A COMPARISON OF ZINC AND CADMIUM UPTAKE VIA THE INTESTINAL TRACT OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

A COMPARISON OF ZINC AND CADMIUM UPTAKE VIA THE INTESTINAL TRACT OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University
September 1999

MASTER OF SCIENCE (1999) **McMASTER UNIVERSITY**
(Biology) **Hamilton, Ontario**

TITLE: A comparison of zinc and cadmium uptake via the intestinal tract of rainbow trout (*Oncorhynchus mykiss*).

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NUMBER OF PAGES:

ABSTRACT

The absorption and distribution of metals via the gut of fish is not well known. Consequently, the aim of the present study was to describe the movement of metals along the gut, their absorption and binding to gut tissues, and their distribution to the internal tissues following model dietary exposure. Two different approaches were employed, an *in vivo* gastric dosing procedure, and an *in vitro* gut bag protocol and two different metals were studied: an essential metal, zinc, and a non-essential (and more toxic) metal, cadmium.

The dietary uptake and distribution of zinc and cadmium to 0.3 kg rainbow trout (*Oncorhynchus mykiss*) was examined at 15°C at 1, 2, 3, or 7 days following a single bolus dose to the stomach of 0.5 mM of radiolabelled metal. After exposure, all internal organs and the remaining carcass were individually counted for radioactivity. Uptake, distribution and excretion of both zinc and cadmium was rapid, occurring largely within the first 24 h of exposure. By 24 h, fish exposed to Zn had absorbed 20.0% of the dose, 21.0% was bound in the gastrointestinal tissues and the remainder was either excreted (38.1%) or was present in the gut lumen (20.9%). Cadmium showed a much different pattern of uptake, with only 2.9% of the dose absorbed after 24 h, and the remainder found either in the gut tissue (30.2%) and the lumen (19.0%) or excreted (47.9%). Over the following six days, very little uptake and internal metal redistribution occurred.

When exposed to higher doses of metal *in vivo* (0.5 - 50 mM), there were distinct differences in the handling of the two metals. Zinc concentrations in the gut tissues continued to rise at higher doses until apparent saturation. In contrast, gut tissues were saturated with cadmium at the lowest dose employed (0.5 mM). Both metals bound most avidly to the distal intestine but all gut tissues had a higher binding capacity for zinc, as compared to cadmium. Target tissues (liver, gills, kidney) all saturated with zinc at high doses. In contrast, cadmium concentrations in these tissues continued to rise in a linear fashion with increasing dose.

In vitro studies revealed that the most important region of the gut for metal uptake in rainbow trout was the mid-intestine. Studies using the metabolic uncoupler, 2,4-DNP, suggested that the transfer of both zinc and cadmium across intestinal cells was passive at the brush border membrane, but was at least partly dependent on ATP for movement across the basolateral membrane. Furthermore, this transport mechanism was not shared by calcium, as the presence of calcium had no inhibitory effect on the transport of either metal.

Mucus within the intestinal lumen appears to have a higher binding affinity but lower capacity for cadmium than zinc. Calcium did not displace cadmium from the mucus layer. In contrast, zinc was displaced by an equimolar exposure to calcium in the medium. Gut mucus apparently impedes the movement of metals along the intestine with the extent of the delay likely being related to the binding affinity of the metal. The impediment was greatest for cadmium, as 10% of the metal remained in the lumen of fish

exposed *in vivo*, after a period of 7 days. In contrast, only 2% of the original dose of zinc remained in the gut lumen after only 3 days.

ACKNOWLEDGMENTS

There are a number of people that I need to thank for getting this done. I am not great with sentimental words so I will make this short.

First I would like to thank Dr. Gord McDonald for all of his support throughout this project. There were many times where I didn't know which way was up and he helped me out every time. The whole process of writing was not fun, but Gord played a great role in helping me to get it done, and make sense, in a very short period of time. Thanks for everything over the last 3 years Gord.

I would also like to thank Dr. Chris Wood, who has given many valuable insights towards this work also. I appreciated his guidance along the way. And thanks for reading my thesis under such short notice.

There a lot of people in the lab who I would like to thank also. Thanks to Sara for guiding me through my undergrad and graduate days at MAC. Thanks to Sue, who has since left, but without her moral support and many ideas, as well as her help with experiments, this would never have gotten done. Also, thanks to Lisa, Wendy, Debi, James, and Julian for both good times and help in the lab when I needed them. You guys will all be missed. Also thanks to the many people in the Wood lab who have helped to keep me sane over my years here. I won't list names, as I am likely to miss a few, given that there are so many of them.

Thanks to all the people who I have shared many beers with at the Phoenix, and

everyone on the Far Slide baseball team for the good times.

And last, but certainly not least, I would like to thank my parents, Ron and Sandra, my brother Darcy and my grandparents for supporting me along the way, even though they think I am crazy to spend so much time in school.

I am certainly going to miss MAC for all the good times and good friends.

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INTRODUCTION

The uptake and toxicity of waterborne metals to fish has been a subject of intense research (see Sorenson, 1991) but until recent years the importance of metals-contaminated diets as a potential for toxicity to fish has received little attention (see review by Handy, 1996).

To date, most studies have examined metal uptake by the gut of fish using prolonged dietary exposures to metals. A number of different metals have been examined including zinc, cadmium, copper and mercury. Two different approaches have been used in these studies to look at metal uptake and accumulation over periods of time ranging from 21 to 220 d in a large variety of fish species at various stages of development, with rainbow trout being the most widely used species. Some authors have chosen to use metal-contaminated invertebrate diets as the source of metal exposure (Woodward *et al*, 1994, 1995; Harrison and Curtis, 1992; Mount *et al*, 1994; Hatakeyama and Yasuno, 1982; Willis and Sunda, 1984; Haesloop and Schirmer, 1985; Milner, 1982). Many others have chosen to utilize contaminated artificial feed as a source of elevated dietary metal (Harrison and Klaverkamp, 1989; Spry *et al*, 1988; Köck and Bucher, 1997; Jeng and Sun, 1981; Farag *et al*, 1994; Gatlin and Phillips, 1989; Hardy and Shearer, 1985; Brafield and Koodie, 1991; Harrison and Curtis, 1992; Handy, 1992; Crespo *et al*, 1986; Overnell *et al*, 1988).

By using these methods, very little is learned of the transit times of metals in the gastrointestinal (GI) tract, the role of mucus in metal uptake, the mechanisms of absorption of metals by the GI tract, the roles of the various compartments of the tract in metal absorption and the acute handling of metals within the body.

Thus, two approaches were utilized in the present study to examine the acute handling of dietary metals in rainbow trout. The first was an *in vivo* technique in which a known amount of radiolabelled metal was given directly into the stomach of the fish via a catheter. The fate of the metal was then followed at various time intervals by dissection of the fish. The second method used was an *in vitro* protocol which looked at uptake by different parts of the GI tract by forming them into gut bags by sealing them off at both ends and infusing metal into the lumen.

The techniques used in the present study have seldom been used in the context of metal absorption across the gut of fish. The *in vivo* dosing method allows for acute metal exposure analysis using a known amount of radiolabelled metal administered directly to the stomach. This technique is very useful since it can be used to examine the transit time of metals along the gastrointestinal tract which may be important in determining the extent of metal exposure. It can also be used to look at binding of metals to the gut tissue and the distribution of metals to all body tissues following absorption after exposures of various time intervals. Acute *in vivo* dosing of metals has also been used by Giblin and Massaro (1973) in the study of methyl mercury uptake and distribution in rainbow trout. But, with the exception of a few authors who have used *in situ* techniques which expose the gut compartments without removing them from the body (Shears and Fletcher, 1983;

Morgan *et al*, 1994), no other studies on the acute handling of metals by the GI tract of fish have been done. Shears and Fletcher (1983) used winter flounder (*Pseudopleuronectes americanus*), a marine teleost, to examine zinc absorption by the various regions of the gut, while Morgan *et al* (1994) looked at caesium influx via the intestine of rainbow trout. There have been no equivalent studies on zinc or cadmium absorption in the gastrointestinal tract of rainbow trout.

