The effects of dietary ligands on zinc uptake at the porcine intestinal brush-border membrane

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Intestinal brush-border-membrane vesicles were prepared from the porcine small bowel by magnesium precipitation and differential centrifugation, and were functionally intact. The influence of dietary ligands on ^{65}Zn uptake was determined using a ^{65}Zn concentration of 5 μM, an incubation time of 1 min and a reaction temperature of 27°, with a rapid filtration technique. At this low Zn concentration the addition of an excess of folate, histidine or glucose had no effect on Zn uptake. Addition of picolinate, citrate and phytate to the incubation medium significantly reduced Zn uptake at all concentrations of ligand examined. Any inhibitory effects of folic acid in vivo may thus be due to a mucosal rather than lumen interaction. Those ligands inhibiting absorption may have done so through the formation of Zn–ligand complexes, which are either insoluble, or which reduce the binding of Zn to its mucosal receptor. This in vitro model of Zn absorption is useful for comparing the effects of potential Zn-binding ligands in the diet.

Zinc absorption: Ligands: Pig

Several studies have shown that, even in the otherwise well-nourished, the dietary intake of Zn may be marginal (Solomons, 1982; Black *et al.* 1986; Southon *et al.* 1988). This precarious Zn balance may be further compromised by conditions of increased demand, such as pregnancy, growth and lactation. Under such conditions, the inclusion in the diet of nutrients that chelate Zn may significantly affect Zn status.

Several models have been used for the study of Zn–nutrient interactions. Brush-border-membrane vesicles are convenient for examining the first phase of nutrient absorption in isolation, and studies on intestinal glucose and calcium transport have shown that the results obtained with brush-border-membrane vesicles are qualitatively comparable to those with intact epithelial preparations (Murer & Kinne, 1980). They have been used previously to study the absorption of several divalent cations including Zn (Menard & Cousins, 1983*a*; Blakeborough & Salter, 1987), the uptake of which is biphasic. The first phase, which is saturable, probably represents carrier-mediated, facilitated diffusion, and this predominates when the Zn concentration is below 10 μM. The second, non-saturable phase results from non-specific binding of Zn to the membrane (Blakeborough & Salter, 1987).

Both citrate (Hurley *et al.* 1979) and picolinate (Evans & Johnson, 1980) have been proposed to enhance the absorption of Zn from milk, but the importance of either in vivo is controversial (Cousins & Smith, 1980; Hurley & Lonnerdal, 1981; May *et al.* 1982), and the precise site at which these ligands act is uncertain. In man, an inhibitory effect of folate on Zn absorption has been suggested (Milne *et al.* 1984; Simmer *et al.* 1987), but not confirmed in all studies (Keating *et al.* 1987). Conversely, glucose (Steinhardt & Adibi,

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1984) and histidine (Scholmerich *et al.* 1987) enhance Zn absorption in man, although their site and mechanism of action are also unclear. In the present study these ligands have been re-examined specifically for their effect at the intestinal brush-border membrane.

MATERIALS AND METHODS

Materials

Carrier-free ^{65}Zn (0·52 μCi/ml, 0·38 μg Zn/ml) was purchased from Amersham International plc, Amersham, Bucks. Polycose (glucose polymer) was obtained from Abbott Laboratories, Queensborough, Kent. 'Analar' grade Zn sulphate was obtained from BDH Ltd, Poole, Dorset, and all other chemicals, including phytic acid (dodecasodium inositol hexaphosphoric acid), folic acid (pteroylglutamic acid) and picolinic acid (2-pyridine carboxylic acid), were obtained from Sigma Chemical Co., Poole, Dorset. All glassware was acid washed and rinsed twice in de-ionized water. Solutions were made up in distilled de-ionized water.

