This assumption is important because Fe absorption is greatly influenced by Fe status (Bothwell *et al.* 1958).

One of the prime regulatory steps in the mechanism of Fe absorption appears to be the initial passage of Fe from the lumen across the brush-border into the mucosal cells, and the amount taken up will inevitably depend on the number of Fe-binding sites available. There is some evidence that the transfer of Fe across the serosal surface of the cell into the blood is the primary site of control of Fe entry into the body (Linder *et al.* 1975) as first postulated by Wheby & Crosby (1963). However, the number of Fe-binding sites in brush-border preparations is increased with Fe-deficiency and decreased by Fe-loading after only 1 or 2 d (Greenberger *et al.* 1969), when a new group of cells with different receptor populations has been formed in the crypts of the mucosa. This suggests that the extent of Fe-loading of the mucosal cells is an important factor influencing uptake of Fe from the lumen. The experiments described here are a preliminary attempt to investigate this

hypothesis in order evaluate critically and improve on the current technique of measuring Fe availability.

## MATERIALS AND METHODS

Three experiments were performed on rats: (a) Expt 1 was designed to determine whether the Fe content of a diet given for 3 d (equivalent to the mean mucosal cell turnover time) influenced the subsequent retention of Fe from a test meal and to measure the distribution of retained Fe within the body; (b) Expt 2 was designed to establish whether a quantitative relationship existed between Fe retention and previous Fe intake; (c) Expt 3 was designed to evaluate the effects of shorter time-periods of different dietary Fe intake on subsequent Fe retention from a test meal.

In all experiments the rats (male Wistars, bought from suppliers at 60–100 g body-weight) were trained to meal-feed for at least 2 weeks on a semi-synthetic diet as described in Table 1, and any that did not consume at least three-quarters of the $^{59}$Fe-extrinsically labelled test meals were excluded from the experiment. Three hours after the test meal they were all given a semi-synthetic diet *ad lib.* Whole-body Fe retention was calculated by measuring the difference in the $^{59}$Fe content of the body immediately post-dosing and 7 d later when all the unretained Fe from the test meal had been excreted.

### *Expt 1*

Twenty rats were given one meal daily of a semi-synthetic diet of low (8 $\mu$g Fe/g)- or high (566 $\mu$g Fe/g)-Fe content (prepared by adjusting the level of ferrous sulphate added to the mineral mix) for 3 d. After a 24 h fast they were given 3 g wholewheat flour (containing 40 $\mu$g Fe/g) made to a paste with distilled water and extrinsically labelled with approximately 0·5 $\mu$Ci $^{59}$Fe ($FeCl_3$, Amersham International, Amersham). After consuming the meal their whole-body radioactivity was monitored in a small-animal counter (NE 8112, Nuclear Enterprises, Edinburgh) and again at regular intervals until the unabsorbed Fe from the $^{59}$Fe-labelled flour had been totally excreted, i.e. the fall in whole-body $^{59}$Fe activity was less than 1% of the original level, and then for a further 7 d. During this period they were all given the control diet described in Table 1. Faeces were collected and monitored for $^{59}$Fe activity at the same time as counting radioactivity in the animal. At the end of the experiment (14 d after the test meal) the rats were killed under diethyl ether anaesthesia and the liver, heart, spleen, kidneys and small intestine removed for counting to measure the distribution of absorbed Fe.

### *Expt 2*

Ninety-six rats were split into six groups of sixteen and given meals of a semi-synthetic diet containing 8, 77, 136, 334, 566 or 1270 $\mu$g Fe/g *ad lib.* for 3 d. After 24 h they were given 3 g $^{59}$Fe-labelled wholewheat flour and Fe absorption was measured as described in Expt 1. Three hours after the test meal they were all given the control diet (Table 1). On day 7 after the test meal haemoglobin (Hb) and packed cell volume (PCV) were measured in whole heparinized blood taken by cardiac puncture.

### *Expt 3*

Ninety-six rats were split into six groups of sixteen. Groups 1–3 were given a 15 g meal of a semi-synthetic diet containing 8, 136, or 1270 $\mu$g Fe/g respectively. Groups 4–6 were given similar meals for 2 d instead of 1 d, group 4 the low-Fe diet, group 5 the medium-Fe diet and group 6 the high-Fe diet. After 24 h they were given a test meal of $^{59}$Fe-labelled wholewheat flour and Fe absorption was measured as before. Three hours after the test meal they were all given the control diet (Table 1).

