ELSEVIER

Journal of Nutritional Biochemistry 12 (2001) 73–80

THE JOURNAL OF Nutritional Biochemistry

Divalent metals inhibit and lactose stimulates zinc transport across brush border membrane vesicles from piglets

Robert F.P. Bertolo[a], William J. Bettger[a], Stephanie A. Atkinson[b,*]

[a]*Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, ON Canada*
[b]*Department of Pediatrics, McMaster University, Hamilton, ON Canada*

Received January 13, 2000; accepted August 28, 2000.

Abstract

Interactions between metals of similar coordination chemistry are of relevance to infant nutrition due to the highly variable metal:metal ratios found in formulas. Using ratios similar to those found in infant formulas, our objectives were to determine the effects of metals and of lactose and other saccharides on Zn^{+2} transport across intestinal brush border membranes. Brush border membrane vesicles prepared from intestines of 5 preweaned piglets were used to determine whether Ca^{+2}, Mg^{+2}, Fe^{+2}, Cu^{+2}, Cd^{+2}, or Mn^{+2} would antagonize Zn^{+2} uptake. $^{65}Zn^{+2}$ uptake by brush border membrane vesicles was measured over 20 min with metal concentrations constant, and at 1 min with increasing metal concentrations. Zn^{+2} bound to the external surface of vesicles was removed with ethylenediamine-tetraacetic acid. Lactose induced Zn^{+2} uptake to a greater extent than glucose polymer, whereas maltose, galactose, or galactose/glucose had no effect. Over 20 min, a 10:1 concentration of Fe^{+2}, Cd^{+2}, Cu^{+2}, and Mn^{+2} lowered Zn^{+2} uptake significantly ($P < 0.05$). Higher concentrations of divalent cation significantly lowered Zn^{+2} (0.2 or 0.1 mM) uptake for all metals tested ($P < 0.05$), except for Mn^{+2} (0.1 mM Zn^{+2}). Inhibition constant determination quantified relative competitive potential with $Mg^{+2} < Ca^{+2} << Mn^{+2} < Fe^{+2} < Zn^{+2} << Cu^{+2}$. Relative amounts of Ca^{+2}, Mg^{+2}, and Fe^{+2} similar to those found in infant formulas reduced Zn^{+2} uptake by at least 40%. Our data demonstrate that dietary minerals compete during brush border membrane transport, and may help explain antagonistic mineral interactions observed in vivo. Divalent metal concentrations and lactose content of milk affect zinc absorption in neonates and must be carefully considered in formula design.

Keywords: mineral interactions; infant formula; zinc transport; intestine; brush border membrane; piglet

1. Introduction

The nutritional implications of metal interactions in humans are just beginning to be appreciated. The ratios of metals are usually fairly stable in a well balanced diet for the adult, but in formulas designed for infants, these ratios are highly variable, and the potential for metal–metal interactions has rarely been investigated. For example, preterm formulas are often supplemented with relatively large amounts of calcium,[1] and North American formulas are frequently fortified with iron [2]. The impact of adding amounts of some metals proportionately in excess of others on metal–metal interactions requires further study.

The potential for some specific metal interactions in infant feedings is expected given the evidence in animals and adult humans for competitive metal uptake in the intestine. In vivo, Ca [3–5] and Fe [6] antagonistically interact with Zn during absorption. In addition, interactions have been described between Cu^{+2} and Zn^{+2} via zinc-induced expression or activities of specific copper transport proteins such as metallothionein.[7,8] Antagonisms between Ca^{+2}–Zn^{+2} [9,10], Cu^{+2}–Zn^{+2} [11], Mg^{+2}–Ca^{+2} [10,12], and Mn^{+2}–Zn^{+2} [9] during transport in brush border membrane vesicles (BBMV) have also been reported. Altogether, these studies support the hypothesis that similar divalent metals may compete with each other for brush border membrane transport mechanisms; indeed, a divalent cation transporter with broad specificity has recently been described in the intestinal brush border membrane [13]. Using ratios similar to those found in infant feedings, we proposed to analyze the effects of various divalent metals on Zn^{+2} uptake.

Lactose versus glucose polymer addition to infant feed-

Presented in part as a minisymposium at the Experimental Biology 1994 Conference, April 27, 1994.

* Corresponding author. Tel.: 905-521-2100 x75644; fax: 905-521-1703.

0955-2863/01/$ – see front matter
PII: S0955-2863(01)00126-1

ings is also controversial with respect to metal absorption. Because lactose has been described to induce absorption of many divalent metals of interest [14–17] via unclear mechanisms, we also studied saccharide–Zn^{+2} interactions at the brush border membrane.

2. Methods and materials

2.1. Piglets

Five 20–24-day-old male Yorkshire piglets (4.5–6.1 kg) were removed from sows at the Arkell Research Farm (Guelph, ON Canada) and brought to the McMaster University Central Animal Facility. The piglets were not littermates and were sow fed. Handling of piglets conformed with the *Guide to the Care and Use of Experimental Animals* [18]. Piglets were immediately killed by euthanyl injection and the proximal and medial jejuna were removed; no differences in Zn^{+2} uptake by BBMV exist between the proximal, medial, or distal small intestine[19]. Mucosa was obtained as described previously[19].

2.2. BBMV preparation and purity

BBMV were obtained by employing a Mg precipitation/differential centrifugation method [20,21]. The final pellet was resuspended in an incubation buffer (112 mM NaCl, 100 mM D-mannitol, 10 mM Tris-HEPES, pH 6.7). Vesicle solutions were used in $^{65}Zn^{+2}$ transport studies the same day to avoid vesicle damage caused by freezing and thawing.