Preparation of intestinal brush-border-membrane vesicles

Pigs (28 d of age) given a normal-Zn diet were obtained from the piggery of the AFRC Institute of Food Research, Shinfield, Berkshire. They were killed and the entire small intestine was immediately excised and rinsed with ice-cold normal saline (9 g sodium chloride/l) before storing at −20°. Vesicles were prepared by the method of Booth & Kenny (1974). The defrosted intestine was cut into small pieces and suspended in 300 mM-mannitol, 12 mM-Tris at pH 7·1. Enterocytes were isolated by vortex mixing and filtration through a 1 mm pore size Buchner funnel. The filtrate was homogenized in a Waring-type blender and diluted 1:6 with water. Brush-border-membrane vesicles were isolated by the addition of magnesium chloride to a final concentration of 10 mM and incubation for 15 min. This results in cross-linkage and precipitation of organelles and basolateral membranes, which were removed by centrifugation at 1500 ***g*** for 12 min. The supernatant fraction was spun at 15000 ***g*** for 12 min and the resultant pellet resuspended in 50 mM-mannitol, 2 mM-Tris buffer, pH 7·1. $MgCl_2$ was again added to 10 mM final concentration and after 15 min incubation the preparation was centrifuged at 2200 ***g*** for 12 min. The resulting supernatant fraction was centrifuged at 15000 ***g*** for 12 min to yield a final pellet of purified brush-border-membrane vesicles, which was resuspended in 50 mM-mannitol–Tris buffer. All procedures were done at 4° and portions of the final preparation stored at −70° before use.

Assays of brush-border-membrane vesicles preparation

Protein content was determined according to Bensadoun & Weinstein (1976), using crystalline bovine serum albumin as the protein standard. The degree of purification of the preparation over the crude homogenate was estimated by determination of the enrichment of alkaline phosphatase (*EC* 3.1.3.1), a marker enzyme of the brush-border membrane (Murer *et al.* 1976).

Uptake studies

Uptake studies were performed as previously described (Blakeborough & Salter, 1987) with slight modifications. Incubation solution (pH 7·5) containing 5·0 μM-zinc sulphate, 150 mM-sodium chloride, 20 mM-Tris and the ligand under investigation were prepared and 0·22 μCi ^{65}Zn/ml added. The ligands used were folic acid (0·05, 0·5, 5·0 and 50·0 μM), citric acid (0·1, 0·5 and 1·0 mM), picolinic acid (25, 250 and 500 μM), phytic acid (250 and 500 μM), glucose (5, 10 and 50 mM), Polycose (5 and 10 mM) and histidine (5 and 20 μM). Uptake was initiated by the addition of brush-border-membrane vesicles containing 50 μg protein to

0·5 ml of the incubation solution at 27°. Reactions were conducted for 1 min and were terminated by adding 5·0 ml ice-cold Tris–NaCl buffer. The incubate immediately underwent rapid filtration through a pre-wetted cellulose nitrate membrane filter (Whatman Ltd, Maidstone, Kent) of pore size 0·45 μm. A further 5 ml buffer was used to ensure transfer of the entire sample to the filter, and the latter washed twice with 5 ml portions of buffer. ^{65}Zn uptake by the vesicles retained on the filter was estimated using an LKB 1282 gamma counter. To allow for adhesion of the isotope to the membrane, filter blanks were treated as described previously but the stop solution added simultaneously with the brush-border-membrane vesicles. Uptake was measured for each ligand using material from three preparations, and for each preparation six replicates and four blanks were performed. Mean activity of the blanks was subtracted from that of the incubated samples and uptake expressed as nmol Zn/mg membrane protein.

Statistical analysis

For each preparation (n3) mean Zn uptake over a range of ligand concentrations was calculated, and by subtracting this value from the mean value of uptake of Zn in the absence of ligand, the mean change in Zn uptake was derived for the three preparations. Its significance was estimated using a paired t test (mean uptake with ligand v. mean uptake without). In addition, by pooling the individual standard deviations from the six replicates of each sample, a residual standard deviation (RSD) was calculated.

