Zinc Uptake by Isolated Rat Enterocytes: Effect of Low Molecular Weight Ligands (42998)

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Abstract. **The luminal phase of zinc intestinal absorption has not been well characterized. This study was intended to elucidate the possible role of low molecular weight (LMW) ligands in zinc intestinal transport in an isolated rat enterocyte system. Under these *in vitro* conditions, zinc uptake by the isolated enterocytes was rapid, leveling off within 1 min. Kinetic analysis revealed that both a mediated and diffusion component were involved in zinc uptake in the absence of LMW ligands by the cells. For the mediated component of zinc transport, the K_t and V_{max} were 64.1 μM and 13.9 nmol/20 sec/mg protein, respectively. Zinc uptake was not affected by the addition of metabolic inhibitors. In the presence of histidine or cysteine (2:1 ligand:zinc molar ratio), zinc uptake was greatly reduced and occurred solely via mediated transport. Zinc uptake was also significantly decreased upon the addition of EDTA to the assay media. Other amino acids tested had no effect on zinc uptake by the cells. Albumin markedly reduced zinc uptake by the cells. Histidine and other potential LMW ligands were unable to facilitate albumin-inhibited zinc uptake. The results of this study suggest that the intestinal absorption of zinc may not be effected in the form of chelates with LMW ligands. Amino acids such as histidine and cysteine significantly reduce the uptake of the metal by isolated rat enterocytes, making questionable their putative role as necessary vehicles in the luminal phase of zinc absorption.** [P.S.E.B.M. 1989, Vol 192]

The nutritional importance of zinc has been well established (1, 2). However, the specific mechanisms by which zinc is absorbed by the gut have not been entirely defined. The first step in the intestinal absorption of zinc is its transport across the brush border into the enterocyte. In this phase of zinc absorption, low molecular weight (LMW) compounds, including amino acids, which can act as ligands for zinc may play a role in its transport into the gut. LMW ligands may facilitate zinc transport by presenting the metal to a transport protein on the cell membrane, or the zinc-ligand chelate may be transported intact (3). Intestinal perfusion studies in rats have revealed that LMW ligands, such as histidine, can enhance zinc transport (4). These studies have also shown that the binding of zinc to large, unabsorbable compounds, such as albumin, significantly decreases its uptake (5).

Isolated enterocyte preparations have been used to study the transport of amino acids, sugars, and bile salts (6, 7). These preparations allow characterization of the uptake of molecules and ions at the cellular level. Also, transport of molecules can be measured for short time intervals under specific assay conditions. Isolated rat enterocytes, therefore, may serve as a suitable model for zinc transport.

The aim of this study was to better define the mechanisms of zinc transport using isolated rat enterocytes and to determine the effects of LMW ligands. The effects of histidine and cysteine on zinc uptake were studied in more detail since these two amino acids are known to have great affinities for zinc (8). The ability of potential LMW ligands to sequester zinc from large, nonabsorbable molecules was examined as well.

Materials and Methods

Animals. Male Wistar rats (Crl:[WI]BR, Charles River Breeding Laboratories, Kingston, NY) weighing 150–250 g were used in this study. The rats were housed for a minimum of 4 days after their arrival. They were fed stock diet (Purina Lab Chow, #5001; Ralston Purina Co., St. Louis, MO) *ad libitum*, which contained 58 ppm of zinc according to the manufacturer, and were provided free access to tap water. The rats were fasted

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the night before they were to be used in these experiments.

