

Chemical factors affecting the intestinal absorption of zinc in vitro and in vivo

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1. Everted sacs of rat duodenum and ileum were used to study the effect of anions and organic ligands on the absorption of zinc. The uptake per unit weight of tissue was greater in duodenum than ileum, and it was influenced by the Zn concentration and pH of the incubation medium.

2. The Zn uptake from inorganic salts in simple buffered medium varied in the order zinc sulphate > zinc chloride > zinc phosphate. Zinc acetate was more effective and zinc citrate less effective than ZnCl₂. Addition of aspartic acid or histidine to ZnCl₂ increased the uptake but galactose or lactose decreased it. 2-Picolinic acid greatly increased the Zn uptake but 4-picolinic acid reduced it.

3. When incubated with intestinal sacs after incorporation into a synthetic rat diet, only ZnSO₄ and 2-picolinic acid increased Zn uptake compared with ZnCl₂, but zinc citrate and 4-picolinic acid still tended to decrease it.

4. Metabolic balance studies showed no significant differences in the faecal excretion, total excretion or retention of Zn between rats receiving diets containing different forms of Zn. ZnSO₄, zinc citrate and particularly 2-picolinic acid increased the urinary excretion of Zn.

5. The significance of these results is discussed in relation to the suitability of methods for investigating Zn absorption and the importance of Zn-binding ligands.

Many methods are available for measuring the absorption of dietary minerals and most of them have been applied to the study of zinc uptake. Although the metabolic balance technique is probably the ultimate standard of reference, it is a time-consuming and expensive procedure, and it does not provide any information about the site of absorption. Alternative methods have therefore been developed to investigate mineral absorption in greater detail. These include the use of artificial membranes (Miller *et al.* 1981), everted gut sacs (Pearson *et al.* 1966), ligated intestinal loops (Schwarz & Kirchgessner, 1978) and intestinal perfusion techniques (Smith *et al.* 1978).

Several dietary constituents have been found to influence the availability of ingested Zn, with substances like calcium, phosphate and phytate reducing it (O'Dell & Savage, 1960; Likuski & Forbes, 1965; House *et al.* 1982). We wanted to examine the effect of a wide range of substances on Zn absorption and used the uptake by everted sacs of rat intestine as an initial screen. The compounds that produced the most interesting changes were then studied more fully by metabolic balance methods in living rats.

EXPERIMENTAL

Materials and methods

All investigations were carried out using adult male Wistar albino rats from the stock colony weighing 220–250 g. They had been housed in galvanized cages and maintained on commercial animal cubes (41B; E. Dixon & Sons Ltd, Ware) and tap water *ad lib*.

The composition of the basic Zn-free diet is given in Table 1. By using casein of low vitamin content (Koch-Light Laboratories Ltd, Colnbrook) and Analar chemicals in the salt mixture a background Zn concentration of 3 mg/kg diet was obtained. Individual diets were prepared from salt mixtures containing sufficient zinc chloride, zinc sulphate, zinc phosphate or zinc citrate to give an additional Zn concentration of 50 mg/kg diet. Further diets were prepared containing ZnCl₂ + histidine, ZnCl₂ + 2-picolinic acid and ZnCl₂ + 4-picolinic acid at a molar ratio, ligand: Zn of 50:1.

Table 1. *Composition (g/kg) of zinc-free diet*

Sucrose	660
Casein	200
Maize oil	80
Salt mixture*	45.3
Cod-liver oil†	20
Vitamin mixture‡	0.77

* Composition (g/kg): CaCO_3 188, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 200, K_2CO_3 89, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5, MgCl_2 80, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 2.6, NaI 0.52, NaF 0.40, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.28.

† Provided the following ($\mu\text{g/kg}$ diet): vitamin A 3600, vitamin D 42.5.

‡ Provided the following (mg/kg diet): choline chloride 360, *p*-aminobenzoic acid 120, *myo*-inositol 120, nicotinic acid 120, pantothenic acid 24, riboflavin 9.6, thiamin 4.8, pyridoxine 4.8, folic acid 2.4, biotin 0.96, menaphthone 0.096, cyanocobalamin 0.048.

