In vitro availability of iron and zinc: effects of the type, concentration and fractions of digestion products of the protein

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An *in vitro* dialysis method was employed to determine the effect on the Fe and Zn absorption of the type (beef, pork and soyabean) and the amount (10 and 30 g/kg) of protein present. In addition, the effects of low- and high-molecular-weight (LMW and HMW respectively) digestion products were investigated. After *in vitro* digestion and dialysis a lower percentage of N, Fe and Zn was found in the LMW fractions from beef, pork and soyabean proteins when the protein level was increased from 10 to 30 g/kg; the higher level of protein being associated with a lower percentage of hydrolysed protein. The highest percentage levels of intrinsic Fe were always found in the HMW fractions, independent of the type and the level of proteins studied, while in the case of Zn, both HMW and LMW fractions gave similar values. An interaction was found between inorganic Zn and non-haem-Fe. The addition of inorganic Zn (10 μg/ml) caused a significant decrease in the *in vitro* availability of Fe from soyabean protein, while it did not affect the dialysability of intrinsic Fe from beef and pork proteins. Our results showed that the type and the level of the protein had a positive effect on the dialysability of extrinsic Fe. We postulate that the effect of a protein on the absorption of extrinsic Fe could be accounted for by free amino acids and/or small peptides released during the digestion process and also by the undigested or partially-digested HMW fractions of hydrolysed proteins which could play a fundamental role in the availability of this essential element.

Iron: *In vitro* availability: Zinc: Protein digestion

The nutritional value of a diet in terms of macrominerals and trace minerals is dependent on much more than their respective contents in the diet. Mineral imbalances can be attributed not only to a deficient intake, but also to the amount of mineral that is bioavailable for physiological processes in the organism. It is well known that many of the interactions between food constituents can result in changes in the bioavailability of nutrients. Several dietary factors have been implicated in changes in the absorption of some trace minerals, for example Fe, Zn, Cu and Mn (Rosenberg & Solomons, 1982; Solomons, 1982; Mills, 1985; Hallberg, 1987; Sandström, 1988; Churella & Vivian, 1989; Wapnir & Devas, 1995). One of the factors which appears to play an important role in Fe and Zn absorption is the amount and type of protein in the diet (Cossack & Prasad, 1983; Snedeker & Greger, 1983; Van Dokkum *et al.* 1986; Greger, 1989; Wapnir, 1989).

There have been a number of reports showing that animal tissues in the diet can increase the bioavailability of dietary Fe; this has been attributed to their protein properties (Layrisse *et al.* 1984; Berner & Miller, 1985; Politz & Clydesdale, 1988; Slatkavitz &

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Clydesdale, 1988; Gordon & Godber, 1989). The increasing rate of consumption of soyabean-based foods in human diets has caused some observers to express concern about a possible unfavourable effect on Fe and Zn bioavailability. Experiments conducted using different animals have shown a reduced absorption of Fe and Zn in presence of soyabean products (Forbes *et al.* 1979; Cook *et al.* 1981; Thompson & Erdman, 1984). However, other studies, which have been carried out on human subjects, have given conflicting results (Greger *et al.* 1978; Van Stratum & Rudrum, 1979; Bodwell, 1983; Cossack & Prasad, 1983; Gillooly *et al.* 1984).

In addition to the influence of food components, there is also the mutual influence of minerals on their individual bioavailabilities. Every year, more examples are published that demonstrate the biological antagonism between minerals. Competitive interactions between Fe and Zn have been revealed by a number of investigations (Solomons & Jacob, 1981; Meadows *et al.* 1983; Valberg *et al.* 1984; Fairweather-Tait & Southon, 1989).

Different types of *in vitro* methods have been developed for the estimation of the absorption of macrominerals and trace minerals (Narasinga Rao & Prabhavathi, 1978; Miller *et al.* 1981; Diepenmaat-Wolters, 1992). *In vitro* digestion of protein-containing meals and subsequent analysis of soluble or dialysed Fe and Zn has been shown to have a good correlation with *in vivo* determinations of Fe and Zn absorption (Schricker *et al.* 1982; Kane & Miller, 1984; Hurrell *et al.* 1988; Vaquero *et al.* 1992; Diepenmaat-Wolters & Schreuder, 1993; Wolters *et al.* 1993; Dahiya & Kapoor, 1994). However, the prediction by *in vitro* methods of the amounts of macrominerals and trace minerals available for absorption is only relative, because it is not possible to simulate all the important physiological conditions prevailing in the stomach and the small intestine. The method developed by Miller *et al.* (1981), which permits discrimination between low (LMW)- and high-molecular-weight (HMW) soluble metal complexes, has been used in the present study.

Different authors have postulated that the absorption of some trace minerals, such as Fe and Zn, is facilitated by LMW-binding ligands (Evans & Johnson, 1980; Lönnerdal *et al.* 1980; Layrisse *et al.* 1984; Taylor *et al.* 1986; Wapnir & Stiel, 1986; Politz & Clydesdale, 1988). However, the chemical composition and the mechanism involved in facilitating absorption are not entirely understood. A better knowledge of the bioavailability of macrominerals and trace minerals, and the factors, such as food components and their digestion products, that may influence this absorption positively or negatively, will contribute to maintaining an optimal mineral balance in animals and man.

