A continuous *in vitro* method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content

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A continuous *in vitro* method for the estimation of the bioavailability of minerals and trace elements is presented. This *in vitro* method is believed to be more representative of *in vivo* physiological conditions than *in vitro* methods based on equilibrium dialysis, because dialysable components are continuously removed from the pancreatic digestion mixture. The continuous *in vitro* method is compared with the equilibrium *in vitro* method with respect to the dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread (varying in phytic acid content). The results show a pronounced effect of continuous removal of dialysable components from the pancreatic digestion mixture on the dialysability of minerals and trace elements. Furthermore, removal of dialysable components influences the effect of phytic acid on the bioavailability of minerals and trace elements. For these two reasons the importance of removal of dialysable components *in vitro* for the estimation of bioavailability *in vivo* needs further investigation. The bioavailability of minerals and trace elements from bread samples is not related to the phytic acid content only. Therefore, the effect of phytic acid on the bioavailability of minerals and trace elements cannot be studied separately from the effects of other components on bioavailability.

Continuous *in vitro* method: Bioavailability: Minerals and trace elements: Phytate

A good mineral balance is of importance to animals and man. Deficiency, overdose, or imbalances between inorganic nutrients have a negative effect on health (Mertz, 1981; Nielsen, 1988; Prasad, 1988). However, it is not the dose of a mineral that is important to maintain balance, but rather the amount that is bioavailable.

In the small intestine several components of our food form soluble or insoluble complexes with minerals and trace elements. These food components may influence the bioavailability of these minerals and trace elements by influencing their availability for absorption. Components that may have a positive effect on the bioavailability of minerals and trace elements are citric acid, ascorbic acid, lactose and some amino acids (Hallberg *et al.* 1986; Hazell & Johnson, 1987*a*, *b*; Sandström & Cederblad, 1987; Saxena & Seshadri, 1988; Schuette *et al.* 1989), while phytic acid, dietary fibre and polyphenolic compounds may have a negative effect (Brune *et al.* 1989; Lönnerdal *et al.* 1989; Spivey Fox & Tao, 1989; Torre *et al.* 1991).

In vivo experiments with experimental subjects are the best way to study the bioavailability of minerals and trace elements to man. *In vivo* experiments, however, are

time-consuming and very expensive, and often quite variable results are obtained which are difficult to interpret. As an alternative, the rat is often used as a model for man (Schricker *et al.* 1981; Hunt *et al.* 1987; Churella & Vivian, 1989; Forbes *et al.* 1989; Lönnerdal *et al.* 1989). Those experiments, however, are limited by uncertainties with regard to differences in metabolism between rat and man. Reddy & Cook (1991) recently reported that rat studies cannot be used to assess the quantitative importance of dietary factors in human Fe nutrition.

In the past few years *in vitro* methods to assess the bioavailability of minerals and trace elements have gained popularity because of their accuracy, speed of analysis and relatively low costs.

The solubility of minerals and trace elements under simulated conditions of the stomach (pH 1–2, 37°) as an index of bioavailability has been studied by Narasinga Rao & Prabhavathi (1978), Lock & Bender (1980) and Forbes *et al.* (1989). In general, correlations with *in vivo* bioavailability were poor. Narasinga Rao & Prabhavathi (1978), Wien & Schwartz (1983), Hunt *et al.* (1987), Sandberg *et al.* (1989), Schwartz & Nevins (1989) and Turnlund *et al.* (1990) have investigated the solubility of minerals and trace elements after simulation of the conditions in the stomach (pH 1–2, 37°) and small intestine (pH 6·5–8, 37°) as a measure of *in vivo* bioavailability. The authors report contradictory results with respect to the correlation between *in vitro* and *in vivo* bioavailability. Miller *et al.* (1981) used the dialysability of Fe under simulated conditions of the stomach and the small intestine as an index for its bioavailability. This method has been the basis for several *in vitro* methods for estimation of the bioavailability of Fe and Zn. Promising correlations between *in vitro* dialysability and *in vivo* bioavailability were reported (Schricker *et al.* 1981; Hazell & Johnson, 1987*b*; Hurrell *et al.* 1988; Forbes *et al.* 1989; Sandström & Almgren, 1989). Miller *et al.* (1981), however, conclude that the prediction of bioavailability with *in vitro* methods is relative rather than absolute because not all important physiological factors can be simulated *in vitro*. Thus, *in vitro* methods may be very useful for ranking purposes.