Some fish researchers have used similar techniques to those used in mammalian research to examine various aspects of gastrointestinal uptake. Stokes and Fromm (1964) utilized an *in vitro* gut perfusion technique to examine glucose uptake by the different regions of the gut. Everted gut sacs (as described by Karasov and Diamond, 1983) have been used to examine gut uptake of amino acids in rainbow trout (Ingham and Arme, 1977; Marcotte and De La Noüe, 1984; Buddington and Diamond, 1987). With the exception of one unpublished study (Handy, 1999), none of these *in vitro* techniques have been used for the study of metal uptake to date and thus very little is known of the exact mechanisms and locations of metal absorption in the gut of fish.

The *in vitro* gut bag method, which is similar to that described by House and Green (1965), allows for the determination of the role of individual gut segments in metal uptake. It can also be used as a means of determining the mechanism of uptake of metals across the gastrointestinal tissue using metabolic inhibitors, competitive ions and binding ligands. Short-term exposures are useful when determining the kinetics of uptake in the different compartments.

The processes by which metals are handled by the gut and within the body may be unique for each metal or there may be shared pathways that exist for a number of different metals of similar size and charge. For this reason an essential and a non-essential metal, Zn and Cd respectively, were chosen as a means of exploring some of the differences that may arise in the handling of different metal species.

Zinc is known to be an essential micronutrient in the diet of fish, necessary for the proper functioning of a large number of enzymes in the body, while cadmium has no known function in fish. Zinc is required in purified rainbow trout diets in quantities of 15-30 μg/g (Ogino and Yang, 1978) but may need to be supplemented up to 150 μg/g of feed depending on the formulation of the diet (Ketola, 1979).

Oral toxicity data for cadmium and zinc in fish is scarce since the levels needed to produce death are very high and fish do not like the taste of highly contaminated food. Rainbow trout exposed to 10 g Cd/kg dry weight of food over a period of 28 days suffered 42% mortality, suggesting that this dose may be close to the oral toxicity limit (Handy, 1996). Oral toxicity data for Zn in fish is non-existent, although it is known from studies in our lab that no mortality occurs when fish are fed 2 g Zn/kg wet weight of diet (Baskin, Clearwater, Matsuda and McDonald, unpublished).

Given that aquatic invertebrates may contain up to 1290 mg/kg dry weight of zinc (Dallinger and Kautzky, 1985) and have cadmium levels as high as 109 mg/kg dry weight (Dallinger and Kautzky, 1985), uptake of these two metals in the diet of rainbow trout could be of great concern.

Though zinc is an essential micronutrient, in excess quantities in the diet or water, it can have deleterious effects. At the gills, free Zn^{+2} ions are very strong inhibitors of the basolateral Ca^{+2}-ATPase and can compete with the apical Ca^{+2} transporter (Hogstrand *et al*, 1996). Zinc also impairs reproduction in fish (Spear, 1981). Cadmium can be very harmful upon consumption in the diet and via the gills of fish. One of the primary toxic actions of Cd in fish is to block basolateral Ca^{+2}-ATPase in the gills (Verbost *et al*, 1988). Cadmium administered branchially can also cause renal failure and demineralization of the skeleton as a result of the disruption of calcium homeostasis (Larsson *et al*, 1981). Viability of eggs (Birge *et al*, 1981) and vitellogenin production (Olsson *et al*, 1995) in female fish are also decreased following exposure to cadmium. In the intestine of rainbow trout, cadmium perturbs the intestinal cells in a number of ways causing increases in mucous cell activity, disruption of the intestinal brush border layer, and increases in the renewal rate of absorptive cells (Crespo *et al*, 1986).

Although there is a dietary essentiality for zinc and toxic effects caused by consumption of both metals, very little is known of the mechanisms of dietary uptake of either zinc or cadmium in fish, and specifically in rainbow trout.

One aspect of gut metal uptake of possible importance is the mucus that lines the intestinal cells. Mucus in the gut is a macromolecule of high viscosity, negative charge and asymmetrical shape (Forstner and Forstner, 1975). It is secreted by goblet cells in the intestine and stomach and forms a continuous layer over the surface epithelium, separating luminal contents from the cells of the mucosa (Forstner and Forstner, 1975). The main constituents of mucus are protein, galactose, hexosamine, fucose, sialic acid,

uronic acid and sulfate (Bella and Kim, 1973). Mucus has lubricative and protective functions within the gut and it may also aid in the breakdown and absorption of nutrients from the diet (Quarterman, 1987). Mucus within the gut may also bind some of the metal ions within the lumen and prevent or aid in the absorption of these ions. To date, very few studies have focussed on the role of mucus in the gut of fish (Noël-Lambot, 1981) or mammals (Bella and Kim, 1973; Forstner and Forstner, 1975) as a means of protection against toxic metals. Little is known of its binding capacity and involvement in the movement of metals along the intestine. Information regarding the roles of mucus in the gut of fish may help to explain the low bioavailability of metals ingested via the diet.

There is little evidence of the regional uptake of metals across the gut of fish. In rainbow trout, the gastrointestinal tract is conventionally divided into the esophagus, stomach, and intestine. Buddington and Diamond (1987) have further divided the intestine into three regions where the proximal intestine is defined as the region from the pyloric sphincter to the last pyloric cecum, the mid-intestine is the region from the last pyloric cecum to the distal intestine, and the distal intestine is distinguished by its larger diameter, darker colour, and annular rings. The proximal intestine contributes 70% of the total postgastric surface area in rainbow trout (Buddington and Diamond, 1987) and thus constitutes a potentially enormous capacity to absorb metals and nutrients. Indeed, research by Buddington and Diamond (1987) has shown the proximal intestine to be the most important of all regions in the absorption of amino acids and glucose in rainbow trout. Metals, however, may not be absorbed over the same regions as nutrients. Shears and Fletcher (1983) have shown that the proximal intestine was the most important

segment for the absorption of zinc in winter flounder (*Pseudopleuronectes americanus*) but Handy *et al* (1999) have recently shown that copper uptake by the GI tract of African catfish *(Clarias gariepinus)*, is highest in mid-intestine, followed closely by distal intestine. No known studies on cadmium absorption in the various regions of the gut exist in the literature. There are also no known studies on zinc absorption in rainbow trout. Furthermore, the gastrointestinal tract of fish is highly variable between freshwater and marine species and between carnivorous, omnivorous and herbivorous fish (Buddington *et al*, 1997; Kapoor *et al*, 1975; Fange and Grove, 1979) making it very difficult to make assumptions concerning metal uptake based on the findings in another species.

Based on findings with regards to metal uptake in the mammalian literature (for reviews see Powell *et al*, 1999; Whitehead *et al*, 1996; Rolfs and Hediger, 1999), metal absorption by the gastrointestinal tract can be characterized as a 3 step process which first involves the movement of metal across the brush border membrane (also referred to as apical, mucosal membrane) of the gut cells. Within intestinal cells, metals are bound to a number of proteins that may have transport or storage functions. The final step is movement across the basolateral (also called serosal) membrane where transport of the metal ions is completed by movement into the extracellular fluid or plasma for transport to any number of different tissues and compartments within the body that may use or accumulate the metal.

In fish, little mechanistic evidence for cadmium and zinc transport exists. Shears and Fletcher (1983) have shown that intestinal Zn uptake in winter flounder,

(*Pseudopleuronectes americanus*) is a two step process that involves a rapid accumulation of zinc by the tissue, followed by a slow transfer to the body. The uptake was not saturable at high luminal concentrations. Other mechanistic evidence in fish is lacking.

Metals may be taken up via specific metal uptake mechanisms but it is generally thought that the uptake of heavy metals is related to that of other essential divalent cations. In winter flounder, zinc uptake is inhibited by a number of other metals including copper, cadmium, cobalt, chromium, nickel, iron, manganese, and mercury (Shears and Fletcher, 1983). It has also been suggested that Ca^{+2} pathways are utilized in the uptake of zinc (Hardy and Shearer, 1985) and cadmium (Shoenmakers *et al*, 1992) in the gut. The role of calcium in the absorption of metals is an area that has received some research attention in fish, but there is not enough evidence to implicate calcium pathways as the means of transport of either zinc or cadmium in the fish gut. Gill uptake of Zn has also been shown to proceed via the same pathway as that of Ca (Hogstrand *et al*, 1996).