The aims of the present study were: (1) to evaluate the effect on Fe and Zn absorption of various purified proteins and their digestion products under simulated gastrointestinal conditions, and (2) to study the possible interaction between these two trace minerals.

MATERIALS AND METHODS

Protein sources and sample preparation

Three purified proteins were used in the present study: extracted beef and pork proteins and soyabean-protein isolate. The two types of fresh meat were obtained from a local supermarket. Soyabean-protein isolate was provided by a manufacturer.

Meat samples were trimmed of visible fat and ground twice through a 3·175 mm plate, mixed and maintained at 0° in plastic bags before protein extraction.

Reagents and materials

Double-distilled and deionized water (DDW) was used. All glassware and the polyethylene bottles used for the samples were washed and rinsed in distilled water, soaked overnight in

10 M-HNO_3, and rinsed again with DDW. Pepsin suspension was prepared from pepsin powder (16 g; from porcine stomach mucosa; Sigma Chemical Co., St Louis, MO, USA) which was suspended in 0·1 M-HCl and brought to 100 ml with 0·1 M-HCl. Pancreatin–bile-extract mixture contained pancreatin (4 g; from porcine pancreas; Sigma Chemical Co.) and bile extract (25 g; porcine; Sigma Chemical Co.) dispersed in 0·1 M-$NaHCO_3$, the mixture being brought to 1 l with 0·1 M-$NaHCO_3$. $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ were used as sources of extrinsic Fe and Zn respectively. The dialysis tubing comprised segments of dialysis tubing manufactured from natural cellulose, with a molecular weight cut-off of 6000–8000, and a diameter of 32 mm (Spectra/Por, Spectrum, Houston, TX, USA). Dialysis tubing segments were washed three times with DDW and soaked overnight in 0·1 M-$NaHCO_3$. All reagents were of analytical grade and were prepared in DDW.

Meat-protein extraction procedure

Meat proteins were extracted by placing 50 g samples of ground meat in a 250 ml Erlenmeyer flask, adding 150 ml of the extraction solution (90 g NaCl/l) and mixing at moderate speed, with a magnetic stirrer, for 6 min. Extracts were separated from particulate matter by centrifugation at 1475 *g* for 10 min. The extracts were decanted and poured through glass-wool filters. Meat samples and extraction solutions and all equipment were equilibrated at the extraction temperature of $7·2 \pm 1°$ (Gillett *et al.* 1977). Finally, the extracts of proteins were lyophilized before analysis of the protein and its utilization in *in vitro* experiments. It has been shown that when the meat used is fresh, uncooked and unfrozen, as in our study, both the extracted proteins obtained by this method and the original meat sources present similar properties in relation to their emulsifying ability (Gillett *et al.* 1977).

In vitro *digestion and separation of the digestion-product fractions*

The *in vitro* digestion method developed by Miller *et al.* (1981) was used, with minor modifications. The Miller *et al.* (1981) method was developed for estimating Fe availability in food mixtures, while in our study we estimated Fe and Zn availability from three purified proteins. The extrinsic minerals (Fe and/or Zn) were added after the digestion process, while in the Miller *et al.* (1981) method the Fe was added before the digestion process. In the original method the minerals were dialysed during the pancreatin digestion, while in our study the minerals were dialysed after both the digestion process and the separation of the digestion-product fractions. We also used different pH values, which were necessary to separate both LMW and HMW digestion-product fractions. The different digestion-product fractions were obtained according to the method of Kane & Miller (1984). The methods involve a two-stage (pepsin and pancreatin) digestion.

Pepsin digestion. An amount of each product containing the equivalent of 1 or 3 g protein was placed in a 250 ml plastic bottle and mixed with distilled water. The pH of the mixture was adjusted to 2·0 using 6 M-HCl and the pepsin suspension (2·5 ml) was added to the mixtures (0·361 g/100 g homogenate). The pH was re-adjusted to 2·0 and the flask contents were brought to a total weight of 100 g. The bottles were incubated at 37° for 2 h in a vigorously-shaking water-bath (rate of oscillation 90/min). Throughout the incubation the pH of the mixtures was maintained at 2·0 (by adding 6 M-HCl when necessary). Pancreatin digestion which followed pepsin incubation was different for each digestion-product fraction. Extrinsic Fe and/or Zn were not added until after the fractions were prepared.

Pancreatin digestion of the whole fraction. An amount of $NaHCO_3$ equivalent to the titratable acidity measured previously (see p. 730) was placed in each bottle. The bottles were then incubated in a vigorously-shaking water-bath (rate of oscillation 90/min) at 37°

for 30 min. This was followed by the addition of pancreatin–bile extract (25 ml/100 g homogenate), and the incubation was continued for a further 2 h. After pancreatin digestion the pH was adjusted to 2·0 with 6·0 M-HCl.

