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# *In vitro* availability of zinc from infant foods with increasing phytic acid contents

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An *in vitro* method was used to determine the availability of Zn from infant foods containing increasing amounts of phytate, and to quantify the effect of the phytate:Zn molar ratio on the availability. During the *in vitro* assay, digestive conditions of infants, younger and older than 4 months of age, were carefully simulated since the solubility of phytate–Zn complexes during digestion is pH dependent. Availability was measured with a continuous flow dialysis *in vitro* procedure with previous intralumen digestive stage. Zn concentrations were determined with flame atomic absorption spectrometry. Phytic acid content was measured with HPLC. Adding phytate to infant formula lowered Zn availability to 2·84 (SD 0·17) % when the phytate:Zn molar ratio increased to 2·2 ($P < 0·05$), as compared with cows' milk-based formula (6·65 (SD 0·55) %). Availability from vegetables (23·83 (SD 2·17) %) significantly decreased ($P < 0·05$) at a ratio $> 7·9$ (15·12 (SD 1·63) %). Zn availability from soyabean-based formula (2·26 (SD 0·36) %) was lower ($P < 0·05$) compared with cows' milk-based formula (6·65 (SD 0·55) %). Availability between soyabean- and cows' milk-based formula was similar ($P > 0·05$) when a phytate:Zn ratio of 2·2 (2·84 (SD 0·17) %) was obtained in the cows' milk formula. The negative effect of phytic acid on Zn availability was dependent on the type of the food and the phytate content, and should be considered when using soyabean-based formulas during early infancy.

**Bioavailability of micronutrients: Zinc: Phytate: Infants: *In vitro* method**

Phytic acid naturally occurs in many foods derived from plants. It is the storage form of P in most seeds. Of the total amount of P in plants, approximately 60–90 % is found as phytate (Cheryan, 1980). Phytic acid forms strong ionic complexes (phytates) with many essential bi- and trivalent metal ions in foods as well as in the intestine (Nolan & Duffin, 1987; Frølich, 1990). The presence of phytate has therefore been shown to have an inhibitory effect on the bioavailability of minerals and trace elements (Reddy *et al.* 1982; Bosscher *et al.* 1998*b*). The influence of phytate on Ca (Heaney *et al.* 1991; De Vizia & Mansi, 1992), Fe (Sandberg *et al.* 1989, 1993), and Zn (Wise, 1995; Rimbach *et al.* 1998) bioavailability has been extensively studied *in vitro* as well as *in vivo*. Zn is reported to be the essential element most adversely affected by phytate (Torre & Rodriguez, 1991; Van Dyck *et al.* 1996). The binding of Zn by phytate is dependent on several factors such as the amount of phytate, pH, and the presence of other metal ions. At raised stomach pH values, as can be found in infants (Vandenplas, 1992), Zn complexes with phytate yet enter the duodenum in an insoluble form (Champagne, 1988). In addition, infant formulas are often supplemented with relatively large amounts of Ca and Fe, which may further interact with Zn absorption, leading to reduced bioavailability (Morris & Ellis, 1980; Bougle *et al.* 1999). Zn deficiency may lead to deficits in children's growth and development and immunological function, which can result in delayed cognitive performance (Black, 1998) and may increase their susceptibility to a variety of pathogens (Shankar & Prasad, 1998).

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A previous study performed in our laboratory has demonstrated that the availability of Zn in soyabean-based infant formula is significantly lower than in whey- or casein-based formula *in vitro*. It was speculated that the low availability of Zn from the soyabean-based formula was due to its phytate content (Bosscher *et al.* 2001). The present study was designed to determine from which baseline phytate level onwards, phytate might inhibit Zn availability. Two different food matrices were used: infant formula and a homogenised vegetable (green beans; *Phaseolus vulgaris*) preparation, to which increasing amounts of phytate were added. The term 'availability' is used throughout this work to describe the bioavailability of Zn from the foods *in vitro*. Bioavailability can be used as a large concept including digestion, absorption, and incorporation into metabolic processes. It can also be used in a narrow sense, meaning that any potentially available part of a nutrient after gastrointestinal digestion should be attributed as bioavailability (Bender, 1989; Jackson, 1997).