RESULTS

Assays

Alkaline phosphatase assay showed an enrichment in specific activity of at least ninefold in all preparations which was considered a sufficient degree of purification for use in uptake studies. Lysis of brush-border-membrane vesicles in Triton X100, at a concentration of 0·5 g/l buffer, resulted in no increase in enzyme activity, indicating that the vesicles were orientated predominantly right side-out (Haase *et al.* 1978).

Zn uptake

Reproducibility. Uptake of Zn alone was examined on two separate occasions in each of seven preparations. Mean values of Zn uptake for the six different preparations used ranged from 5·57 to 12·48 nmol/mg protein per min. The percentage error of the assay performed on two occasions was 0·1–5·5 (mean 3%) and the within-run coefficient of variation was 5·6–29% (mean 12%).

Effects of ligands. Generally, although the preparations differed quantitatively in the absolute amount of Zn taken up, qualitatively they behaved similarly when ligands were added to the incubate.

The addition of folic acid to the incubation medium did not significantly change Zn uptake over a range of folate concentrations from 0·05 to 50 μM (Fig. 1(*a*)). Assuming a daily uptake of Zn of 15 mg and folic acid of 400 μg (US recommended daily allowances; National Research Council, 1980), all the concentrations tested were in excess of the 1:250 molar folate:Zn ratio present in the normal diet.

Citric acid (100–1000 μM) markedly reduced Zn uptake at all concentrations studied (Fig. 1(*b*)). Inhibition was maximal with 1 mM-citrate, a citrate:Zn molar ratio of 200:1. At this concentration the 60% reduction in mean Zn uptake differed significantly from the control value (n 3, $P < 0{\cdot}05$).

Incubation with 250 and 500 μM-dodecasodium phytate which, unlike calcium phytate, is soluble at pH 7·5, also markedly inhibited Zn uptake (Fig. 1(*c*)). A phytate:Zn molar ratio