Table 1. *Composition (g/kg) of semi-synthetic control diet*

| | |
|---|---|
| Starch | 326 |
| Sucrose | 326 |
| Casein | 168 |
| Mineral mix* | 40 |
| Vitamin mix† | 20 |
| Solka floc | 40 |
| Maize oil | 80 |

* Mineral mix (g/kg diet): $CaHPO_4$ 13·0, $CaCO_3$ 8·2, KCl 7·03, $Na_2HPO_4$ 7·4, $MgSO_4 . H_2O$ 4·0, $MnSO_4 . H_2O$ 0·18, $ZnCO_3$ 0·10, $FeSO_4 . 7H_2O$ 0·144, $CuSO_4$ 0·015, $KIO_3$ 0·001.

† Vitamin mix (mg/kg diet): Nicotinic acid 60, cyanocobalamin in mannitol 50, calcium-D-pantothenate 40, thiamin hydrochloride 10, riboflavin 10, pyridoxine 10, pteroylmonoglutamic acid 5, D-biotin 1, menadione 1, Rovimix E50 (Roche) 150, Rovimix A-500 (Roche) 25, Rovimix $D_3$500 (Roche) 15, choline bitartrate 1800.

### *Use of the whole-body counter*

The NE 8112 small animal 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. Four 130 mm photomultipliers collect the light output from the outside face of the well-bottom. The whole assembly is shielded by lead with an outer casing of low-activity steel. Rats were placed in a ventilated perspex box 240 mm 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 is not more than 1% of the over-all mean, and reproducibility is better than 99%. The time required for counting is normally less than 1 min and the counting efficiency for $^{59}Fe$ is approximately 50%.

### *Atomic absorption spectrophotometry*

Diets were analysed for total Fe by ashing in silica crucibles at 480° for 48 h, taking the ash up in warm concentrated hydrochloric acid and diluting to an appropriate volume with distilled water. The Fe content of the resultant solution was measured by flame spectrophotometry using a Varian Atomic Absorption Spectrophotometer (Varian Associates, Walton-on-Thames) with background correction.

### *Hb and PCV*

The blood Hb was measured using an AO haemoglobinometer (American Optical Corporation, Buffalo, New York) and PCV by the microhaematocrit method.

### *Statistical calculations*

Differences between groups were compared using unpaired Student's *t* test (Snedecor & Cochrane, 1973). One way analysis of variance was performed on values from Expt 3 and regression analysis on values from Expts 2 and 3 using the Biomedical Data Package computer program (University of California, 1981).

## RESULTS

### *Expt 1*

The mean (with SE) weights (g) of rats given the low- and high-Fe diets were 119 (4) and 114 (3) respectively on the day of the test meal. Fe retention from the test meal of 3 g wholewheat flour is shown in Fig. 1. The proportion of Fe retained (mean with SE) was 0·46 (0·04) in the group given the low-Fe diet and 0·15 (0·01) in the group given the high-Fe diet.

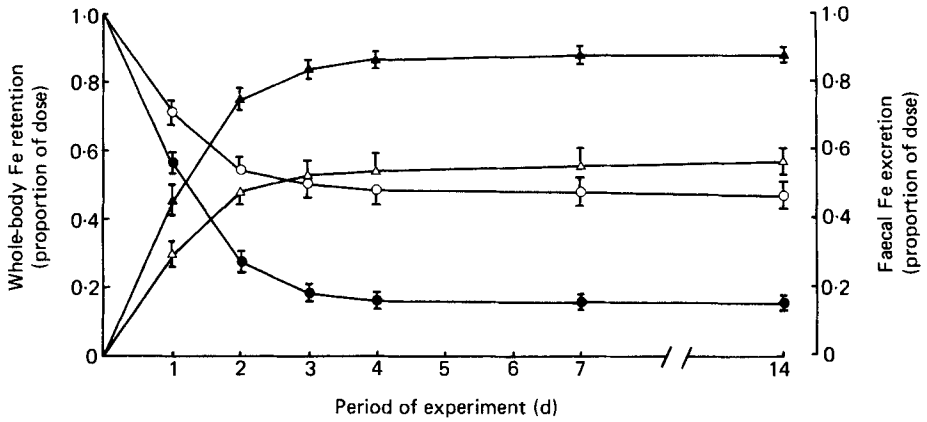


Fig. 1. Expt 1. Whole-body iron retention (○, ●) and cumulative faecal excretion (△, ▲) from 3 g wholewheat flour extrinsically labelled with $^{59}$Fe (proportion of dose) in rats given a low-Fe 8 $\mu$g Fe/g (○, △) or high-Fe, 566 $\mu$g Fe/g (●, ▲) diet for 3 d before the test meal. Points are mean values with their standard errors represented by vertical bars.

Table 2. *Expt 1. Distribution of absorbed $^{59}$Fe (proportion of total amount present in body) in various tissues*

(Mean values with their standard errors)

| | Low-Fe diet | | High-Fe diet | |
|---|---|---|---|---|
| | Mean | SE | Mean | SE |
| Liver | 0·076 | 0·004 | 0·072 | 0·005 |
| Kidneys* | 0·015 | | 0·013 | |
| Heart* | 0·008 | | 0·008 | |
| Spleen* | 0·006 | | 0·007 | |
| Small intestine* | 0·006 | | 0·006 | |

* Measured as a group rather than individually.