Isolation of Rat Enterocytes. Enterocytes were obtained from the rats using hyaluronidase by a modification of the method of Kimmich (9). In brief, the rats were anesthetized with urethane (1.3 g/kg ip) and a 30-cm piece of the small intestine, distal to the ligament of Treitz, was excised and rinsed in cold saline. The rats were euthanized by exsanguination from the aorta. Immediately after its removal, the intestine was everted, both ends tied and placed in a modified Hanks' salt solution with 1.5 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO). The intestinal segment was incubated in a shaking water bath at 37°C for 20 min. Following this incubation, the segment was transferred to a hyaluronidase-free buffer where the cells were released by gentle agitation with a plastic pipette. The cells were filtered through a 200-μm nylon mesh, washed, and resuspended in the incubation buffer which contained the following: 10 m*M* Hepes, 142 m*M* NaCl, 5 m*M* KCl, and 5.6 m*M* glucose (pH 7.4). The viability was assessed by trypan blue exclusion. The cells were also tested for their ability to actively transport glucose as a criteria of their functional integrity. The morphology of the isolated enterocytes was examined under the electron microscope, using standard techniques.

Zinc Uptake Assays. Measurements of zinc uptake were performed at 25°C, using cells at a final protein concentration of 2–4 mg/ml. A 100-μl aliquot of the cell suspension was added to 100 μl of the incubation medium which contained ^{65}Zn (0.5 μCi/ml) and $ZnCl_2$. The incubation was terminated by spinning the mixture through 100 μl of a 3:1 mixture of dibutyl phthalate:dinonyl phthalate in 400-μl polyethylene microcentrifuge tubes in a Beckman Microfuge E. The bottom of the tubes containing the cell pellets were cut off and counted in a gamma counter (MINAXI Auto Gamma 5000, Packard). To determine the volume of adherent fluid that is pelleted with the cells, the cells were incubated with [^{14}C]inulin. The amount of extracellular medium brought down with the cells was found to be negligible.

Kinetics of Zinc Uptake. Zinc uptake by the isolated enterocytes was determined at time points ranging from 10 to 120 sec. The concentration of $ZnCl_2$ in these experiments was 160 μM. When histidine was present, the histidine:zinc ratio was 2:1. For kinetic analysis of zinc uptake, the zinc concentration in the incubation medium was adjusted with $ZnCl_2$ (10–720 μM). The cells were incubated for 20 sec at 25°C and the assays were terminated as described above.

To assess the energy requirements of zinc uptake, isolated enterocytes were preincubated with ouabain or dinitrophenol at concentrations of 1.0 and 0.1 m*M* for 5 min. The cells were then assayed for zinc uptake as described at a $ZnCl_2$ concentration of 80 μM for 2 min at 25°C.

Effect of Histidine, Cysteine, and Other LMW Ligands on Zinc Uptake. Isolated enterocytes were incubated with ^{65}Zn and concentrations of $ZnCl_2$ ranging from 10 to 640 μM in the presence of histidine or cysteine. The amino acid:zinc molar ratio in these experiments was 2:1. Uptake was terminated after 20 sec.

The effect of other amino acids and potential ligands on zinc uptake by the cells was determined at a $ZnCl_2$ concentration of 80 μM. The cells were incubated with the ligand for 20 sec at 25°C. The amino acids tested included histidine, cysteine, proline, glutamine, and glycine. The hydrolysis-resistant dipeptide glycylsarcosine and EDTA were also tested for their effect on zinc uptake by isolated enterocytes. In these experiments, the ligand concentration was 160 μM (2:1 ligand:zinc ratio). Histidine was also tested at a concentration of 1.6 m*M* (20:1 ligand:zinc ratio).

Effect of Albumin on Zinc Uptake. To test the effect of large, nonabsorbable molecules on zinc uptake by the cells, isolated enterocytes were incubated with 80 μM $ZnCl_2$ and ^{65}Zn in the presence of 0–80 μM albumin. The cells were incubated for 20 sec at 25°C.

Effect of Histidine and Other Potential Zinc Ligands on Albumin-Inhibited Zinc Uptake. To assess the ability of histidine to mobilize zinc from large molecules for uptake by the isolated enterocytes, the cells were incubated with 80 μM $ZnCl_2$ and histidine (0–640 μM) in the presence or absence of 20 μM albumin. The cells were incubated for 20 sec at 25°C.