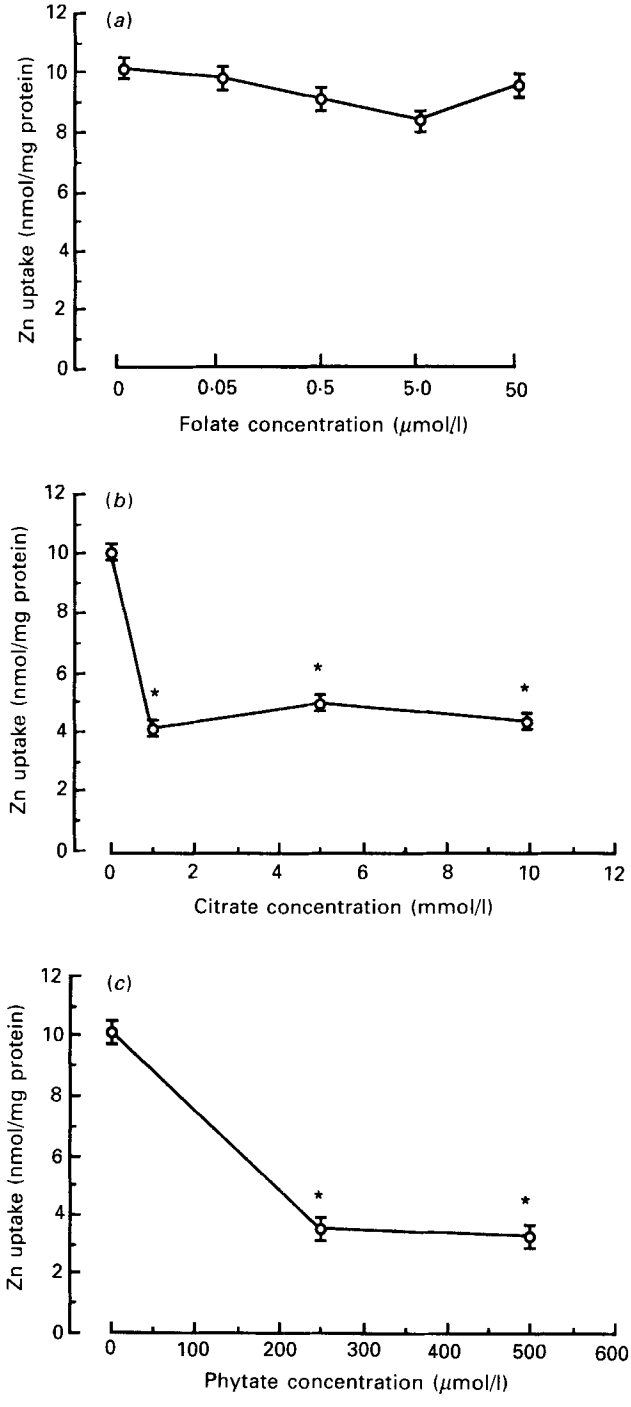

Fig. 1(*a–c*). For legend see opposite.

