The Journal of Experimental Biology 205, 141–150 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3607

In vivo characterisation of intestinal zinc uptake in freshwater rainbow trout

Chris N. Glover[1,*] and Christer Hogstrand[1,2]

[1]*Division of Life Sciences, King's College, London, London SE1 9NN, UK* and [2]*T. H. Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506–0225, USA*

*e-mail: chris.glover@kcl.ac.uk

Accepted 19 October 2001

Summary

Knowledge of the uptake mechanisms and metabolism of metals is essential for understanding the factors governing metal toxicity, discerning means by which acclimation and homeostasis may be achieved and characterising interactions between the metal of interest and other environmental moieties. Zinc is both an important aquatic contaminant and a vital micronutrient. The physiological characterisation of dietary zinc absorption in fish has, therefore, important implications for environmental protection and aquaculture. The present study aimed to elucidate the mechanism of intestinal zinc uptake in freshwater rainbow trout (*Oncorhynchus mykiss*), using an *in vivo* cannulation technique. Only a saturable component of zinc uptake, with a concentration giving half-maximal rate of accumulation ($K_{0.5}$) of 309 μmol l^{-1}, and a maximal rate of accumulation (J_{max}) of 933 nmol kg^{-1} h^{-1}, was described. This characterised the intestine as a low-affinity, high-capacity zinc absorption pathway. Physiological mechanisms appear to regulate zinc uptake. Intestinal mucus was one important regulatory locus, promoting zinc uptake at low concentrations yet buffering the animal against high luminal zinc loads. Regulatory mechanisms also seemed to limit subepithelial zinc accumulation. Experiments using ethylene glycol tetraacetic acid (EGTA) to wash the intestinal lumen following zinc perfusion exhibited a higher proportion of loosely associated zinc at higher perfused concentrations. This was attributed to saturation of the uptake process or efflux from the subepithelium. Two distinct pathways for passage of zinc across the epithelium were discerned, with post-intestinal transfer possibly mediated by sulphydryl groups, as illustrated by *N*-ethylmaleimide perfusion experiments. Putative roles of zinc transporters and/or intracellular-binding proteins are discussed.

Key words: dietary metal absorption, kinetics, heavy metal accumulation, mucus, tissue distribution, fish, zinc homeostasis.

Introduction

For aquatic biota, as for other organisms, zinc is a vital micronutrient (Watanabe et al., 1997) and must be assimilated from the environment. However, in contaminated ecosystems zinc is also an important toxicant (Eisler, 1993) and therefore presents an additional physiological challenge. While the absorption of zinc needs to persists, mechanisms must also exist for limiting assimilation and/or toxicity. Homeostatic control of accumulation may be the key to survival in contaminated waters.

Zinc absorption occurs *via* two major routes. The branchial uptake pathway for waterborne zinc is well characterised, owing to its mediation of acute toxicity. The passage of zinc through an apical Ca^{2+} channel in the chloride cells of gills accounts both for the uptake and for the potentially lethal hypocalcaemia induced upon zinc exposure (Spry and Wood, 1985; Hogstrand et al., 1996, 1998).

Dietary zinc absorption is less well characterised. It is known, however, that gastrointestinal uptake is likely to be of greater importance to zinc body burden than the branchial route (Hogstrand and Wood, 1996). This is a consequence of zinc speciation within aquatic systems. Most zinc is sequestered within sediments while water column zinc is almost entirely particulate, adsorbed to dissolved organic and inorganic compounds (Florence et al., 1992; Rozan et al., 2000). It is within the alimentary canal, where interactions with digestive secretions may cause desorption of metals from food and ingested particulate matter (Powell et al., 1999), that bioavailability is increased. It is therefore not surprising that orally assimilated metals have been implicated in chronic toxicity (Woodward et al., 1994; Farag et al., 1999).

Despite the obvious nutritional and toxicological value in studying dietary zinc uptake, there have been relatively few studies on this topic. In freshwater rainbow trout (*Oncorhynchus mykiss*) dietary zinc supplementation caused dose-dependent increases in body zinc (Wekell et al., 1983). Significant absorptive interactions of zinc with other dietary cations have been hypothesised (Knox et al., 1984; Hardy and Shearer, 1985; Wekell et al., 1986), although the mechanisms by which this could occur have not been investigated. Feeding zinc as an organic, *versus* an inorganic, chelate have also been

studied, with either a beneficial (Paripatananont and Lovell, 1995; Hardy et al., 1987) or no (Li and Robinson, 1996) effect upon body zinc status. These studies have mainly focussed on quantitative endpoints and have not attempted to address the question of how zinc is absorbed.

In contrast the mechanism of zinc absorption in a marine fish (winter flounder: *Pseudopleuronectes americanus*) has been addressed more directly (Shears and Fletcher, 1979, 1983). Two components to intestinal zinc uptake were noted, with a saturable uptake pathway superimposed upon a linear, diffusive route that was prominent at higher luminal concentrations. In addition, the intracellular metal-binding protein metallothionein (MT) was found to play an important role. Passage of zinc across the basolateral membrane was believed to be passive.

Zinc absorption in the mammalian intestine follows a similar pattern to that described by Shears and Fletcher for marine fish. Two uptake components exist, while intracellular binding moieties such as MT have important roles (Lönnerdal, 1989). The nature of the basolateral transfer of zinc is controversial although recent localisation of the ZnT-1 zinc exporter to the basolateral membrane suggests a mediated event (McMahon and Cousins, 1998).

In fact much recent research has focussed upon the potential roles of membrane transporters in zinc uptake. Molecular approaches have led to the cloning of many different metal transporters in a wide variety of organisms. Transporters such as the zinc transporter (ZnT) group (Palmiter and Findlay, 1995; Cousins and McMahon, 2000), the ZRT/IRT-related protein (ZIP) family (Guerinot, 2000) and divalent metal transporter-1 (DMT-1; Nramp2) (Gunshin et al., 1997) are likely to play important roles in the uptake of zinc in a variety of species, tissues and cellular organelles.

The present investigation utilised an *in vivo* perfusion technique to examine zinc uptake in *Oncorhynchus mykiss*. This procedure permitted close control of the gut environment in a physiologically responsive system. The *in vivo*, flow-through nature of the method provided insights into potential homeostatic regulation of the uptake process. Basic kinetic characterisation of zinc uptake was followed by investigation of specific aspects of the absorptive process, notably the potential role of sulphydryl moieties. This yielded information about the mechanism of zinc absorption in a freshwater fish species, and allowed comparison to uptake in mammals and marine teleosts. The establishment of this technique has permitted subsequent examination of other aspects of zinc uptake such as the role of low molecular weight ligands (Glover and Hogstrand, 2002), and interactions of zinc with other dietary metals (C. N. Glover and C. Hogstrand, in preparation).

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 73–443 g, mean ± S.D. 191±9 g, *N*=85) were obtained from Wolf Creek Dam National Fish Hatchery, Kentucky, USA and transported to holding facilities at the University of Kentucky. Fish were maintained in 400 l fibreglass tanks with flowing, aerated and dechlorinated Lexington city tap water. Water temperature was maintained at 11–15 °C and food was withheld from fish for at least 5 days before experimentation.

Surgical preparation

Anaesthesia was induced by immersion of fish in 3-aminobenzoic acid ethylester (MS-222: 0.4 g l^{-1}, pH 8.0; Sigma) and maintained by perfusion of aerated MS-222 (0.1 g l^{-1}, pH 8.0) across the gills for the duration of the procedure.