Pancreatin digestion of the low- and high-molecular-weight fractions. Segments of dialysis tubing containing 200 ml distilled water and an amount of $NaHCO_3$ equivalent to the titratable acidity measured previously were placed in each bottle. The bottles were then incubated in a vigorously-shaking water-bath (rate of oscillation 90/min) at 37° for 30 min. This was followed by the addition of pancreatin–bile extract (25 ml/100 g homogenate), and the incubation was continued for a further 2 h. At the end of the incubation period the retentate (material outside the dialysis tube) from the *in vitro* digestion procedure was dialysed against 0·01 M-HCl for 48 h at 4° (to remove the LMW fraction and purify the HMW fraction). The HCl was changed every 6 h (HMW fraction). The dialysis sacs were rinsed with DDW and blotted after removal from the bottles to remove contaminating dialysate. The dialysate from the *in vitro* digestion procedure was lyophilized and brought to half its original volume with water and sufficient 6·0 M-HCl to bring the final pH to 2·0 (LMW fraction). Re-adjustment of the pH used in the experimental process does not occur in the *in vivo* situation. However, this re-adjustment of the pH is a necessary step in the separation method of the digestion-product fractions (method of Kane & Miller, 1984).

Iron and zinc dialysis experiments

Samples of the digestion-product fractions (19·6 ml) were transferred to 100 ml plastic bottles. Fe and/or Zn solutions (0·2 ml) were added, giving extrinsic Fe and/or Zn concentrations of 10 μg/ml. Dialysis tubes containing 25 ml $NaHCO_3$ solution of sufficient strength to produce a final pH of 7·0 were added to each bottle. After gentle shaking (rate of oscillation 100/min) at room temperature for 2 h, the dialysis tubes were removed and for each bottle the pH of retentate and for each dialysis tube the weight of dialysate was measured, rinsed with DDW and blotted. Dialysates were analysed for Fe and Zn.

Blanks containing no added Fe and Zn (but otherwise prepared as described previously) were run to determine the contribution of intrinsic Fe and Zn to dialysable Fe and Zn. Blanks (without protein and minerals) containing the components used in the digestion process (enzymes and/or bile extract) were also run throughout the experiment (digestive, dialysis and analytical phases).

Titratable acidity was determined on a 20 g portion of the pepsin digest to which 5 ml of the pancreatin–bile-extract mixture was added. Titratable acidity was defined as the number of equivalents of NaOH required to titrate the combined pepsin-digest–pancreatin–bile-extract mixture to pH 7·0 (0·5 M-NaOH was used in the titration).

The dialysability was expressed as a percentage of the total amount of trace mineral (Fe and Zn) present in the sample. The percentage was calculated according to the following equation:

$$\text{dialysability (\%)} = \frac{2 \times D}{A} \times 100,$$

where D is the amount of mineral dialysed (mg) and A is the initial amount of mineral present in the sample portion before the dialysis process (mg).

Analytical methods

Analysis of protein sources, purified proteins, and digestion-product fractions. The Fe and Zn contents were determined by atomic absorption spectrophotometry (model 5000; Perkin-Elmer Corp., Norwalk, CT, USA). Protein contents were determined by the Kjeldahl method; the N to protein conversion factor was 6·25. Phytate (*myo*-inositol hexaphosphate)

Table 1. *Composition of raw meats, soyabean-protein isolate and extracted meat proteins*

	Raw meat		Isolated soyabean protein	Extracted protein	
	Beef	Pork		Beef	Pork
Protein (mg/g)	233	231	789	12·5	10·9
Fe (μg/g)	13·4	9·2	117·0	1·10	0·65
Zn (μg/g)	34·0	20·0	33·0	1·30	0·90

Table 2. *Contents of phytic acid in the low (⩽ 6–8000 Da; LMW) and high-molecular-weight (> 6–8000 Da; HMW) fractions from soyabean-protein isolate* at 10 and 30 g protein/kg levels after the digestion and dialysis processes†*

	Phytic acid (%)	
Protein level (g/kg)	HMW fraction	LMW fraction
10	65·1	34·9
30	75·7	24·3

* Phytic acid (*myo*-inositol hexaphosphate) 11·75 mg/g protein.
† For details of procedures, see pp. 728–731.

contents of the soyabean-protein isolate and its digestion-product fractions were determined using the method of Bos *et al.* (1991), which is a chromatographic method combined with an EDTA treatment of the extracts.

Analysis of dialysates. The Fe and Zn contents of the dialysates were determined spectrophotometrically using two reagent kits (catalogue no. 14761 and 14832 respectively; E. Merck, Darmstadt, Germany). The procedures and the reagents for each kit are different, and the complex formed with each mineral (Fe or Zn) is specific for that mineral; therefore, there should be no interference between the mineral complexes. Fe was determined using the Ferrospectral colour reagent (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine, disodium salt) and ammonium thioglycolate. In an alkaline solution, Zn ions react with pyridylazonaphthol to form a chelate complex which is extracted with isobutyl methyl ketone. Absorbance was measured at 565 nm and Fe and Zn were determined by comparison with a standard curve of 0, 0·1, 0·25, 0·5, 1·0, 1·5 and 2·0 mg/l using $Fe(NO_3)_3.9H_2O$ in 0·5 M-HNO_3 and $Zn(NO_3)_2.4H_2O$ in 0·5 M-HNO_3 respectively.

Statistical analysis

Results are given as means of four or six observations with the standard error of the mean. Statistical evaluation was performed by ANOVA for the type of protein and the presence of Fe or Zn. Student's *t* test was conducted for comparisons between groups when a significant variation was demonstrated by ANOVA. A probability level of $P < 0·05$ was considered significant.

