Measurement of chemically-available iron in foods by incubation with human gastric juice in vitro

BY SUSAN LOCK AND A. E. BENDER

Department of Food Science and Nutrition, Queen Elizabeth College, University of London, Campden Hill, London W8 7AH

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1. The proportion of iron liberated from twenty foods was measured after incubation in vitro with human gastric juice.

2. Results with samples of gastric juice obtained from one subject on ten occasions and from sixteen subjects on a single occasion showed good agreement, the proportions of Fe liberated covering the entire range from zero for egg to 1·0 for wine.

3. Where there were small variations between the results obtained with different samples of gastric juice attempts were made to correlate these with peptic activity, various proteins and mucoproteins, pH and total and free acid; the only factors that varied with the proportions of Fe solubilized were the various measures of acidity.

4. The results correlated highly with those of Layrisse *et al.* (1969) for the in vivo absorption of Fe from similar foods, although the samples used were not the same.

5. The amounts of Fe solubilized when the foods were mixed with bran, oats or egg (known to reduce the in vivo absorption of Fe from foods) were less than calculated from the sum of each, with two exceptions.

6. The results obtained with human gastric juice differed from those obtained with in vitro methods previously reported, namely treatment with dilute hydrochloric acid and double incubation with pepsin plus HCl at pH 2·5 followed by adjustment to pH 7·5.

7. The amount of Fe solubilized from soya-bean flour by gastric juice was compared with that solubilized by pepsin plus HCl; both systems were shown to be pH dependent but the amount of Fe liberated with pepsin-HCl was much greater than that known to be absorbed by man in vivo.

8. It is suggested that incubation with human gastric juice may permit in vitro analyses of 'chemically-available' Fe in foods and thus be of value in food composition tables in terms of 'Fe equivalents' even though the amounts absorbed in vivo are subsequently influenced by other ingredients of the diet and by the Fe status of the individual.

The problem of determining the proportion of iron that is absorbed from the diet, biologically available, is well recognized. Three distinct groups of factors are involved: (1) the chemical form of the Fe in the food, about which little is known, apart from haem-Fe in meat; (2) the presence in the intestine of other food components, such as fructose, ascorbic acid and cysteine which increase the amount of Fe absorbed, and oxalate, phosphate and phytate which reduce the amount absorbed; (3) the physiological status of the individual, such as the size of Fe stores and possibly the acidity of the gastric juice.

Consequently, it is not surprising to find that even carefully conducted experiments such as those of Layrisse & Martinez-Torres (1971) carried out under similar conditions on identical foods show a wide range in Fe absorption. For example, the proportion of Fe absorbed from soya bean ranged from 0·002 to 0·422 in a group of twenty-eight individuals.

Attempts have been made in the past to measure chemical availability by determining the proportion of ionizable Fe in the ferrous state (believed to be biologically available) extracted in water (Kohler *et al.* 1936) and saline (Sanford, 1960). More recently the solubility of Fe in foods has been measured in pepsin and hydrochloric acid (Jacobs & Greenman 1969) and in pepsin-HCl adjusted to pH 6–7 (Mg-Mg-Thwin *et al.* 1975; Narasinga Rao & Prabhavathi, 1978).

In the present work it was shown that incubation of a range of foods with human gastric juice liberated Fe in amounts similar to those generally regarded as biologically available.

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METHODS

Preparation of food samples

The foods were prepared in the form, raw or cooked, in which they would be eaten and if cooked, they were dried at 50° before being ground to pass through a sieve (no. 30).

Almonds, ground (Safeway Stores)	Untreated
Wheat bran (Prewett's)	Untreated
Cocoa powder (Rowntrees)	Untreated
Curry powder (British Pepper & Spice Co.)	Untreated
Eggs	Boiled hard
Lentils, *Lens esculenta*, Lebanese (Safeway Stores)	Boiled for 1 h in 10 vol. water
Oats (Porage; Scotts)	Boiled for 5 min in 3 vol. water
Peas, *Pisum sativum* (Mushy Processed; Batchelors)	Boiled for 5 min
Soya-bean flour (defatted)	Boiled for 5 min in 10 vol. water
Spinach, *Spinacea oleracea* (canned puree; Smedley)	Boiled for 5 min
Watercress, *Nasturtium officinale*, fresh	Freeze-dried
Wine (Tonic, without added iron; Sanatogen)	
Black beans, *Phaseolus vulgaris*	Soaked overnight and boiled for 2 h in 10 vol. water
Mackerel, *Scomber scombrus*	Grilled, dried, defatted with acetone
Lettuce, *Lactuca sativa*, fresh	Freeze-dried
Liver, ox	Fried for 10 min
Maize flour	Boiled for 5 min in 5 vol. water
Rice, white	Boiled for 20 min
Veal muscle	Fried for 10 min
Wheat (wholemeal flour)	Boiled for 5 min in 5 vol. water

Estimation of total Fe

Samples were ashed in a muffle furnace at 450° for 18 h. At this temperature losses of Fe are negligible (Basson & Böhmer, 1972). The ash was dissolved in 5 ml warm concentrated HCl (artisar) and the solution and washings made up to 25 ml with distilled water: final strength of acid 2 M.