Incubation of intestinal sacs

Rats in the post-absorptive state were killed by cervical dislocation at 09.30 hours, the whole small intestine removed immediately and rinsed free of contents with distilled water. Three segments of 30–40 mm length were cut from each region of the intestine. Duodenal segments were taken from the first 120 mm posterior to the pylorus and ileal segments from the region 120–240 mm anterior to the ileo-caecal junction. They were placed immediately in previously-aerated 0.15 M-Tris-Krebs buffer, pH 7.3, having the following composition (mM): Tris-hydrochloride 15.5, sodium chloride 120.7, potassium chloride 5.6, calcium chloride 2.5, magnesium chloride 1.2, glucose 11.5.

Each segment was inverted on a Pasteur pipette and one end tied with cotton. The sac was filled with Zn-free 0.15 M-Tris-Krebs buffer, pH 7.3, from a syringe until slightly distended and the other end tied. Sacs were incubated for 30 min at 37° in the same buffer containing different Zn salts at a constant Zn concentration of 3×10^{-4} M. Organic compounds were always added at a concentration of 1.5×10^{-2} M. The pH of each medium was checked and if necessary adjusted to 7.3. All media were previously saturated with air and bubbled continuously with air during incubation; the incubation commenced within 15 min of removing the intestine. At the end of the incubation period sacs were sectioned and the contents drained for analysis. Trial measurements were made with incubation media at pH 6.4 and 8.3 using the same medium, except that in the former case the Tris-hydrochloride buffer was replaced by HEPES-potassium hydroxide.

The seven synthetic diets were suspended in 0.15 M-Tris-Krebs buffer, pH 7.3, and incubated with intestinal sacs in the same way. Measurements were made with dietary concentrations of 40 and 400 g/l medium, which gave Zn concentrations of 3×10^{-5} and 3×10^{-4} M respectively. The sacs were filled with Zn-free buffer as described previously.

Metabolic balance measurements

Rats were housed individually in stainless-steel metabolism cages at 20° in a room with 12 h light–12 h dark periods. They were allocated randomly to different groups and each rat received 18 g of the appropriate diet/d with distilled water *ad lib*. Preliminary trials had shown that this was the maximum amount of food they would totally consume. After 7 d equilibration, balance collections were made over a 4 d period and the rats were weighed at the beginning and end of this period. The daily urine and faecal collections from each rat were pooled over the balance period. At the end of the balance, blood was obtained by cardiac puncture and heparinized plasma was separated for Zn determination.

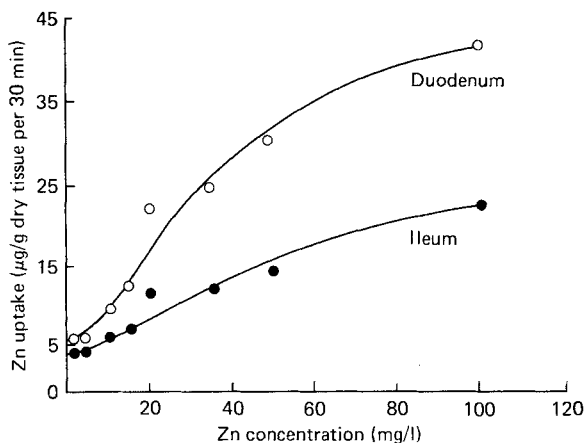


Fig. 1. Effect of zinc concentration (mg/l) in the medium on Zn uptake ($\mu\text{g/g}$ dry weight of tissue per 30 min) by everted sacs of rat duodenum and ileum incubated *in vitro*.

Analytical methods

Intestinal sacs were dried overnight at 105° to obtain their dry weight. Food and faecal collections were dried in the same way, then ashed in silica crucibles by heating in a muffle furnace at 500° for 16 h; the ash was dissolved in 2 M-HCl. Plasma was deproteinized with hydrochloric and trichloroacetic acids as described by Gubler *et al.* (1952).

Zn was determined in sac contents, urine, solutions of food and faecal ash, and deproteinized plasma with a Pye Unicam SP 90 atomic absorption spectrophotometer; all solutions contained 0.1 M-HCl to prevent interference by other constituents of the samples. Glucose was estimated in some sac contents by the glucose oxidase method (Fleming & Pegler, 1963). The statistical significance of differences was assessed by Student's *t* test.