As the absorption of minerals and trace elements is taking place in the complex environment of the small intestine, simulation of the conditions prevailing in the small intestine is probably the most critical step for *in vitro* methods aiming at prediction of the bioavailability of minerals and trace elements. *In vitro* methods based on the method of Miller *et al.* (1981) use equilibrium dialysis of minerals and trace elements across a semipermeable membrane as a model for the passage across the intestinal wall. It is assumed that the minerals and trace elements that are dialysable are available for absorption in the small intestine. In contrast with the situation *in vivo*, however, components that pass the membrane are not removed. As we expect these components to influence the equilibrium dialysis of minerals and trace elements, we hypothesize that a dynamic *in vitro* method taking removal of dialysable components into account leads to a better estimate of bioavailability *in vivo*. Therefore, we developed an *in vitro* method for continuous dialysis of minerals and trace elements based on a hollow-fibre system. In this way it is possible to remove dialysable components continuously from the pancreatic digestion mixture.

In the present paper the continuous *in vitro* method is described in detail. Possible interactions between the hollow-fibre membrane and minerals and trace elements were investigated. As we expected an influence of pH on dialysability, the influence of pH during pancreatic digestion was studied for both the continuous *in vitro* method presented here and the equilibrium *in vitro* method described by Miller *et al.* (1981). The results of the continuous *in vitro* method described here were compared with the results of the equilibrium *in vitro* method with respect to the bioavailability of Ca, Mg, Fe, Cu and Zn

from different types of bread. The bread samples were chosen so as to contain variable phytic acid contents. The influence of phytic acid on the dialysability of Ca, Mg, Fe, Cu and Zn was investigated with both *in vitro* methods.

MATERIALS AND METHODS

Bread samples

White bread, brown bread, wholemeal wheat bread, rye bread, brown bread with sunflower seeds, white bread with hazelnuts, sour-dough fermented brown bread and sour-dough fermented brown bread with sunflower seeds were bought in local stores. The breads were chosen so as to have varying contents of phytic acid (between 0·1 and 8·2 g/kg dry matter). The breads were dried at 60° for 24 h and milled on a 0·5 mm sieve.

Pepsin suspension: 8 g pepsin (*EC* 3.4.23.1) powder (from porcine stomach mucosa; Sigma Chemical Co., Poole, Dorset) was suspended in 50 ml 0·1 M-HCl.

Pancreatin–bile extract mixture: 1 g pancreatin (from porcine pancreas; Sigma) and 6·25 g porcine bile extract (Sigma) were dispersed in 250 ml 0·1 M-$NaHCO_3$. In the *in vitro* method with continuous dialysis the pancreatin–bile extract mixture was used at twice this concentration.

In vitro *method with equilibrium dialysis*

The *in vitro* method with equilibrium dialysis was performed according to Miller *et al.* (1981) and Hazell & Johnson (1987*b*) with slight modifications. The method consists of three parts: peptic digestion, pH adjustment, and pancreatic digestion with equilibrium dialysis.

Peptic digestion. Dry food sample (25 g) was suspended in 200 ml Milli Q water (Millipore Co., Etten-Leur, The Netherlands) in a plastic bottle. After adjusting the pH to 2·1 with HCl, 7·5 ml pepsin suspension was added. The pH was adjusted to $2{\cdot}00 \pm 0{\cdot}03$, the weight of the sample was brought to 250 g with Milli Q water and the sample was incubated in a shaking water-bath at 37° for 2 h. The pH was adjusted to 2·00 every 30 min.

pH-adjustment for pancreatic digestion. The titratable acidity was determined as described by Hazell & Johnson (1987*b*). The suspension after peptic digestion was divided into five portions of 20 g each which were transferred into plastic bottles. Segments of dialysis tubing (molecular weight cut-off 12000–14000, diameter 28·6 mm; Spectra/Por, Spectrum, Houston, TX, USA) containing an amount of $NaHCO_3$ (60 g/l) equivalent to the titratable acidity filled up to 25 ml with Milli Q water were placed in each bottle. The bottles were incubated in a shaking water-bath for 30 min at 37°. For one bottle the incubation was stopped at this moment (*t* 0).