## Materials and methods

### *Samples, reagents, and materials*

The infant formulas were soyabean- and cows' milk-based that are frequently used during the first months of infancy. Infant formulas are currently supplemented with minerals and essential trace elements during the manufacturing process (Table 1). From the age of 4 months on, babies are generally fed homogenised solid foods. A green bean preparation was chosen as a control food because of its low phytic acid content (Table 1). The energy and nutrient composition of the samples is given in Table 1. The energy, protein, fat, and carbohydrate content were taken from manufacturer's information. The % DM was calculated after lyophilisation of the food. The Ca and Zn contents were measured by atomic absorption spectrometry and the phytic acid content was measured by HPLC (Sandberg & Adherinne, 1986; H De Rycke, M Seynaeve and R De Wilde, unpublished results). Phytate (as magnesium potassium salt, P 7660) was obtained from Sigma (St Louis, MO, USA). All chemicals (Merck, Darmstadt, Germany) were of analytical grade. Bi-distilled water (MilliQ; Millipore, Bedford, MA, USA) was used throughout the study. Digestive enzymes and bile salts were purchased from Sigma and Merck. Pepsin (P-7000, from porcine stomach mucosa), pancreatin (107133 0500, porcine) and bile salt (B-8631, porcine) concentrations were specified according to the developing stage of the gastrointestinal tract that had to be simulated *in vitro* (Bosscher *et al.* 1998*a*). For the preparation of infant formulas (method 1): a pepsin solution was made by dissolving 1·5 g pepsin in 15 ml 0·1 M-HCl. The pancreatic–bile mixture contained 0·3 g pancreatin and 0·7 g bile in 100 ml 0·1 M-$NaHCO_3$. For the experiments with green beans (method 2): the pepsin solution contained 3 g pepsin in 15 ml 0·1 M-HCl and the pancreatic–bile mixture was prepared by adding 5·6 g pancreatin and 2·1 g bile to 100 ml 0·1 M-$NaHCO_3$.

The dialysis bags (10–12 kDa, Visking 3-20/32; Medicell Ltd, London, UK) were free from trace metal impurities by boiling in 0·24 M-$NaHCO_3$ with 0·01 M-EDTA solution and 0·003 M-SDS, followed by thorough washing with bi-distilled water.

### *Equipment*

Dialysis cells (model 8200, 200 ml) and related dialysis membranes (molecular weight cut off (MWCO) 1000) were purchased from Amicon Ltd (Beverly, CA, USA). Prior to use, the cells were rinsed with 1·44 M-$HNO_3$ and washed with bi-distilled water. Dialysis membranes were soaked in 0·1 M-NaOH and washed with bi-distilled water.

### *Continuous flow dialysis method with preliminary digestive stage* in vitro

The method consisted of an intralumen digestive stage (Bosscher *et al.* 2000), adapted to the gastrointestinal conditions of infants younger or older than 4 months of age (Bosscher *et al.* 1998*a*), followed by a dialysis procedure in which dialysable food components were continuously removed from the digest (Minihane *et al.* 1993; Shen *et al.* 1994). The method consisted of two phases: a gastric and an intestinal stage. Prior to the gastric stage, the pH of the food sample was lowered to pH 2·0 (infants older than 4 months of age, method 1) or 4·0 (younger than 4 months of age, method 2) with 6 M-HCl, and 3 ml freshly prepared pepsin

**Table 1.** Gross energy and nutrient content of the samples*

| | DM (g) | Gross energy (kJ) | Protein (g) | Fat (g) | Carbohydrate (g) | Phytic acid (mg)† | Ca (mmol) | | Fe (μmol) | | Zn (μmol) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Mean | SD | Mean | SD | Mean | SD |
| Infant formula‡ | | | | | | | | | | | | |
| Cows' milk-based (/l) | 131 | 2800 | 14 | 35 | 75 | – | 13·5 | 0·4 | 97·8 | 3·5 | 85·3 | 1·5 |
| Soyabean-based (/l) | 127 | 2760 | 18 | 36 | 67 | 73·5 | 16·5 | 0·4 | 233 | 3 | 122 | 4 |
| Vegetables | | | | | | | | | | | | |
| Green beans (*Phaseolus vulgaris*) (/kg) | 103 | 1800 | 22 | 2 | 110 | 11·8 | 10·2 | 0·5 | 234 | 2 | 40·6 | 4·1 |