The intestinal cannulation method was modified from Wilson et al. (1996). Transverse incisions in the body wall and then the stomach were made and a flanged inflow catheter (Microline: i.d. 1.02 mm, o.d. 1.75 mm, Cole Parmer) was pushed through the pyloric sphincter and into the anterior intestine. The catheter was secured in place by a ligature, taking care not to occlude intestinal blood vessels. A second body wall incision near the anal opening exposed the posterior intestine. A small slit was created in the intestine and the outflow cannula [flared 10–200 µl pipette tip attached to a urethral catheter (Bard)] was inserted and secured in place. Undigested food was cleared from the intestine by sluicing with saline (77 mmol l^{-1} NaCl). Following incision closure the exteriorised ends of both cannulae were secured to the body wall *via* ligatures at the base of the dorsal fins. This allowed the fish to adopt a normal upright posture following recovery. The entire surgical procedure lasted 15–20 min.

Experimental procedure

Following surgery, individual fish were placed in flux chambers (Wood et al., 1996) supplied with flowing aerated and dechlorinated Lexington tapwater. Chambers consisted of a clear, perforated acrylic box (35 cm×11 cm×8 cm) fixed inside a larger black acrylic box (40 cm×20 cm×14 cm). Both boxes contained fitted lids with anterior and posterior perforations. This construction allowed the fish to be isolated from outside disturbances yet permitted unobstructed experimenter access to both cannulae. Once placed in the flux chamber, approximately 1 h was allowed for recovery from surgery. Experiments were performed on unfed, sensory deprived animals to minimise stress. The stress of surgery and confinement, however, were unavoidable, but consistent for treated and control animals. During recovery the inflow cannula was connected to a peristaltic pump supplying a 77 mmol l^{-1} NaCl saline and perfused at experimental flow rates.

Perfusion of experimental solutions commenced once consistent outflow was achieved. Previous experimenters noted inconsistency of output at high flow rates (Wilson et al., 1996). In the present study higher flow rates appeared to stimulate mucus secretion, obscuring flow. Lower perfusion rates did not allow consistent flow. Therefore outflow rates averaging 9.0±0.15 ml h^{-1} (mean ± S.E.M.) were used, independent of fish

mass. Flow was calculated by collection and weighing 10 min periods of efferent outflow. Fish were checked for signs of intestinal fluid leakage and intestinal oedema (subepithelial tissue water content). Furthermore, in initial experiments a fluid budget was able to account for total perfusate volume, suggesting that intestinal integrity was maintained throughout the experiment.

Experimental perfusion solutions consisted of ^{65}Zn(II) (as $ZnCl_2$: approx. 4 kBq ml^{-1}, New England Nuclear), with Zn(II) added as $ZnSO_4{\cdot}7H_2O$ (Sigma) to the stated concentration (5, 50, 150, 300, 400 or 500 μmol l^{-1}), in saline (77 mmol l^{-1} NaCl). In experiments to investigate the role of sulfhydryl group blockade, *N*-ethylmaleimide (NEM: 1 mmol l^{-1}; Sigma) was added in solution with 50 μmol l^{-1} Zn(II). To allow the physiological control of pH by the fish, pH buffers were not used. Consequently the pH of the inflowing perfusate ranged from 6.0 to 6.4, while outflowing perfusate generally showed a slight but insignificant increase.

Ethylene glycol tetraacetic acid (EGTA; Sigma) is a strong chelator of Zn(II). Experiments were conducted using EGTA to determine the nature of the Zn(II) bound to the intestine. EGTA should remove loosely bound Zn(II) without an effect on internalised metal levels. These experiments were conducted in an identical manner to those described above. Fish were perfused with either 50 μmol l^{-1} or 500 μmol l^{-1} Zn(II). Following euthanasia the intestine was removed with cannulae intact and solutions [60 ml EGTA-free (deionised) water; 50 ml 1 mmol l^{-1} or 5 mmol l^{-1} EGTA followed by 10 ml deionised water] were flushed through the intestine at a rate of approximately 10 ml min^{-1}.

All experimental perfusions lasted 3 h, during which time ^{65}Zn(II) reached a steady state in collected outflow samples. Upon dissection the intestine was removed and split longitudinally. The exposed mucosal surface was scraped with a glass microscope slide and dried to constant mass. At the conclusion of the experiment, outflow samples, plasma, blood cells, 'epithelium' (mucus and epithelial cells), 'subepithelium' (scraped intestine) and duplicate homogenised samples of remaining body tissue were assayed for ^{65}Zn activity *via* gamma-counting (Canberra Packard 3600). Plasma chloride (CMT10, Radiometer) and haematocrit were determined as indicators of iono- and osmoregulatory integrity.

Calculations

The specific ^{65}Zn(II) activity of perfusate solutions was calculated as follows:

$$SA = A / [\mathrm{Zn}] \times 1000 , \quad (1)$$

where SA is the specific activity (cts min^{-1} nmol^{-1}), [Zn] is the Zn(II) concentration (nmol l^{-1}) and A is the activity (gamma cts min^{-1} ml^{-1}) of the perfusate.

Net influx (F) was calculated using the following formula:

$$F = [(\mathrm{cpm_{in}} - \mathrm{cpm_{out}}) \times Q] / (m \times SA) , \quad (2)$$

where $\mathrm{cpm_{in}}$ is the gamma counts per minute of the inflowing perfusate, while $\mathrm{cpm_{out}}$ is a calculated average of ^{65}Zn(II) activity from the efferent cannula over the last 30 min of the experiment. Flow rate (Q) is the calculated average of outflow volume over the final 30 min of perfusion and is expressed in ml h^{-1}. m is the fish mass in g, and SA is the specific activity of the perfused solution. Net influx (F) is expressed as nmol g^{-1} h^{-1}, and is a measure of the net disappearance of ^{65}Zn(II) from the perfused solution, at steady state.

Accumulated tissue Zn(II) concentrations were calculated:

$$Z = \mathrm{cpm_{tissue}} / SA , \quad (3)$$

where accumulation (Z) is nmol. Accumulation rates $\dot{Z}$ were also determined:

$$\dot{Z} = \mathrm{cpm_{tissue}} / (m \times t \times SA) , \quad (4)$$

where tissue mass (m) is in g and time (t) is in h, to give accumulation rates ($\dot{Z}$) of nmol g^{-1} h^{-1} for all tissues. $\dot{Z}$ is a measure of the appearance of Zn(II) in tissues. This differs from F, which was based upon the difference between inflow and outflow radioactivity.

The percentage of perfused Zn(II) retained (Z_R) by the fish after a 3 h perfusion was determined as follows:

$$Z_R = Z_T / [Z_T + (\textstyle\sum \mathrm{cpm_{out}} / SA)] , \quad (5)$$

where total accumulation Z_T is the sum of accumulation in each tissue (nmol). Mucus and epithelial cells are continuously sloughed into the lumen and excreted. It can therefore be considered that epithelial Zn(II) accumulation (Z_E) does not necessarily represent Zn(II) that is absorbed. Consequently the absorbed Zn(II) fraction (Z_A) was used as a measure of post-epithelial absorption:

$$Z_A = Z_T - Z_E / [Z_T + (\textstyle\sum \mathrm{cpm_{out}} / SA)] , \quad (6)$$

where Z_E is in nmol.