Following absorption by intestinal cells, metals are distributed to the other tissues of the body for excretion, storage, or use by the cells. Acute tissue distribution of metals following uptake by the gut has only been published in one study of which I am aware (Hardy *et al*, 1987). Experiments performed in this study only involved Zn distribution following a 72 h exposure period to a single oral dose of the metal. There is no knowledge of the acute tissue distribution of Cd in fish. By developing a better understanding of the major tissues of accumulation and of changes within these organs over time, valuable insight into the mechanisms of toxicity may be achieved.

In order to understand the uptake and distribution of metals completely, it is essential that we explore both acute and chronic uptake and distribution of metals via the gut. Inferences concerning the movement of metal within the fish and its excretion from the body may be made using acute dosing studies. Key differences between essential and non-essential metals may also become more apparent with acute knowledge of metal distribution. To assess the effects of excess metals in the diet of rainbow trout *(Oncorhynchus mykiss)*, it is necessary to first establish a model of dietary metal absorption mechanisms and accumulation in the tissues of this species.

Given the number of gaps in the current literature concerning acute zinc and cadmium uptake by the fish intestine, their mechanisms of transport by the gut and the distribution of each in the gut tissues and internal organs, the goals of the present research were to use the *in vitro* and *in vivo* methods described above to improve our present knowledge of:

a) transit time of zinc and cadmium in the gut with relation to the roles of mucus.

b) the role of gut tissue binding of zinc and cadmium in the uptake and elimination in the four segments of the GI tract.

c) compartmental differences in metal transport along the GI tract.

d) transepithelial transport mechanisms of both Cd and Zn in the gut with relation to Ca.

e) the acute tissue distribution and exchange of metals within the body following intestinal absorption.

f) the similarities and differences between Cd, a highly toxic metal, to Zn, an essential metal for all characteristics above.

MATERIALS AND METHODS

Experimental Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Trout Farm, Thamesford, Ontario (average weight 259.63 ± 4.60 g) and held in circular 500 L holding tanks with constant flow of dechlorinated aerated Hamilton, Ontario tap water at a temperature range of 13-16°C. Fish were kept under a natural photoperiod in dim light and fed a maintenance ration of Martin's commercial dried trout pellet feed 5 times per week at 1% body weight per feeding. The zinc content of the food was determined, as described below, to be 201.52 ± 7.45 μg/g, which is close to what is thought to be the optimal dietary zinc level for rainbow trout (150 μg/g; Ketola, 1979). Cadmium content in the food was determined to be 0.83 ± 0.06 μg/g. All trout were starved for 2 d prior to experimentation to allow for gut contents to empty. For each experiment, trout were randomly selected from the holding tank.

Baseline Zinc and Cadmium Levels in Tissues and Food

Fish (n=6) were terminally anaesthetized in 1 g/L MS-222, all internal organs including gastrointestinal tract (divided into stomach, proximal intestine, mid-intestine, and distal intestine) dissected out, and weighed. All tissues were dissolved at 60°C in 1N HNO_3 in 15 mL falcon tubes for 1 d, and centrifuged for 10 min. in an IEC clinical centrifuge at 2800 rpm to separate solution. The remaining carcass was homogenized in

2 parts $_{DD}H_2O$ per 1 part carcass. Samples of homogenized carcass (approx. 6 g) were placed in 15 mL falcon tubes with 5 mL acid and treated as above organs. Pellets of food (approx. 1 g) were also weighed, dissolved and centrifuged using the same protocol. For the determination of zinc and cadmium levels in the tissues, each supernatant was diluted in 10 mL of $_{DD}H_2O$ into 20 mL scintillation vials. For analysis of Zn concentrations, each sample was then aspirated on a Varian AA-1275 series Atomic Absorption Spectrophotometer. Cadmium concentrations were determined using the Cd program on a Varian GTA-95 Graphic Tube Atomizer.

In Vivo Single Oral Dose Experiments

Surgery

Each trout was anaesthetized prior to surgery in MS-222 (0.08 g/L) with $NaHCO_3$ (0.16 g/L) as a buffer (pH ~7.2) in dechlorinated Hamilton, Ontario tap water. After anaesthesia was attained, fish were transferred to a surgical table, where the gills were irrigated with anaesthetic during the entire procedure. A catheter (30 cm, PE 50 polyethylene tubing) was inserted directly into the stomach via the esophagus using a blunt probe. It was then stitched to the roof of the mouth, and the free end was surgically inserted through the roof of the mouth. A similar method of fluid infusion into the gut is also described by Giblin and Massaro (1973) in the administration of methyl mercury to rainbow trout. The surgical procedure took about 20 min., after which the fish were revived in dechlorinated tap water and allowed to recover in individual 2 L flux

chambers, with aerated, continuous water flow for a period of approximately 48 h prior to experimentation.

GI Absorption of Zn and Cd Over Time

A dose of 0.5 mM of either radio-labelled Cd^{109} or Zn^{65} in the form of $ZnSO_4$ and $Cd(NO_3)_2$ (these compounds were used throughout the experiments) was administered in 1 mL 0.1 M glucose via the stomach catheter. After exposure periods of 24, 48, 72, or 168 h, fish were terminally anaesthetized, blood samples were taken (1 mL) and all internal organs (liver, kidney, spleen, gall bladder, heart, swim bladder, gills, and gonads) were removed and weighed. Samples of muscle and structural fat (the fatty layer surrounding the internal organs) were also taken. Blood samples were centrifuged at 1500 x g in a Fisher microcentrifuge for 2 min. to separate plasma and hematocrit. Gastrointestinal tissues were divided as characterized by Buddington and Diamond (1987), sliced open and rinsed of all contents in 10 mL of deionized water (in 20 mL scintillation vials) and weighed. Radioactivity was separately measured in gut tissues and rinsed gut contents. The remaining carcass was then sliced into pieces and divided into 20 mL scintillation vials in order to count it for radioactivity in its entirety. All samples were counted for radioactivity using a Canberra-Packard MINAXI γ Auto-Gamma 5000 series Gamma Counter for 5 min. per sample.

Dose Dependent Absorption of Zn and Cd from the Gut

Four concentrations of each metal were used for this experiment. Fish were infused with 0.5, 5, or 50 mM Cd^{109} or 0.5, 5, 12, or 25 mM Zn^{65}. After an exposure period of 7 days, fish were terminally anaesthetized and dissected as described above. Samples were counted for radioactivity as above.

Effect of Feeding on Cd and Zn Absorption

Fish were catheterized and treated as in previous *in vivo* experiments prior to infusion of the dose of metal. For this experiment, all fish were also fin clipped for identification. Fish were dosed either 0.5 mM Cd^{109} or 0.5 mM Zn^{65}. After 4 h, catheters were removed and all fish were transferred to 2 large free swimming holding tanks. Fish in one of the tanks were fed Martin's floating pellet feed to apparent satiation later that day and daily for the next 2 d following infusion of the metal. Fish in the second tank was not fed. 72 h post-infusion, fish were terminally anaesthetized, dissected and counted for radioactivity as above.