RESULTS

Conditions affecting Zn uptake by intestinal sacs

Preliminary studies using duodenal and ileal sacs containing buffer without either glucose or Zn showed that the preparations maintained active glucose uptake for 2.5 h and the relationship between the rate of Zn uptake and the period of incubation was linear for approximately 45 min. Fig. 1 shows that the Zn uptake was dependent on the external concentration of Zn and was always greater with duodenal than with ileal sacs. In view of these observations, standard conditions involving an incubation period of 30 min with an external Zn concentration of 20 mg/l (3×10^{-4} M) were adopted for comparative purposes to obtain the most sensitive response.

Further trials showed that the uptake from ZnCl_2 was influenced by the pH of the incubation medium. Lowering the pH from 7.3 to 6.4 reduced the Zn uptake by duodenal sacs (mean with SE) from 23.4 (0.9) to 15.2 (1.5) $\mu\text{g/g}$ dry weight of tissue per 30 min ($P < 0.001$) and raising the pH from 7.3 to 8.3 lowered the uptake by ileal sacs from 13.0 (0.5) to 8.6 (1.1) $\mu\text{g/g}$ dry weight of tissue per 30 min ($P < 0.01$). A uniform pH of 7.3 was therefore used for measurements with different Zn salts and organic compounds.

Effect of anions and organic ligands on uptake by intestinal sacs

Table 2 shows that the rate of Zn uptake from different salts varied considerably. Taking ZnCl_2 as the reference, because it is likely to be the predominant matrix after exposure to

Table 2. Zinc uptake by duodenal and ileal sacs incubated in buffer containing different Zn salts and organic ligands

Chemical form of Zn	No. of observations	Zn uptake ($\mu\text{g/g}$ dry weight tissue in 30 min)			
		Duodenum		Ileum	
		Mean	SEM	Mean	SEM
ZnCl ₂	50	23.4	0.9	13.0	0.5
ZnSO ₄	16	34.5***	2.1	15.4*	1.0
Zn ₃ (PO ₄) ₂	8	14.7***	1.3	13.2	0.9
Zn acetate	8	35.5***	4.7	14.2	1.0
Zn citrate	6	11.9***	1.0	9.6**	0.2
ZnCl ₂ + aspartic acid	8	27.4*	1.8	17.1**	1.3
ZnCl ₂ + cysteine	8	19.3	2.3	13.5	1.0
ZnCl ₂ + glutamic acid	8	22.3	1.9	13.4	1.2
ZnCl ₂ + histidine	8	35.7***	2.1	17.7**	2.9
ZnCl ₂ + tryptophan	8	20.3	1.6	10.7	0.7
ZnCl ₂ + galactose	8	15.5***	1.7	12.3	0.6
ZnCl ₂ + lactose	8	15.8***	0.8	12.8	1.2
ZnCl ₂ + 2-picolinic acid	5	94.8***	12.2	33.3***	2.2
ZnCl ₂ + 4-picolinic acid	4	11.1***	1.6	8.2**	0.7

Value significantly different from that for ZnCl₂: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. Zinc uptake by duodenal and ileal sacs incubated with synthetic diets containing different Zn salts and organic ligands

(Mean values with their standard errors for eight observations in each group)

Diet concentration (g/l medium)†...	Chemical form of Zn	Zn uptake ($\mu\text{g/g}$ dry weight tissue in 30 min)							
		Duodenum				Ileum			
		40		400		40		400	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
	ZnCl ₂	6.36	0.22	9.43	0.35	5.25	0.23	5.36	0.12
	ZnSO ₄	8.87***	0.29	10.51*	0.19	5.42	0.10	8.17**	0.20
	Zn ₃ (PO ₄) ₂	6.68	0.24	9.40	0.19	5.31	0.21	5.28	0.08
	Zn citrate	5.40*	0.19	9.23	0.37	5.10	0.07	5.39	0.19
	ZnCl ₂ + histidine	6.40	0.42	10.23	0.27	5.54	0.27	6.08	0.35
	ZnCl ₂ + 2-picolinic acid	10.48***	0.41	41.75***	2.14	11.69***	0.42	27.15***	3.12
	ZnCl ₂ + 4-picolinic acid	5.35**	0.14	9.15	0.23	5.29	0.19	5.43	0.15

Value significantly different from that for ZnCl₂: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† Synthetic diet was suspended in 0.15 M-Tris-Krebs buffer, pH 7.3.

the gastric juice in vivo, sulphate and acetate significantly increased the uptake in duodenum, but phosphate and citrate decreased it. Among the organic constituents of foods that were examined, aspartic acid and histidine enhanced Zn uptake, galactose and lactose depressed it, and cysteine, glutamic acid and tryptophan had no significant effect.