Fe was determined by atomic absorption spectrophotometry (AAS) using Fe standards containing similar amounts of HCl.

Collection of gastric juices

Subjects presented themselves for gastric juice collection after an overnight fast. A Ryles tube was passed into the stomach and the subject then swallowed 500 ml distilled water. After 15–20 min the gastric contents were removed with the aid of a syringe and filtered through Whatman no. 41 filter paper into a plastic container. The volume of gastric juice collected varied from 50 to 500 ml depending on gastric emptying time and the extent of discomfort of the subject.

In vitro gastric digestion

Duplicate 0·2 g portions of the foods were incubated in plastic centrifuge-tubes with 5 ml gastric juice at 37° for 1·5 h. The mixture was then centrifuged at 18000 *g* to precipitate any

ferric hydroxide that might have been present in suspension. The supernatant fraction was removed for determination of solubilized Fe by AAS.

Analysis of gastric juice

pH was measured using a Corning EEL pH meter. Free acid was determined by titration with 0·1 M-sodium hydroxide using Topfers reagent, and total acid using phenolphthalein indicator. Peptic activity was assayed by measurement of the concentration of peptides released from haemoglobin substrate when incubated for a fixed time with gastric juice (Anson, 1938). One unit of activity is equivalent to the absorbance resulting from reaction of the reagent with 0·001 M-tyrosine.

Fe content was estimated using AAS.

Proteins and mucoproteins were determined by electrophoresis on polyacrylamide gel by a modification of the method of Davis *et al.* (1969). Samples of gastric juice were applied to polyacrylamide gels together with cytochrome C as a marker and the proteins and mucoproteins separated by sodium decyl sulphate electrophoresis.

The gels were stained with coomassie blue for proteins and with Schiffs reagent for mucoproteins. Known molecular weight markers together with cytochrome C were run on separate gels and thus the approximate molecular weights of the proteins and mucoproteins were calculated. The gels were scanned in a densitometer and the concentration of each protein and mucoprotein in the gastric juice calculated.

Simulated gastric digestion

Portions of 0·2 g raw soya-bean flour were incubated at 37° for 1·5 h with a range of solutions of pepsin (5 ml, 5 g/l), adjusted with HCl at intervals of 0·2 units to pH values ranging from 1·4 to 2·8. After centrifuging the amount of Fe solubilized was measured as described previously.

Statistical significance was examined by the Mann–Whitney test and correlations by Pearson's method.

RESULTS AND DISCUSSION

The Fe solubilized from the twelve foods by gastric juice collected from one female subject (age 27 years) on ten occasions is shown in Table 1; the total Fe content of the foods is included in Table 2. The results expressed as the proportion of total Fe present ranged from zero for egg to nearly 1·0, complete liberation, from the sample of wine.

The amounts liberated bore no relation to the total amount of Fe present which ranged from 4 μg/g wine to 830 μg/g curry powder.

Since the composition of human gastric juice varies from sample to sample and, as shown in Table 1, the pH, free and total acid, pepsin activity and Fe content differed in each of the ten samples the close agreement of the replicated results suggests that it was the intrinsic chemical properties of the Fe in the various foods that was being measured.

Table 2 shows the amounts of Fe solubilized from the same foods by gastric juice obtained from sixteen additional subjects (seven male, nine female, age range 19–38 years). The mean values were similar to those shown in Table 1 for the one female subject and the ranking order of the twelve foods was almost identical.