The extent of brush border purification was determined by periodically measuring changes in the specific activity of sucrase (EC 3.2.1.48) [22]. Orientation of vesicles was assessed by measuring sucrase activity before and after treatment with 1% Triton X-100 for 30 min [20].

Residual Mg associated with the final BBMV suspension was measured by digesting BBMV in 5 mL of concentrated nitric acid at 120°C for 24 hr. Solutions were then diluted with deionized water and Mg was measured by atomic absorption spectrometry (Perkin-Elmer, Model 703, Norwalk, CT USA). In addition, a Mg washout experiment, using ethylenediamine-tetraacetic acid (EDTA), was conducted to determine whether Zn uptake was altered by any residual Mg associated with the BBMV during preparation. Final suspensions were exposed to either buffer or 3 mM EDTA for 1 hr and then diluted and centrifuged at 48,000 *g* for 15 min. Zn^{+2} uptake at 1, 3, 10, and 20 min (37°C) was measured by BBMV from both preparations.

2.3. Zn^{+2} uptake experiments

Three wash buffers were tested for their ability to remove Zn^{+2} bound to the external surface of vesicles by employing Zn^{+2} saturation curves[12]. BBMV (~120 μg protein) were added to a solution containing 3.7 kBq $^{65}Zn^{+2}$ (New England Nuclear Corp., Dupont, Boston, MA USA) and Zn^{+2} concentrations ranging from 0.1 to 0.5 mM (final volume 200 μL). A 100-μL aliquot of this solution was removed and filtered (0.45 μm filters, Millipore, Groton, CT USA) under vacuum after a 1-min incubation at 25°C. The filtered vesicles were rinsed with 200 μL of incubation buffer alone, with 5 mM EDTA, or with 5 mM ethyleneglycotetraacetic acid (EGTA). The triplicate filters at each time point were collected and assayed for radioactivity by a gamma counter (Beckman Gamma 5500, Irvine, CA USA). Samples were prepared in triplicate with a blank (no BBMV) included to correct for background radioactivity. Uptake rates (J) were expressed as nmol $Zn^{+2} \cdot min^{-1} \cdot mg$ total vesicular $protein^{-1}$. Total protein was measured by the procedure of Bradford[23] using crystalline bovine serum albumin as a standard. An Eadie-Hofstee transformation of the EDTA wash data was performed using linear regression to obtain kinetic parameters [24]. All other uptake studies employed the EDTA wash buffer.

For time curves, a BBMV suspension in incubation buffer was added to stock solutions (incubation buffer with 0.2 mM $ZnCl_2$, 15 kBq $^{65}Zn^{+2}$, and 2 mM of the appropriate divalent cation as chloride) at 37°C. Sixty microliters (~40 μg vesicular protein) of stock solution were removed in triplicate at various time points and filtered under vacuum. All solutions were corrected to pH 6.7 after metal additions. All filters were rinsed and counted as above.

For inhibition curves, divalent metal solutions were prepared for each metal of interest. The osmolar contribution of the large amounts of Ca^{+2} and Mg^{+2} used was corrected for by removing D-mannitol and adjusting NaCl. The Ca^{+2} solution (57 mM $CaCl_2$, 90 mM NaCl, 10 mM Tris-HEPES) and Mg^{+2} solution (21.4 mM $MgCl_2$, 132 mM NaCl, 10 mM Tris-HEPES) were isoosmolar (0.32 Osm/L) and were diluted to lower concentrations with incubation buffer. The other divalent metal buffers were not adjusted because the amounts of divalent metal salts added did not alter osmolarity appreciably. Divalent metal concentration ranges (mM) used were $CaCl_2$: 2–15, $MgCl_2$: 2–15, $FeCl_2$: 0.02–1.0, $CuCl_2$: 0.02–0.20, and $MnCl_2$: 0.02–0.80. Approximately 120 μg of vesicular protein were added to a solution containing the divalent metal solution, 3.7 kBq $^{65}Zn^{+2}$, and either 0.2 mM $ZnCl_2$ or 0.1 mM $ZnCl_2$ for a total volume of 200 μL. A 100-μL aliquot of this solution was removed and filtered after a 1-min incubation at 25°C. Incubations were performed in 96-well plates and could not be incubated at 37°C. Filters were treated as described above.

2.4. K_i calculations

Inhibition constants (K_i) represent the affinity of an inhibitor for the binding site of a protein and were calculated

using the equation $K_i = [v_i/(v - v_i)] \times \{[K_m \times (I)]/[(S) + K_m]\}$, where v and v_i are the rates of transfer in the absence and presence, respectively, of inhibitor; K_m is the Michaelis-Menten constant for Zn^{+2} determined via Eadie-Hofstee plot; (I) is the concentration of inhibitor; and (S) is the substrate (Zn^{+2}) concentration [24]. A K_i was determined for each inhibited uptake datum and the mean ± SEM was calculated for each metal. K_i values were also determined using Dixon plots to verify calculations; linear regression was applied to all reciprocal data for each Zn^{+2} concentration and the resulting equations were solved for x (with y values equal).