* Energy, protein and fat contents were taken from the manufacturer's information. DM was calculated after lyophilisation of a sample. Calcium and zinc were measured by atomic absorption spectrometry and phytate by HPLC (for details, see p. 243).
† Phytic acid: inositol hexaphosphate.
‡ Calculations were based on normal reconstitution of infant formula powder with water (thirty spoons powder + 850 ml water to give 1 litre formula).

solution was added. The sample was incubated in a shaking water bath for 2 h at 37°C (120 strokes/min). The intestinal stage was performed in a dialysis cell with a dialysis membrane (MWCO, 1000) under a pressure of 350 kDa for continuous flow. The dialysis cell contained a dialysis bag (10–12 kDa), with an amount of $NaHCO_3$ to gradually increase the pH to 7 as the chyme left the stomach and entered the intestine. After 30 min of dialysis, the pancreatic–bile mixture was added to the cell and dialysis was continued for another 2 h. The whole procedure was undertaken four times for each sample and/or blank investigated.

### *Acid destruction of food samples*

Before acid destruction of the food, various aliquots of about 100 g were freeze-dried (GTL, Leybold, Heraeus, Germany). About 0·4 g lyophilised material was placed into a Teflon vial of a polypropylene destruction bomb. Bi-distilled water (1 ml), $H_2O_2$ (suprapure, 300 ml/l, 500 μl), and $HNO_3$ (suprapure, 650 ml/l, 2 ml) were added and the closed vessel was placed in a microwave digestion oven with a turntable (Hendrix *et al.*1998). The lyophilisation procedure was performed in duplicate and acid destructions of the food in quadruplicate.

### *Atomic absorption spectrometry*

The Zn concentration of the samples and dialysate fractions was determined by flame atomic absorption spectrometry. A Perkin-Elmer Analyst 300 atomic absorption spectrometer (Perkin-Elmer; Norwalk, CT, USA) was used in all measurements.

### *HPLC*

The content of phytic acid and inositol phosphates was determined by HPLC with a pulsed electrochemical detector (Dionex, Sunnyvale, CA, USA), as described by H De Rycke, M Seynaeve and R De Wilde, unpublished results.

### *Calculation of the phytate:zinc molar ratio and (phytate×calcium):zinc ratio*

To calculate the phytate:Zn molar ratio of the infant food, the amount of phytate (mg) present in 100 g infant food was divided by the molecular mass of the inositol phosphate, and the result was divided by the total amount of Zn (mmol) in 100 g of the same food. To find the (phytate×Ca):Zn molar ratio, the total amount of Ca (mmol) in 100 g infant food was multiplied by the phytate:Zn ratio. The final result was then recalculated per 1 kg.

### *Calculation of the zinc availability*

The availability of the element was calculated from the amount of element in the dialysate (corrected for blank), in proportion to the total elemental content of the original infant food sample. The following equation was used:

$$\text{Zn availability } (\%) = \frac{(D - Bl)}{W \times A} \times 100,$$

where D was the amount of element in the dialysate after digestion (μg), Bl was the amount of element in the blank dialysate after digestion (μg), W was the dry weight of the food sample used for intestinal digestion (g), and A was the concentration of element in the food sample (μg/g).

### *Assessment of the analytical performance of the* in vitro *procedure*

Initial standardisation was achieved by preparing two aqueous Zn solutions at concentrations of 0·08 mmol/l (sample 1) and 0·15 mmol/l (sample 2), and by using these solutions during digestion and following dialysis to determine recovery of the procedure as described for infants younger (method 1) or older (method 2) than 4 months of age. The repeatability of both procedures was calculated from the Zn availability of the infant formula and of the vegetable preparation on four occasions over 1 d (intra-batch precision). Blanks were taken through the entire procedure and Zn content was measured to correct for element contamination from reagents, equipment or enzymes.