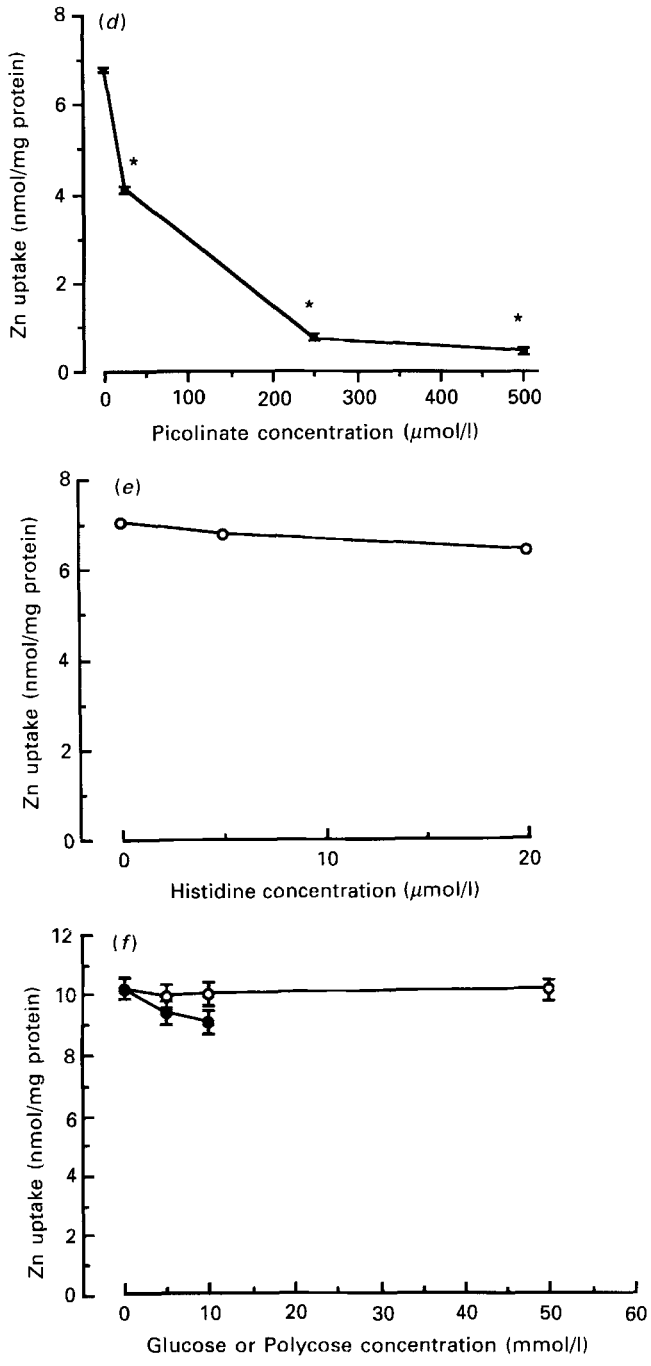

Fig. 1. The effects of increasing concentrations of nutrient ligands on zinc uptake by intestinal brush-border-membrane vesicles. (*a*) Folic acid; (*b*) citric acid; (*c*) phytate; (*d*) picolinic acid; (*e*) histidine, (*f*) glucose (○–○) or Polycose (●–●). Brush-border-membrane vesicles (50 μg protein) were incubated for 1 min (pH 7·5) in buffers containing varying ligand concentration. Results are means of eighteen experiments (six replicates of three preparations). Residual standard deviations (RSD) are shown by vertical bars (where no bars are shown, the RSD falls within the symbols). Uptake in the presence of ligand differed significantly from control values (Student's *t* test): $*P < 0{\cdot}05$. For details of methods, see p. 734.

of 50:1 inhibited mean uptake by 65%, and doubling this ratio did not increase inhibition. The mean change in absorption at both molar ratios was statistically significant (n 3, $P < 0.05$).

A dose-related inhibition of uptake was found with 25–500 μM-picolinic acid (Fig. 1(*d*)). Maximal inhibition occurred at a picolinate: Zn molar ratio of 100:1, at which point mean Zn uptake was reduced by 93%.

Thus, on a mole-for-mole basis, the potency of inhibition was ranked picolinate > phytate > citrate.

Zn uptake was unaffected by histidine at either 5·0 or 20 μM concentration (Fig. 1(*e*)). Similarly, glucose had no effect over the range 5–50 mM. Mean changes on incubation with Polycose at 5–10 mM were also not significant, although there was a trend to impaired uptake with increasing Polycose concentrations (Fig. 1(*f*)).

DISCUSSION

The presence of dietary ligands in the intestinal lumen is a critical factor in determining the availability of ingested Zn. Zn–ligand complexes may vary not only in their solubility and stability, but also in their site of action. In the present study the effects of nutrient ligands have been investigated entirely at the lumen–mucosa interface. Using porcine brush-border-membrane vesicles prepared by an identical technique, Blakeborough & Salter (1987) have shown that ^{65}Zn accumulation is osmotically sensitive over a 1 min incubation period and at an extravesicular Zn concentration of 5 μM. Thus, the values we measured for Zn accumulation represent true uptake, rather than non-specific binding to the vesicle membrane. The pig was chosen as the source of intestinal mucosa because the anatomy and physiology of the porcine gastrointestinal tract is similar to that of man (Clarke & Hardy, 1971; Dodds, 1982).

Folate

Not only are folates dietary constituents, but their intake is also frequently supplemented in two groups prone to Zn deficiency, namely pregnant women (Meadows *et al.* 1981) and patients with sickle cell anaemia (Prasad, 1984). In the present study no interaction in vitro between folate and Zn was detected at pH 7·5, even when folate was present in amounts equivalent to those administered therapeutically. Folates readily form complexes with trace metals, but these tend to be insoluble only at low pH (Albert, 1953). The effect of folate on Zn absorption in vivo is controversial. Folate-feeding studies in man (Milne *et al.* 1984) and intestinal perfusion studies in the rat (McMaster *et al.* 1985; Ghishan *et al.* 1986) suggest that folate in the intestinal lumen impairs Zn absorption by the formation of insoluble folate–Zn complexes, although in the feeding studies, overall Zn balance was unchanged. Conversely, when pregnant rats on both low and adequate Zn diets were given an excess of folate no change in tissue Zn levels was found in the dams or pups (Fuller *et al.* 1988), while indices of Zn status were normal in children on high-dose folate therapy (Krebs *et al.* 1988). The present findings do not preclude an adverse effect of folate on Zn absorption, but indicate that its site may be mucosal rather than luminal. Support for this hypothesis comes from two studies using the oral Zn tolerance test. Simmer *et al.* (1987) found reduced Zn absorption in patients given folate up to 24 h previously, whereas simultaneous administration of folate with the Zn had no effect (Keating *et al.* 1987).