Zn uptake was consistently lower with ileum than duodenum (Table 2, $P < 0.001$) and the influence of different anions and organic substances was less marked. With ileum only sulphate, citrate, aspartic acid and histidine produced significant effects, but the changes were consistent with those observed in duodenum.

Table 4. *Metabolic balance results for rats receiving the same amounts of synthetic diets containing different zinc salts and organic ligands*(Mean values with their standard errors for ten rats in each group, except ZnCl₂ where twenty rats were used)

Chemical form of Zn	Faecal Zn (% intake)		Urinary Zn (% intake)		Total Zn excretion (% intake)		Net Zn absorption (% intake)		Zn retention (% intake)		Wt gain of rats (g)		Plasma Zn concentration (mg/l)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ZnCl ₂	91.9	2.1	1.43	0.06	93.3	2.9	8.14	1.83	6.71	2.85	16.7	0.5	1.85	0.03
ZnSO ₄	98.1	4.3	1.84**	0.18	99.9	4.2	1.80	4.29	0.06	4.15	17.6	0.7	1.78	0.03
Zn ₃ (PO ₄) ₂	97.6	4.6	1.62	0.08	99.2	4.6	2.41	4.60	0.79	4.64	14.7**	0.4	1.78	0.03
Zn citrate	87.0	3.6	2.04**	0.21	89.0	3.6	12.97	3.59	10.93	3.59	15.6	0.6	1.82	0.04
ZnCl ₂ + histidine	85.6	3.2	1.45	0.06	87.1	3.2	14.40	3.20	12.94	3.20	16.2	0.9	1.71**	0.03
ZnCl ₂ + 2-picolinic acid	87.0	2.4	6.70***	0.65	93.7	2.6	12.97	2.41	6.27	2.59	15.6	0.5	1.78	0.03
ZnCl ₂ + 4-picolinic acid	91.9	3.8	1.51	0.15	93.4	3.8	8.08	3.78	6.58	3.76	14.1**	0.7	1.71**	0.03

Value significantly different from that for ZnCl₂: * P < 0.05, ** P < 0.01, *** P < 0.001.

The contrasting response to the two isomers of picolinic acid (pyridine carboxylic acid) is striking. The 2-isomer approximately trebled Zn uptake by both duodenal and ileal sacs, but the 4-isomer almost halved it within both regions of the intestine.

After incorporation into the whole diet the effects of the different Zn salts and organic ligands were less marked than those observed in simple buffer solution. Only the sulphate and 2-picolinic acid increased Zn uptake by both duodenum and ileum (Table 3), although citrate and 4-picolinic acid decreased the uptake by duodenum.

Measurement of Zn balance in vivo

The Zn concentration determined by analysis was almost constant in all seven diets at 53.2 (SE 0.3) mg/kg. This gave a mean Zn intake during the 4 d balance period of 3.83 mg/rat. The amounts of Zn retained and excreted by different routes have been related to the dietary intake in Table 4 in order to facilitate interpretation.

Faecal output was the predominant route of Zn loss, averaging 91.0 (SE 1.8)% of the intake, and the urinary excretion only amounted to 1–2% of the intake in most cases. No statistically-significant differences from ZnCl₂ were observed between the faecal excretion, total excretion or retention of Zn on the different diets. The only significant differences found in Zn metabolism were increased rates of urinary excretion by rats receiving the sulphate, citrate or particularly 2-picolinic acid. This increased excretion did not, however, correlate with the lowered plasma levels of Zn in rats receiving histidine or 4-picolinic acid. All rats were in positive balance for Zn and increasing in body-weight although the rate of growth was reduced in those receiving diets containing Zn₃(PO₄)₂ or 4-picolinic acid.