The results obtained with different samples of gastric juice showed a degree of variation. In order to ascertain the reason(s) for differences in Fe solubility, a range of gastric juice variables was measured. These were pH of gastric juice, free and total acid, pepsin activity, proteins and mucoproteins present (as revealed by gel electrophoresis), Fe content of gastric juice, sex and age of subject and the degree of Fe solubilization in each food. The

Table 1. *The proportion of total iron solubilized from foods with gastric juice samples from subject no. 1 on ten occasions*

Food samples*	Mean	SD
Almonds	0·200	0·034
Bran	0·237	0·030
Cocoa powder	0·067	0·019
Curry powder	0·020	0·007
Eggs	0·00	0·00
Lentils	0·013	0·009
Oats	0·038	0·015
Peas	0·061	0·041
Soya-bean flour	0·132	0·026
Spinach	0·308	0·013
Watercress	0·023	0·015
Tonic wine	0·971	0·037
pH	1·88	0·13
Free acid	14·3	5·2
Total acid	19·3	6·3
Pepsin (units/ml × 10^3)†	1·16	0·19
Fe (μg/ml)	0·037	0·047

* For details of samples and their preparation, see p. 414.

† One unit of activity was defined as the absorbance resulting from 0·001 M-tyrosine under conditions of the assay.

Table 2. *Total iron and proportion solubilized from foods with gastric juice samples from sixteen subjects*

(Seven males, nine females, ages 19–39 years)

Food sample*	Total iron (μg/g)	Proportion solubilized		
		Mean	Range	SD
Almonds	42·5	0·166	0·05–0·32	0·062
Bran	160·3	0·199	0·08–0·29	0·051
Cocoa powder	177·0	0·044	0·02–0·10	0·021
Curry powder	831·3	0·015	0·01–0·03	0·007
Eggs	76·3	0·013	0·00–0·06	0·018
Lentils	87·5	0·025	0·00–0·13	0·031
Oats	61·7	0·028	0·00–0·05	0·019
Peas	64·0	0·099	0·04–0·28	0·060
Soya-bean flour	157·1	0·167	0·12–0·27	0·040
Spinach	375·0	0·321	0·27–0·45	0·051
Watercress	617·5	0·019	0·01–0·05	0·011
Tonic wine	4·1	0·985	0·90–1·04	0·038

* For details of samples and their preparation, see p. 414.

results were then analysed by partial correlation to see whether any pair of variables were related when the effects of the others had been eliminated. The only factors that were related to the amounts of Fe solubilized were the various measures of acidity.

The foods fell into two groups: (1) those in which the proportion of Fe solubilized increased with increasing acidity, i.e. bran (r 0·64, $P < 0·05$) cocoa powder (r 0·64, $P < 0·01$) and curry powder (r 0·68, $P < 0·001$); (2) those in which it decreased with increasing acidity, i.e. soya-bean flour ($r -0·57$, $P < 0·05$), lentils ($r -0·76$, $P < 0·001$), egg ($r -0·76$, $P < 0·05$) and peas ($r -0·66$, $P < 0·001$).

There were no differences between males and females for any of the variables measured; age correlated positively with pH (r 0·64; $P < 0·01$) and hence negatively with free acid ($r -0·57$, $P = 0·02$) total acid ($r -0·57$, $P = 0·02$) and pepsin ($r -0·77$, $P < 0·001$).

Table 3. *Iron (μg) solubilized from foods with 5 ml gastric juice (pH 2·2) with and without bran, oats and egg (total weight of food 0·2 g)*

Treatment ...		Plus bran		Plus oats		Plus eggs	
Food sample*	Food alone	Calculated	Actual	Calculated	Actual	Calculated	Actual
Egg	0·54	—	—	—	—	—	—
Bran	6·75	—	—	—	—	—	—
Oats	0·26	—	—	—	—	—	—
Black beans	0·39	3·75	2·21	0·33	0·13	—	—
Fish	0·13	3·44	0·52	0·20	0·00	—	—
Lettuce	1·43	4·09	2·08	0·85	1·95	—	—
Liver	6·38	6·57	6·51	3·32	0·65	—	—
Maize flour	0·13	3·44	1·82	0·20	0·00	—	—
Rice	0·00	3·88	1·70	0·13	0·00	—	—
Soya-bean flour	3·06	4·91	1·83	1·66	0·69	1·80	1·01
Spinach	8·59	7·67	9·11	4·43	6·77	4·57	3·29
Veal muscle	0·39	3·57	1·17	0·33	0·13	0·72	0·00
Tonic wine (2 ml)	8·20	—	—	—	—	4·37	1·12
Wheat	0·39	3·57	3·38	0·33	0·13	—	—

* For details of samples and their preparation, see p. 414.