2.5. Vesicle aggregation experiments

Possible aggregation of BBMV was measured using a mild centrifugation technique [25]. Under time curve conditions, 150 μL of vesicles (6 mg/mL) were added to 600 μL of incubation buffer with 2 mM divalent metal salt. A control without divalent metal was included as well as a sample with 0.2 mM $ZnCl_2$. After 20 min at 37°C, the samples were centrifuged at 2,000 × *g* for 15 min and the protein was analyzed using the Bradford assay [23]. Similarly, under inhibition curve conditions, 150 μL of vesicles were added to 600 μL of buffer with respective maximum divalent metal concentrations (from ranges above) and incubated for 1 min at 25°C before pellet protein analysis. All data were expressed as percentage of total vesicular protein and compared to Na^+ buffer control.

2.6. Saccharides and Zn^{+2} uptake

Approximately 120 μg BBMV protein were added to base incubation buffer of 112 mM NaCl, 10 mM Tris-HEPES, 50 mM D-mannitol, pH 6.7 (final volume 200 μL) with either 50 mM D-mannitol (control), 50 mM maltose, 50 mM D-galactose, 25 mM D-galactose with 25 mM D-glucose, 50 mM (18 g/L) lactose (0.36 mOsm/L), 18 g/L glucose polymer (0.31 mOsm/L; Amaizo, Wyeth-Ayerst, Philadelphia, PA USA), 18 g/L glucose polymer with 5.2 g/L D-mannitol (0.36 mOsm/L), or 9 g/L glucose polymer with 9 g/L lactose. All data were expressed as percentage of control.

2.7. Statistics

Due to the high variation among animals, the data for uptake measurements were standardized. For the time curves, the approximate point of inflection (5-min point) for the control Na^+ curve of each piglet was set at 100% and all other time points were expressed as a percentage of this point. Total area under each piglet's time curve was calculated and each metal group was compared to Na^+ control using one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons (SigmaStat, Jandel Scientific, San Rafael, CA USA).

Inhibition curve uptake data were standardized by setting each piglet's control (0 divalent metal:0.2 mM Zn^{+2}) as 100% and expressing all other data as a percentage of control. One-way ANOVA was applied to the combined data (excluding control) to determine a treatment effect of inhibitor concentration on Zn^{+2} uptake. Data are presented as mean ± SEM and differences were considered significant if $P < 0.05$.

In the saccharide experiments, because control data had a SEM of 0, data were considered different if SEM bars did not overlap control data (100% line).

3. Results

3.1. Membrane purity

Periodic sucrase assays ($n = 16$) before and after the BBMV preparation demonstrated 12.9 ± 0.5-fold increases in specific activity that are consistent with previous studies using this particular BBMV preparation method [19,21,26]. In addition, BBMV were >95% right-side-out, which was similar to results by Kessler et al. [20].

Residual Mg associated with the BBMV after preparation amounted to approximately 150 nmol Mg/mg total protein (range 101–187, $n = 6$), similar to that found by Tacnet et al. [26]. This value corresponds to approximately 0.15 mM Mg in the final volume used in our experiments, which is negligible compared to the 2–15 mM Mg^{+2} used in the Mg^{+2} inhibition curves. Furthermore, Zn^{+2} uptake (at 1, 3, 10, and 20 min) by EDTA-washed BBMV (64, 102, 144, and 165% of 3-min control uptake, respectively) was not different compared to control BBMV (73, 100, 151, and 164%, respectively, $n = 3$), suggesting that the residual Mg did not interfere with Zn^{+2} uptake mechanisms.

To account for extravesicular binding of Zn^{+2}, Zn^{+2} saturation curves with different wash buffers were analyzed. EDTA and EGTA buffers similarly reduced vesicle-associated Zn^{+2} by 70–80% compared to incubation buffer; this EDTA-removable Zn^{+2} is assumed to be bound to the external surface of vesicles [12,26].

3.2. Metal:Zn^{+2} interactions at 10:1 over time

Time curves for Zn^{+2} uptake by BBMV in the presence of various divalent metals are shown in *Figure 1*. The uptake of Zn^{+2} (0.2 mM) over time was depressed significantly ($P < 0.05$) by Cd^{+2}, Cu^{+2}, Mn^{+2}, and Fe^{+2} at 10× concentrations. Because this study addressed divalent metal competition, we estimated the Fe^{+2} content of our solutions using phenanthroline chelation. In the buffer, approximately 80% of 2 mM Fe was in divalent form after 2 hr at room temperature, whereupon experiments were begun.

3.3. Inhibition curves

The inhibition curve studies attempted to use divalent metal concentrations that reflected ratios of metals in North Ameri-

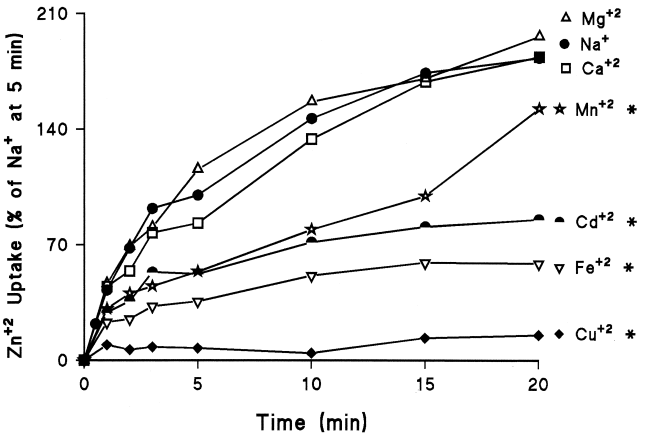

Figure 1 Zn^{+2} (0.2 mM) uptake at 37°C by brush border membrane vesicles with extravesicular Ca^{+2}, Cu^{+2}, Cd^{+2}, Mg^{+2}, Fe^{+2}, or Mn^{+2} concentrations of 2 mM. Na^{+} represents control uptake without any other divalent metal added. In each piglet, the 5-min value for Na^{+} buffer was set as 100% control and all other data were expressed as a percentage of control. Data points represent means for 5 piglets (to simplify figure, error bars are not presented). An asterisk represents areas under curves that were statistically different compared to Na^{+} control ($P < 0.05$).

can infant formulas. However, with Cu^{+2} and Mn^{+2}, the highest ratios found in formulas were too low to affect Zn^{+2} uptake. So higher concentrations of Cu^{+2} and Mn^{+2} were tested so that inhibition of Zn^{+2} was achieved and a K_i calculable.