RESULTS

The protein, Fe and Zn contents of the raw meats used as protein sources and those of the soyabean-protein isolate and extracted meat proteins are given in Table 1. The phytic acid (*myo*-inositol hexaphosphate) contents of soyabean-protein isolate and its digestion-product fractions are given in Table 2.

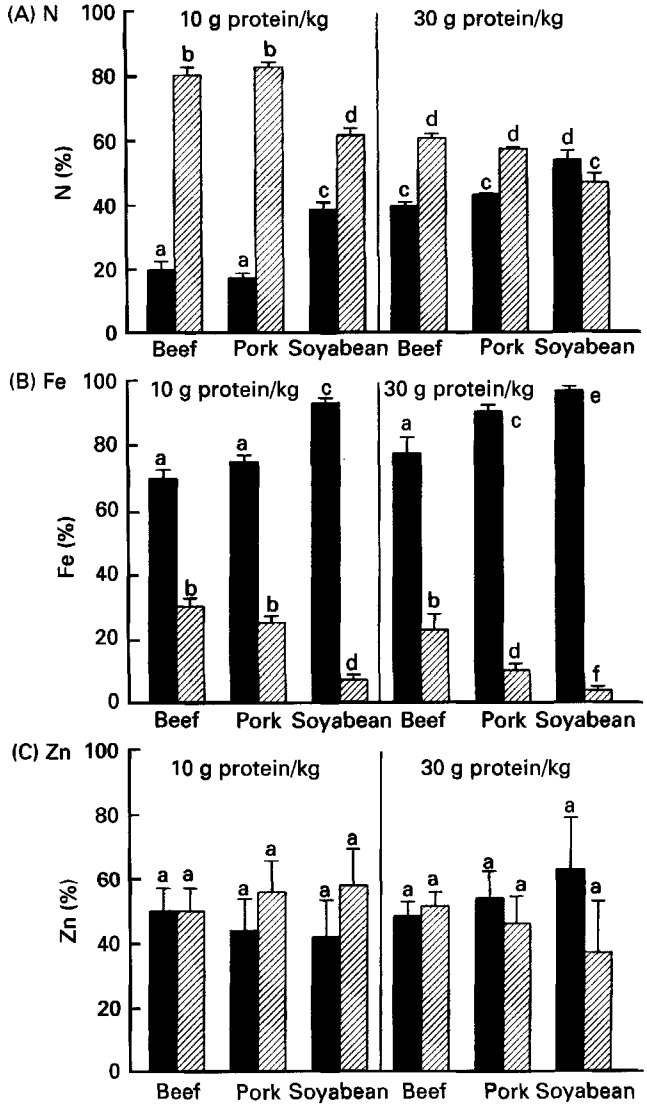

Fig. 1. Percentage distribution of nitrogen (A), iron (B) and zinc (C) from beef, pork and soyabean proteins, at 10 and 30 g protein/kg levels, in low (▨) and high (■)-molecular-weight fractions after the digestion and dialysis processes. Values are means with their standard errors represented by vertical bars for four determinations. [a, b, c, d, e, f] Means with unlike superscript letters were significantly different (Student's t test): $P < 0{\cdot}05$. For details of procedures, see pp. 728–731.

Fig. 1 shows the percentage distribution of N, Fe and Zn in the digestion-product fractions from *in vitro* digestion, separated on the basis of molecular weight (LMW fraction $\leqslant$ 6–8000 Da and HMW fraction $>$ 6–8000 Da), for the three types of protein at the two levels of protein tested (beef, pork and soyabean proteins; 10 and 30 g/kg). The percentage of hydrolysed protein decreased when the protein level was increased from 10 to 30 g/kg, independent of protein type used. At both protein levels, soyabean protein showed a lower N content in the LMW fraction than the other two proteins (Fig. 1A). When the protein

Table 3. *Percentage dialysability of iron from the beef, pork and soyabean proteins at 10 and 30 g protein/kg levels in presence and absence of added zinc (10 μg/ml)**

(Mean values with their standard errors for four determinations)

Type of protein	Dialysability of intrinsic Fe (%)			
	10 g protein/kg		30 g protein/kg	
	Mean	SE	Mean	SE
Beef	11·62[a]	1·30	14·05[a]	1·73
Beef + Zn (10 μg/ml)	12·94[a]	1·52	14·59[a]	1·67
Pork	12·97[a]	1·89	16·19[a]	2·00
Pork + Zn (10 μg/ml)	12·71[a]	2·30	17·21[a]	2·99
Soyabean	3·17[b]	0·14	2·22[b]	0·18
Soyabean + Zn (10 μg/ml)	0·30[c]	0·54	0·97[c]	0·14

[a, b, c] Means with unlike superscript letters were significantly different (Student's *t* test): $P < 0·005$.

* For details of procedures, see pp. 728–731.

Table 4. *Percentage dialysability of zinc from the beef, pork and soyabean proteins at 10 g protein/kg level in presence and absence of added iron (10 μg/ml)**

(Mean values with their standard errors for four determinations)

Type of protein	Dialysability of intrinsic Zn (%)	
	Mean	SE
Beef	55·42[a]	7·23
Beef + Fe (10 μg/ml)	54·70[a]	8·30
Pork	30·13[b]	4·84
Pork + Fe (10 μg/ml)	31·09[b]	4·25
Soyabean	28·65[b]	2·84
Soyabean + Fe (10 μg/ml)	22·38[b]	2·91

[a, b] Means with unlike superscript letters were significantly different (Student's *t* test): $P < 0·001$.