Pancreatic digestion with equilibrium dialysis. To each of the four remaining bottles 5 ml pancreatin–bile extract mixture was added and the samples were incubated in a shaking water-bath at 37° for 0·5, 1, 2·5 or 4 h (*t* 0·5, *t* 1, *t* 2·5 and *t* 4) respectively. Depending on the buffering capacity of the food samples, the resulting pH after dialysis against $NaHCO_3$ and addition of the pancreatin–bile extract mixture varied between 6·7 and 7·0. At the end of the pancreatic digestion the pH was measured; during pancreatic digestion the pH remained fairly constant.

In the dialysates the concentrations of Ca, Mg, Fe, Cu and Zn were determined. A blank was run in each experiment to correct for small amounts of dialysable minerals and trace elements from the reagents.

The method is based on the formation of an equilibrium across a semipermeable membrane. In general, an equilibrium was reached after 2·5 h. Consequently, the amount of dialysed Ca, Mg, Fe, Cu and Zn was calculated as the mean value of *t* 2·5 and *t* 4. As the volumes at both sides of the membrane are equal, the amounts of dialysed Ca, Mg, Fe,

Cu and Zn represent only half the amounts dialysable. For this reason the amounts of dialysable minerals were calculated as twice the amount dialysed. The dialysability was expressed as a percentage of the amounts of Ca, Mg, Fe, Cu and Zn present in the food sample. The dialysability was calculated according to the following equation:

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

where D is the amount of mineral dialysed, calculated as the mean of the values at t 2·5 and t 4 (mg), W is the dry weight of the food sample used for pancreatic digestion (g), A is the concentration of mineral present in the dry food sample (mg/g).

In vitro *method with continuous dialysis using a hollow-fibre*

The *in vitro* method with continuous dialysis was partly based on the *in vitro* methods described by Miller *et al.* (1981) and Hazell & Johnson (1987*b*). The simulation of the small intestine was different. Furthermore, samples and reagents were used at twice the concentration of those described for the method with equilibrium dialysis because otherwise the levels of minerals and trace elements in the (continuous) dialysate are sometimes too low to allow accurate determination. The continuous *in vitro* method consisted of three parts: peptic digestion, pH adjustment, and pancreatic digestion with continuous dialysis.

Peptic digestion. A dry food sample (50 g) was suspended in 175 ml Milli Q water (Millipore Co.) in a plastic bottle. After adjustment of the pH to 2·1 with HCl, 15 ml pepsin suspension was added. The pH was adjusted to $2{\cdot}00 \pm 0{\cdot}03$, the weight of the sample was brought to 250 g with Milli Q water and the sample was incubated in a shaking water-bath at 37° for 2 h. The pH was adjusted to 2·00 every 30 min.

pH adjustment for pancreatic digestion. Titratable acidity was determined as described by Hazell & Johnson (1987*b*). After peptic digestion 20 g suspension was transferred into a reaction vessel. A segment of dialysis tubing (molecular weight cut-off 12000–14000, diameter 28·6 mm; Spectra/Por, Spectrum, Houston, TX, USA) containing $NaHCO_3$ (60 g/l) equivalent to the titratable acidity filled up to 5 ml with Milli Q water was placed into the reaction vessel. The reaction vessel was incubated for 30 min at 37° in a shaking water-bath. After this stage the contents of the dialysis tubing were added to the reaction vessel. The dialysis tubings were rinsed with 5 ml Milli Q water which was also added to the reaction vessel.

Pancreatic digestion with continuous dialysis. Pancreatin–bile extract mixture (5 ml) was added to the reaction vessel and the mixture was incubated for 4 h at 37°. During this pancreatic digestion the mixture was led through a hollow-fibre system. Every 30 min the dialysate was collected. As in the *in vitro* method with equilibrium dialysis, the resulting pH after addition of $NaHCO_3$ and pancreatin–bile extract mixture varied between 6·7 and 7·0. During pancreatic digestion the pH in the reaction vessel decreased by 0·1–0·4 units (depending on the type of sample).

The concentrations of Ca, Mg, Fe, Cu and Zn in the dialysates were determined. Every four or five experiments a blank experiment was carried out to correct for small amounts of dialysable minerals and trace elements from the reagents.