Inhibition curves are shown in *Figures 2a–e* for Ca^{+2}, Mg^{+2}, Fe^{+2}, Cu^{+2}, and Mn^{+2}, respectively. Significantly lower Zn^{+2} uptake over increasing metal concentrations ($P < 0.05$) was observed for all curves except Mn^{+2} with 0.1 mM Zn^{+2}. For Ca^{+2}, only 4 mM was required to lower Zn^{+2} (0.2 mM) uptake to 42% of control uptake; higher Ca^{+2} concentrations had only moderate additional effect. Indeed, 30 mM Ca^{+2} did not lower Zn^{+2} uptake below 25% of control (not shown). In contrast, a 15 mM Mg^{+2} concentration was needed to reduce Zn^{+2} uptake to 40–45% of control at both Zn^{+2} concentrations. A more potent inhibitor, Fe^{+2} at a concentration of 0.4 mM, lowered Zn^{+2} uptake to 55–60% of control at both Zn^{+2} concentrations. This inhibitory effect was even greater with an Fe^{+2} concentration of 1.0 mM. Using phenanthroline chelation, over 92% of 1.0 mM Fe was divalent at the time of BBMV incubation. The most potent inhibitor was Cu^{+2}, which reduced Zn^{+2} uptake (at both concentrations) to 40–45% of control at a concentration of only 0.10 mM; however, this amount of Cu^{+2} is not achieved in formulas. Furthermore, an equimolar concentration of Cu^{+2} reduced Zn^{+2} uptake to 20–25% of control when Zn^{+2} was introduced at either 0.2 or 0.1 mM. As with Cu^{+2}, exceedingly high concentrations of Mn^{+2} were used to inhibit Zn^{+2} uptake. Even with a 4× concentration, Mn^{+2} only lowered Zn^{+2} uptake to 63% of control.

3.4. K_i calculations

K_i values are presented in *Figure 2* legend. K_m for Zn^{+2} was 0.23 mM, as determined by Eadie-Hofstee plot (not shown). K_i values (mM) determined from Dixon plots were within 99% confidence intervals (CI) of algebraically calculated values for Ca^{+2} (4.0) and Fe^{+2} (0.39), and within 95% CI for Mg^{+2} (8.2), Cu^{+2} (0.019), and Mn^{+2} (0.42). All regression analyses for Dixon plots were significant ($P < 0.05$).

3.5. BBMV aggregation

Aggregation of BBMV in the presence of the various metals under both inhibition and time curve conditions averaged approximately 20% of total protein and were not different among metals.

3.6. Saccharide effects on Zn^{+2} uptake by BBMV

Zn^{+2} uptake in transport buffers of various saccharides were compared (*Figure 3*). In the presence of lactose, Zn^{+2} uptake was 240–255% of control, whereas its monosaccharide components (galactose + glucose) as well as maltose had slightly negative effects. Glucose polymer induced Zn^{+2} uptake to 175–181% of control. In addition, the lactose + glucose polymer buffer effected similar Zn^{+2} uptake rates (251 ± 19, 222 ± 14, and 205 ± 13% at 1, 10, and 30 min, respectively; not shown) to lactose alone, whereas Zn^{+2} uptake in the glucose polymer + mannitol buffer (197 ± 7, 157 ± 7, and 174 ± 10% at 1, 10, and 30 min, respectively; not shown) was similar to rates in polymer alone. The galactose buffer had no effect on Zn^{+2} uptake (98 ± 14, 107 ± 19, and 111 ± 16% at 1, 10, and 30 min, respectively; not shown).

4. Discussion

All of the metals studied were capable of inhibiting Zn^{+2} uptake by BBMV at various concentrations. These results suggest that Ca^{+2}, Mg^{+2}, Fe^{+2}, Cu^{+2}, and Mn^{+2} may compete with Zn^{+2} for transport across the brush border membrane. Furthermore, Zn^{+2} uptake was inhibited by Ca^{+2}, Mg^{+2}, and Fe^{+2} at concentrations similar to those found in commercial infant formulas, whereas Cu^{+2} and Mn^{+2} reduced Zn^{+2} uptake only at much higher concentrations than those present in infant feedings.