* For details of procedures, see pp. 728–731.

level was increased, there was also a lower percentage of Fe and Zn in the LMW fraction (Fig. 1(B and C)).

The dialysability of Fe from the three purified proteins at the two levels (10 and 30 g/kg) and the effect of added Zn (10 μg/ml) on the dialysability of intrinsic Fe are shown in Table 3. Proteins from animal tissues showed the same percentage of dialysable Fe, and these values were significantly higher than that from soyabean protein ($P < 0·005$). There were no differences between the protein levels in the dialysability of intrinsic Fe. The addition of inorganic Zn (10 μg/ml) had no effect on the dialysability of intrinsic Fe from animal proteins, but it had a significant decreasing effect ($P < 0·005$) on the dialysability of intrinsic Fe from soyabean protein when it was used at 10 and 30 g protein/kg levels.

Table 4 shows the percentage of dialysable Zn from the beef, pork and soyabean proteins (10 g protein/kg), in presence and absence of added Fe (10 μg/ml). The dialysability of Zn from beef protein was significantly ($P < 0·005$) higher than that from the other proteins

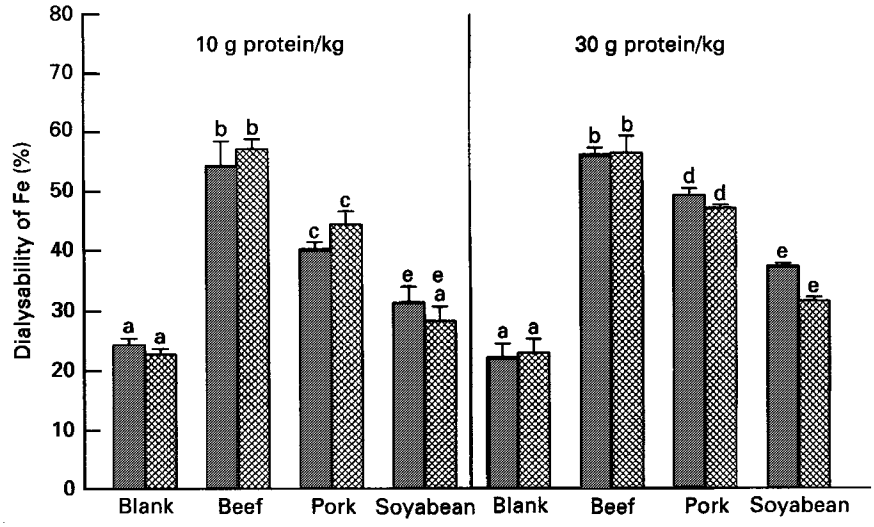

Fig. 2. The effect of the type and level of protein (10 and 30 g/kg) on the dialysability of extrinsic iron (10 μg/ml), in presence (▩) and absence (⊠) of zinc (10 μg/ml), added as $ZnSO_4$ after *in vitro* digestion (combined digestion-product fractions). Values are means with their standard errors represented by vertical bars for four determinations. [a, b, c, d, e] Means with unlike superscript letters were significantly different (Student's *t* test): $P < 0{\cdot}05$. For details of procedures, see pp. 728–731.

studied. The dialysability of intrinsic Zn from soyabean isolate was the lowest of the three proteins tested, but it did not differ significantly from that from pork protein. The addition of Fe had no effect on the dialysability of intrinsic Zn.

Fig. 2 shows the effect of the type and level of protein on the percentage dialysability of extrinsic Fe, added as Fe^{2+} (10 μg/ml). The three proteins had a significant enhancing effect (beef and pork proteins $P < 0{\cdot}001$, soyabean protein $P < 0{\cdot}05$) on the dialysability of extrinsic Fe in relation to the blank (only the enzymes and bile extract were present during digestion). The addition or inorganic Zn had no effect on the dialysability of extrinsic Fe. As can be seen also from Fig. 2, when the protein level was increased, the percentage dialysability of extrinsic Fe tended to increase, but was only significant ($P < 0{\cdot}002$) in the case of pork protein.

The effects on the dialysability of extrinsic Fe of the digestion-product fractions from the three proteins at each of the two levels (10 and 30 g/kg) are shown in Fig. 3. The LMW fractions of the different types and levels of proteins had no effect on the dialysability of extrinsic Fe, while for the HMW fraction the effect was significantly different ($P < 0{\cdot}001$) between both the types and the levels of protein used.

Fig. 4 shows the effects of the type of protein and its digestion-product fractions on the dialysability of extrinsic Zn, added as $ZnSO_4$ at 10 μg/ml, in presence and absence of added Fe (10 μg/ml). There were no differences in the dialysability of extrinsic Zn between the proteins. The addition of inorganic Fe had no effect on the dialysability of extrinsic Zn. As can be seen from Fig. 4, in all cases the percentage dialysability of extrinsic Zn in the presence of the LMW fraction was significantly higher than that with the HMW fraction ($P < 0{\cdot}001$).