The hollow-fibre system is represented schematically in Fig. 1.The reaction vessel (1) was placed in a water bath at 41° (the temperature inside the reaction vessel was 37°); in this reaction vessel the pancreatic digestion takes place. The suspension was pumped via a peristaltic pump (2) through the suction tube (3) into the hollow-fibre (4) (Amicon, molecular weight cut-off 10000, Type H1P3-20; Amicon Division, W. R. Grace & Co.,

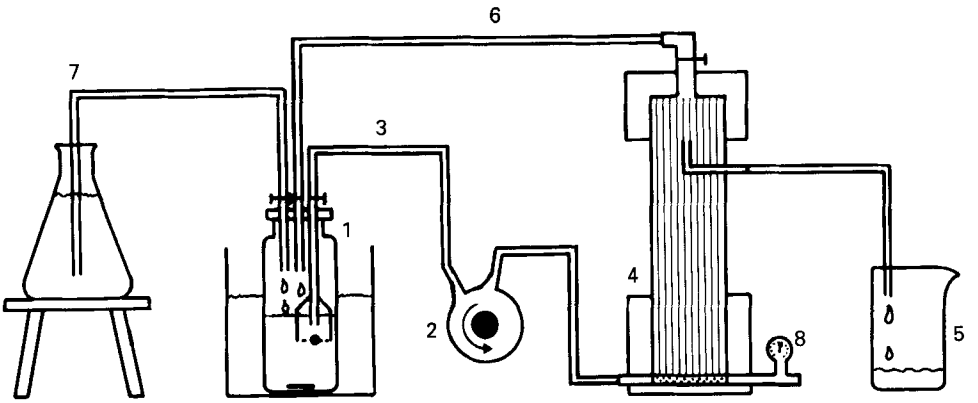

Fig. 1. Schematic representation of hollow-fibre system for continuous dialysis. For details of procedure, see pp. 852–853.

Danvers, USA). Components in the suspension that could pass the hollow-fibre membrane were dialysed and collected in a plastic bottle (5). The dialysis flow was 2 ml/min. That part of the suspension that could not pass the hollow-fibre membrane was pumped back into the reaction vessel via the recycle tube (6). In the reaction vessel these components could be digested further. The recycle flow was 50 ml/min. The volume in the reaction vessel was kept constant by a siphon (7). The siphon vessel was filled with Milli Q water (pH 7·0). The suction tube had a plastic cap on the end, this cap had some holes on the top to maintain a constant pressure inside and outside the cap. A fine filter cloth was stretched over the plastic cap to prevent large food particles from entering the hollow-fibre system. A magnetic stirrer was put inside the plastic cap to prevent clogging of the filter cloth. A filter was placed in front of the hollow-fibre membrane to prevent clogging of the membrane. The pressure on the hollow-fibre membrane could be measured by a manometer (8); this pressure may not exceed 170 kPa. After each experiment the hollow-fibre system was cleaned by pumping successively 0·1 M-HCl and water through the system.

The amount of dialysed material (dialysability) was expressed as a percentage of the total amount present in the food sample. The dialysability was calculated according to the following equation:

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

where D is the total amount of mineral dialysed in 4 h (mg), W is the dry weight of food sample used for pancreatic digestion (g), A is the concentration of mineral present in dry food sample (mg/g).

Investigation of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn

The possibility of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn was investigated by dialysing pure salts. $CaCl_2$ was dissolved in Milli Q water (pH 7·0), $MgSO_4$ was dissolved in 0·1 M-phosphate buffer (pH 7·0) and $FeSO_4$ was dissolved in 0·1 M-phosphate buffer (pH 7·0). In the last case a precipitate was formed immediately. The supernatant fraction was filtered and the Fe content determined. As the amounts of $CuSO_4$ and $ZnCl_2$ soluble in phosphate buffer of pH 7·0 were too small to be dialysed and analysed accurately, these salts were dissolved in 0·1 M-Tris buffer (pH 7·0).

The clear solutions were put in the reaction vessel of the hollow-fibre system and dialysed for 4 h. Other reagents were not added. The siphon vessel contained Milli Q water during

the experiment with Ca, 0·1 M-phosphate buffer (pH 7·0) during the experiments with Mg and Fe, and 0·1 M-Tris buffer (pH 7·0) during the experiments with Cu and Zn. Ca, Mg, Fe, Cu and Zn were determined in the dialysate and in the reaction vessel after dialysis.

Analytical methods

Phytic acid was determined after extraction with dilute HCl by ion-exchange chromatography with post-column derivatization and u.v. detection as described by Bos *et al.* (1991). Ca, Mg and Zn were determined with flame atomic absorption spectroscopy. Fe and Cu were determined with graphite-furnace atomic absorption spectroscopy.