Inhibition constants reflect the affinities of each metal for binding sites and, hence, their competitive or antagonistic potentials. Including the affinity calculated for Zn^{+2} (K_m = 0.23 mM), relative affinities were $Mg^{+2} < Ca^{+2} << Mn^{+2} < Fe^{+2} < Zn^{+2} << Cu^{+2}$. These affinities were consistent with the results from the time curves with constant metal:Zn^{+2} ratios. At a 10× concentration, Ca^{+2} and Mg^{+2} had no effect on Zn^{+2} uptake, whereas Cu^{+2} almost blocked

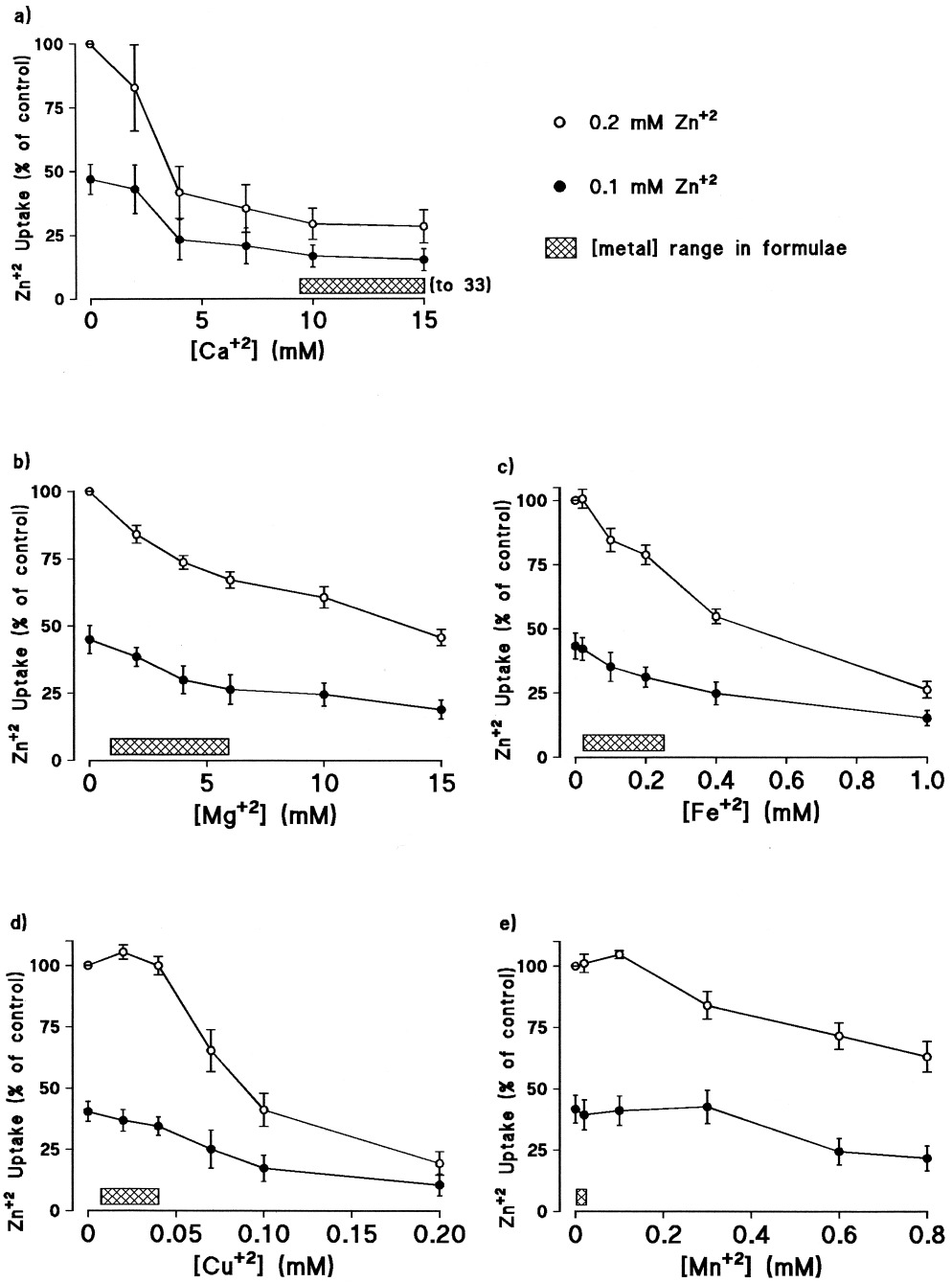

Figure 2 Inhibition curves showing initial rates of Zn^{+2} uptake (1 min, 25°C) by brush border membrane vesicles with extravesicular Zn^{+2} concentration at 0.2 mM or 0.1 mM and extravesicular divalent metal [(a) Ca^{+2}, (b) Mg^{+2}, (c) Fe^{+2}, (d) Cu^{+2}, (e) Mn^{+2}] concentrations as indicated on abscissa. Controls were set at 100% uptake (0 mM divalent metal and 0.2 mM Zn^{+2}) for each piglet and data expressed as percentage of control. Data represent the standardized mean ± SEM for vesicles from 5 piglets. Significantly lower Zn^{+2} uptake over divalent metal concentrations (analysis of variance, $P < 0.05$) was observed for all curves except Mn^{+2} with 0.1 mM Zn^{+2}. K_i constants (mM) calculated for each metal were (a) Ca^{+2}: 3.3 ± 0.4, (b) Mg^{+2}: 6.9 ± 0.3, (c) Fe^{+2}: 0.34 ± 0.03, (d) Cu^{+2}: 0.053 ± 0.008, and (e) Mn^{+2}: 0.66 ± 0.06.