In Vitro Gut Bag Experiments

Preparation of the Gut Bags

Rainbow trout were killed with an overdose of MS-222 (1 g/L). A ventral incision from the gills to the anus was then used to remove the entire gastrointestinal tract from the esophagus to the anus (including the liver and gall bladder). The tract was then immediately placed in a large petri dish for dissection in Cortland's saline solution. Any

gut contents were gently squeezed from the stomach and intestine. Visceral fat was then removed from the entire gastrointestinal tract. A pre-weighed catheter (PE 50) with flared tip (approximately 5-6 cm in length) was then inserted (flared end inside) into the posterior end of the distal intestine. This was tied securely in place using suture silk (size 00) as close to the end of the tissue as possible. At the junction of the mid-intestine and the distal intestine suture silk was used to tie off the intestine securely. These two sections were then separated to make a gut bag from the distal portion of the intestine. A similar procedure was used to isolate the mid-intestine with the anterior end tied off immediately posterior to the final caeca. The liver and gall bladder were then removed after tying off the bile duct that enters just posterior to the stomach. A flared catheter was then inserted into the posterior end of the proximal intestine and tied securely. At the anterior end, suture silk was tied just posterior to the pyloric sphincter of the stomach. Separation of the stomach and proximal intestine was then accomplished by slicing through a small portion of the pyloric valve just anterior to the tie. Gut bags were then weighed and infused with 140 mM NaCl containing 1.5 μmol/mL of either radiolabelled Zn^{65} or Cd^{109} using a 23 gauge needle. The catheter was then plugged using a wax filled 22 gauge needle and the bags were placed in glass scintillation vials with Cortland's saline (at 14°C) and bubbled with a 0.3% CO_2, 99.7% O_2 mixture. Weights and details of the bath and infusate volumes are shown in Table 1.

Table 1.

Details concerning the setup of *in vitro* gastrointestinal gut bags used in the determination of regional absorption of zinc and cadmium. Means ± SE. N numbers for each compartment are noted.

Tissue	N (Cd, Zn)	Ave. Mass (g ± SE)	Mucosal Volume (mL)	Serosol (Bath)Volume (mL)
Stomach	(6,10)	1.830 ± 0.136	0.5	15
Proximal Intestine	(6,4)	2.373 ± 0.307	0.5	20
Mid-Intestine	(4,7)	0.245 ± 0.026	0.5	15
Distal Intestine	(5,8)	0.456 ± 0.039	0.5	15

mucosal solution: 140 mM NaCl with 1.5 mM Cd or Zn
serosal (bath) solution: Cortland's saline bubbled with 99.7% O_2, 0.3% CO_2.

Sampling Protocol and Analysis

To assess transepithelial transport of metal by each gut bag, 0.5 mL samples of the serosal bathing medium were taken at 0 time (immediately following infusion to assess leakage from bags), 15 min, 30 min, 1 h, 2 h, and 4 h periods. Bathing medium was replaced with Cortland's saline after each sample was taken to maintain serosal volume. Samples were each counted for radioactivity to measure metal uptake by the different compartments.

Following the 4 h flux period, gut bags were removed from the bathing medium and all mucosal contents were then emptied using a 23 gauge needle via the catheter. The entire volume of these contents was counted for remaining radioactivity. Following this, the gut bag was cut open and rinsed of all remaining contents and any fluid or mucus on the serosal surface by gently washing in deionized water. Each gut tissue was then counted for radioactivity to estimate the amount of metal bound to the gastrointestinal cells.

Incremental Dosing Experiments

Due to their high uptake capacity (as shown in previous experiments) and the relative ease with which they were formed into gut bags, the midintestine was chosen for the remainder of the *in vitro* gut bag metal uptake experiments. Midintestine gut bags were produced using the protocol described earlier. Each midintestine gut bag was infused with 0.3 mL of mucosal fluid (140 mM NaCl) instead of the 0.5 mL previously used. This change ensured that the bags did not burst but was enough to maintain an

adequate stretch on the walls of the intestine. Radiolabelled Zn^{65} or Cd^{109} were infused in 7 concentrations ranging from 0.8 to 28 mM. Samples of the serosal solution (0.5 mL) were again taken at 0, 15, 30, 60, 120 min intervals with saline replacement. The compartmental uptake studies (as described above) had shown that a 120 min flux period was sufficient to see trends in metal uptake and thus the flux protocol was changed for all midintestine gut bag exposures. Following this 120 min flux period, midintestines were removed from their baths, emptied of fluid contents using a 23 gauge needle, sliced open and gently scraped on the mucosal surface with a blunt pair of forceps to remove any mucus, weighed and traced onto graph paper for surface area calculations. The tissue, fluid contents and serosal samples were then counted for radioactivity.

Ca^{+2} Competition Studies

Midintestine gut bags were used for these experiments. Gut bags were prepared as described earlier except that midintestines were divided into 2 equally sized gut bags in order to provide paired control-treatment groups. For the two treatment groups, either 1 mM Ca^{+2} or 10 mM Ca^{+2} in the form of $CaCl_2$ was added to the 140 mM NaCl infusion solution previously described. The concentrations of radiolabelled Zn and Cd to be infused were chosen to be 10 mM. Thus, there were 3 treatment groups for each metal: 10 mM metal only as control, 10 mM metal plus 1 mM Ca^{+2}, and 10 mM metal plus 10 mM Ca^{+2}. Since the bags were smaller, the infusion amount was decreased to 0.15 mL and the bathing solution of Cortland's saline was decreased to only 10 mL. Bathing solution samples (0.5 mL) were taken as before at 0, 15, 30, 60, and 120 minute intervals.

Following the 120 min flux period, gut bags were treated as before, except that all mucus was gently removed from the mucosal surface of the intestine, weighed and counted for radioactivity to determine the amount of metal bound to the mucus layer.

Metabolic Inhibition Experiment

This experiment again utilized paired control-treatment midintestine gut bags. The control infusion was the same as above. Treatment infusions consisted of 1 mM 2,4-Dinitrophenol (DNP) in 140 mM NaCl plus 10 mM radiolabelled Zn or Cd. Bathing solution was again 10 mL Cortland's saline. Gut bags were sampled as described in the Ca^{+2} competition studies above.

Statistical Analysis

All statistical analysis was performed using Quattro Pro 8 for Windows 95. All numbers are expressed with standard error. Paired t-tests were performed on paired mid-intestine control and treatment groups for Ca^{+2} and DNP experiments for differences in tissue binding, mucus concentration, rinse concentration. Paired t-tests were also performed to determine differences between mucus and rinse concentrations within the same treatment group. The slope of the lines for DNP and control groups were compared using analysis of variance in SAS JMP 2.0.5 for Macintosh. Unpaired t-tests were also performed using Quattro Pro 8 for Windows 95 to determine differences between fed and unfed tissue burdens.

RESULTS

Tissue and Food Zn and Cd Concentrations in Unexposed Fish

Zinc and cadmium levels in the tissues and food of naive rainbow trout are reported in Table 2. The distal and mid-intestine, spleen and gonads contained the highest concentrations of Zn, while the liver, gills, plasma, muscle, fat and carcass had very low Zn levels. Cadmium concentrations in all tissues were 100-1000 fold lower than concentrations of Zn. As with zinc, the distal and mid-intestine, spleen and gonads had high concentrations of cadmium, but the bile, kidney, heart and swim bladder also had high concentrations.

Pellet food contained Zn at a concentration of 3.1 μmol/g which is comparable to the optimal dietary zinc level for rainbow trout (2.31 μmol/g; Hardy and Shearer, 1985). The cadmium concentration in the food was roughly 400 fold lower than zinc at 7.4 nmol/g.

The concentrations of zinc used in the present experiments (0.5 to 25 mmol/L) were similar to the concentrations of zinc found in naive gut tissues (0.35 to 3.2 mmol/kg) and pellet food (3.1 mmol/kg). Cadmium concentrations were elevated 1000-10000 fold beyond those found in the tissues but were equal to those of zinc so that the two metals could be compared equally.

Figure 1 gives a quick comparison of the relationship between zinc and cadmium tissue concentrations in unexposed fish. The plot illustrates that there was a strong

Table 2.

Measurements of the Cd and Zn concentrations in all tissues and pellet food of unexposed fish. Note the difference in units between zinc (μmol/g) and cadmium (nmol/g) levels. Means ± SE. n=6.