RESULTS AND DISCUSSION

To estimate the bioavailability of minerals and trace elements a continuous *in vitro* method was developed as an alternative to the equilibrium *in vitro* method developed by Miller *et al.* (1981). In the equilibrium *in vitro* method components that pass the membrane are not removed, in contrast with the situation *in vivo*. As we expected these dialysable components to influence the equilibrium dialysis of minerals and trace elements, we studied the influence of the continuous removal of dialysable components from the pancreatic digestion mixture on the dialysability of Ca, Mg, Fe, Cu and Zn.

Investigation of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn

The hollow-fibre membranes were made of polysulphone and should be inert. However, we investigated whether Ca, Mg, Fe, Cu or Zn bound to the hollow-fibre membrane, because this might lead to errors. Possible interactions were investigated by dialysis of pure salts.

It was found that the Ca from the $CaCl_2$ solubilized in water (pH 7·0), the Mg from $MgSO_4$ solubilized in phosphate buffer (pH 7·0), the Cu from $CuSO_4$ and the Zn from $ZnCl_2$ solubilized in Tris buffer (pH 7·0) were completely recovered. Dialysis experiments with $FeSO_4$ were hampered because the phosphate buffer appeared to be largely contaminated with Fe. When only this phosphate buffer was dialysed in the hollow-fibre system the Fe from this buffer was completely recovered. No evidence was found for binding of Ca, Mg, Fe, Cu or Zn to the hollow-fibre membrane at pH 7. This, in combination with the inert character of the polysulphone membrane, led to the conclusion that dialysability measurements are not likely to be disturbed by binding of minerals or trace elements to the hollow-fibre membrane.

Repeatability of the continuous in vitro *method*

The repeatability of the continuous *in vitro* method was tested with a sample of wholewheat meal. The dialysability of Ca, Mg, Fe, Cu and Zn was tested in triplicate; the mean values obtained (%) were: Ca 35 (SD 4), Mg 57 (SD 3), Fe 21 (SD 3), Cu 76 (SD 5), Zn 24 (SD 6). It is concluded that the repeatability of the determination of the dialysability of Ca, Mg, Fe, Cu and Zn with the continuous *in vitro* method is good.

Influence of pH during pancreatic digestion on dialysability

The solubility of minerals and trace elements decreases and the binding of minerals and trace elements by dietary fibre and phytic acid increases with increasing pH (Fernandez & Phillips, 1982; Sri Kantha *et al.* 1986; Martin & Evans, 1986, 1987; Champagne, 1988; Champagne & Phillippy, 1989; Sandberg *et al.* 1989). Therefore, it is expected that pH influences the bioavailability of minerals and trace elements. We investigated the influence of pH during pancreatic digestion on the dialysability of Ca, Mg, Fe, Cu and Zn from a

Table 1. *Influence of pH on dialysability (% of the amount present) of Ca, Mg, Fe, Cu and Zn from rye bread*

Element	pH	Dialysability: *In vitro* method with equilibrium dialysis	Dialysability: *In vitro* method with continuous dialysis
Ca	6·2	74	—
	6·6	68	73
	6·9	50	—
	7·1	44	33
	7·4	42	—
Mg	6·2	68	—
	6·6	69	66
	6·9	68	—
	7·1	68	55
	7·4	69	—
Fe	6·2	22	—
	6·6	21	30
	6·9	22	—
	7·1	18	23
	7·4	18	—
Cu	6·2	38	—
	6·6	40	95
	6·9	39	—
	7·1	38	59
	7·4	41	—
Zn	6·2	46	—
	6·6	48	50
	6·9	47	—
	7·1	46	48
	7·4	49	—

sample of rye bread. Dialysability was measured with both the *in vitro* method with continuous dialysis and the *in vitro* method with equilibrium dialysis. The dialysability with the equilibrium *in vitro* method was studied at pH 6·2, 6·6, 6·9, 7·1 and 7·4, while the dialysability with the continuous *in vitro* method was studied at pH 6·6 and 7·1. The results are shown in Table 1. For the *in vitro* method with equilibrium dialysis there was a marked decrease in the dialysability of Ca and a slight decrease in dialysability of Fe with increasing pH. The dialysability of Mg, Cu and Zn was not affected by pH. For the *in vitro* method with continuous dialysis there was a decrease in the dialysability of Ca, Mg, Fe and Cu with increasing pH. The dialysability of Zn was not affected.