### *Statistical analysis*

One-way ANOVA procedures were applied by using Sigma Stat (SPSS Inc. Software and Services; San Rafall, CA, USA). Differences were considered statistically significant at $P < 0{\cdot}05$. Values are means and standard deviations ($n$ 4).

## Results

### *Validation criteria for the atomic absorption spectrometric technique*

Accuracy of the technique for Ca and Zn was checked before the start of every assay by analysing non-fat milk powder (NBS 1549; National Bureau of Standards, Gaithersberg, FL, USA), which yielded values of 46·93 (SD 0·02) μg Zn/g and 13·3 (SD 0·25) mg Ca/g that fell between the boundaries of the certified value (46·1 (SD 2·2) μg Zn/g, 1·30 (SD 0·05) mg Ca/g). Precision of the atomic absorption spectrometric technique was tested on a standard solution for Ca (2 mg/l) and Zn (0·2 mg/l), and yielded values of 0·98 % for Ca and 1·01 % for Zn.

### *Validation criteria for the HPLC technique*

To determine accuracy of the technique standard solutions of 10 ($n$ 4), 25 ($n$ 5), 50 ($n$ 10), 60 ($n$ 4), and 100 mg/l ($n$ 10) were measured individually on subsequent days and linearity of the regression curve was determined. Linearity was highly significant in the range of 10–100 mg/l ($r^2$ 0·996, $P < 0{\cdot}001$). Precision of the HPLC technique was tested on a 50 mg/l standard solution ($n$ 10) and yielded a CV of 4 %.

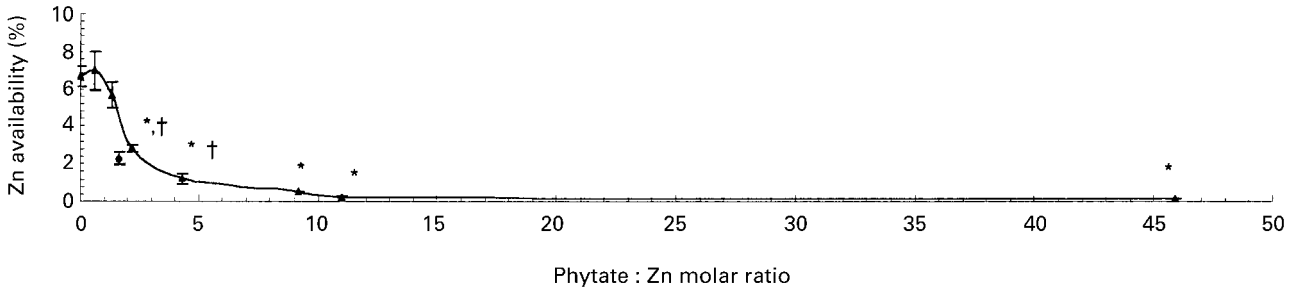


**Fig. 1.** Effect of phytate:zinc molar ratio on zinc availability from cows' milk-based (▲) and soyabean-based (●) infant formulas. Values are means for four determinations with standard deviations represented by vertical bars. For details of procedures, see p. 242. Mean values were significantly different from the non-supplemented cows' milk-based formula: * $P < 0{\cdot}05$. Mean values were not significantly different from the soyabean-based formula: † $P > 0{\cdot}05$.

### *Assessment of the analytical performance of the* in vitro *procedure*

Recovery of Zn from sample 1 was 110 (SD 5) % and from sample 2 was 107 (SD 5) %. The repeatability of the method was 8·22 % for the infant formula and 9·11 % for the vegetable preparation ($N$ 4). Zn concentrations in the blank dialysates of method 1 were 0·038 (SD 0·005) μg/ml, and were subtracted from the infant formula dialysates. Zn concentrations in the blank dialysates in method 2 were 0·314 (SD 0·021) μg/ml, and were subtracted from the green-bean dialysates.