Tissue	Average Wt. (g)	Cd (nmol/g)	Zn (μmol/g)
Liver	2.46 ± 0.19	0.904 ± 0.083	0.139 ± 0.016
Gall Bladder + Bile	0.48 ± 0.07	3.521 ± 0.218	0.257 ± 0.031
Stomach	3.34 ± 0.15	0.616 ± 0.092	0.572 ± 0.058
Proximal Intestine	5.61 ± 0.73	0.494 ± 0.065	0.354 ± 0.090
Mid-Intestine	0.60 ± 0.08	5.614 ± 0.715	3.163 ± 0.413
Distal Intestine	1.39 ± 0.21	3.341 ± 0.363	1.078 ± 0.281
Gills	6.29 ± 0.34	0.213 ± 0.016	0.165 ± 0.023
Kidney	1.42 ± 0.13	1.348 ± 0.083	0.195 ± 0.022
Plasma		0.113 ± 0.037	0.019 ± 0.003
Muscle	3.59 ± 0.21	0.360 ± 0.021	0.031 ± 0.003
Spleen	0.38 ± 0.04	6.250 ± 1.276	1.540 ± 0.361
Heart	0.29 ± 0.02	5.189 ± 0.308	0.724 ± 0.100
Structural Fat	4.51 ± 0.02	0.049 ± 0.023	0.027 ± 0.004
Carcass	204.34 ± 8.09	0.233 ± 0.029	0.107 ± 0.011
Gonads	0.28 ± 0.06	5.419 ± 1.656	2.718 ± 0.250
Swim Bladder	0.40 ± 0.02	4.551 ± 0.680	0.704 ± 0.087
Pellet Food	1.03 ± 0.01	7.380 ± 0.529	3.100 ± 0.115

relationship between stable zinc and cadmium levels in naive rainbow trout. The tissues with the highest metal levels for zinc (mid-intestine, gonads, spleen, distal intestine) were similar to those that contained high levels of cadmium (mid-intestine, gonads, spleen, heart). Likewise, those with lowest concentrations of zinc (fat, plasma), also had low concentrations of cadmium.

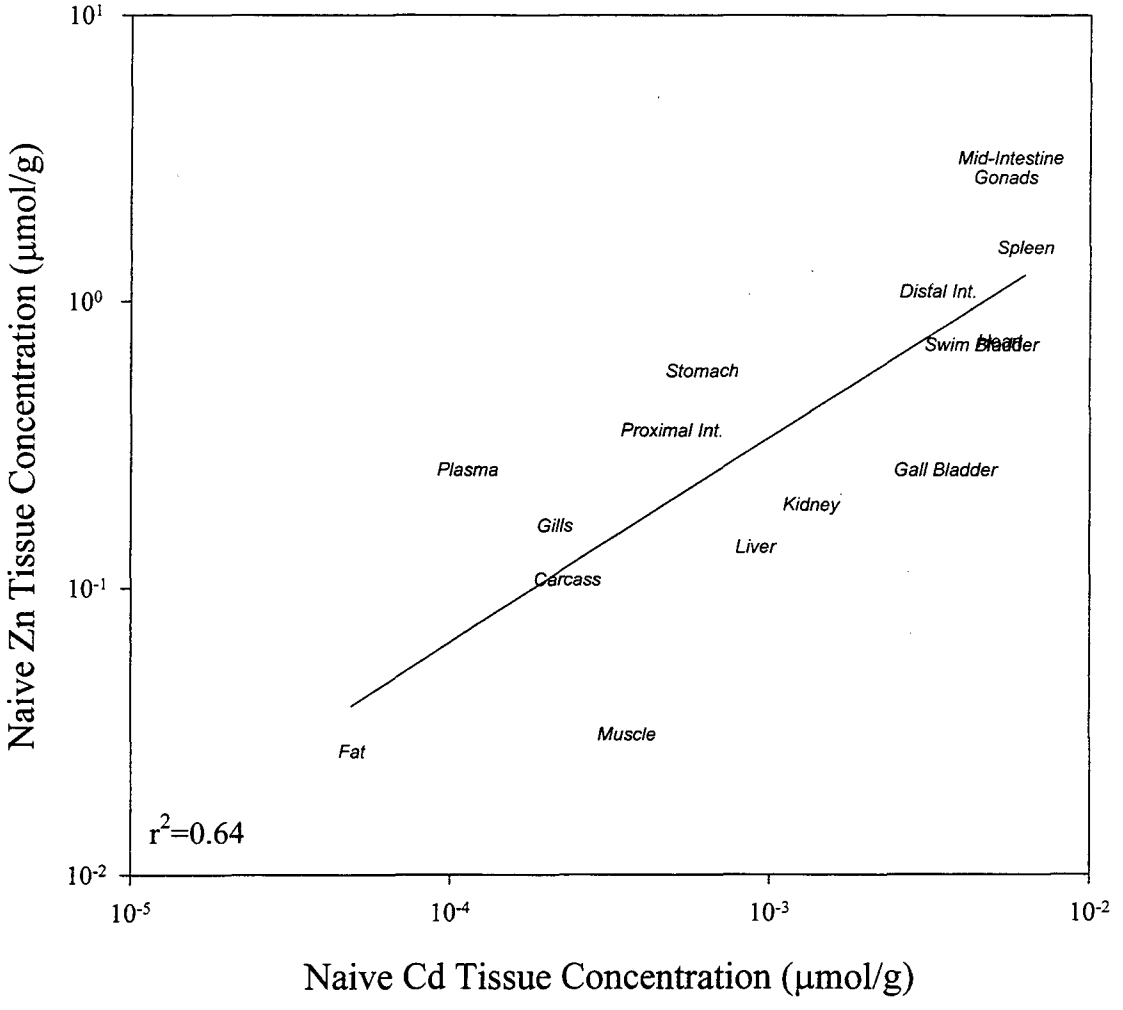
In Vivo Metal Infusion

Volume of the Gut Lumen

It was determined *in vitro*, that the total volume of the gastrointestinal tract was approximately 2-3 mL, since infusion of 0.5 mL of fluid into each of the stomach, proximal intestine, mid-intestine and distal intestine resulted in significant tension on the walls of the gut bags. *In vivo*, the infusion of 1 mL of fluid did not cause vomiting or coughing and thus it is likely that a portion of the infusate moved directly from the stomach into the proximal intestine immediately following dosing. The volume of fluid in the GI tract of a starved fish is not known, however, very little fluid was observed upon dissection of fish *in vivo*, even with a 1 mL fluid load. It can be assumed that there was only 0.5-1 mL of fluid in the GI tract based on this observation. Thus the concentration of metal infused *in vivo* could be diluted as much as 50% within the GI tract.

Figure 1.

A comparison of the tissue distribution of zinc and cadmium in unexposed rainbow trout. r^2 of the regression line = 0.64. Values are taken from Table 1. Note the differences in scale between the two axes. Also note that each value occurs in the center of the text label.


Naive Zn Tissue Concentration (µmol/g)
10¹
10⁰
10⁻¹
10⁻²
Mid-Intestine
Gonads
Spleen
Distal Int.
Swim Bladder
Stomach
Proximal Int.
Plasma
Gall Bladder
Kidney
Gills
Liver
Carcass
Muscle
Fat
r²=0.64
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Naive Cd Tissue Concentration (µmol/g)

Overall Distribution of Metal

Preliminary observations suggested that the distribution of metal could be separated into 4 major compartments: the gut lumen (which included any mucus and other contents), the gut tissues (which may include a microlayer of mucus), the internalized fraction (the total amount in the body with the exception of the gut tissues) and the portion excreted (100% of dose minus the recovered fractions). Figure 2 illustrates the fate of single oral doses of zinc and cadmium (0.5 mM) over time to these compartments: the gut lumen (A), the gut tissues (B), the body (C), and the excreted portion (D).

At the first sampling period of 24 h, the zinc dose was distributed evenly between the gut lumen, gut tissues and body tissues, with the remaining portion being excreted. The distribution of zinc dose was as follows: GI lumen-21%, gut tissues-21%, internalized-20%, excreted-38% (Fig. 2). The distribution of Cd following 24 h of exposure was quite different. The major difference between the two metals was that Cd was not internalized. Instead, more Cd was present in the gut tissue and a larger portion of the metal was excreted. After 24 h, cadmium was distributed as follows: GI lumen-19%, gut tissue-30%, internalized-3%, excreted-48% (Fig. 2).