Throughout the text 'epithelial' refers to the tissue removed from scraping of the intestine following perfusion. It therefore includes both mucus and mucosal intestinal cells. 'Subepithelial' denotes intestinal tissue remaining following scraping. Blood variables were calculated assuming that a 1 kg rainbow trout has a blood volume of 50 ml (Milligan and Wood, 1982). 'Body' represents tissue remaining following excision of the intestine, and blood sampling. 'Post-intestinal' parameters are those calculated using pooled blood and body data.

Data throughout the text and in figures are expressed as means ± S.E.M. Significant effects ($P<0.05$) of treatments were tested using analysis of variance (ANOVA), unless otherwise stated.

Results

Characterisation of Zn(II) uptake

The passage of Zn(II) through the intestine at experimental flow rates was rapid, with ^{65}Zn(II) detected in outflow within the first 10 min collection period (Fig. 1). An initial rise in outflow ^{65}Zn(II) activity was followed by a sharp linear phase of output, the slope being independent of Zn(II) concentration.

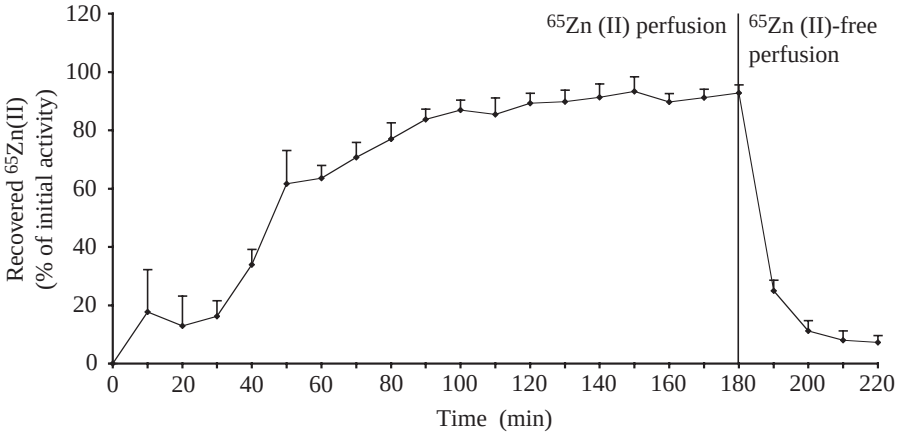

Fig. 1. Profile of ^{65}Zn(II) activity obtained from the efferent cannula following perfusion of the intestine with Zn(II) (50 μmol l^{-1}). Perfusion rates averaged 9.0±0.15 ml h^{-1}. The vertical line indicates when the solution was switched to ^{65}Zn(II)-free medium. Values are means ± S.E.M. (*N*=10, Zn(II) perfusion; *N*=4, Zn(II)-free perfusion).

A gradual realization of steady state was achieved during the second hour of perfusion. An exponential decay of activity from the intestine was recorded following the subsequent perfusion of the intestine with a Zn(II)-free solution at experimental rates.

Zn(II) stimulated mucus production at all concentrations tested (Fig. 2). An increase in epithelial mass above that of Zn(II)-free perfused controls indicated mucus production, and an increase in intestinal mucus was observed upon dissection. Control experiments yielded a mean epithelial mass of 0.07 g compared with mean epithelial masses ranging from 0.17 to 0.32 g with Zn(II) perfusion. The magnitude of the mucus response was independent of the perfused Zn(II) concentration and was induced even at the lowest Zn(II) concentration tested (5 μmol l^{-1}).

The retained Zn(II) fraction (the proportion of perfused Zn(II) retained by the epithelial, subepithelial and post-intestinal compartments) ranged from 20.9±2.4 to 32.9±5.0 % at perfusion concentrations of 500 μmol l^{-1} and 400 μmol l^{-1}, respectively (Fig. 2). With the exception of the 400 μmol l^{-1} treatment group there was a trend of decreased retention with increasing perfused Zn(II) concentration. The absorbed fraction (proportion of perfused Zn(II) accumulated in post-epithelial compartments) exhibited a similar trend. At 5 μmol l^{-1}, 11.6 % of perfused Zn(II) was absorbed beyond the epithelium, while at 500 μmol l^{-1} the proportion dropped to 6.4 %. The absorbed Zn(II) fraction was significantly correlated with the retained Zn(II) fraction (P<0.05), while the retained Zn(II) fraction was significantly correlated with epithelial dry mass (P<0.05). This relationship was made especially apparent by the 400 μmol l^{-1} treatment group, where a peak in epithelial mass coincided with a sharp increase in retained Zn(II) fraction.

Net influx, the disappearance of Zn(II) from the perfusate,

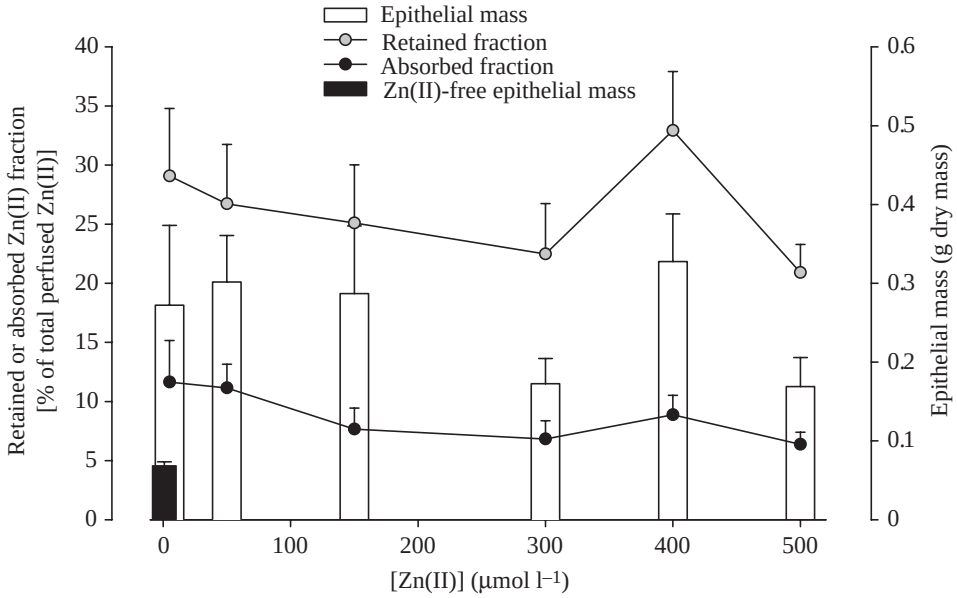

Fig. 2. The influence of perfused Zn(II) concentration upon epithelial mass, retained Zn(II) fraction and absorbed Zn(II) fraction. The value for epithelial mass (g dry mass) resulting from perfusion of Zn(II)-free solutions (filled bar) is the mean ± S.E.M. of 4 experiments; epithelial mass of Zn(II) perfused solutions (open bars), retained Zn(II) fraction (open circles), and absorbed Zn(II) fraction (filled circles) are means ± S.E.M. of 7–11 experiments. Control and Zn(II)-exposed intestines were perfused for 3 h, and retained and absorbed fractions are expressed as percentage of total Zn(II) perfused over the 3 h period. Epithelial mass is the mass of material scraped from the intestinal mucosal surface and includes both epithelial cells and mucus. Retained Zn(II) fraction is the proportion of perfused Zn(II) retained in the animal, while absorbed Zn(II) fraction represents the proportion of Zn(II) retained in post-epithelial tissues (intestine, blood, body).