To test the effect of other potential zinc ligands on albumin-inhibited zinc uptake, the cells were incubated with 80 μM $ZnCl_2$, 20 μM albumin, and 160 μM of the potential zinc ligand. These included cysteine, EDTA, proline, glycylsarcosine, and a protein hydrolysate. The cells were incubated for 20 sec at 25°C.

Kinetic Analysis. The mediated and nonmediated components of zinc uptake were determined by the method of Neame and Richards (10). In brief, this consisted of extrapolating to the origin of abscissas the linear portion of the plot obtained at higher concentrations of substrate and transposing the line to the origin of the ordinates. The values thus obtained are attributable to diffusion or nonmediated transport. The points representing the mediated transport are obtained by subtraction of the diffusion from the overall uptake. The K_t and V_{max} values were obtained by using the Hofstee transformation (11).

Statistical Analysis. The differences between the means of absorption rates of two groups were analyzed by a Student's t test. The minimum significance was set at $P < 0.05$. For comparisons between the control group and experimental groups, a one-way analysis of variance was used in conjunction with the Dunnett's

test, at a minimum significance level of 0.05 (12). Kinetic parameters (K_t and V_{max}) were compared using Student's t test.

Results

Isolated Enterocytes. Cell aggregates and single cells were obtained by the isolation procedure. In all experiments, viability of the isolated cells and aggregates was greater than 90% as determined by trypan blue exclusion. A microscopic analysis of the small intestine treated with hyaluronidase showed removal of epithelial cells from the villus tips, while mid-villus and crypt cells remained attached to the villi. Examination of the cells with the electron microscope revealed that the isolated cells possessed microvillus brush borders (Fig. 1). The isolated cells actively transported glucose.

Time Course of Zinc Uptake by Isolated Enterocytes. As seen in Figure 2, zinc uptake by the isolated rat enterocytes was a rapid process, leveling off within 1 min. Rapid uptake of zinc was observed in the presence and absence of histidine.

Kinetics of Zinc Uptake by Isolated Enterocytes. The curvilinear shape of the uptake/concentration plot in Figure 3 indicates that, in the absence of a ligand, zinc uptake by these cells can be split into a diffusion and saturable component. The diffusion component was subtracted as described in Materials and Methods. The K_t and V_{max} for the saturable component were obtained from the Hofstee plot (Fig. 4) and were 64.1 μM and 13.9 nmol/20 sec/mg protein, respectively. The K_d for the diffusion component was 0.09 ml/20 sec/mg protein.

Effect of Histidine and Cysteine on Zinc Uptake. In the presence of histidine or cysteine, the diffusion component of zinc uptake was minor or nonexistent (Fig. 3). The K_t and V_{max} obtained for zinc uptake in the presence of histidine were 72.5 μM and 13.6 nmol/20 sec/mg protein, respectively. In the presence of

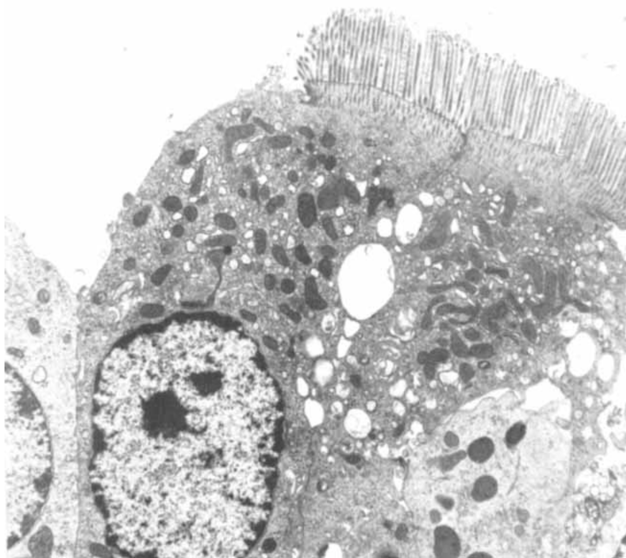

Figure 1. Electron photomicrograph of isolated rat enterocytes. Note intact brush border (original magnification ×8000).