Table 4. *Comparison of 'chemically-available' iron as solubilized by gastric juice with in vivo absorption in man (Layrisse* et al. 1969)

Food* (dried)	Present study Total μg Fe/g	Layrisse *et al.* (1969) Total μg Fe/g	Solubilization (% total)	In vivo absorption (% total)†
Black beans	76	85–90	0·85	3·2
Fish				
Mackerel	43	—	4·6	—
Brycon whitei / *Microptens salmonoides*	—	11–17	—	18·3
Lettuce	209	188–543	4·1	5·8
Liver				
Ox	777	—	4·9	—
Veal	—	163–407	—	14·5
Maize flour	25	21	1·4	5·9
Rice	11	10	0·0	0·9
Soya-bean flour	148	86–140	10·8	17·9
Spinach‡	377	77	10·7	1·7
Veal muscle	51	44–49	6·6	20·3
Wheat	49	85	4·1	7·9

* For details of samples and preparation, see p. 414.
† Values calculated from those quoted by Layrisse *et al.* (1969).
‡ Samples differed considerably in Fe content therefore omitted from calculations.

It is suggested that the Fe solubilized by gastric juice is available for absorption and an attempt was made to validate this suggestion by adding bran, oats and egg to the foods before incubating them with gastric juice. These foods reduce the amount of dietary Fe that is absorbed (Sharpe *et al.* 1950; Callender *et al.* 1970; Bjorn-Rasmussen, 1974). Table 3 shows the effect of mixing 0·1 g oats, bran or egg with 0·1 g of the food before incubating as described earlier. The sum of the Fe solubilized from the food alone and the bran, oats and egg alone provides the values for the calculated amount of Fe solubilized from the mixture. The results shown in Table 3 indicate that in all but two foods there was a substantial reduction in the amounts of Fe liberated. The two exceptions were lettuce and spinach and it is possible that residual ascorbic acid may have affected the results by chelating with

Table 5. *Iron solubilized from foods* (%) *by dilute hydrochloric acid* (*pH* 2·5), *gastric juice* (*pH* 2·5) *or gastric juice* (*pH* 2·5) *followed by adjustment of pH to* 7·5

Food	Dilute HCl (pH 2·5)	Gastric juice	
		pH 2·5	pH 7·5
Black beans	2·4	0·9	0·0
Fish	2·8	4·6	0·0
Lettuce	4·0	4·1	3·1
Liver	4·6	4·9	2·0
Maize flour	0·0	1·4	0·0
Rice	0·0	0·0	0·0
Soya-bean flour	23·9	10·9	8·1
Spinach*	10·4	10·7	10·3
Veal muscle	4·7	6·6	2·9
Wheat	3·7	4·1	0·0

* Omitted from statistical calculations, see Table 4.

Fe. Bran had no effect on the Fe solubilized from liver but oats substantially reduced the amount.

Another attempt to validate the findings was made by comparing the results with those reported on similar foods by Layrisse *et al.* (1969) for the absorption of Fe by human subjects. Such a comparison may be unreliable for two reasons. First the samples were not the same. For example, as shown in Table 4 the Fe content of some of the foods used by Layrisse *et al.* (1969) varied considerably (lettuce ranged from 188 to 542 μg Fe/g and soya-bean flour ranged from 86 to 140 μg) and the foods used in the present work differed from these in the total Fe content. Additionally we used different types of fish and liver. However, it is possible that the chemical form of the Fe and hence the amounts solubilized and absorbed may be similar in foods of the same type.

Secondly, the present work is attempting to measure the Fe that is 'chemically available' whereas Layrisse *et al.* (1969) measured the amount absorbed, which would thus include the effects of other ingredients of the diet and also the nutritional status of the subjects.

With these limitations the results in Table 4 show a Pearson correlation coefficient of 0·82 ($P < 0·01$). The results for spinach were omitted since the sample used here contained five times as much Fe as that reported by Layrisse *et al.* (1969).

Mg-Mg-Thwin *et al.* (1975) suggested that available Fe could be measured by incubating the food with HCl or by a double incubation with pepsin-HCl followed by adjusting the pH to 7·5 to simulate the conditions in the duodenum.

Table 5 shows the effect of treating the foods with dilute HCl at pH 2·5, with gastric juice at pH 2·5 as used to obtain the results shown in Tables 1 and 2, and by incubating with gastric juice at pH 2·5, then adjusting pH to 7·5 with NaOH and incubating again before centrifuging and measuring the Fe solubilized. Considerably less Fe was solubilized by the double incubation. HCl alone liberated more Fe than did the gastric juice for two foods, less from three foods and approximately the same amount for the remaining five foods.

When the results in Table 5 are compared with those of Layrisse *et al.* (1969) given in Table 4 for the proportion of food iron absorbed in vivo the following Pearson correlation coefficients were obtained: dilute HCl, r 0·52, not significant; gastric juice at pH 2·5, r 0·82 ($P < 0·01$); double incubation with gastric juice at pH 2·5 then adjusted to 7·5, r 0·51, not significant.