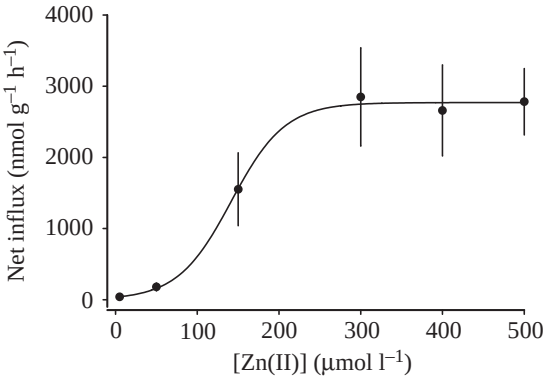

Fig. 3. Net influx of Zn(II) (nmol g^{-1} h^{-1}) from the perfused medium into the tissues, measured as disappearance of Zn(II) from perfusate. Values are means ± S.E.M. (N=7–11). Net influx was calculated over the final 30 min of a 3 h perfusion.

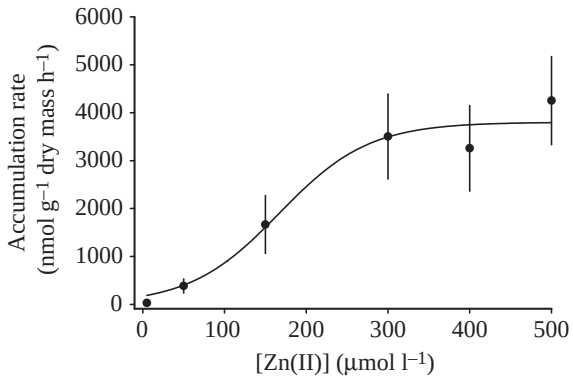

Fig. 4. Epithelial Zn(II) accumulation rate (nmol g dry mass^{-1} h^{-1}) following a 3 h intestinal perfusion of the intestine with Zn(II) solutions of varying concentration. Values are means ± S.E.M. (N=7–11). Epithelium represents tissue scraped from intestinal mucosal surface and includes epithelial cells and surface mucus.

exhibited a distinct sigmoidal pattern (Fig. 3). At lower Zn(II) concentrations a linear influx was noted which accelerated rapidly between 50 and 300 μmol l^{-1}, and then levelled out to approx. 2750 nmol g^{-1} h^{-1} at higher perfusate concentrations. A three-parameter sigmoidal curve best described the data (r^2=0.62, P<0.001, fitted to all data points).

Increasing Zn(II) concentration increased total Zn(II) accumulation (based upon Zn(II) appearance in the fish), although saturation was apparent at higher experimental concentrations (Table 1). This was reflected in most tissue accumulation profiles, with the exception of plasma, which exhibited a linear increase in accumulation with increasing perfusate Zn(II) concentration. The intestinal epithelial tissue (epithelial cells and mucus from intestinal scrapings) was the largest Zn(II) compartment accounting for 56–74 % of ttotal Zn(II) accumulation. The gut as a whole (epithelium and subepithelium) contained 76–88 % of total retained Zn(II). There was a tendency at higher concentrations for proportionally less Zn(II) to accumulate in the blood (specifically in erythrocytes) and body (carcass remaining following dissection), and concomitantly greater accumulation in the epithelial compartment. There were no significant dose-dependent differences in percentage accumulation for plasma or subepithelial (intestinal tissue remaining following scraping) compartments.

Epithelial accumulation rate followed a very similar pattern

Table 1. *Accumulation of Zn(II) into various compartments following* in vivo *intestinal perfusion of ^{65}Zn(II) solutions of increasing concentration*

	Zn(II) accumulation (nmol)					
[Zn(II)] (μmol l^{-1})	5	50	150	300	400	500
Epithelium	20±4 (64±7)	176±31 (56±3)	664±140 (72±3)†	1198±223 (71±6)†	2302±294 (74±3)†	1945±254 (68±4)†
Subepithelium	5±2 (14±2)	56±7 (20±2)	119±22 (13±2)	246±54 (17±5)	412±79 (13±3)	427±76 (16±4)
Erythrocyte	1±0.3 (4±2)	6±1 (2±0.5)	10±2 (1±0.2)*	18±3 (1±0.4)*	61±18 (2±0.5)	48±11 (2±0.3)*
Plasma	2±1 (6±2)	23±6 (8±2)	53±12 (7±1)	74±15 (6±1)	143±38 (7±1)	183±51 (7±2)
Body	4±2 (12±4)	41±8 (14±2)	62±12 (7±1)†	96±25 (7±2)†	166±39 (5±1)†	202±30 (8±1)†
Total	33±6	302±45	902±168	1622±236	3088±367	2787±234

Values are means ± S.E.M. (N=7–11); values in parentheses represent the percentage contribution of each compartment towards total accumulation.

Significant differences in Zn(II) distribution (P<0.05) were tested by ANOVA, with *post-hoc* differences determined by Fisher's LSD test: * significantly different from the 5 μmol l^{-1} treatment group; † significantly different from 50 μmol l^{-1} treatment group.

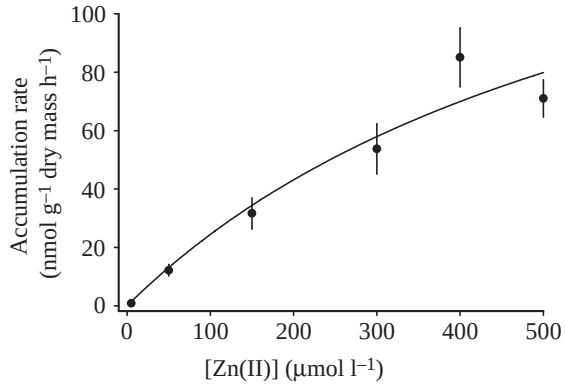

Fig. 5. Subepithelial Zn(II) accumulation rate (nmol g^{-1} wet mass h^{-1}) following a 3 h perfusion of the intestine with Zn(II) solutions of varying concentration. Values are means ± S.E.M. (N=7–11). Subepithelium represents intestinal tissue remaining following mucosal scraping.

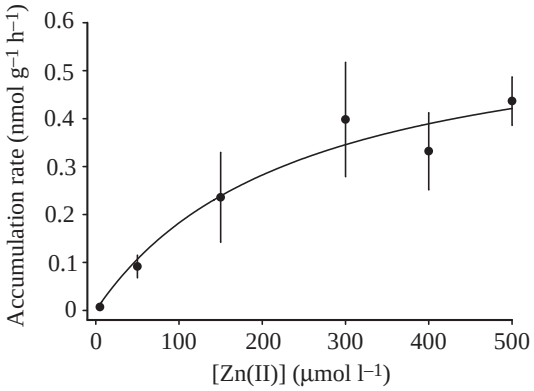

Fig. 6. Body Zn(II) accumulation rate (nmol g^{-1} wet mass h^{-1}) following a 3 h perfusion of the intestine with Zn(II) of varying concentration. Values are means ± S.E.M. (N=7–11). Body represents the tissues remaining following intestinal excision.

to net influx data (Fig. 4). An initial linear accumulation followed by a faster uptake rate at intermediate concentrations was described. Epithelial accumulation rate appeared to saturate at approx. 3750 nmol g^{-1} h^{-1}. As with influx, the raw data gave the best fit (r^2=0.41, P<0.001) when fitted to a three-parameter sigmoidal curve.