Zn^{+2} uptake. With Mn^{+2}, Fe^{+2}, and Cd^{+2} intermediate in their ability to inhibit Zn^{+2} uptake, these results suggest relative affinities: Mg^{+2}, $Ca^{+2} < Mn^{+2}$, Cd^{+2}, $Fe^{+2} < Cu^{+2}$. Interestingly, trace metals with the lowest dietary abundance had the highest affinity, possibly allowing successful competition in the presence of large quantities of macrominerals such as Mg and Ca. Although we hypothesize that these divalent metals compete for a common transporter with broad specificity, such as that recently discovered in the duodenum [13], our data do not preclude the possibility that multiple transporters are involved in the various metal–metal interactions observed.

The objective in choosing the metal concentrations and

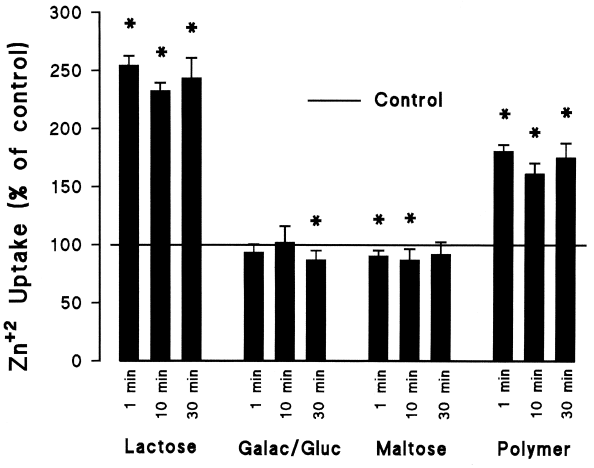

Figure 3 Zn^{+2} (0.2 mM) uptake by brush border membrane vesicles in buffers with various saccharide compositions (25°C). Buffers contained either 50 mM mannitol (Control), 50 mM lactose (18 g/L), 25 mM D-galactose + 25 mM D-glucose (Galac/Gluc), 50 mM maltose, or 18 g/L glucose polymer (Polymer). Bars represent mean + SEM for 4 piglets (except for lactose, where $n = 5$). An asterisk indicates data were different ($P < 0.05$) than control uptake (100% line).

ratios for this in vitro experiment was to determine whether there was the potential for metal–metal interactions that might be of nutritional significance in formulas and human milk fortifiers designed for feeding of premature and term infants. In infant feedings, Zn concentrations range from 0.06 to 0.96 mM, whereas Ca concentrations range from 9 to 33 mM; Ca:Zn ratios range from 80:1 to 450:1. In this study, at a 0.2 mM Zn^{+2} concentration, a Ca^{+2}:Zn^{+2} ratio of 20:1 (4 mM Ca^{+2}) reduced Zn^{+2} uptake by BBMV to less than 50% of control, and a ratio of 50:1 Ca^{+2}:Zn^{+2} (10 mM Ca^{+2}) reduced it to one third of control. Using a similar BBMV system, but from adult rats, Gunshin et al. [9]. also observed a 50% reduction in Zn^{+2} uptake when incubated in a Ca^{+2}:Zn^{+2} medium of 50:1 (10 mM Ca^{+2}:0.2 mM Zn^{+2}). Conversely, in BBMV from pigs (no reported age), Ca^{+2} did not inhibit Zn^{+2} uptake over the range of 20:1 to 50:1 Ca^{+2}:Zn^{+2} [26], but the incubation time was only 2 sec compared to 1 min for our and other studies [9], and Zn^{+2} concentrations were much higher at up to 3.5 mM. In addition, Ca^{+2} uptake by BBMV from young rats was inhibited by Zn^{+2} at a molar ratio of 0.1:1 Zn^{+2}:Ca^{+2} (0.05:0.5 mM), but over 60 min [10]. It has been suggested that Ca^{+2} and Zn^{+2} directly compete for transporter binding sites [9,10]. The present study supports such a mechanism and goes further by observing other divalent metal effects.

Mg^{+2} may antagonistically interact with Zn^{+2} as shown by a 30–45% reduction in Zn^{+2} uptake at Mg^{+2} concentrations similar to those found in formulas (6 mM). Very few studies have addressed Mg^{+2}–Zn^{+2} interactions at physiological concentrations; however, Mg^{+2} has been shown to lower Ca^{+2} uptake by rat BBMV [10,12]. As a macromineral, Mg^{+2} and its interactions with other chemically similar metals should be further studied.

The observation of an inhibitory effect of Fe^{+2} on Zn^{+2} uptake by 20–30% at Fe^{+2} concentrations comparable to the upper concentrations found in formulas (0.25 mM) is consistent with previously described interaction effects of these metals in human nutrition [6]. In rat BBMV, equimolar Fe^{+2} (0.2 mM) reduced Zn^{+2} uptake by 18%, whereas equimolar Zn^{+2} and Mn^{+2} lowered Fe^{+2} uptake by 42% and 19%, respectively [27]. These data suggest that these divalent metals may compete with each other for transport across intestinal brush border membranes with affinities of Mn^{+2}, $Fe^{+2} < Zn^{+2}$. Using a perfused intestine system, Hamilton et al. [28] found that Fe and Zn competed for binding sites at the uptake step; with a 10:1 (1.0:0.1 mM) Fe:Zn molar ratio in iron-replete mice, Zn^{+2} uptake was 60% of control compared to our 35% at the same Fe:Zn ratios. These studies identify a need for caution when modifying Fe concentrations in infant formulas because both studies used Fe:Zn ratios well within those found in commercial infant feedings.