DISCUSSION

The results of the present investigation show that the rate of Zn uptake per unit weight of tissue was consistently greater with duodenal than with ileal sacs, usually by a factor of two. This observation together with the effect of pH on Zn uptake, which was greater at 7.3 than at either 6.4 or 8.3, indicates that Zn is transferred across the gut wall more efficiently in the duodenum than the ileum and is likely to be most efficient in the part of the duodenum where the pH of the lumen contents approaches neutrality. However, this conclusion does not necessarily imply that the duodenum is the most important site of Zn absorption in the living animal, because the greater length of the ileum and the longer period of its contact with the intestinal contents could quantitatively outweigh the more efficient uptake in the duodenum. This problem has been investigated by several groups using a variety of techniques *in vivo* (Van Campen & Mitchell, 1965; Methfessel & Spencer, 1973; Davies, 1980) and it appears that the duodenum is the major site of Zn absorption.

Incubation with intestinal sacs and particularly duodenal sacs *in vitro* appears to provide a rapid and useful method for screening substances that may influence Zn absorption. The relative effects of the various anions and organic ligands were qualitatively consistent during the three stages of the present study, but their quantitative effects were progressively reduced on moving from incubation with sacs in simple buffer solution, to incubation with sacs when incorporated in a complete diet, to metabolic balance studies *in vivo*. This was probably due to interaction with components other than Zn when mixed in the diet and the reduced sensitivity obtained during balance studies in whole animals compared with the simpler intestinal sac procedure.

Wide variations in Zn absorption have been reported in metabolic balance studies (Becker & Hoekstra, 1971) but the range of net absorption from 2 to 15%, with a mean value of 9%, that we observed is consistent with the results of other studies in rats (Ballou & Thompson, 1961; Heth & Hoekstra, 1965). Measurements of this type will include the faecal excretion of endogenous Zn, which may be considerable (Weigand & Kirchgessner, 1978;

Evans *et al.* 1979), and they therefore underestimate the true absorption of ingested Zn. Another limitation of the balance technique is the probable experimental error of 5–10%. With a substance like Zn, where the faecal excretion amounts to approximately 90% of the dietary intake, it will require a very large proportionate increase in the net absorption to make the difference statistically significant. Although the metabolic balance is usually regarded as the standard of reference, because it is the only procedure that uses intact animals, it is probably too insensitive in this type of situation to detect differences that are likely to be of biological significance.

ZnSO₄ seems to be the inorganic salt that is most efficiently utilized. Its uptake by intestinal sacs was greater than from the chloride in both simple buffer solution and after incorporation into the diet, and the increased urinary excretion of Zn observed during the balance studies in rats receiving ZnSO₄ is consistent with this. The effect of amino acids that have the potential to chelate Zn is surprisingly variable. Only histidine and to a lesser extent aspartic acid increased its uptake from simple buffer, which confirms the influence of histidine reported by Giroux & Prakash (1977) and Schwarz & Kirchgessner (1975, 1978), but the effect was masked after incorporation into the diet. Cysteine, tryptophan and glutamic acid had no significant effect in our system, but Schwarz & Kirchgessner (1975) and Oestreicher & Cousins (1982) have obtained conflicting evidence about the effect of the first two of these amino acids on Zn uptake. The inhibitory influence of galactose and lactose on Zn uptake contrasts with their stimulatory effect on the absorption of calcium and magnesium.

In view of the controversy about the roles of citrate (Lönnerdal *et al.* 1980) and picolinate (Evans & Johnson, 1980) in enhancing the availability of Zn from human milk we examined their effects during the present study. The 2-isomer of picolinic acid, which is the metabolite of tryptophan, greatly increased Zn uptake by duodenal and ileal sacs from both simple buffer and complete diet, whereas the 4-isomer had the opposite effect. Citrate, however, inhibited Zn uptake from buffer in both regions of the intestine and showed a similar but less marked tendency after incorporation into the diet. These results conflict with the observations of Jackson *et al.* (1981) who found that citrate increased the absorption of ⁶⁵Zn whereas picolinic acid did not. It may be significant that their studies were conducted with fasting rats while the tissues we used were always obtained from animals in the normal post-absorptive state.

The results from our intestinal sac studies therefore indicate that 2-picolinic acid has the potential to enhance the absorption of dietary Zn in the rat whereas citric acid does not, irrespective of their occurrence in milk. The marked increase in urinary Zn excretion by rats receiving diets containing 2-picolinic acid, however, suggests that this ligand may merely enhance the turnover of Zn rather than increase its utilization in the body.

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