Subepithelial accumulation rate exhibited a hyperbolic curve (Fig. 5). As the Zn(II) concentration increased, the Zn(II) accumulation rate rose in a relatively rapid manner, levelling off at higher concentrations. The rate of Zn(II) accumulation into the body (carcass remaining following dissection) followed a similar pattern, albeit of lesser magnitude (Fig. 6). At low concentrations a rapid rate of accumulation existed, whilst at higher concentrations a saturation effect occurred. This saturation was more pronounced for the body than for the subepithelium.

Michaelis–Menten kinetics were calculated from an Eadie–Hofstee plot of post-intestinal accumulation rate (pooled blood and body accumulation rates). Post-intestinal rate was used as this represents the Zn(II) considered to be truly absorbed. A maximal rate of accumulation (J_{max}) of 933±232 nmol kg^{-1} h^{-1} was determined, while the concentration required to reach half this maximal rate ($K_{0.5}$) was calculated to be 309±117 μmol l^{-1}. A linear phase of uptake could not be determined.

A strong and significant linear relationship between subepithelial and post-intestinal accumulation was noted (Fig. 7). As subepithelial accumulation increased so too did post-intestinal accumulation.

Post-perfusion flushing

Flushing of excised intestine with saline or EGTA-saline reduced epithelial Zn(II) accumulation rate. Accumulation rates dropped to 52–115 nmol g^{-1} h^{-1}, with no significant difference between experiments conducted at 50 and 500 μmol l^{-1} (Fig. 8A). The presence of EGTA in the rinse solution had no additional effect compared to EGTA-free solutions. Flushing removed 30–50 % of epithelial mass, and 46–97 % of epithelial Zn(II) accumulation.

There was also a significant flushing effect on subepithelial Zn(II) accumulation rate (Fig. 8B). Flushing in EGTA-free medium reduced subepithelial Zn(II) accumulation rate by 85 % to 16 nmol g^{-1} h^{-1} at 500 μmol l^{-1}. The resulting accumulation rate was not significantly different from flushed intestine at 50 μmol l^{-1}. EGTA solutions reduced subepithelial Zn(II) accumulation to between 3.6 and 6.5 nmol g^{-1} h^{-1}, independent of Zn(II) concentration initially perfused.

Effects of NEM in perfusate

A significant and highly pronounced effect of NEM was noted (Table 2). Transport of Zn(II) into post-intestinal compartments virtually ceased, with a 91–92 % decrease in accumulation rate. There was no significant effect of NEM on accumulation rates into epithelial or subepithelial

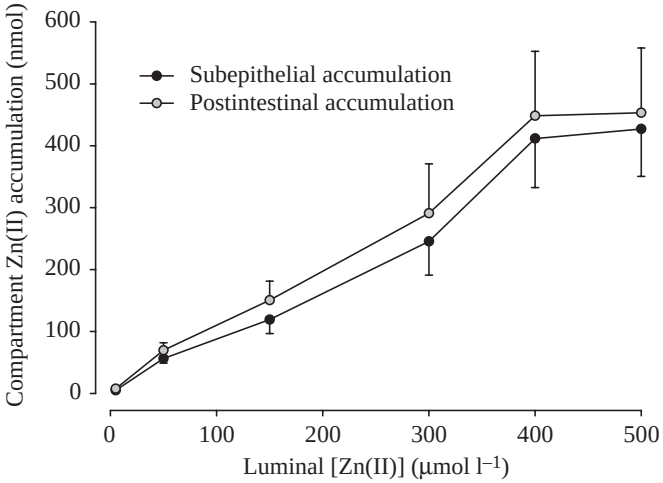

Fig. 7. Relationship between subepithelial (intestinal tissue following mucosal scraping) and post-intestinal (pooled blood and body) Zn(II) accumulation at different concentrations of perfused Zn(II). Values are means ± S.E.M. (N=7–11).

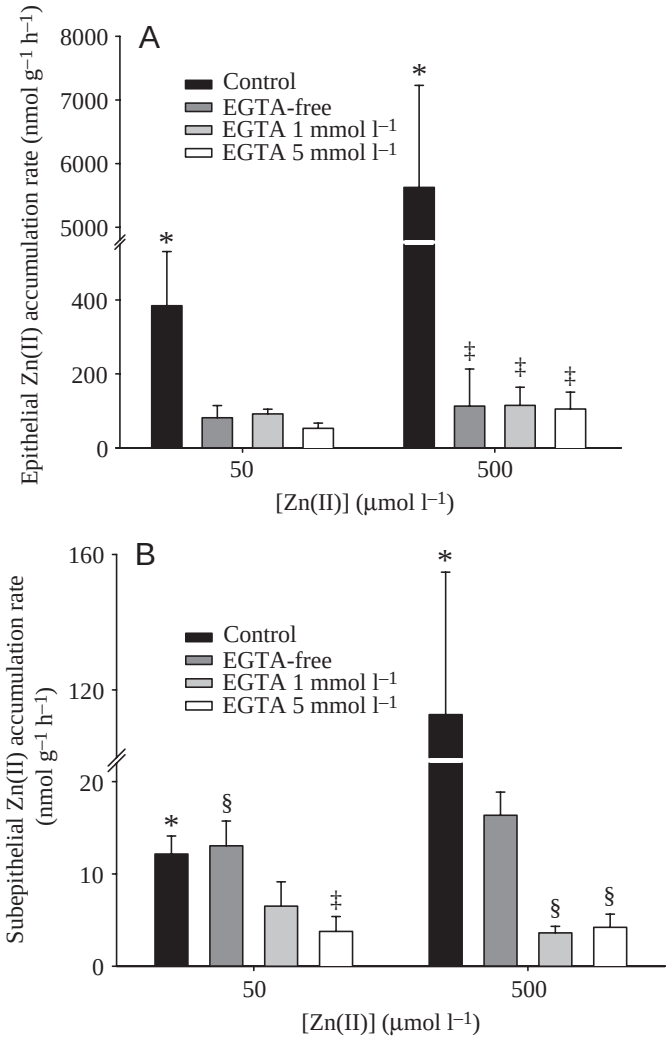

Fig. 8. Effect of post-perfusion flushing of excised intestine on epithelial (A) and subepithelial (B) Zn(II) accumulation rate (nmol g^{-1} h^{-1}). Values are means ± S.E.M. (N=3–4). Significant differences * between perfused Zn(II) concentrations; ‡ between control and flushed treatments; § between EGTA-free and EGTA solutions, where indicated, are at P<0.05.

compartments. Markers of osmo- and ionoregulatory pertubation to the animal (haematocrit and plasma chloride concentration) did not differ from control values following NEM perfusion.

Discussion

Kinetic characterisation of intestinal Zn(II) uptake

An *in vivo* perfusion technique was used to characterise intestinal Zn(II) uptake in freshwater rainbow trout. Important differences to established patterns of Zn(II) uptake in other organisms were revealed. Most significantly only a saturable component of absorption appeared to exist over the physiologically reasonable Zn(II) concentrations examined. While saturable Zn(II) uptake is an established phenomenon in the intestine of other animals, it is usually accompanied by a diffusive linear component that was absent in the present study (Lönnerdal, 1989). Kinetic analysis of the saturable component yielded a J_{max} of 933 nmol kg^{-1} h^{-1} and a $K_{0.5}$ of 309 μmol l^{-1}. Other authors using a variety of techniques in rats and in human intestinal cell lines have reported $K_{0.5}$ values in the range of approx. 30–380 μmol l^{-1} (Menard and Cousins, 1983; Oestreicher and Cousins, 1989; Hoadley et al., 1987; Fleet et al., 1993), which include the $K_{0.5}$ determined in the present study.