As the influence of pH may depend on the type of sample, we investigated the influence of pH on the dialysability of Ca, Mg, Fe, Cu and Zn from carrots for the *in vitro* method with equilibrium dialysis (values not shown). For carrots the dialysability of Ca, Mg, Fe and Zn was influenced by pH. Only the dialysability of Cu was not influenced. This demonstrates that the influence of pH is dependent on the type of food sample.

These results clearly show that there is a great influence of pH during pancreatic digestion on the dialysability of minerals and trace elements. The actual influence depends on the type of food sample and on the *in vitro* method used. Miller *et al.* (1981) found no or only a very small influence of pH during pancreatic digestion on the dialysability of Fe

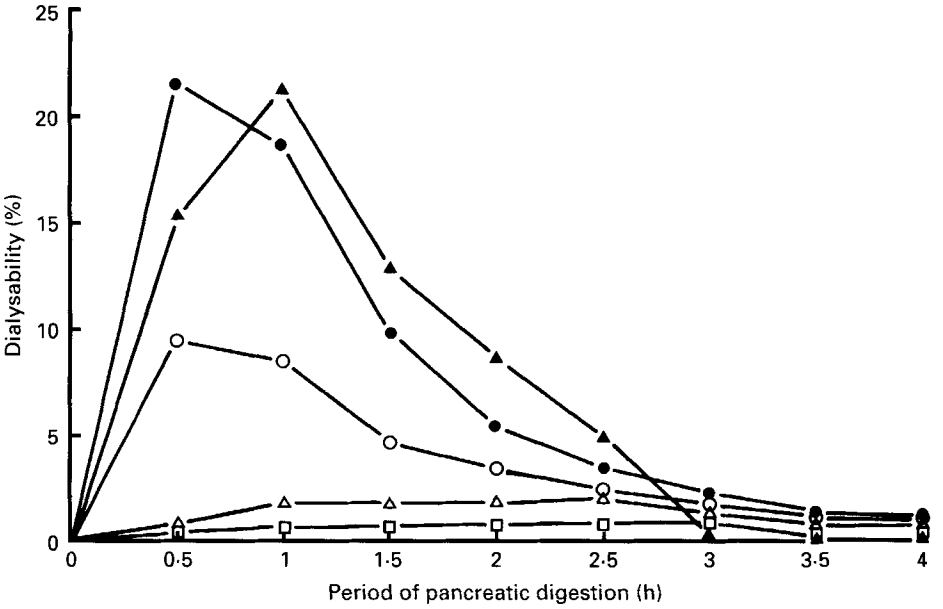

Fig. 2. Continuous dialysis of Ca (○–○), Mg (●–●), Fe (△–△), Cu (▲–▲) and Zn (□–□) from wholemeal wheat bread. For details of procedures, see pp. 852–854.

as estimated with the *in vitro* method with equilibrium dialysis. This may be explained by the type of samples studied by Miller *et al.* (1981) (complex meals).

Dialysability of Ca, Mg, Fe, Cu and Zn from several types of bread: comparison between equilibrium dialysis and continuous dialysis

The dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread was determined with both the equilibrium *in vitro* method and the continuous *in vitro* method reported in the present paper. Fig. 2 shows the results of the continuous dialysis of Ca, Mg, Fe, Cu and Zn from wholemeal wheat bread. The dialysis was almost complete after 3 h. The dialysability was calculated as the total amount of Ca, Mg, Fe, Cu or Zn dialysed in 4 h.

In the first hour the amount of dialysed minerals and trace elements was very high. This may have been due partly to the high initial concentration of minerals and trace elements and partly to the pH. At *t* 0, the pH varied between 5 and 5·5 and the pancreatin–bile extract mixture was added. In the next 30 min the pH rose to 7. As is shown in Table 1, a lower pH generally resulted in a higher dialysability. A similar pH course was found in the equilibrium *in vitro* method. The pH course observed *in vitro* agrees well with that in the *in vivo* situation: the pH in the duodenum is lower than in the jejunum where the pH is about 7 (Clemens *et al.* 1975). In the duodenum a large part of the minerals and trace elements is absorbed (Avioli, 1988; Fairbanks & Beutler, 1988; Shils, 1988; Solomons, 1988; Wilson & Greene, 1988), but it is not clear whether the higher initial concentration and the lower pH are the only important factors here.