Finally, Fig. 5 shows the effects on the dialysability of extrinsic Fe and Zn of the different components used in the *in vitro* digestion process (enzymes, pepsin and pancreatin, and bile extract), as well as those of their digestion-product fractions. Dialysability for Fe was very high for the enzymes and the LMW fractions, intermediate for the combined fractions (all

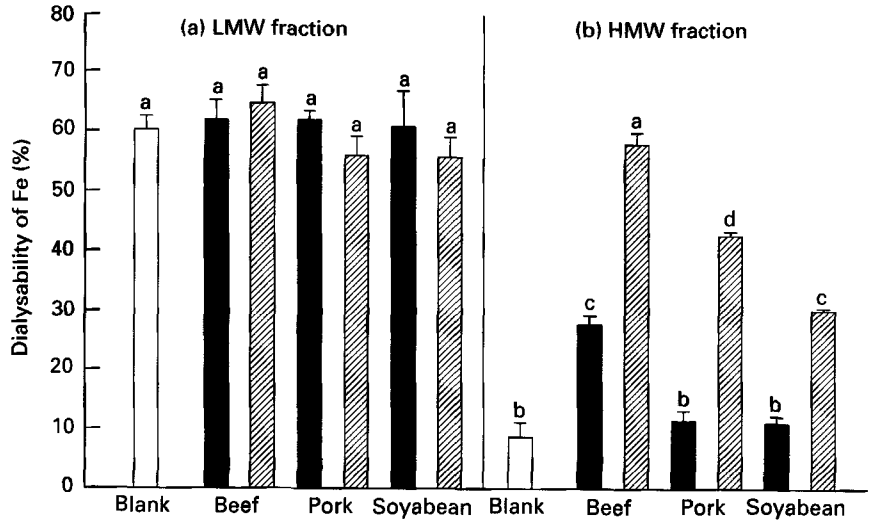

Fig. 3. The effect of the type and level of protein (10 (■) and 30 (▨) g/kg) after *in vitro* digestion on the dialysability of extrinsic iron (10 μg/ml) added as $FeSO_4$. (a) Low ($\leq$ 6–8000 Da; LMW) and (b) high (> 6–8000 Da; HMW)-molecular-weight digestion-product fractions. (□), Blank (only the enzymes, bile extract and extrinsic Fe). Values are means with their standard errors represented by vertical bars for six determinations. [a, b, c, d] Means with unlike superscript letters were significantly different (Student's *t* test): $P < 0{\cdot}001$. For details of procedures, see pp. 728–731.

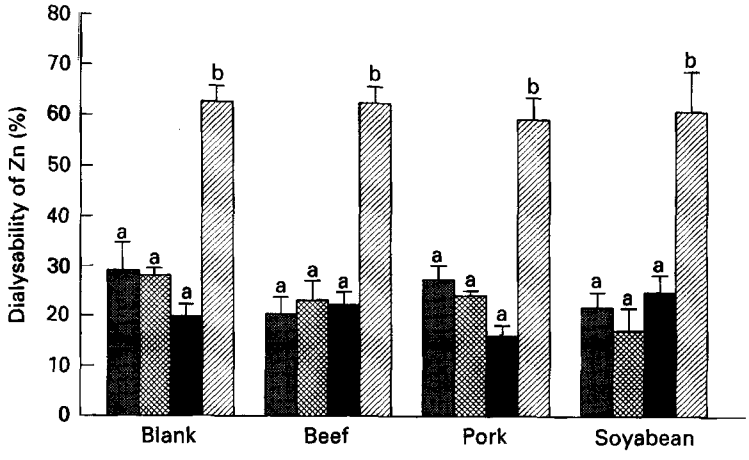

Fig. 4. The effect of the type of protein 10 g/kg level after *in vitro* digestion on the dialysability of extrinsic zinc (10 μg/ml). (▩) Combined digestion-product fractions; (▨), combined digestion-product fractions in presence of iron (10 μg/ml), added as $FeSO_4$; (■) high-molecular-weight fraction (> 6–8000 Da); (▨), low-molecular-weight fraction ($\leq$ 6–8000 Da). Values are means with their standard errors represented by vertical bars for four or six determinations. [a, b] Means with unlike superscript letters were significantly different (Student's *t* test): $P < 0{\cdot}001$. For details of procedures, see pp. 728–731.

components together), and very low for bile extract and HMW fractions ($P < 0{\cdot}005$). Dialysabilities for Zn were the same from the combined and HMW fractions and both values were significantly lower than that from the enzymes, the bile extract and the LMW fraction ($P < 0{\cdot}005$). Bile salts had a strongly inhibiting effect on the dialysability of Fe ($P < 0{\cdot}005$), but not that of Zn.