Table 2. *Effect of* N-*ethylmaleimide on tissue Zn(II) accumulation rate*

	Accumulation rate		
	Control	+NEM	Decrease (%)
Epithelium	384±146	357±67	7
Subepithelium	12.1±2.0	11.7±3.0	4
Blood	1.2±0.3	0.11±0.02	91*
Body	0.09±0.02	0.006±0.002	92*

NEM, *N*-ethylmaleimide (1 mmol l^{-1}).

Accumulation rate is nmol g^{-1} h^{-1} over a 3 h period of intestinal perfusion with 50 μmol l^{-1} Zn(II). Values are means ± S.E.M.; N=7–11 experiments for controls, N=3 experiments for +NEM.

*Significant differences from control values (P<0.05), determined by Student's t-test.

The intestine and gill are the two major uptake routes for Zn(II) in fish. Kinetic analysis of branchial Zn(II) uptake has been performed previously in rainbow trout. The J_{max} of intestinal Zn(II) uptake in the present study was two to three times greater, while the $K_{0.5}$ was nearly two orders of magnitude larger than corresponding values measured previously for the gill (J_{max} 240-410 nmol kg^{-1} h^{-1}, $K_{0.5}$ 3.6–7.8 μmol l^{-1}) (see Hogstrand et al., 1995). The intestine, relative to the gill, can therefore be described as a low-affinity, high-capacity Zn(II) transport pathway.

It is unlikely that a diffusive component of uptake exists in the *in vivo* preparation used. The similarity of the piscine rate constant to those determined in mammals suggests that the calculation of $K_{0.5}$ was not unduly affected by an undetermined diffusive component. Furthermore the Zn(II) concentrations used in the present study were sufficiently high to detect such a component should it exist (Steel and Cousins, 1985; Fleet et al., 1993). The remarkable cross-species similarity of rate constants for the saturable uptake pathway highlights the generally conserved nature of intestinal Zn(II) transport.

The conservation of transport across species and tissues is likely to result from the actions of Zn(II) transporter proteins. In mammalian intestine, a number of transporters with proposed roles in Zn(II) absorption have been discerned. DMT-1 (Nramp-2) is an apically located transporter (Tandy et al., 2000) known to be capable of transporting Zn(II) (Gunshin et al., 1997), while ZnT-1 is likely to be involved in Zn(II) efflux across basolateral surfaces of intestinal epithelia (Cousins and McMahon, 2000). Within cells ZnT-2 (Palmiter

et al., 1996) and ZnT-4 (Murgia et al., 1999) may play vital roles in Zn(II) compartmentalisation with putative functions in vesicular Zn(II) sequestration. Recent studies have demonstrated the existence of ZnT-1 homologues in piscine intestine (S. Balesaria and C. Hogstrand, unpublished). Many of the results in the present study could be explained by the actions of the transporters described above.

Regulation of subepithelial Zn(II) accumulation

One such transporter-mediated role could be in the regulation of subepithelial Zn(II) accumulation. Flushing of excised intestines removed considerable quantities of epithelial Zn(II), independent of perfused Zn(II) concentration or the presence of EGTA in the rinse solution. This is notable for three reasons. First, it suggests that most Zn(II) was present in the loosely bound fraction of the epithelium (mucus and sloughing epithelial cells). Second, EGTA, which would be expected to remove extracellularly bound Zn(II) (Tacnet et al., 1990), had no effect above that of flushing alone. This implies that extracellular binding to the epithelial surface did not play an important role in the uptake process. Third, the lack of any difference in Zn(II) accumulation rate between 50 and 500 $\mu mol\,l^{-1}$ suggested intracellular compartments of the epithelium were saturated with Zn(II) at the lower concentration.

This evidence is suggestive of a mechanism controlling uptake. Owing to the probable lack of a diffusive, paracellular pathway for Zn(II) uptake, EGTA-removable Zn(II) is likely to be sourced from the subepithelial tissue. Such an action could be consistent with the presence of a basolateral efflux protein such as ZnT-1 and suggests that the passage of Zn(II) into the subepithelial tissue is subject to regulation. The decreased proportion of retained and absorbed Zn(II) with increasing perfused concentration (Fig. 2), a pattern also noted in marine fish and mammals (Lönnerdal, 1989; Shears and Fletcher, 1983), would also hint at a regulated Zn(II) uptake process.

Important roles for mucus

One of the most notable aspects of the present investigation was the massive intestinal mucosecretory response induced by the perfusion of Zn(II). Heavy metals have previously been shown to stimulate mucus secretion in both fish and mammalian systems (e.g. Lock and Van Overbeeke, 1981; Bandyopadhyay and Bandyopadhyay, 1997). This is purported to have significance for the protection of fish from metal pollutants. The polyanionic nature of mucus at neutral pH bestows it with a large metal binding capacity (Handy et al., 1989). In the present study the flow-through nature of the technique used resulted in the appearance of shed mucus in the outflow fractions. The epithelial compartment (mucus and epithelial cells) accumulated up to 74 % of the retained Zn(II), indicating the shedding of mucus-bound metal and epithelial cells greatly reduced tissue exposure to this potential toxicant. The role of mucus in binding, precipitating and preventing the potential toxicant from reaching the uptake surface has also been hypothesised to occur at other piscine uptake surfaces (Shephard, 1994). This suggests that mucus is likely to contribute to the regulation of intestinal Zn(II) absorption.

Mucus hypersecretion may also explain the lack of diffusive Zn(II) uptake. At high mucus polymer content it is possible for epithelial mucus to exclude water passage across the uptake surface (Shephard, 1994). This appeared to be the situation in the present study where no intestinal oedema was determined. In other studies using a similar *in vivo* perfusion method, intestinal oedema has been reported (Bogé et al., 1981). Using the same protocol, luminal composition can be modified to reduce intestinal mucus secretion, also resulting in intestinal oedema (C. N. Glover and C. Hogstrand, in preparation). If Zn(II) absorption is linked to water or solute passage (Wapnir et al., 1989) the mucus layer would obstruct both water absorption and diffusive Zn(II) uptake. The linear uptake component observed in mammalian studies may therefore be an artefact of *in vitro* techniques. In experimental systems lacking the natural mucosecretory response, a diffusive pathway to Zn(II) may become available that would not exist *in vivo*.

Mucus binding of metals is proposed to have an additional role in mammalian gut, potentially acting as the first step in assimilation. At low, nutritionally relevant Zn(II) levels, mucus may trap the metal close to the epithelium, generating high local Zn(II) concentrations at the uptake surface. This would explain why the subepithelial Zn(II) accumulation curve exhibits a hyperbolic uptake at low Zn(II), while the slope of epithelial accumulation remains relatively slight (Figs 4, 5). The ability of mucus to bind, sequester and then donate Zn(II) to the epithelial cells is attributed to the mucus microclimate itself. Within the mucus layer pH gradients may act to facilitate the transfer of Zn(II) from the mucus to the epithelial cells (Powell et al., 1999). It has been demonstrated that affinity of Zn(II) for mucus is at its lowest at the pH of the mucosal microclimate in mammalian intestine (Powell et al., 1999), a scenario that would promote Zn(II) donation to the epithelium. The dual roles of intestinal mucus in both facilitating and hindering Zn(II) absorption suggest this moiety has an important regulatory influence upon dietary metal uptake. However, in providing an increased barrier to uptake mucus secretion may result in decreased assimilation of nutrients (Forstner and Forstner, 1994). As Woodward et al. (1994) observed, environmental contaminants need not be absorbed to cause harm.