### *Zinc availability from the infant food samples*

Zn availability from infant formula with phytic acid decreased as the phytate:Zn molar ratio (and (phytate×Ca):Zn ratio) increased (Figs. 1 and 2). One-way ANOVA (with a Tukey *post-hoc* test for multiple comparisons) indicated a significantly lower *in vitro* Zn availability from infant formula when a phytate:Zn molar ratio 2·2 ((phytate×Ca):Zn ratio 256) was obtained (2·84 (SD 0·17) %) as compared with the formula without phytate (6·65 (SD 0·55) %) ($P < 0{\cdot}05$). The test statistics also demonstrated significantly lower Zn availability from the soyabean-based formula (2·26 (SD 0·36) %) compared with the availability from the cows' milk-based formula (6·65 (SD 0·55) %) ($P < 0{\cdot}05$). If the phytate:Zn molar ratio of the infant formula increased to 2·2 ((phytate×Ca):Zn 256), Zn availability (2·84 (SD 0·17) %) was similar to the soyabean-based formula (2·26 (SD 0·36) %) ($P > 0{\cdot}05$).

The availability of Zn from an infant vegetable preparation decreased with increasing phytate:Zn molar ratio (and (phytate×Ca):Zn ratio) (Figs. 3 and 4). From the one-way ANOVA (Tukey *post-hoc* test) a significant decrease in Zn availability was found when the phytate:Zn molar ratio increased to the value of 7·9 ((phytate×Ca):Zn ratio 791) (15·12 (SD 1·63) %), when compared with the vegetable preparation without added phytate (23·83 (SD 2·17) %) ($P < 0{\cdot}05$).

## Discussion

From this *in vitro* model Zn availability was 6·65 (SD 0·55) % from cows' milk-based formula, and 2·26 (SD 0·35) % from soyabean-based infant formula. These results correspond well with data from *in vivo* studies. Ziegler *et al.* (1989) found net $^{70}$Zn absorption by infants of 9·1 (SD 8·7) % from extrinsically labelled formulas. Lönnerdal (1994) and Hambidge *et al.* (1979) both showed significant lower plasma Zn levels in infants fed soyabean-based formula than infants fed cows' milk-based formula.

Using an *in vitro* method, we demonstrated that addition of phytate to cows' milk-formula (phytate:zinc 2·2; (phytate×Ca):Zn 256) at a level similar to that of soyabean-based formula (phytate:Zn molar ratio 1·6; (phytate×Ca):Zn ratio 208) caused a significant reduction in Zn availability so that it was similar to that from

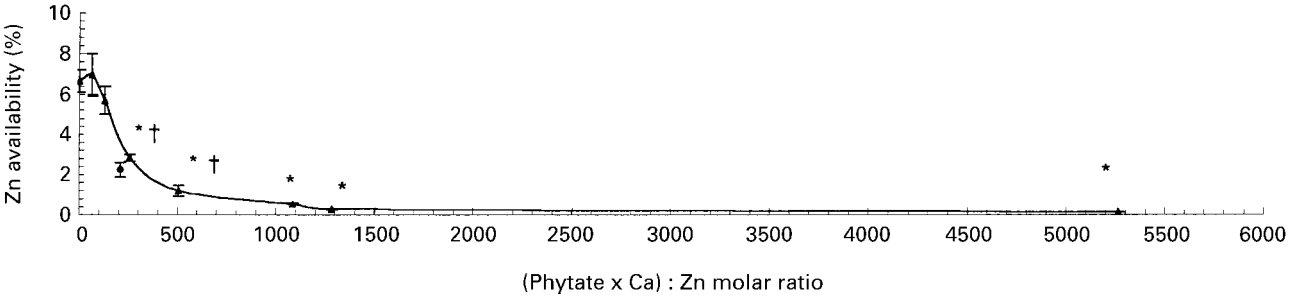


**Fig. 2.** Effect of (phytate×calcium):zinc molar ratio on zinc availability from cows' milk-based (▲) and soyabean-based (●) infant formulas. Values are means for four determinations with standard deviations represented by vertical bars. For details of procedures, see p. 242. Mean values were significantly different from the non-supplemented cows' milk based formula: * $P < 0{\cdot}05$. Mean values were not significantly different from the soyabean-based formula: † $P > 0{\cdot}05$.