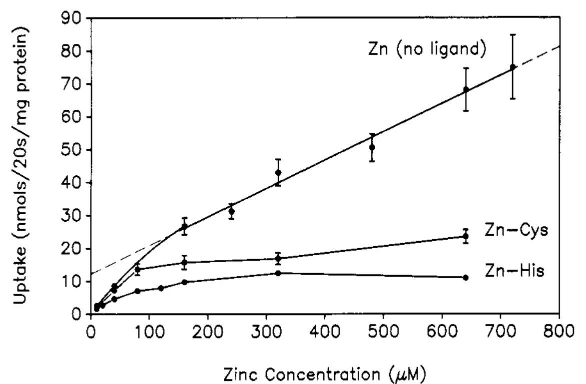

Figure 3. Concentration dependence of zinc uptake by isolated enterocytes in the absence of ligand or in the presence of histidine or cysteine. Cells were incubated with varying concentrations of zinc for 20 sec at 25°C. Ligands were present at a 2:1 molar ratio to zinc, when present. Each point represents the mean of least three different cell preparations.

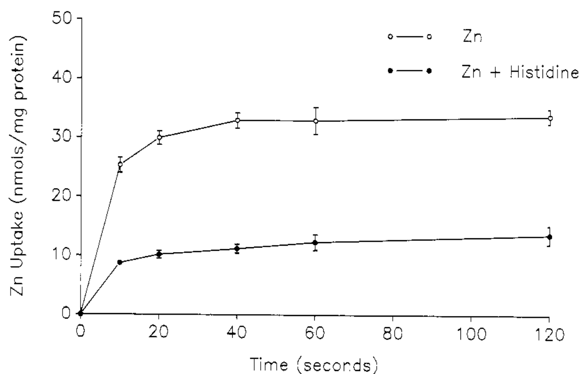

Figure 2. Time dependence of zinc uptake (160 μM) by isolated rat enterocytes in the presence and absence of histidine (320 μM). Assays were performed at 25°C. Each point represents the mean of at least three different cell preparations.

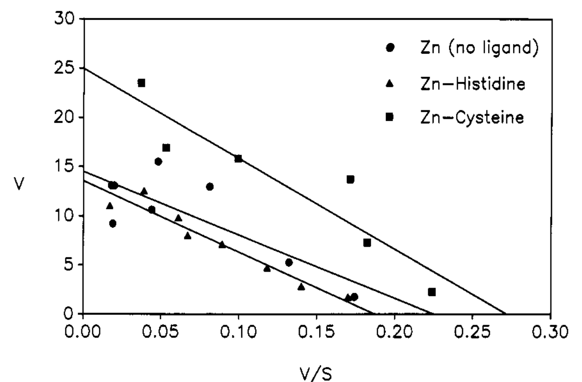

Figure 4. Hofstee plot of zinc uptake in the absence of a ligand or in the presence of histidine or cysteine. The intercept with the ordinate represents the V_{max}. The slope of the lines are indicative of the respective K_t.

cysteine, the K_t and V_{max} obtained were 92.6 μM and 24.9 nmol/20 sec/mg protein, respectively. The K_t's for zinc uptake in the absence of a ligand or in the presence of histidine or cysteine were not statistically different from one another. The V_{max} obtained for zinc uptake in the presence of cysteine was significantly greater than the V_{max}'s obtained for zinc uptake in the absence of a ligand or in the presence of histidine.

Effect of Inhibitors on Zinc Uptake. Zinc uptake was not significantly affected by the inhibitors tested (Table I). The mediated component of zinc uptake by these cells is apparently not energy dependent.

Effect of Other Potential LMW Ligands on Zinc Uptake. Isolated enterocytes were incubated with zinc at a concentration of 80 μM and various LMW ligands for 20 sec (Table II). None of the ligands tested significantly enhanced zinc uptake. The presence of histidine, at molar ratios of 2:1 and 20:1 to zinc, caused a significant decrease in zinc uptake. In the presence of EDTA, zinc uptake was nearly obliterated.