As a consequence of the continuous removal of dialysable components in the continuous *in vitro* method, the pH during the 4 h pancreatic digestion decreased slightly. This is in agreement with the situation found in *in vivo* experiments with pigs: the pH in the ileum is slightly lower than that in the jejunum (Clemens *et al.* 1975). In the equilibrium *in vitro* method, on the other hand, the pH remained rather constant during pancreatic digestion.

The dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread as determined by the *in vitro* method with equilibrium dialysis and the *in vitro* method with continuous dialysis is presented in Table 2. In general, the dialysability of Ca, Mg, Fe and Cu determined by the continuous *in vitro* method is higher than that determined with the equilibrium *in vitro* method (up to a factor 3–4). For Zn both methods gave comparable dialysabilities. Linear regression analysis showed a positive linear correlation between the dialysabilities of Fe, Cu and Zn as determined by the two *in vitro* methods (r 0·84, 0·89 and 0·96 respectively). For Mg and Ca no correlation was found.

These results show that removal of dialysable components from the pancreatic digestion mixture has a marked influence on the dialysability of minerals and trace elements. Whether removal of dialysable components leads to better estimates of bioavailability *in vivo* is currently being investigated.

The influence of phytic acid on the dialysability of Ca, Mg, Fe, Cu and Zn

The breads were chosen to contain variable amounts of phytic acid because phytic acid is known to have a strong negative influence on the bioavailability of some minerals and trace elements. The contents of phytic acid, Ca, Mg, Fe, Cu and Zn in the breads are presented in Table 3. Table 2 shows the dialysability of Ca, Mg, Fe, Cu and Zn from the breads.

The phytic acid content increased in the order white bread < brown bread < wholemeal wheat bread, whereas the dialysability of Fe and Zn as determined with the equilibrium *in vitro* method decreased dramatically. There was a smaller decrease in the dialysability of Cu. For the continuous *in vitro* method the dialysability of Ca, Fe and Zn decreased with increasing phytic acid content. There was a smaller decrease in dialysability of Mg. These results agree with other studies where *in vitro* and *in vivo* experiments have shown a strong negative influence of phytic acid on the bioavailability of Ca, Fe and Zn, and a less marked influence on the bioavailability of Mg and Cu (Spivey Fox & Tao, 1989; Torre *et al.* 1991).

Addition of sunflower seeds to brown bread led to a 3-fold increase in phytic acid content. This led to a decrease in the dialysability of Ca, Mg, Fe and Zn as determined by the equilibrium *in vitro* method. The dialysability of Cu increased. Apart from a small increase in Ca dialysability, the continuous *in vitro* method resulted in similar dialysabilities for brown bread and brown bread with sunflower seeds. This shows that phytic acid is not the only component influencing the bioavailability of minerals and trace elements from bread samples. Therefore, the effect of phytic acid cannot be studied separately from the effects of other components on the bioavailability of minerals and trace elements.

Addition of hazelnuts to white bread led to an increase in phytic acid content. At the same time the dialysability of Fe and Cu as determined with the equilibrium *in vitro* method decreased. When determined by the continuous *in vitro* method the dialysability of Ca, Mg and Fe decreased. Despite the rise in phytic acid content, both *in vitro* methods showed an increase in Zn dialysability.

Sour-dough fermentation of bread resulted in a significant reduction in phytic acid content. This has been reported previously by van Lonkhuijsen & van Gelderen (1985). Although the equilibrium *in vitro* method showed a small increase in Ca dialysability for sour-dough fermented brown bread and a small increase in Fe and Zn dialysability for sour-dough fermented brown bread with sunflower seeds, neither the equilibrium *in vitro* method nor the continuous *in vitro* method showed a clear positive influence of the decrease in phytic acid content on the dialysability of Ca, Mg, Fe, Cu and Zn. In general, reduction of the phytic acid content by sour-dough fermentation is believed to increase the bioavailability of minerals and trace elements because the phytic acid content is reduced. However, the phosphate produced from the phytic acid during sour-dough fermentation may also have a negative effect on the bioavailability of minerals and trace elements

Table 2. *Dialysability of Ca, Mg, Fe, Cu and Zn (% of the amount present) from eight different types of bread determined with the equilibrium* in vitro *method and the continuous* in vitro *method**

(Means of duplicate analyses)