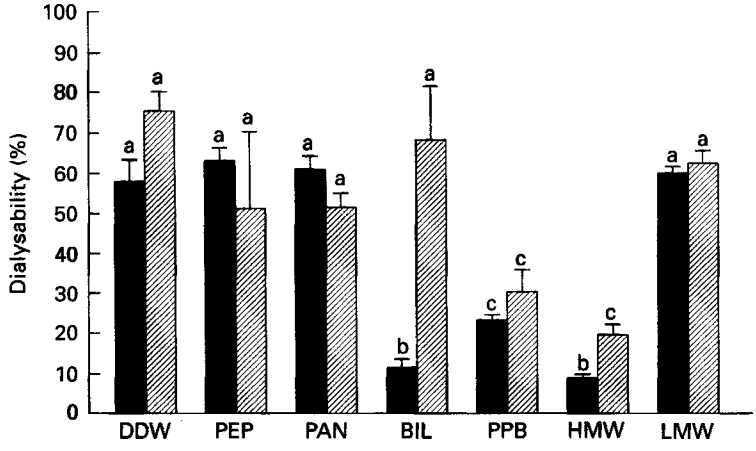

Fig. 5. Effect of the different components used in the digestion process (blank) on the dialysability of extrinsic iron (■) and zinc (▨). DDW, double-distilled and deionized water; PEP, pepsin; PAN, pancreatin; BIL, bile extract; PPB, all components, LMW, HMW, low (≤ 6–8000 Da)- and high (> 6–8000 Da)-molecular-weight fractions from all components. Values are means with their standard errors represented by vertical bars for four determinations. [a, b, c] Means with unlike superscript letters were significantly different (Student's t test): $P < 0{\cdot}005$.

DISCUSSION

Proteins and the availability of intrinsic iron and zinc

Studies reported in the literature have suggested that the absorption of some trace minerals can be related to the affinity that some products which are formed and/or released during the protein digestion process have for these minerals (Layrisse *et al.* 1984; Berner & Miller, 1985; Politz & Clydesdale, 1988; Slatkavitz & Clydesdale, 1988; Hurrell *et al.* 1989; Wapnir, 1989). The results from the present study on *in vitro* availability support these observations for Fe, but not for Zn.

After the *in vitro* digestion and dialysis processes, a lower percentage of N, Fe and Zn was found in the LMW fractions where the protein level was increased from 10 to 30 g/kg. This difference in distribution between LMW and HMW fractions was independent of the type of protein used. Because the concentration of enzymes and the duration of the digestion processes in these experiments were the same for both protein levels, we suggest that these events are related to a lower degree of protein hydrolysis in the experiment with the highest protein level. Fig. 1 shows that most of the Fe is found in the HMW fraction. It is known from the literature that HMW peptides can bind, or include, macrominerals and trace minerals (Politz & Clydesdale, 1988; Slatkavitz & Clydesdale, 1988; Wapnir, 1989). Our results are consistent with those of Hurrell *et al.* (1989), who conducted a study to compare the effect of casein and whey proteins on the dialysability of Fe under simulated gastrointestinal conditions. They observed that when intact milk-protein products were replaced by enzyme-hydrolysed preparations, the percentage of dialysable Fe increased markedly and in proportion to the extent of hydrolysis.

Fe and Zn distribution in the LMW and HMW fractions differed markedly. The highest percentage intrinsic Fe was always found in HMW fractions, independent of the type and level of protein studied. In the case of Zn, it was found at similar percentages in the LMW and HMW fractions. The difference in behaviour of the trace minerals can partly explain the different *in vitro* absorption observed in Tables 3 and 4. The dialysability of Zn was always higher than that of Fe (22–55% for intrinsic Zn and 0·3–13% for intrinsic Fe, depending on the protein type).

At both protein levels studied the lowest level of N in the LMW fractions was found in the soyabean-protein-isolate experiments. Our data also showed that the *in vitro* availability of Fe from soyabean-protein isolate was lower than that from animal proteins. This could be due to the presence of phytate in this protein. Our data on the distribution of phytic acid in the LMW- and HMW-peptide fractions also support this observation; it was higher in the HMW fraction at 30 g/kg than that at 10 g protein/kg. It has been reported that phytic acid can be found in the form of complexes with essential minerals and/or proteins (Erdman, 1979; Cheryan, 1980). Many of these complexes are insoluble and are not bioavailable under normal physiological conditions (deRham & Jost, 1979; Nosworthy & Caldwell, 1988). In addition, proteins bound to phytate are less subject to attack by proteolytic enzymes than the free proteins (Rodríguez *et al.* 1985; Champagne & Phillippy, 1989).

Conversely, the dialysability of intrinsic Zn from pork and soyabean proteins were similar, while in the beef-protein experiment it was significantly higher. These findings suggest that phytic acid did not have a negative effect on the dialysability of intrinsic Zn from soyabean protein. Apparently, the higher availability of Zn from beef protein also suggests the presence of an enhancing factor(s) in this meat.

Interactions between iron and zinc

As mentioned previously, competitive interactions occur between Zn and Fe at the level of absorption in human subjects and experimental animals. It was demonstrated that Zn inhibits Fe absorption, and *vice versa*, when the minerals are administered together (Solomons & Jacob, 1981; Solomons *et al.* 1983; Sandström *et al.* 1985; Fairweather-Tait & Southon, 1989). These interactions may be associated with ligands (LMW substances that bind inorganic ions) and/or their common transport sites in the bowel wall. In an *in vitro* study, as has been shown here, it is only possible to explore the first interaction (with ligands). This is not the only limitation that the *in vitro* method presents; as mentioned previously, the *in vitro* methods are only an approximation of the *in vivo* situation. *In vitro* methods cannot be considered, therefore, as an excellent alternative to *in vivo* techniques. However, because animal and human studies are expensive, time-consuming and difficult to control, it appears that there could be some advantage in screening different foods and their constituents for mineral availability using an *in vitro* technique.