Evidence for two uptake pathways across the epithelium

A direct relationship existed between subepithelial Zn(II) accumulation and Zn(II) accumulation in blood and body (post-intestinal accumulation). A similar observation was made for winter flounder (Shears and Fletcher, 1983). Two possibilities may explain this correlation. Either post-intestinal accumulation is dependent upon subepithelial accumulation (Shears and Fletcher, 1983), or passage of Zn(II) from the epithelium to the subepithelium or the circulation is proportionally partitioned. Recent studies show that post-

intestinal Zn(II) accumulation can be affected independently of subepithelial accumulation (and *vice versa*) by altering luminal composition (Glover and Hogstrand, 2002) (C. N. Glover and C. Hogstrand, in preparation; see also Table 2). Such evidence favours the latter hypothesis.

The actions of NEM elucidate a potential mechanism for this transfer of Zn(II) into post-intestinal compartments. NEM is a membrane-permeant alkylating agent that targets reactive thiol groups in proteins (Riordan and Vallee, 1972). Consequently NEM and other sulfhydryl blocking reagents have often been used in uptake studies to show that functional thiol groups are important for Zn(II) uptake (Ackland et al., 1988; Bobilya et al., 1992). NEM severely reduced accumulation of Zn(II) in post-intestinal compartments, without inducing an effect on subepithelial accumulation. A similar scenario was described in rat intestinal loops using iodoacetate, another -SH group blocker (Sahagian et al., 1967).

The NEM thiol-blocking action could be mediated upon a number of intracellular moieties, each with potential roles in Zn(II) transfer. Inhibition of intracellular binding entities (e.g. metallothionein, glutathione), a basolateral transporter or an epithelial vesicular trafficking pathway (Calakos and Scheller, 1996) could explain the action of NEM, as each of these has been proposed to play an important role in the absorption of Zn(II) (e.g. Weber et al., 1992; Fleet et al., 1993; Jiang et al., 1998; Cousins and McMahon, 2000). Another possibility is that NEM caused a general inhibition of energy metabolism (Oestreicher and Cousins, 1989). One implication of the NEM data is that the transfer of Zn(II) into the circulation was a mediated process, while subepithelial accumulation was energy-independent. Such a mechanism has recently been suggested for copper uptake in freshwater rainbow trout (Clearwater et al., 2000).

Knowledge of absorption pathways for metals such as Zn(II) is of crucial importance. Our understanding of nutrient assimilation, metal toxicity, and the factors responsible for mediating acclimation to environmental metals are dependent upon knowledge of uptake and metabolism. Using an *in vivo* intestinal perfusion technique a saturable component of intestinal Zn(II) uptake was discerned and kinetically characterised. The similarity of kinetic constants between mammals and fish suggests that intestinal Zn(II) uptake is a conserved process. However, important differences, perhaps a result of the technique used, suggest that fish may have the ability to regulate intestinal Zn(II) assimilation. Such differences could be a consequence of the many roles of Zn(II) in normal physiological processes as well as a function of inhabiting environments where the animal can be exposed to Zn(II) *via* both waterborne and dietary uptake pathways.

Thanks to the Environmental Physiology and Toxicology Research Cluster at the University of Kentucky for provision of facilities, and especially to Dr Joseph R. Shaw for useful discussions. Also thanks to the US Fish and Wildlife staff of the National Fish Hatchery at Wolf Creek Dam, Kentucky for fish supply. This research was sponsored by the US Environmental Protection Agency grant #R826104 awarded to C.H. Experiments presented here were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. We are grateful to the anonymous referees whose comments improved the quality of the manuscript.

References

Ackland, M. L., Danks, D. M. and McArdle, H. J. (1988). Studies on the mechanism of zinc uptake by human fibroblasts. *J. Cell. Physiol*. **135**, 521–526.

Bandyopadhyay, B. and Bandyopadhyay, S. K. (1997). Protective effect of zinc gluconate on chemically induced gastric ulcer. *Ind. J. Med. Res.* **106**, 27–32.

Bobilya, D. J., Briske-Anderson, M. and Reeves, P. G. (1992). Zinc transport into endothelial cells is a facilitated process. *J. Cell. Physiol.* **151**, 1–7.

Bogé, G., Rigal A. and Pérès, G. (1981). A study of *in vivo* glycine absorption by fed and fasted rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **91**, 285–292.

Calakos, N. and Scheller, R. (1996). Synaptic vesicle biogenesis, docking, and fusion: a molecular description. *Physiol. Rev*. **76**, 1–29.

Clearwater, S. J., Baskin, S. J., Wood, C. M. and McDonald, D. G. (2000). Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* **203**, 2455–2466.

Cousins, R. J. and McMahon, R. J. (2000). Integrative aspects of zinc transporters. *J. Nutr.* **130**, 1384S-1387S.

Eisler, R. (1993). *Zinc Hazards to Fish, Wildlife, and Invertebrate: A Synoptic Review*. Biological Report 10. Washington, DC: US Department of Interior, Fish and Wildlife Service.

Farag, A. M., Woodward, D. F., Brumbaugh, W., Goldstein, J. N., MacConnell, E., Hogstrand, C. and Barrows, F. T. (1999). Dietary effects of metals-contaminated invertebrates from the Coeur d'Alene River, Idaho, on cutthroat trout. *Trans. Am. Fish Soc*. **128**, 578–592.

Fleet, J. C., Turnball, A. J., Bourcier, M. and Wood, R. J. (1993). Vitamin D-sensitive and quinacrine-sensitive zinc transport in human intestinal cell line Caco-2. *Am. J. Physiol*. **264**, G1037–G1045.

Florence, T. M., Morrison, G. M. and Stauber, J. L. (1992). Determination of trace element speciation and the role of speciation in aquatic toxicity. *Sci. Tot. Environ.* **125**, 1–13.

Forstner, J. F. and Forstner, G. G. (1994). Gastrointestinal mucus. In *Physiology of the Gastrointestinal Tract*, 3rd edition (ed. L. R. Johnson), pp. 1255–1283. New York: Raven Press.

Glover, C. N. and Hogstrand, C. (2002). Amino acid modulation of *in vivo* intestinal zinc absorption in freshwater rainbow trout. *J. Exp. Biol*. **205**, 151–158.

Guerinot, M. L. (2000). The ZIP family of metal transporters. *Biochim. Biophys. Acta* **1465**, 190–198.

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.

Handy, R. D., Eddy, F. B. and Romain, G. (1989). *In vitro* evidence for the ionoregulatory role of rainbow trout mucus in acid, acid/aluminium and zinc toxicity. *J. Fish Biol.* **35**, 737–747.

Hardy, R. W. and Shearer, K. D. (1985). Effect of dietary calcium phosphate and zinc supplementation on whole body zinc concentration of rainbow trout (*Salmo gairdneri*). *Can. J. Fish. Aquat. Sci.* **42**, 181–184.