Type of bread	*In vitro* method	Dialysability				
		Ca	Mg	Fe	Cu	Zn
White bread	Equilibrium	12	52	36	50	27
	Continuous	53	76	46	64	27
Brown bread	Equilibrium	22	56	14	44	11
	Continuous	30	64	27	67	15
Wholemeal wheat bread	Equilibrium	17	51	7	41	5
	Continuous	34	66	11	63	7
Rye bread	Equilibrium	33	66	11	25	4
	Continuous	43	63	13	57	10
Brown bread with sunflower seeds	Equilibrium	12	41	7	61	7
	Continuous	40	59	29	71	16
White bread with hazelnuts	Equilibrium	16	50	6	37	38
	Continuous	31	62	9	60	44
Sour-dough fermented brown bread	Equilibrium	32	63	9	47	13
	Continuous	35	68	12	72	12
Sour-dough fermented brown bread with sunflower seeds	Equilibrium	15	45	13	63	14
	Continuous	42	61	25	78	17

* For details of procedures, see pp. 851–854.

Table 3. *Contents of phytic acid (g/kg dry matter (DM)), Ca, Mg, Fe, Cu and Zn (mg/kg DM) in various types of bread*

Type of bread	Phytic acid	Ca	Mg	Fe	Cu	Zn
White bread	0·1	360	320	17	2	11
Brown bread	2·9	470	890	32	4	20
Wholemeal wheat bread	4·3	880	1020	44	4	27
Rye bread	2·0	430	1100	35	4	38
Brown bread with sunflower seeds	8·2	570	1670	47	6	27
White bread with hazelnuts	1·6	620	480	22	4	12
Sour-dough fermented brown bread	0·5	360	760	34	3	17
Sour-dough fermented brown bread with sunflower seeds	4·0	470	1380	40	7	29

(Simpson *et al.* 1981; Nävert *et al.* 1985; Hallberg, 1987; Hallberg *et al.* 1987). Moreover, some lower inositol phosphates may influence the bioavailability of minerals and trace elements (Lönnerdal *et al.* 1989; Sandberg *et al.* 1989; Simpson & Wise, 1990).

It should be noted that although the relative dialysability of minerals and trace elements is lower for certain breads, the absolute bioavailability may be higher due to the higher absolute content of minerals and trace elements present in those breads (Table 2).

Our results show that for white bread, brown bread and wholemeal wheat bread there is a marked negative effect of phytic acid on the dialysability of Ca, Fe and Zn. Results for brown bread with sunflower seeds, white bread with hazelnuts and the two types of sour-dough fermented bread, however, show that the bioavailability of minerals and trace

elements from bread samples is not related to the phytic acid content only. Therefore, it is concluded that the effect of phytic acid on the bioavailability of minerals and trace elements cannot be studied separately from the effects of other components on this bioavailability. Removal of dialysable components as performed in the continuous *in vitro* method appears to have a marked effect on the influence of phytic acid on the bioavailability of minerals and trace elements. This once more stresses the importance of a better understanding of the removal of dialysable components.

Conclusions

The *in vitro* method with continuous dialysis for the estimation of the bioavailability of minerals and trace elements presented here takes continuous removal of dialysable components into account. Experiments showed that there is no interaction between the hollow-fibre membrane used in the continuous *in vitro* method and Ca, Mg, Fe, Cu and Zn ions. Therefore, dialysability measurements are not likely to be disturbed by binding of minerals or trace elements to the hollow-fibre membrane. There is a large influence of pH during pancreatic digestion on the dialysability of Ca, Mg, Fe, Cu and Zn determined by both *in vitro* methods. The actual influence depends on the type of sample and on the *in vitro* method used.

For most types of bread the continuous *in vitro* method leads to higher dialysabilities of Ca, Mg, Fe and Cu than the equilibrium *in vitro* method. The dialysability of Zn is for most breads comparable for the two methods. It is concluded that removal of dialysable components has a marked influence on the dialysability of minerals and trace elements. However, the importance for the estimation of bioavailability *in vivo* needs further investigation.

Phytic acid has a negative effect on the bioavailability of Ca, Fe and Zn. However, the effect of phytic acid cannot be studied separately from effects of other components on the bioavailability of minerals and trace elements from breads. It has been shown that removal of dialysable components *in vitro* influences the effect of phytic acid on the bioavailability of minerals and trace elements.

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