Citrate and picolinate

In demonstrating a potent inhibitory effect on Zn absorption of these organic acids, the results largely confirm the findings of Menard & Cousins (1983*b*). They noted that both ligands reduced Zn uptake by rat brush-border-membrane vesicles but, in contrast to the

present study, they found citrate more potent in this respect. This disparity may either represent a species difference, or reflect the higher Zn (200 μM) and lower citrate (380 μM) concentrations used by Menard & Cousins (1983*b*). At this level of Zn the contribution to uptake by non-specific binding may have been substantial. The conditions of the present study match more closely those of mature human milk, which contains Zn at approximately 6 μM (Solomons, 1982) and citrate at 2–6 mM (Duncan & Hurley, 1978). Using everted gut sacs, Seal & Heaton (1983) found citrate reduced Zn uptake, but 2-picolinic acid markedly increased it. It is not clear why the present findings differ with respect to picolinate but, interestingly, in non-inverted porcine gut sacs picolinate reduced Zn uptake (Hill *et al.* 1987). Thus it is possible that although a given ligand may impair Zn uptake at the brush-border membrane, it may still permit overall absorption either by maintaining ingested Zn in solution or by acting at another site, such as the basolateral membrane. For example, citrate in vivo reduces ^{65}Zn binding to the gut wall but increases its transfer into the carcass (Jackson *et al.* 1981).

Phytate

The present findings support the well-established inhibitory effect of phytate on Zn absorption (Fairweather-Tait, 1988) and confirm that this occurs intraluminally. The lowest phytate:Zn molar ratio used in these studies was 50:1, at which point marked inhibition of Zn uptake occurred; but ratios as low as 6:1 can impair absorption (Lonnerdal *et al.* 1988). The close agreement between the in vitro studies reported here and extensive in vivo studies provides some validation of the brush-border-membrane vesicles model. Unlike citrate and picolinate, which may reduce Zn uptake by brush-border-membrane vesicles by the formation of complexes that prevent Zn binding to its membrane receptor, phytate reduces absorption by the formation of poorly soluble complexes (Kratzer & Vohra, 1986).

Histidine

Histidine:Zn molar ratios of 1:1 and 4:1 had no effect on Zn uptake. Histidine is generally reported to enhance Zn absorption at both low and high ligand:Zn ratios (Seal & Heaton, 1983; Wapnir *et al.* 1983). Although the difference may be concentration related, Scholmerich *et al.* (1987) found a Zn:histidine ratio of only 1:2 to enhance absorption in comparison with zinc sulphate in man. However, in their study the rate of absorption was unchanged, and so histidine may maintain Zn in solution under conditions where it might precipitate or bind to ligands of low bioavailability, and not have a direct effect on membrane Zn transport.

Glucose

The lack of a direct effect of glucose on Zn uptake was expected. Although monomeric glucose stimulates mineral absorption (Steinhardt & Adibi, 1984), it is probable that rather than acting as a ligand, the absorbable carbohydrate has a non-specific effect in vivo arising, for example, from altered gastric emptying (Griessen *et al.* 1989). The present findings support this hypothesis. The slight negative effect of Polycose may have been a result of increased extravesicular osmolarity leading to reduced intravesicular volume. Polycose consists of chains of $\alpha(1 \rightarrow 4)$ glucose of variable length, and is hydrolysed at the brush-border membrane to glucose (Jones *et al.* 1983). It is a constituent of enteral nutrition products and enhances the absorption of Zn, calcium and magnesium in vivo (Bei *et al.* 1986), possibly in a manner analogous to glucose.

Finally, these findings confirm that brush-border-membrane vesicles are a useful model for the investigation of lumen interactions between nutrients and trace metals. Although the results are difficult to extrapolate to the complex situation in vivo when many

competing ligands are present in the intestinal lumen, nevertheless this model provides insight on mechanisms and sites of interaction previously observed in the intact animal.

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