Hardy, R. W., Sullivan, C. V. and Koziol, A. M. (1987). Absorption, body distribution, and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*). *Fish Physiol. Biochem.* **3**, 133–143.

Hoadley, J. E., Leinart, A. S. and Cousins, R. J. (1987). Kinetic analysis of zinc uptake and serosal transfer by vascularly perfused rat intestine. *Am. J. Physiol.* **252**, G825–G831.

Hogstrand, C., Reid, S. D. and Wood, C. M. (1995). Ca^{2+} *versus* Zn^{2+} transport in the gills of freshwater rainbow trout and the cost of adaptation to waterborne Zn^{2+}. *J. Exp. Biol.* **198**, 337–348.

Hogstrand, C., Verbost, P. M., Wendelaar Bonga, S. E. and Wood, C. M. (1996). Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. *Am. J. Physiol.* **270**, R1141–R1147.

Hogstrand, C., Webb, N. and Wood, C. M. (1998). Covariation in regulation of affinity for branchial zinc and calcium uptake in freshwater rainbow trout. *J. Exp. Biol.* **201**, 1809–1815.

Hogstrand, C. and Wood, C. M. (1996). The physiology and toxicology of

zinc in fish. In *Toxicology of Aquatic Pollution* (ed. E.W. Taylor), pp. 61–84. Cambridge: Cambridge University Press.

Jiang, L. J., Maret, W. and Vallee, B. L. (1998). The glutathione redox couple modulates zinc transfer from metallothionein to zinc-depleted sorbitol dehydrogenase. *Proc. Natl. Acad. Sci. USA* **95**, 3483–3488.

Knox, D., Cowey, C. B. and Adron, J. W. (1984). Effects of dietary zinc intake upon copper metabolism in rainbow trout (*Salmo gairdneri*). *Aquaculture* **40**, 199–207.

Li, M. H. and Robinson, E. H. (1996). Comparison of chelated zinc and zinc sulfate as zinc sources for growth and bone mineralization of channel catfish (*Ictalurus punctatus*) fed practical diets. *Aquaculture* **146**, 237–243.

Lock, R. A. C. and Van Overbeeke, A. P. (1981). Effects of mercuric chloride and methylmercuric chloride on mucus secretion in rainbow trout, *Salmo gairdneri* (Richardson). *Comp. Biochem. Physiol.* **69**, 67–73.

Lönnerdal, B. (1989). Intestinal absorption of zinc. In *Zinc in Human Biology* (ed. C. F. Mills), pp. 33–55. Berlin: Springer-Verlag.

McMahon, R. J. and Cousins, R. J. (1998). Mammalian zinc transporters. *J. Nutr.* **128**, 667–670.

Menard, M. P. and Cousins, R. J. (1983). Zinc transport by brush border membrane vesicles from rat intestine. *J. Nutr.* **113**, 1434–1442.

Milligan, C. L. and Wood, C. M. (1982). Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *J. Exp. Biol.* **99**, 397–415.

Murgia, C., Vespignani, I., Cerase, J., Nobili, F. and Perozzi, G. (1999). Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells. *Am. J. Physiol.* **277**, G1231–G1239.

Oestreicher, P. and Cousins, R. J. (1989). Zinc uptake by basolateral membrane vesicles from rat small intestine. *J. Nutr.* **119**, 639–646.

Palmiter, R. D. and Findlay, S. D. (1995). Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J.* **14**, 639–649.

Palmiter, R. D., Cole, T. B. and Findley, S. D. (1996). ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. *EMBO J.* **15**, 1784-1791.

Paripatananont, T. and Lovell, R. T. (1995). Chelated zinc reduces the dietary requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture* **133**, 73–82.

Powell, J. J., Jugdaohsingh, R. and Thompson, R. P. H. (1999). The regulation of mineral absorption in the gastrointestinal tract. *Proc. Nutr. Soc.* **58**, 147–153.

Riordan, J. F. and Vallee, B. L. (1972). Reactions with *N*-ethylmaleimide and *p*-mercuribenzoate. *Meth. Enzymol.* **25**, 449–456.

Rozan, T. F., Lassman, M. E., Ridge, D. P. and Luther, G. W., III. (2000). Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxic rivers. *Nature* **406**, 879–882.

Sahagian, B. M., Harding-Barlow, I. and Perry, H. M., Jr (1967). Transmural movements of zinc, manganese, cadmium and mercury by rat small intestine. *J. Nutr.* **93**, 291–300.

Shears, M. A. and Fletcher, G. L. (1979). The binding of zinc to the soluble proteins of intestinal mucosa in winter flounder (*Pseudopleuronectes americanus*). *Comp. Biochem. Physiol.* **64B,** 297–299.

Shears, M. A. and Fletcher, G. L. (1983). Regulation of Zn^{2+} uptake from the gastrointestinal tract of a marine teleost, the winter flounder (*Pseudopleuronectes americanus*). *Can. J. Fish. Aquat. Sci.* **40** (Suppl. 2): 197–205.

Shephard, K. L. (1994). Functions for fish mucus. *Rev. Fish Biol. Fish.* **4**, 401–429.

Spry, D. J. and Wood, C. M. (1985). Ion flux rates, acid–base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. *Can. J. Fish. Aquat. Sci.* **42**, 1332–1341.

Steel, L. and Cousins, R. J. (1985). Kinetics of zinc absorption by luminally and vascularly perfused rat intestine. *Am. J. Physiol.* **248**, G46–G53.

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.

Tandy, S., Williams, M., Leggett, A., Lopez-Jimenez, M., Dedes, M., Ramesh, B., Srai, S. K. and Sharp, P. (2000). Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. *J. Biol. Chem.* **275**, 1023–1029.

Wapnir, R. A., Lee, S.-Y. and Stiel, L. (1989). Intestinal absorption of zinc: sodium–metal–ligand interactions. *Biochem. Med. Metab. B.* **42**, 146–160.

Watanabe, T., Kiron, V. and Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture* **151**, 185–207.

Weber, D. N., Eisch, S., Spieler, R. E. and Petering, D. H. (1992). Metal redistribution in largemouth bass (*Micropterus salmoides*) in response to restrainment stress and dietary cadmium: role of metallothionein and other metal-binding proteins. *Comp. Biochem. Physiol.* **101C**, 255–262.

Wekell, J. C., Shearer, K. D. and Gauglitz, Jr, E. J. (1986). Zinc supplementation of trout diets: tissue indicators of body zinc status. *Prog. Fish Cult.* **48**, 205–212.

Wekell, J. C., Shearer, K. D. and Houle, C. R. (1983). High zinc supplementation of rainbow trout diets. *Prog. Fish Cult.* **45**, 144–147.

Wilson, R. W., Gilmour, K. M., Henry, R. P. and Wood, C. M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid–base balance? *J. Exp. Biol.* **199**, 2331–2343.

Wood, C. M., Hogstrand, C., Galvez, F. and Munger, R. S. (1996). The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. The effects of ionic Ag^{+}. *Aquat. Toxicol.* **35**, 93–109.

Woodward, D. F., Brumbaugh, W. G., De Lonay, A. J., Little, E. E. and Smith, C. E. (1994). Effects on rainbow trout fry of a metals-contaminated diet of benthic invertebrates from the Clark Fork River, Montana. *Trans. Am. Fish. Soc.* **123**, 51–62.