At the concentrations used in the present study (10 μg/ml, for both trace minerals), there was only an interaction between inorganic Zn and non-haem-Fe. The addition of inorganic Zn reduced the dialysability of Fe from soyabean protein (non-haem-Fe), while it did not affect the dialysability of Fe from beef and pork proteins (about 40% haem-Fe). These results are probably related to the presence of two chemical forms of Fe in animal and vegetable foods. It is well known that the bioavailability of non-haem-Fe is influenced by other dietary factors, while that of haem-Fe is relatively unaffected (Martínez-Torres & Layrisse, 1971; Sayers *et al.* 1973; Hallberg, 1981; Gillooly *et al.* 1984; Monsen, 1988). Our results are in agreement with those of Solomons & Jacob (1981), who found a competitive interaction between non-haem-Fe and Zn, but no interaction in the case of haem-Fe. We found no interaction between the minerals when they were both used in the inorganic form. The findings described here do not exclude interactions between inorganic forms of Fe and Zn at other molar ratios, as observed by other authors (Meadows *et al.* 1983; Valberg *et al.* 1984; Fairweather-Tait & Southon, 1989).

Proteins and the availability of extrinsic iron and zinc

Results for the combined fractions (all components of the *in vitro* digestion process) show that the effect on the dialysability of extrinsic Fe differed from protein-to-protein. In all

cases, the presence of protein increased the dialysability of extrinsic Fe in relation to the blank (where only the enzymes, bile extract and extrinsic Fe were present). This enhancing effect was greater for pork and beef proteins than for soyabean protein. One possible explanation for these findings is that the factor(s) which enhance the availability of extrinsic Fe is (are) present in higher concentrations and/or may have a greater effect in meat proteins than that in soyabean protein. An alternative explanation for these findings is a possible negative effect of phytic acid in soyabean protein. Conversely, the dialysability of extrinsic Zn was not affected by the presence or absence of protein.

In order to explain the findings of our *in vitro* study, for each protein we separated two fractions, on the basis of molecular weight, during the digestion process and studied their separate effects on the dialysabilities of Fe and Zn. Results for LMW and HMW fractions showed different data for extrinsic Fe and Zn dialysability.

Our findings on the effect of both the LMW and HMW fractions on the dialysability of Zn suggest that the type of protein had no effect on its absorption. The data also demonstrated that the dialysability of Zn, added as $ZnSO_4$, was the same with soyabean-protein isolate as that with animal proteins. This finding is consistent with those of other *in vivo* studies (Greger *et al.* 1978; Van Stratum & Rudrum, 1979; Bodwell, 1983; Miles *et al.* 1987).

On the other hand, in the case of Fe the behaviour of the proteins tested was different. Results shown in Fig. 3 suggest that the LMW fractions do not account for the enhancing effect of proteins observed in Fig. 2, and that only HMW fractions play an important role in this effect. However, the percentage dialysability of extrinsic Fe was higher when both the LMW and HMW fractions were present than when the HMW fractions alone were present. Similar observations have been reported also by Slatkavitz & Clydesdale (1988). In addition, the effect of the HMW fraction on the dialysability of Fe depended on its concentration; when the concentration of the HMW fraction was higher, the effect on availability was higher.

We postulate that both the LMW and HMW digestion-product fractions are of importance in the enhancing effect of a protein on the availability of extrinsic Fe, but that their effect is associated with the presence of one or more factors which have an inhibitory effect. This view is supported by the data from Fig. 5. When we studied the individual effect of each of the components used in the *in vitro* digestion process (blank experiments) on the dialysability of extrinsic Fe, we found that one of these components (bile extract) had a strong inhibitory effect on the dialysability of Fe. These findings also suggest that after the digestion and dialysis processes the negative effect from the bile extract was only present in the HMW fraction. The absence of a negative effect associated with bile salts and, in the case of the soyabean protein the low content of phytic acid, could explain why free amino acids and/or small peptides (released during the digestion of beef, pork and soyabean proteins (LMW fractions)) did not appear to affect the dialysability of extrinsic Fe in our experiments.

The mechanism by which HMW fractions solubilize Fe and facilitate its transport across the dialysis membrane is not clear, it is possible that these HMW digestion products from proteins can, in some way, prevent the binding of Fe by bile salts. The formation of insoluble trace mineral–bile salt complexes at pH 7 has been suggested by Champagne (1989).

In summary, although our *in vitro* results cannot be directly applicable to the 'real diet', our findings show the possible effects of dietary protein on the absorption of Fe and Zn. The results of the present study suggest that the effect of a protein on the absorption of Fe *in vitro* could not be attributed only to the presence of free amino acids and/or small peptides released during the digestion process. They also suggest that the undigested or

partially-digested HMW fraction of hydrolysed proteins plays a fundamental role in the absorption of Fe. It appears from our findings that the net effect of a protein on the availability of extrinsic Fe is a combination of the effects of the formation of Fe complexes and/or binding of Fe by both the LMW and HMW digestion-product fractions. The affinity of these compounds for Fe could determine the dialysability of Fe and, hence, its bioavailability for absorption.

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