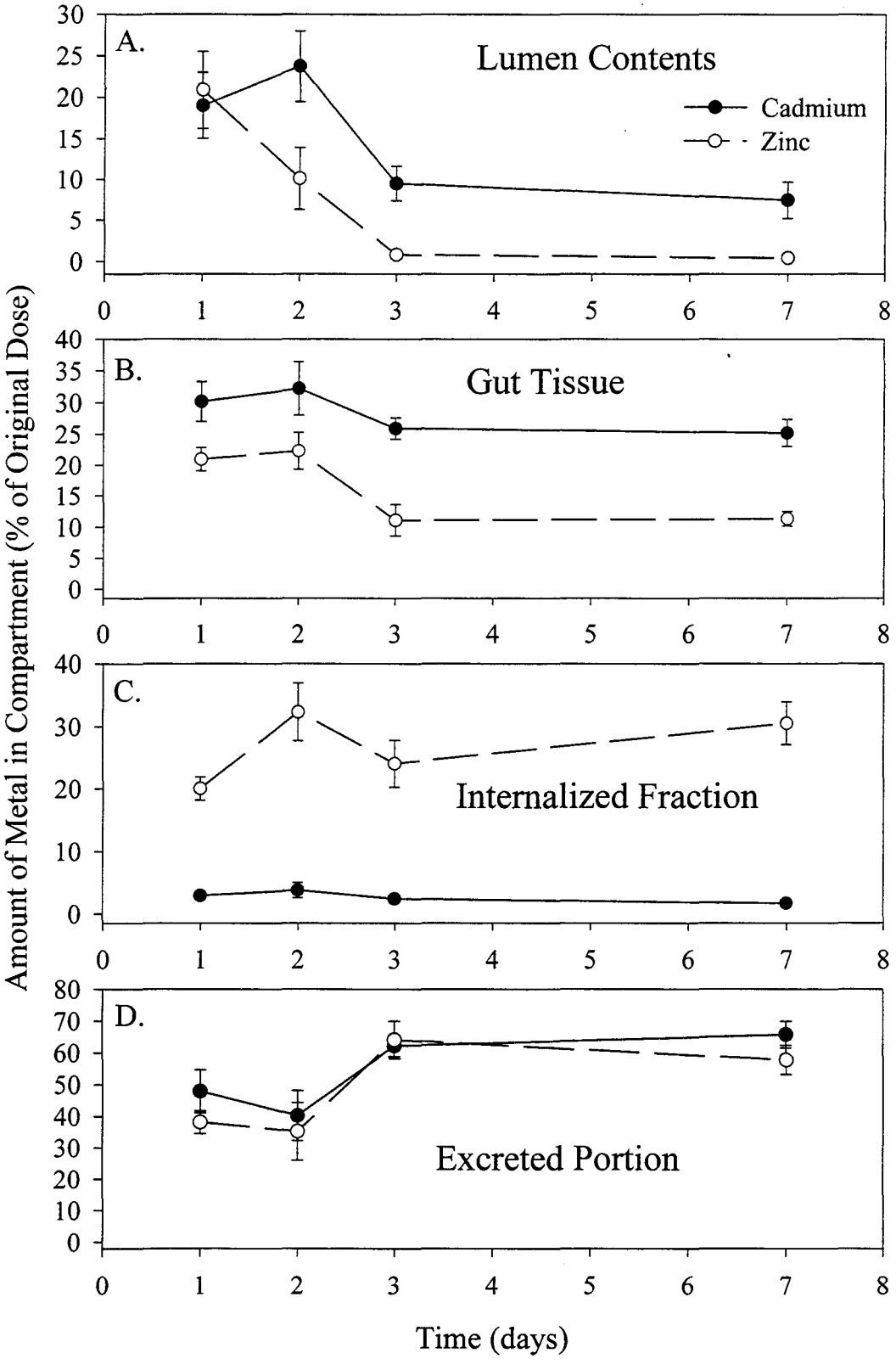
Over the subsequent 144 h (ie 6 days), there was further redistribution of both metals, but the changes were much smaller than those occurring over the first 24 h (Fig. 2). There was no further internalization of zinc but the GI lumen was emptied, resulting in an increase in the percentage of excreted zinc. The zinc concentration in the gut tissues also decreased. After 7 days the compartmental distribution of zinc was: GI

Figure 2.

The fate of orally exposed Cd^{109} and Zn^{65} (0.5 mM) in rainbow trout after exposure times of 24, 48, 72, and 168 h at 15 °C. Means ± SE. n=6.

A. Amount remaining in GI luminal contents (includes mucus and fluids).
B. Amount bound to GI tissue.
C. Internalized (bioavailable) fraction of the metal.
D. Excreted portion of the metal (determined by subtraction).

--●-- Cadmium
--○-- Zinc


A.
Lumen Contents
Cadmium
Zinc
B.
Gut Tissue
C.
Internalized Fraction
D.
Excreted Portion
Amount of Metal in Compartment (% of Original Dose)
Time (days)

lumen-0.4%, gut tissues-11.3%, internalized fraction-30.5%, excreted-57.7% (Fig. 2). Movement of cadmium followed a very similar pattern to that of zinc, with the percentage of internalized metal remaining the same and the lumenal contents and gut tissues dropping. Cadmium, however, did not empty entirely from the GI lumen. 10% of the dose remained after 168 h. Gut Cd was also 2.5 fold higher than gut Zn at this time. The distribution of Cd at 7 days was as follows: GI lumen-7.5%, gut tissues-25.2%, internalized fraction-1.7%, excreted metal-65.7% (Fig. 2). Although the distribution values for the 7th day are given above, there were no changes in the distribution of either metal between the 3rd day and the 7th day of exposure.

The above observations suggest that the uptake and distribution of zinc and cadmium occurs in two phases; a fast phase and a slow phase. The fast phase was complete by 24 h and it was during this phase that the majority of metal movement occurred. During this time, the total amount of zinc and cadmium in the GI lumen dropped from 100% to only 20% (Fig. 2A). The stomach was almost completely emptied of both metals, and both were distributed along the entire length of the GI tract (Fig. 3). Metal binding to the gut tissues had reached maximal values by 24 h, and the majority of uptake had occurred by this time (Fig. 2B, C). Excretion of each metal was also high, approximately 45% of each metal dose (Fig. 2D).

Movement of Zinc and Cadmium Along the Gut During the Slow Phase

The slow phase of zinc movement in the lumen was characterized by a complete emptying of the lumen within 72 h (Fig. 2A). For cadmium, emptying of the entire gut

lumen did not occur even after 168 h (Fig. 2A). The specific movement of zinc and cadmium along the GI tract is shown in Figure 3. There was virtually no zinc (only 1.8% of initial dose) or cadmium (0.6%) remaining in the stomach following the fast phase (Fig. 3A). In the case of zinc, the proximal and mid-intestinal compartments showed a steady decrease in luminal metal from 24 to 72 h (Fig. 3B, C). Zinc movement from the distal intestine lumen did not occur until after 48 h (Fig. 3D). After 72 h, less than 2% of the original dose of zinc was present in the lumen of the gastrointestinal tract. The majority of this (75%) was present in the distal intestine.

The slow phase was similar for cadmium, although cadmium showed greater retention times within the lumen along the entire tract (Fig. 3). The majority of luminal movement of cadmium in the slow phase occurred within the first 72 h of exposure, as with zinc, however, complete emptying never occurred, and the rate of movement along the tract was slower than zinc. Even after 168 h the total amount of cadmium in the tract was still 7.5% of the original dose, as compared to zinc which was less than 0.5% of the original dose. Like zinc, the majority of luminal cadmium was present in the distal intestine (75%), which showed very little emptying during the slow phase.