Little is known about Cu^{+2}–Zn^{+2} interactions at the brush border membrane. In a study from our laboratory [11], Zn^{+2} was shown to reduce Cu^{+2} uptake by BBMV when introduced at molar ratios of 5:1 and 10:1 Zn^{+2}:Cu^{+2}. However, the present study is the first, to our knowledge, to assess the effects of Cu^{+2} on Zn^{+2} uptake at the brush border membrane. Of the divalent metals studied here, Cu^{+2} is the most similar to Zn^{+2} chemically; this is exemplified in our study because Cu^{+2}, with the lowest K_i, was the most effective at antagonizing Zn^{+2} uptake.

The K_i for Mn^{+2} was found to be 80% lower than that for Ca^{+2}. Consistent with this finding, Gunshin et al. [9] found that Mn^{+2} (10 mM) was more potent than equimolar Ca^{+2} in reducing Zn^{+2} uptake by rat BBMV. Although Mn^{+2} and Cu^{+2} were introduced to BBMV at concentrations not physiologically relevant to infant feedings, their abilities to inhibit Zn^{+2} uptake is interesting with respect to metal interaction mechanisms.

The in vitro BBMV model is appropriate for this study because we were interested in elucidating metal:Zn^{+2} interactions at the brush border membrane. However, several potential limitations of the system have been reported. The extensive extravesicular binding previously reported for Zn[26] and Ca[12] was accounted for in this study by using the EDTA wash solution. Furthermore, vesicle-bound residual Mg from the BBMV preparation technique did not alter Zn^{+2} uptake mechanisms. We also showed that the metals and their concentrations in this study did not aggregate BBMV as reported previously in renal BBMV [29]. Potent redox metals such as Cu and Fe may damage vesicle membrane integrity in the system used. However, the typically saturable uptake of Zn^{+2} over 20 min in the presence of Cu and Fe (*Figure 1*) contraindicates general or nonspecific membrane damage. Furthermore, some oxidative damage by Fe and Cu to the brush border membrane during Zn^{+2} transport may be part of the antagonism among these metals observed in vivo.

Lactose has been reported to stimulate intestinal absorption of many divalent metals including Ca [14], Mg [15], Fe [16], Zn [14], and Mn [17], among other metals. Several mechanisms have been proposed to explain the effects of lactose on Ca [14,15] but none have been widely accepted. We found that lactose stimulated Zn^{+2} uptake by BBMV, suggesting that the effect may be at least partially localized to the brush border membrane. Also, because galactose or galactose + glucose did not affect Zn^{+2} uptake, lactose effects may be exerted primarily by the disaccharide. Using a BBMV model, Gunshin et al. [30] showed that Ca^{+2} uptake was also enhanced with lactose, but not maltose; however, they did observe a slight induction by galactose + glucose. Many infant formulas and human milk fortifiers use glucose polymers to reduce osmolarity and to compensate for low lactase activity in premature infants[31]. The inductive effect of glucose polymer on Zn^{+2} uptake (irrespective of osmolarity) is interesting, but not easily explained given that maltose was ineffective. Stathos et al. [32] found that glucose polymers enhanced Ca^{+2} absorption in preterm infants, whereas lactose had no effect. The effects of glucose polymers on metal absorption require further study given their importance in infant formula formulation. Clearly, saccharides are not inert with respect to metal absorption in neonates; the mechanisms involved in saccharide–metal interactions in the intestine may include brush border membrane transport of metals, as indicated in this study.

It is important to note that although we used metal concentrations that are relevant to infant formulas, whether these concentrations or ratios exist at the brush border membrane during formula feeding is unknown. Indeed, macronutrients from a meal will interact with dietary minerals differently, thereby diminishing or exacerbating the interactions we have observed, depending on the net effect at the brush border membrane level of absorption [33]. Our overall goal was to show that dietary metals interact at the level of brush border membrane transport independent of luminal interactions with other meal nutrients; these findings may help explain antagonistic mineral interactions observed in vivo [3,5,6,28,33]. The developing preterm infant is especially vulnerable to metal interactions due to an immature gut and an increased need for intakes of nutrients greater than term infants in order to facilitate rapid growth. Understanding of all levels of metal interactions is needed to manipulate and maintain metal ratios in a way that will optimize metal bioavailability from infant formulas and feedings.

Acknowledgments

This research was supported by a grant from the Dairy Bureau of Canada to Stephanie A. Atkinson.