This difference was significant ($P < 0{\cdot}001$). The distribution of $^{59}$Fe in the various tissues is shown in Table 2 where it can be seen that there were no differences between the two groups when counts were expressed as a proportion of the whole-body level.

*Expt 2*

The mean (with SE) weights of rats from each group on the 1st day of the different diets were as follows (g): group 1, 190 (4); group 2, 196 (4); group 3, 188 (5); group 4, 202 (5); group 5, 187 (4); group 6, 194 (4). The proportions of Fe retained (mean with SE) from the wholewheat flour were as follows: group 1, 0·60 (0·02); group 2, 0·34 (0·02); group 3, 0·30 (0·02); group 4, 0·20 (0·02); group 5, 0·17 (0·02); group 6, 0·09 (0·01). It was shown by regression analysis that Fe retention from the test meal was a function of the logarithm of the Fe concentration of the diet consumed during the 3 d before the test meal ($R - 0{\cdot}997$, $P < 0{\cdot}0001$), as shown in Fig. 2, where the Fe retained ($\mu$g) $= 95 - 28 \log_{10}$ Fe concentration of diet. This relationship was highly significant over the range of Fe concentrations studied, i.e. 8–1270 $\mu$g Fe/g diet. Although the animals were allowed *ad lib.* access to food for 1 h

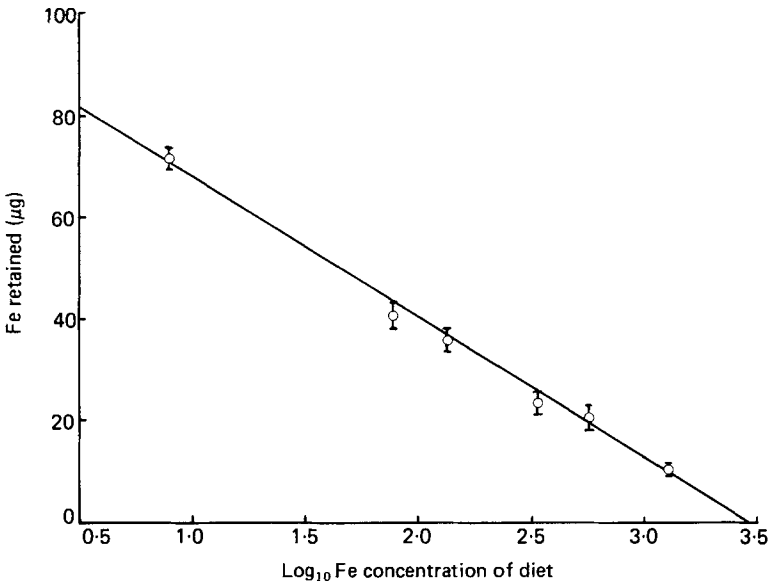


Fig. 2. Whole-body iron retention ($\mu$g) from 3 g wholewheat flour in rats given diets of different Fe concentration (8, 77, 136, 334, 566 or 1270 $\mu$g Fe/g) before the test meal. Points are mean values with their standard errors represented by vertical bars.

Table 3. *Expt 3. Proportion of iron retained from 3 g wholewheat flour*
(Mean values)

| Group* | Proportion of Fe retained |
|---|---|
| 1 | 0·45[a] |
| 2 | 0·25[b] |
| 3 | 0·13[c] |
| 4 | 0·47[a] |
| 5 | 0·31[b] |
| 6 | 0·18[c] |
| SEM | 0·016 |

[a, b, c] Values with different superscript letters were significantly different ($P < 0{\cdot}01$ for groups 2 and 6, $P < 0{\cdot}001$ for all others).

* Groups 1–3: one meal containing 8, 136 or 1270 $\mu$g Fe/g respectively before the test meal. Groups 4–6: two meals containing 8, 136 or 1270 $\mu$g Fe/g respectively before the test meal.

daily they ate very similar amounts over the 3 d, the mean (with SE) daily intake being 13 (0·2) g.

There were no differences between the groups' Hb and PCV values 7 d after the test meal. The mean Hb (with SE) was 14·7 (0·1) and PCV 0·44 (0·004).

*Expt 3*

The mean (with SE) weights of rats given the different diets were as follows (g): group 1, 393 (9); group 2, 392 (5); group 3, 409 (11); group 4, 412 (15); group 5, 396 (9); group 6, 396 (12). Nearly all the 15 g meal was eaten by all but two rats: the mean intake was

14·6 g/d. One animal from group 1 and one from group 4 were excluded because they did not consume the 15 g meal offered to them.