Redistribution of Metal in the Gut Tissue During the Slow Phase

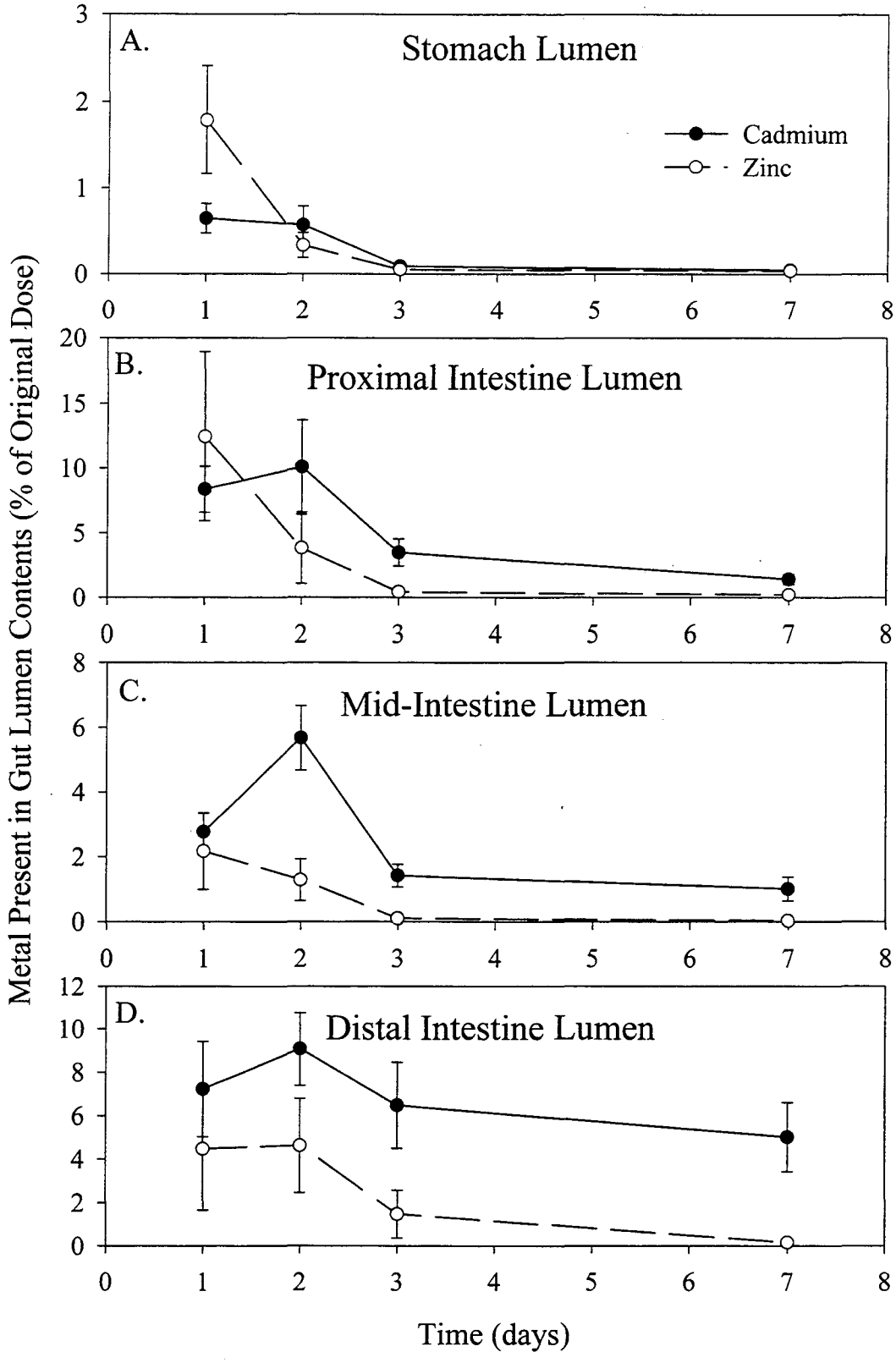
During the slow phase there was a slow movement of zinc from the gut tissue. This occurred during the period from 24 to 72 h and ceased thereafter (Fig. 2B). 10% of the zinc dose remained in the gut tissue at 168 h of exposure. Cadmium, on the other

Figure 3.

A comparison of the rate of movement of a 0.5 mM dose of Cd^{109} or Zn^{65} along the gastrointestinal tract of rainbow trout *in vivo*. Temperature = 15 °C. Means ± SE. n=6.

A. Stomach.
B. Proximal Intestine.
C. Mid-Intestine.
D. Distal Intestine.

--●-- Cadmium
--○-- Zinc


A.
Stomach Lumen
Cadmium
Zinc
B.
Proximal Intestine Lumen
C.
Mid-Intestine Lumen
D.
Distal Intestine Lumen
Metal Present in Gut Lumen Contents (% of Original Dose)
Time (days)

hand, showed very little movement in the gut tissue during the slow phase with only a 5% drop.

Upon examining the individual gut tissues, it was clear that each tissue (with the exception of the stomach) handled the two metals differently (Fig. 4). The distal intestine showed the greatest difference between the two metals (Fig. 4D). The zinc concentration in the distal intestine decreased over the first 2 days of the slow phase and remained the same thereafter. However, the cadmium concentration in the distal intestine nearly doubled in the first 24 h of the slow phase (with a corresponding drop in the proximal intestine), and the concentration remained high (Fig. 4D). In the proximal and mid-intestinal compartments, there were increases in the zinc tissue concentration 24 h following the fast phase. These increases coincided with the drop in luminal zinc content (Fig. 3). Following these increases, the zinc concentrations dropped in all intestinal tissues over the next 24 h and did not change further over the following 96 h. In contrast, there was a drop in cadmium concentration in the proximal intestine over the first 24 h of the slow phase, but no changes following this. The mid-intestine showed similar movement of cadmium as zinc, although Cd concentrations in the tissue were higher.

Differences in Gut Binding Between Zn and Cd

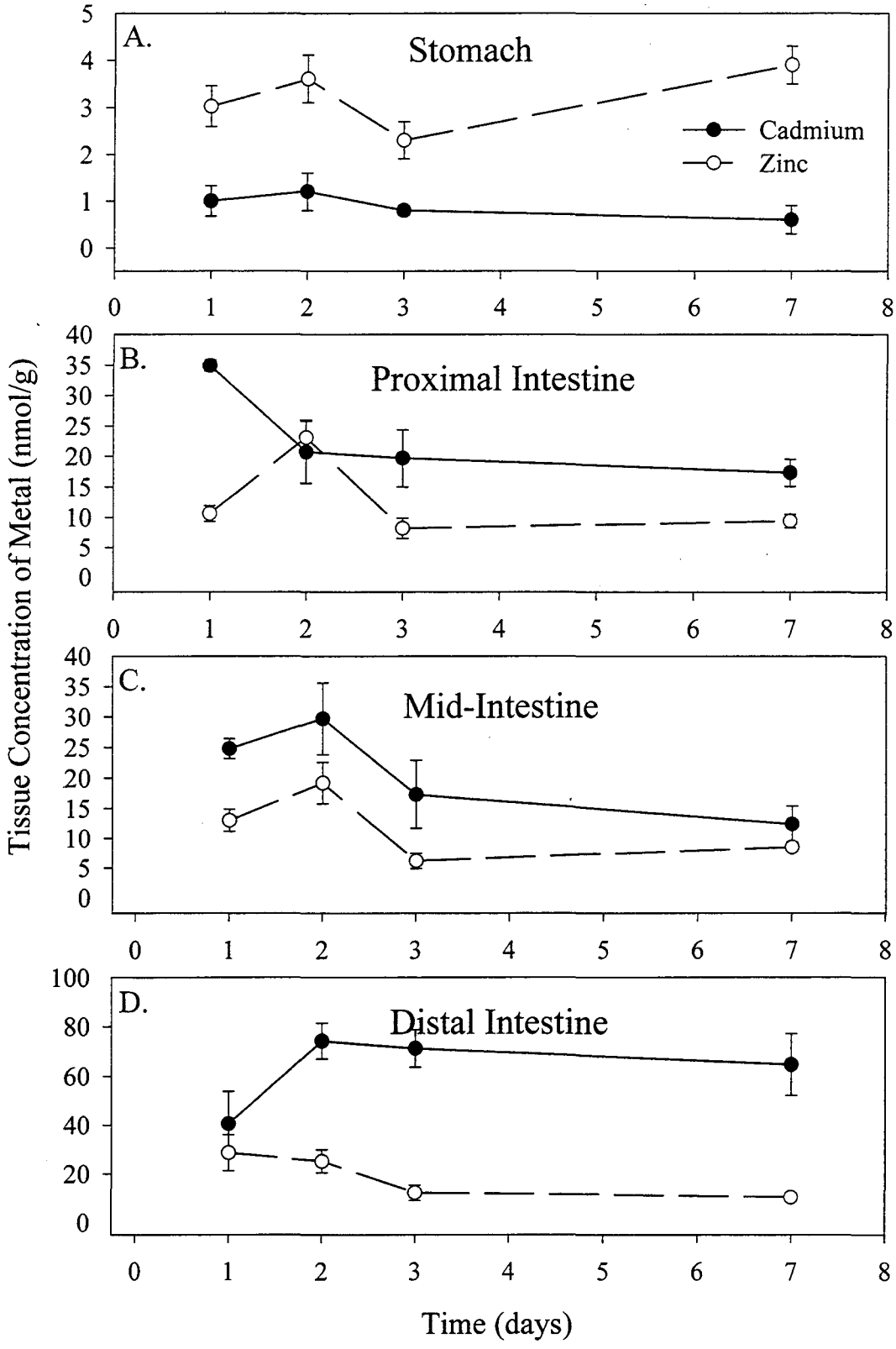
At the end of the 168 h exposure to a 0.5 mM dose, cadmium concentrations in each intestinal tissue were higher than those of zinc, with the distal intestine having the highest binding difference (10.4 nmol/g Zn vs. 64.7 nmol/g Cd)(Fig. 4). At higher doses,