Effect of Albumin on Zinc Uptake. Increasing concentrations of the protein albumin inhibited zinc uptake by the isolated enterocytes (Fig. 5). At 20 μM albumin, zinc uptake was reduced to about 50%.

Effect of Histidine and Other Potential LMW Ligands on Albumin-Inhibited Zinc Uptake. Cells were incubated with increasing concentrations of histidine in the presence or absence of 40 μM albumin. As seen in Figure 6, increasing concentrations of histidine, in the absence of albumin, inhibited zinc uptake by these cells. Increasing concentrations of histidine in the presence of 40 μM albumin did not facilitate albumin-inhibited zinc uptake.

None of the other ligands tested significantly enhanced albumin-inhibited zinc uptake (Table III). The only ligand to have any effect on zinc uptake in the presence of 20 μM albumin was EDTA, which almost totally inhibited zinc uptake.

Discussion

In this study, isolated rat enterocytes were used to study zinc absorption at the cellular level. The uptake of zinc by these cells was extremely rapid. Kinetic analysis of zinc uptake revealed a mediated and non-mediated component. This is in agreement with what has been observed in intestinal perfusion studies (5) and with brush border membrane vesicles (13). Zinc uptake by isolated rat hepatocytes was also found to occur via mediated transport and diffusion (14). The K_t obtained for zinc uptake in this study (64.1 μM) was similar to what has been obtained *in vivo* (54 μM) by Steel and Cousins (15). The inhibitors tested in the present study (ouabain and dinitrophenol) had no effect on zinc uptake, therefore, the mediated component of zinc uptake by these cells is probably energy independent. Since all surfaces (brush border and basolateral membranes) of the isolated enterocytes are exposed to the substrate, it is impossible to determine with certainty at what site zinc uptake actually occurs. However, the similarities between the K_t values obtained in this study and those determined *in vivo* (15) suggests that similar uptake mechanisms are involved.

The uptake of zinc observed in this study most likely represents, both, binding of the metal to the cell membranes as well as its transport into the cell. Significant binding was found to occur as soon as the incubation is started. This has also been observed with brush border and basolateral membrane vesicles (13, 16). Since saturable kinetics are observed, it is unlikely that this binding is purely nonspecific. Nonspecific binding would be dependent on the zinc concentration in the incubation medium.

It has been proposed that LMW ligands which bind zinc can facilitate its transport into the enterocyte. In this study, the presence of histidine or cysteine in the assay buffer resulted in a significant decrease in zinc uptake. Much of the observed decrease in zinc uptake was due to the loss of the nonmediated component of zinc uptake. In the presence of histidine, the mediated

Table I. Effect of Metabolic Inhibitors on Zinc Transport by Isolated Rat Enterocytes[a]

Inhibitor	Zinc uptake (nmol/2 min/mg protein)
Control	25.2 ± 3.4
DNP	
1.0 mM	24.8 ± 3.4
0.1 mM	25.0 ± 3.5
Ouabain	
1.0 mM	25.7 ± 3.3
0.1 mM	24.8 ± 3.3

[a] The cells were preincubated for 5 min with the inhibitor, then assayed for zinc uptake with 80 μM $ZnCl_2$ for 2 min at 25°C. Each value represents the mean ± SE of three separate cell preparations.

Table II. Effect of Other Potential (LMW) Ligands on Zinc (80 μM) Uptake[a]

Ligand	Zinc uptake (nmol/20 sec/mg protein)
None	11.9 ± 1.6
160 μM Histidine	6.9 ± 1.0*
1.6 mM Histidine	1.6 ± 0.2**
160 μM Cysteine	13.7 ± 1.7
160 μM Proline	11.2 ± 1.4
160 μM Glutamine	11.3 ± 1.2
160 μM Glycine	11.0 ± 1.4
160 μM EDTA	0.3 ± 0.1**
160 μM Glycylsarcosine	10.7 ± 1.5

[a] Cells were incubated with 80 μM $ZnCl_2$ plus the indicated ligand for 20 sec at 25°C. Values represent the mean ± SE of four separate cell preparations. Symbols next to values indicate that the values are statistically different from the control (no ligand). * $P < 0.05$, ** $P < 0.01$.