The proportions of Fe retained from the 3 g test meal of wholewheat flour are shown in Table 3. There was a highly-significant difference in Fe retention from the test meal in animals given diets of different Fe concentration on either the previous day or on the previous 2 d, but there were no differences between animals given the same diet for 1 or 2 d. As in Expt 2 regression analysis showed a highly-significant relationship between mean Fe retention and the logarithm of the previous dietary Fe concentration. In groups 1–3, Fe retained ($\mu$g) = $69 - 17 \log_{10}$ Fe concentration of diet ($\mu$g/g) ($R - 0{\cdot}998$, $P < 0{\cdot}05$) and, in groups 4–6, Fe retained ($\mu$g) = $71 - 16 \log_{10}$ Fe concentration of diet ($\mu$g/g) ($R - 0{\cdot}999$, $P < 0{\cdot}05$).

## DISCUSSION

These experiments clearly demonstrate that the amount of Fe retained by rats from a test meal of wholewheat flour depends on the previous dietary Fe intake. The amount retained closely followed a log-linear relationship and was dependent on the age of the animals. For example, a greater proportion of Fe was absorbed from the test meal in rats in groups 1 and 4 of Expt 2 (mean weights 190 and 202 g) than in rats in groups 1 and 4 of Expt 3 (mean weights 393 and 412 g), hence the different coefficients of regression found in Expts 2 and 3.

It was only necessary to feed the animals one meal of different Fe content 24 h before the test meal to obtain a significant difference in response. Prefeeding for longer periods did not affect the magnitude of the response. The reason for this effect cannot be deduced from these experiments but if the results in rats are applicable to man then the implication is that some Fe absorption studies reported in the literature may have been subject to misinterpretation. Day-to-day variations in individuals have been noted (Moore, 1964; Callender, 1970) and could be related to differences in previous dietary Fe intake. Further work is required to test the hypothesis in man.

Hegenauer *et al.* (1977) have suggested that the previous dietary Fe content regulates subsequent Fe absorption not by affecting the level of an Fe transport system but by affecting the biosynthesis of certain haem-containing microsomal monoxygenases in the intestinal mucosa. They found an immediate response of the absorptive capacity in mice to a change in dietary Fe concentration which reached its new level within 24 h. Pollack *et al.* (1964), on the other hand, found that it was necessary to deprive rats of Fe for 5 d before getting a change in Fe absorption. Since the mean turnover time for mucosal cells in rats is 3 d (Quastler *et al.* 1959) the 5 d time-period required to change Fe absorption is probably unrelated to mucosal cell loading or number of Fe-binding sites, or both, and may in fact be the result of a short-term change in body Fe status. Changes in the absorptive capacity for Fe in shorter time-periods are much more likely to be caused by changes in mucosal Fe-binding sites.

Pearson *et al.* (1967) showed that the Fe content of rat gut varied with the Fe content of the diet, with the more proximal sections of the gut being more sensitive to the dietary Fe level than the distal portions. The Fe concentration in the total gut did not relate linearly to the Fe content of the diet over the range studied (20–2500 $\mu$g Fe/g diet) and the gut Fe levels of rats given a diet containing 200 $\mu$g Fe/g were the same as those given a diet containing 2500 $\mu$g Fe/g. They suggested that the Fe content of mucosal cells has an important local influence on the amount of Fe actually absorbed but they did not find a stochiometric relationship between gut Fe levels and percentage absorption. In contrast, the results from Expts 2 and 3 showed a highly-significant relationship between the logarithm of the previous dietary Fe concentration and the amount of Fe ($\mu$g) retained from

the test meal, although as the gut Fe levels were not measured the results cannot be directly compared with those of Pearson *et al.* (1967). There is also the additional problem of possible alteration in specific activity of the Fe in the mucosal cells depending on the amount of Fe already present in the cells. Further work is required to assess whether this takes place and to what extent it influences the interpretation of results of studies using a tracer to label food Fe in order to measure bioavailability.

The initial objective of these studies was to test the hypothesis that differences in Fe absorption exist depending on the previous Fe intake, and to use any information obtained to improve on the techniques of Fe absorption measurement. In view of the results described in the present paper it is probable that the current practice of standardizing conditions in Fe availability studies by fasting subjects overnight before giving test substances is wholly inadequate. Indeed, Solomons *et al.* (1983) found that consecutive-day administration of therapeutic doses of Fe to human subjects progressively reduced the rise in plasma Fe following a dose of $FeSO_4$, although alternate-day dosing had no effects on the plasma Fe response to $FeSO_4$. Once enough information has been obtained regarding the effect of previous Fe intake on mucosal uptake of Fe it should be possible to improve on the standardization procedure by controlling dietary Fe intakes for the requisite time-period, which may only be 24 h, before administration of test substances. Further research is required to establish the time-span of the effect in man and to investigate the mechanisms involved.

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