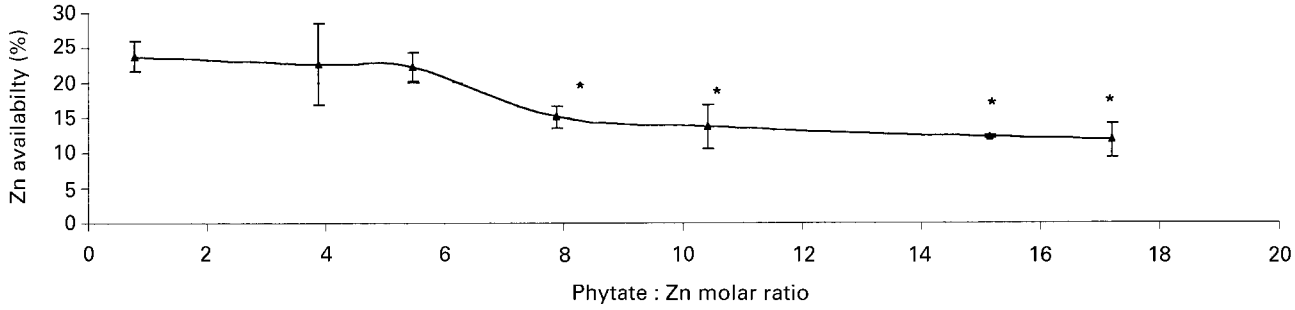


**Fig. 3.** Effect of phytate:zinc molar ratio on zinc availability from vegetables (green beans; *Phaseolus vulgaris*). Values are means for four determinations with standard deviations represented by vertical bars. For details of procedures, see p. 242. Mean values were significantly different from the non-phytate-supplemented vegetables: * $P < 0{\cdot}05$.

soyabean-based formula (Figs. 1 and 2). These findings strongly suggest that the low availability of Zn from foods based on soyabean proteins may be attributed to their phytate content. These data are in agreement with studies performed by O'Dell & Savage (1960) and Sandström *et al.* (1983*a*,*b*).

From this *in vitro* model it appeared that each step in increasing the phytate content of the infant food resulted in a stepwise decrease in Zn availability. These data are in agreement with studies performed by Lönnerdal *et al.* (1988), Rimbach *et al.* (1995) and Couzy *et al.* (1998), who have determined Zn absorption after intake of phytate-containing foods. Our experiments on infant formula indicate that phytate:Zn molar ratios $>1{\cdot}5$, or (phytate×Ca):Zn molar ratios $>200$, can negatively affect Zn availability *in vitro* (Figs. 1 and 2). These results correspond well with the findings of Ellis *et al.* (1987), who indicated that human subjects who have a (phytate×Ca):Zn ratio $>200$ may have increased risk of impaired Zn bioavailability. From our *in vitro* results, Zn availability from the vegetable preparation markedly decreases if the phytate:Zn molar ratio increases to 7·9, or when the ratio (phytate×Ca):Zn increases to 791 (Figs. 3 and 4). In the study of Bindra *et al.* (1986) serum Zn levels of Canadian omnivores were compared with those of Punjababi Sikhs having a diet that contained 90 % more phytate, and 35 % more Ca, but 12 % less Zn. Serum Zn levels of the Sikhs were much lower compared with the omnivores. The authors concluded that diets containing phytate:Zn ratios of 17·7, or (phytate×Ca):Zn ratios $>500$, should be considered as problematic for man. Due to the different composition of both diets, it was argued that the Ca-potentiating effects, expressed as (phytate×Ca):Zn, could have been responsible, but also the phytate content by itself.

In our present study, a whey protein-based infant formula was used as a vehicle for phytate because of its similar content compared with the soyabean protein-based infant formula, especially considering Ca. In addition, the absence of phytate in this formula makes it possible to study the effect of phyate:Zn ratios $<1{\cdot}6$, without interference from inositol phosphate intermediaries.