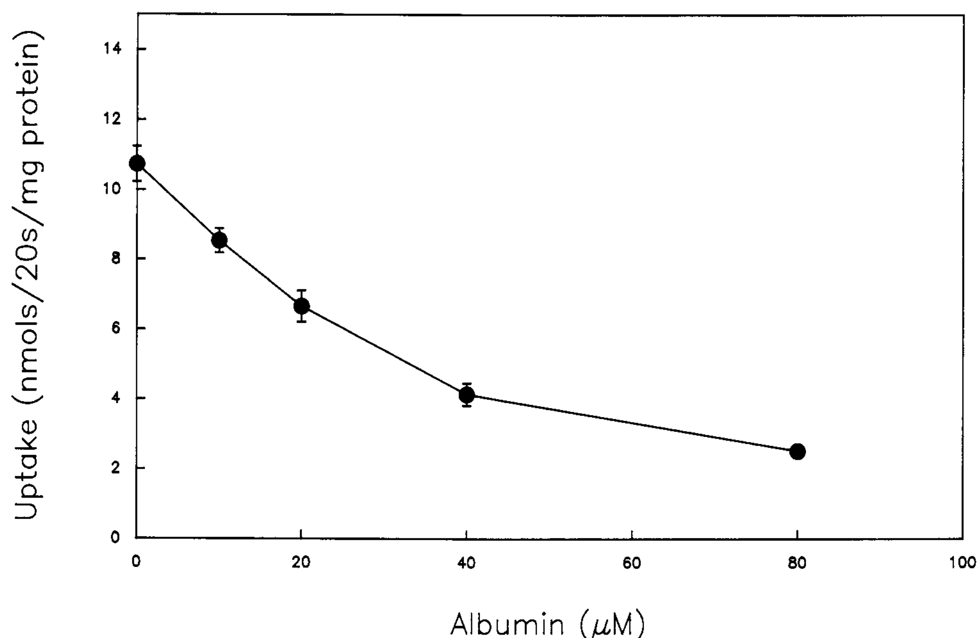

Figure 5. Isolated enterocytes were incubated with 80 μM $ZnCl_2$ and increasing concentrations of albumin (0–80 μM). The cells were incubated for 20 sec at 25°C. Each point represents the mean of at least three separate cell preparations.

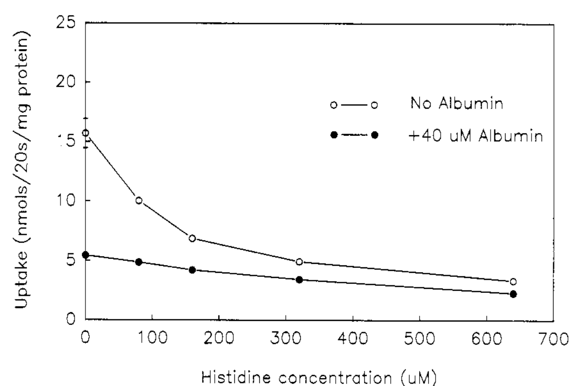

Figure 6. Isolated enterocytes were incubated with 80 μM $ZnCl_2$ in the presence or absence of 40 μM albumin. Increasing concentrations of histidine were added to test the effect of the amino acid on albumin-inhibited zinc uptake. The cells were incubated for 20 sec at 25°C. Each point represents the mean of at least three separate cell preparations.