References

1 Atkinson, S.A. (1994). Calcium and phosphorus needs of premature infants. *Nutrition* **10,** 66–68
2 Schulz-Lell, G., Buss, R., Oldigs, H.-D., Dorner, K., and Schaub, J. (1987). Iron balances in infant nutrition. *Acta Paediatr. Scand.* **76,** 585–591
3 Atkinson, S.A., Shah, J.K., Webber, C., Gibson, I.L., and Gibson, R.S. (1993). A multi-element isotopic tracer assessment of true fractional absorption of minerals from formula with additives of calcium, phosphorus, zinc, copper and iron in young piglets. *J. Nutr.* **123,** 1586–1593
4 Atkinson, S.A. and Shah, J.K. (1990). Calcium and phosphorus fortification of preterm formulas: drug–mineral and mineral–mineral interactions. In *Mineral Requirements for the Premature Infant* (L. Hillman, ed.), p. 58, Excerpta Medica, New York, NY USA
5 Pecoud, A., Donzel, P., and Schelling, J.L. (1975). Effect of foodstuffs on the absorption of zinc sulfate. *Clin. Pharm. Therap.* **17,** 469-474
6 Solomons, N.W. (1986). Competitive interaction of iron and zinc in the diet: consequences for human nutrition. *J. Nutr.* **116,** 927–935
7 Reeves, P.D., Briske-Anderson, M., and Johnson, L. (1998). Physiologic concentrations of zinc affect the kinetics of copper uptake and transport in the human intestinal cell model Caco-2. *J. Nutr.* **128,** 1794–1801
8 Cousins, R.J. (1985). Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol. Rev.* **65,** 238–309
9 Gunshin, H., Noguchi, T., and Naito, H. (1991). Effect of calcium on the zinc uptake by brush border membrane vesicles isolated from the rat small intestine. *Agric. Biol. Chem.* **55,** 2813–2816
10 Roth-Bassell, H.A. and Clydesdale, F.M. (1991). The influence of zinc, magnesium, and iron on calcium uptake in brush border membrane vesicles. *J. Am. Coll. Nutr.* **10,** 44–49
11 Wang, Z. (1993). *Factors affecting zinc and copper metabolism during development in the piglet model.* Ph.D. Thesis, McMaster University, Hamilton, ON Canada
12 Merrill, A.R., Proulx, P., and Szabo, A.G. (1986). Studies on calcium binding to brush-border membranes from rabbit small intestine. *Biochim. Biophys. Acta* **859,** 237–245
13 Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., and Hediger, M.A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388,** 482–488
14 Ghishan, F.K., Stroop, S., and Meneely, R. (1982). The effect of lactose on the intestinal absorption of calcium and zinc in the rat during maturation. *Pediatr. Res.* **16,** 566–568
15 Heijnen, A.M.P., Brink, E.J., Lemmens, A.G., and Beynen, A.C. (1993). Ileal pH and apparent absorption of magnesium in rats fed on diets containing either lactose or lactulose. *Br. J. Nutr.* **70,** 747–756
16 Amine, E.K. and Hegsted, D.M. (1975). Effect of dietary carbohydrates and fats on inorganic iron absorption. *J. Agric. Food Chem.* **23,** 204
17 Fournier, P. and Fournier, A. (1972). Influence de l'ingestion de lactose sur l'absorption et la retention du manganese. *Comput. Rend. Soc. Biol.* **166,** 29–31
18 Olfert, E.D., Cross, B.M., and McWilliam, A.A. (eds.) (1993). *Guide to the Care and Use of Experimental Animals* (2nd ed.). Canadian Council on Animal Care, Government of Canada, Ottawa, ON Canada
19 Wang, Z., Atkinson, S.A., Bertolo, R.F.P., Polberger, S., and Lonnerdal, B. (1993). Alterations in intestinal uptake and compartmentalization of zinc in response to short-term dexamethasone therapy or excess dietary zinc in piglets. *Pediatr. Res.* **33,** 118–124
20 Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., and Semenza, G. (1978). A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of

D-glucose and choline transport systems. *Biochim. Biophys. Acta* **506,** 136–154
21 Davidson, L.A. and Lonnerdal, B. (1988). Specific binding of lactoferrin to brush border membrane: ontogeny and effect of glycan chain. *Am. J. Physiol.* **254,** G580–G585
22 Dahlqvist, A. (1968). Assay of intestinal disaccharidases. *Anal. Biochem.* **22,** 99–107
23 Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 254–260
24 Neame, K.D. and Richards, T.G. (1972). *Elementary Kinetics of Membrane Carrier Transport.* Blackwell Scientific Publications, Oxford, UK
25 Watkins, D.W., Chenu, C., and Ripoche, P. (1989). Zinc inhibition of glucose uptake in brush border membrane vesicles from pig small intestine. *Pflugers Arch.* **415,** 165–171
26 Tacnet, F., Watkins, D.W., and Ripoche, P. (1990). Studies of zinc transport into brush-border membrane vesicles isolated from pig small intestine. *Biochim. Biophys. Acta* **1024,** 323–330
27 Wien, E.M., Glahn, R.P., and Van Campen, D.R. (1994). Ferrous iron uptake by rat duodenal brush border membrane vesicles: effects of dietary iron level and competing minerals (Zn^{+2}, Mn^{+2}, and Ca^{+2}). *J. Nutr. Biochem.* **5,** 571–577
28 Hamilton, D.L., Bellamy, J.E.C., Valberg, J.D., and Valberg, L.S. (1978). Zinc, cadmium and iron interaction during intestinal absorption in iron-deficient mice. *Can. J. Physiol. Pharmacol.* **56,** 384–388
29 Kirschbaum, B.B. (1982). Aggregation of renal brush border membranes by concanavalin A and heavy metals. *Toxicol. Appl. Pharmacol.* **64,** 10–19
30 Gunshin, H., Noguchi, T., and Naito, H. (1991). Lactose-enhanced uptake of calcium by isolated brush border membrane vesicles from the rat small intestine. *Agric. Biol. Chem.* **55,** 1919–1921
31 Shulman, R.J., Feste, A., and Ou, C. (1995). Absorption of lactose, glucose polymers, or combination in premature infants. *J. Pediatr.* **127,** 626–631
32 Stathos, T.H., Shulman, R.J., Schanler, R.J., and Abrams, S.A. (1996). Effect of carbohydrates on calcium absorption in premature infants. *Pediatr. Res.* **39,** 666–670
33 Rossander-Hulten, L., Brune, M., Sandstrom, B., Lonnerdal, B., and Hallberg, L. (1991). Competitive inhibition of iron absorption by manganese and zinc in humans. *Am. J. Clin. Nutr.* **54,** 152–156