Recently, the WHO included the phytate:Zn ratio of the food as criteria for categorising diets according to the potential availability of their Zn content (World Health Organization, 1996). Diets are characterised by low, moderate, or high bioavailablity according to their composition. Diets from which availability of Zn is low may contain high phytate, soyabean-protein products or have a phytate:Zn molar ratio $>15$. Moreover, high intakes of inorganic Ca salts, as is the case in soyabean-protein based infant formulas, are found to potentate the inhibitory effects of these low bioavailable diets (World Health Organization, 1996). Because of synergistic effects between phytate and high Ca on Zn absorption, the (phytate×Ca):Zn molar ratio of the diet is also frequently used to express Zn bioavailability (Oberleas & Harland, 1981; Forbes *et al.* 1983).

It has been described that during industrial processing of soyabean proteins, phytates may be partly degraded into inositol phosphate intermediates (Liener, 1993), and that only inositol hexa- and pentaphosphates have a negative impact on Zn absorption (Lönnerdal *et al.* 1989; Sandström

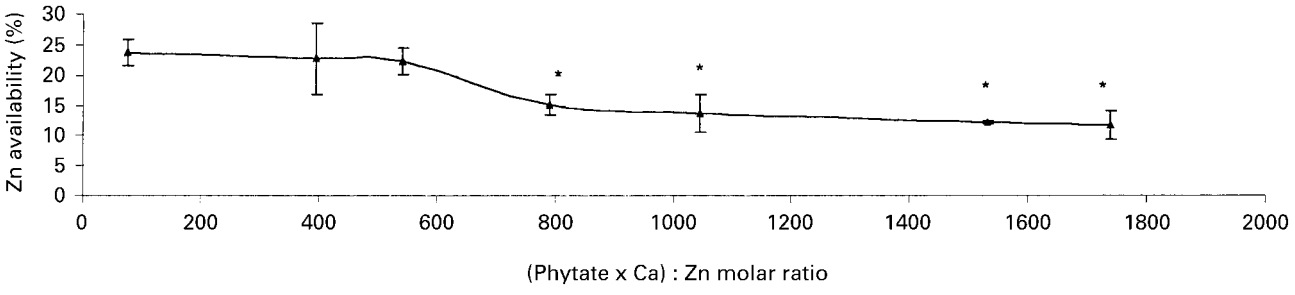


**Fig. 4.** Effect of (phytate×calcium):zinc molar ratio on zinc availability from vegetables (green beans; *Phaseolus vulgaris*). Values are means for four determinations with standard deviations represented by vertical bars. For details of procedures, see p. 242. Mean values were significantly different from the non-phytate-supplemented vegetables: * $P < 0{\cdot}05$.

& Sandberg, 1992). Thus, knowledge about the levels of inositol hexa- and pentaphosphates may give some information about Zn availability. When considerable amounts of lower inositol phosphates (inositol tri- and tetraphosphates) are also present this seems to be an oversimplification. It appears that a mixture of inositol phosphates may have other effects than the pure fractions. Lower inositol phosphates (inositol tri- and tetraphosphates) may then also contribute to the inhibitory effect on Zn absorption (Sandström *et al.* 1987). According to Sandberg *et al.* (1993), Zn absorption may be correlated to the sum of inositol tri-phosphates to hexaphosphates in a number of composite meals. Because of the presence of appreciable amounts of lower inositol phosphate intermediaries in our soyabean protein-based formula, (phytate×Ca):Zn ratios were calculated from the sum of inositol triphosphates to hexaphosphates. However, since the addition of pure penta- or hexaphosphates has a major impact on Zn availability from phytate-enriched foods, (phytate×Ca):Zn ratios in whey-based formula and green beans were calculated using only both higher inositol phosphate intermediaries.

In general, it appears that phytate:Zn ratios $>1{\cdot}5$ may inhibit Zn availability in small infants. After 6 months of life, this ratio increases to approximately 8.

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