Table III. Effect of Potential Zinc Ligands on Albumin-Inhibited Zinc Uptake by Isolated Rat Enterocytes[a]

Condition	Uptake (nmol Zn/ mg/20 sec)
80 μM Zinc + 20 μM albumin	10.4 ± 0.54
80 μM Zinc + 20 μM albumin + 160 μM cysteine	10.5 ± 0.42
80 μM Zinc + 20 μM albumin + 160 μM EDTA	0.6 ± 0.04
80 μM Zinc + 20 μM albumin + 160 μM proline	9.9 ± 0.81
80 μM Zinc + 20 μM albumin + 160 μM glycylsarcosine	10.5 ± 0.35
80 μM Zinc + 20 μM albumin + 160 μM protein hydrolysate	9.6 ± 0.56

[a] The cells were incubated with 80 μM $ZnCl_2$ and 20 μM albumin plus the indicated ligand (160 μM). Values represent the mean ± SE of at least three separate cell preparations.

component of zinc uptake was also reduced. Cysteine did not seem to affect the mediated uptake of zinc by these cells. The K_i's obtained for the mediated component of zinc uptake in the presence or absence of histidine or cysteine were not significantly different from one another, suggesting that the same transport mechanisms are involved in the mediated component of zinc uptake in the presence or absence of LMW ligands. The reduction of zinc uptake by the isolated enterocytes in the presence of histidine and cysteine is not in concordance with what has been observed in some *in vivo* studies. Histidine and cysteine are known to form stable chelates with zinc. Both amino acids have been found to enhance zinc absorption in intestinal perfusion studies at a ligand:zinc molar ratio of 2:1 (4). Also, histidine:zinc complexes were found to be absorbed more readily than zinc sulfate in humans (17).

Other investigators have found that the presence of histidine and other amino acids inhibits zinc absorption in perfused rat intestine (18). However, the ligand:zinc molar ratio was relatively high in these studies (>13:1). Ghishan *et al.* (19) reported a decrease in zinc uptake in perfused rat intestine when folic acid was present in the perfusate with zinc at a 4.5:1 ratio. Hence, LMW ligand excess can result in decreased zinc absorption.

Other amino acids tested and the dipeptide glycylsarcosine had no significant effect on zinc uptake by

the isolated enterocytes. None of these compounds are known to bind zinc with affinities as great as histidine or cysteine. EDTA, which has a great affinity for divalent cations, significantly decreased zinc uptake by the cells. In a study using the ligated loop technique, zinc uptake by the duodenum was markedly reduced by EDTA (20). In contrast to these results, EDTA was found to enhance zinc absorption in vascularly perfused rat intestine (18).

Large molecules which bind zinc can decrease its bioavailability. Albumin significantly inhibited zinc uptake by the isolated enterocytes. To examine the possible role LMW ligands may have in mobilizing zinc from large, nonabsorbable molecules such as albumin, the ligands were tested for their ability to mobilize zinc from albumin for uptake by the cells. Histidine and other potential LMW ligands tested were unable to enhance zinc absorption in the presence of albumin. In contrast, albumin-inhibited copper uptake by isolated rat hepatocytes was found to be facilitated by histidine (21).

At the cellular level, in an isolated, experimental environment, LMW ligands which bind zinc may decrease its uptake by (i) forming complexes too large to diffuse through the cell membrane and (ii) by competing with the zinc-binding molecules for the available zinc. It should be pointed out that zinc may be absorbed via paracellular pathways *in vivo*. It is possible that LMW ligands can exert their effects by altering the permeability of the cell junctions (22).

When assessing the effect of LMW ligands on zinc absorption, it is important to consider (i) the experimental model employed and (ii) the ligand:zinc molar ratios used. This study has revealed that LMW ligands with high affinity for zinc decrease its uptake by isolated rat enterocytes, even at a low ligand:zinc ratio. These findings suggest that zinc is more readily absorbed in a noncomplexed form. Since the concentration of free zinc ions will not exceed the solubility product of zinc hydroxide at physiologic pH (1.8×10^{-14}), it is possible that an electrochemically neutral form of hydrated zinc hydroxide exists under the experimental conditions in this study. The mechanisms by which certain LMW ligands, such as histidine, enhance zinc absorption *in vivo* remain unclear.

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