The effect of pepsin-HCl on the solubilization of Fe from soya-bean flour is shown in Fig. 1 and compared with the results of gastric juice at various levels of acidity. Both

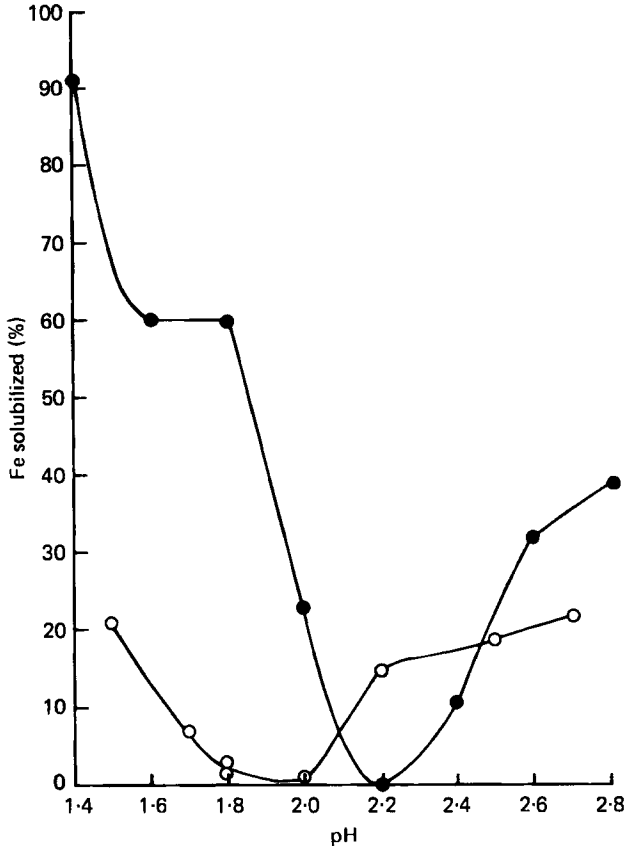

Fig. 1. Percentage of total iron solubilized from defatted raw soya-bean flour by pepsin (5 g/l) – hydrochloric acid (●——●) and human gastric juice at various levels of acidity (adjusted with HCl) (○——○).

systems are pH dependent but the amount of Fe liberated from the soya-bean flour was very much greater with pepsin-HCl than that available to human subjects measured in vivo according to Layrisse *et al.* (1969). It would thus appear that the Fe liberated by the pepsin–HCl system does not correspond to available Fe.

CONCLUSIONS

It is stated in the Report on Recommended Daily Intakes (Department of Health & Social Security, 1969) that the Committee considered whether it would be possible to express the Fe content of diets as 'Fe equivalents' using weighting factors based on the source of the Fe, but at that time there was not enough information about the availability of Fe in different foods and in meals of different composition to justify such a procedure.

The method described here, namely the amount of Fe solubilized in vitro by human gastric juice, might serve such a purpose. The amount of Fe liberated from a food in the stomach might be that which is available for absorption, though the amount absorbed subsequently depends on the presence of other dietary factors as well as on the Fe status of the subject. If the Fe content of foods were expressed as 'available' Fe such a method would be similar to that adopted for vitamin A, in which the amount in the food is expressed as retinol equivalents. As is the situation with Fe, vitamin A occurs in foods in different

chemical forms that differ in their biological potency although the amount subsequently absorbed depends on the presence of other dietary factors such as vitamin E and fats, and the conversion of carotene and transport in the blood depend also on the protein status of the subject. It is difficult to validate the in vitro method described here. The values differ from those given by in vitro methods previously suggested, such as solubility in HCl or in HCl-pepsin (Jacobs & Greenman, 1969) or after incubation in gastric juice at pH 2·5 then at 7·5 (Mg-Mg-Thwin *et al.* 1975), and also Narasinga Rao & Prabhavathi (1978). The values correlate well with the accepted values for Fe absorption from a range of foods reported by Layrisse *et al.* (1969) although the latter values must include the effects of other dietary factors and the Fe status of the subjects as well as the availability of the Fe in the foods.

The difference between pepsin-HCl and gastric juice has not been explained and does not appear to be due to the proteins or the mucoproteins present in gastric juice.

There is some variability between results obtained on repeated samples of gastric juice from the same subject which appears to be pH-dependent and it may be necessary to standardize the pH to make the method reproducible. It will clearly be necessary to examine a much greater variety of foods under standardized conditions if the method is to be considered for use in standard food composition tables.

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