Figure 4.

A comparison of the individual gut tissue compartmentalization over time following single oral dose exposures of 0.5 mM Cd^{109} and Zn^{65} in rainbow trout at 15°C. Means ± SE. n=6.

A. Stomach.
B. Proximal Intestine.
C. Mid-Intestine.
D. Distal Intestine.

--●-- Cadmium
--○-- Zinc


A.
Stomach
Cadmium
Zinc
B.
Proximal Intestine
C.
Mid-Intestine
D.
Distal Intestine
Tissue Concentration of Metal (nmol/g)
Time (days)

however, additional differences in the gut binding of zinc and cadmium became apparent. Nonetheless, the gut compartment with the highest binding of both metals remained the distal intestine (Fig. 5, 6). The concentration of zinc in all intestinal compartments increased 10 fold with a 10 fold increase in dose from 0.5 to 5 mM but reached saturation at higher concentrations (Fig. 5). For zinc, the distal intestine had the highest binding capacity (0.12 μmol/g) but the proximal (0.09 μmol/g) and mid-intestines (0.08 μmol/g) were not significantly lower. In contrast, cadmium apparently reached saturation in all intestinal tissues at the lowest dose (0.5 mM). The binding capacity of the distal intestine for Zn and Cd was equal, but the binding capacities for zinc in the proximal and mid-intestine were 5 and 7 fold, respectively, those of cadmium. Zinc binding to the stomach also saturated but the binding capacity was half that of the intestinal tissues. In contrast, the stomach was the only tissue that did not exhibit saturation at high doses of cadmium and reached a tissue concentration about half that of the proximal intestine and 4 fold lower than the zinc saturation concentration in the stomach (Fig. 6).

In an attempt to find the saturation concentration for the stomach and other internal tissues (see later), oral doses of 500 and 5000 mM cadmium were used. However, all fish died within 48 h of administering the dose.

Internal Distribution of Zinc and Cadmium in the Fast Phase

All internalization of zinc and cadmium occurred during the fast phase. This fraction of the dose has been regarded as the bioavailable fraction (ie binding to gut tissue is excluded). As stated earlier, the bioavailability of zinc was 20%, while that of

Figure 5.

The distribution of Zn^{65} to the gastrointestinal tract tissues following exposures to 0.5, 5, 12.5, and 25 mM oral doses after 168 h *in vivo*. Temperature = 15°C. Means ± SE.

--●-- Stomach.
--○-- Proximal Intestine.
--▼-- Mid-Intestine.
--▽-- Distal Intestine.

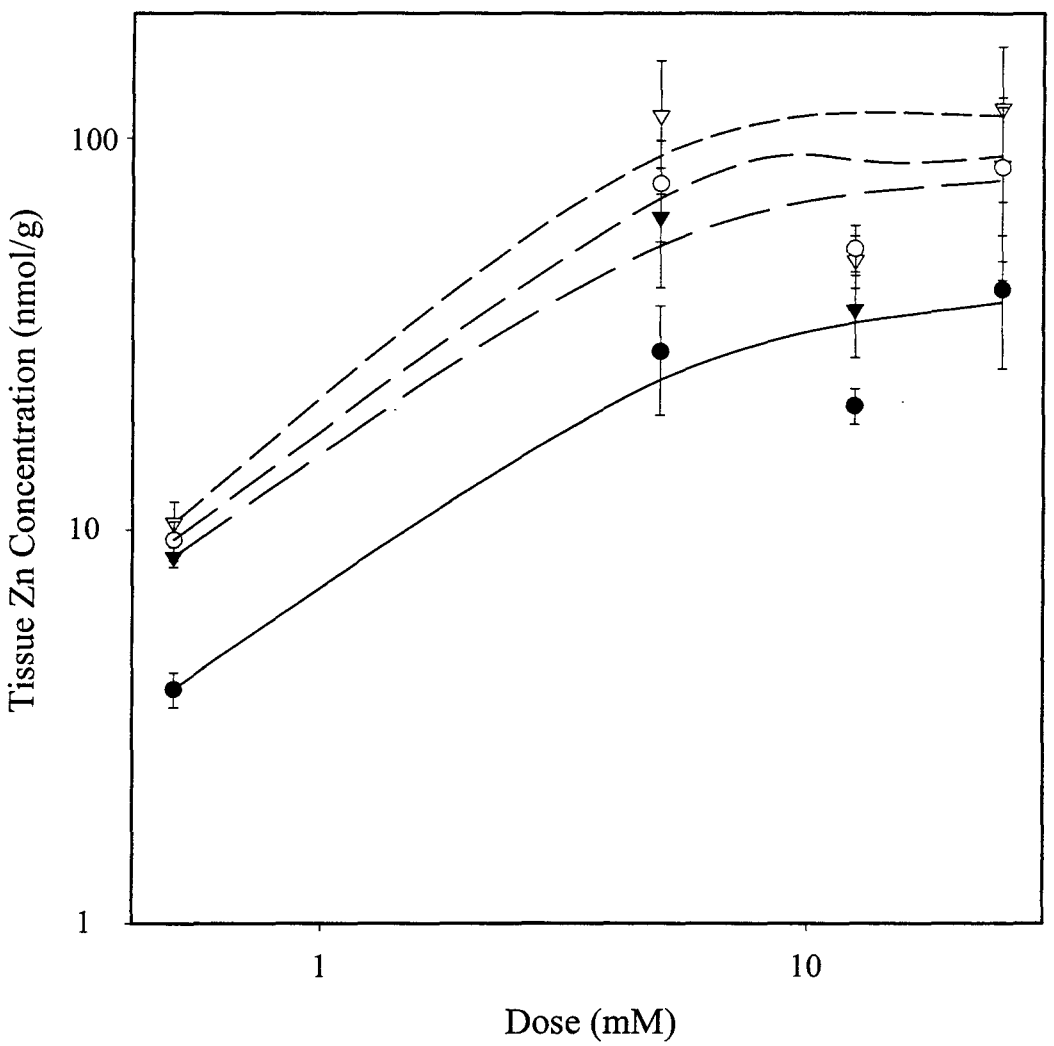
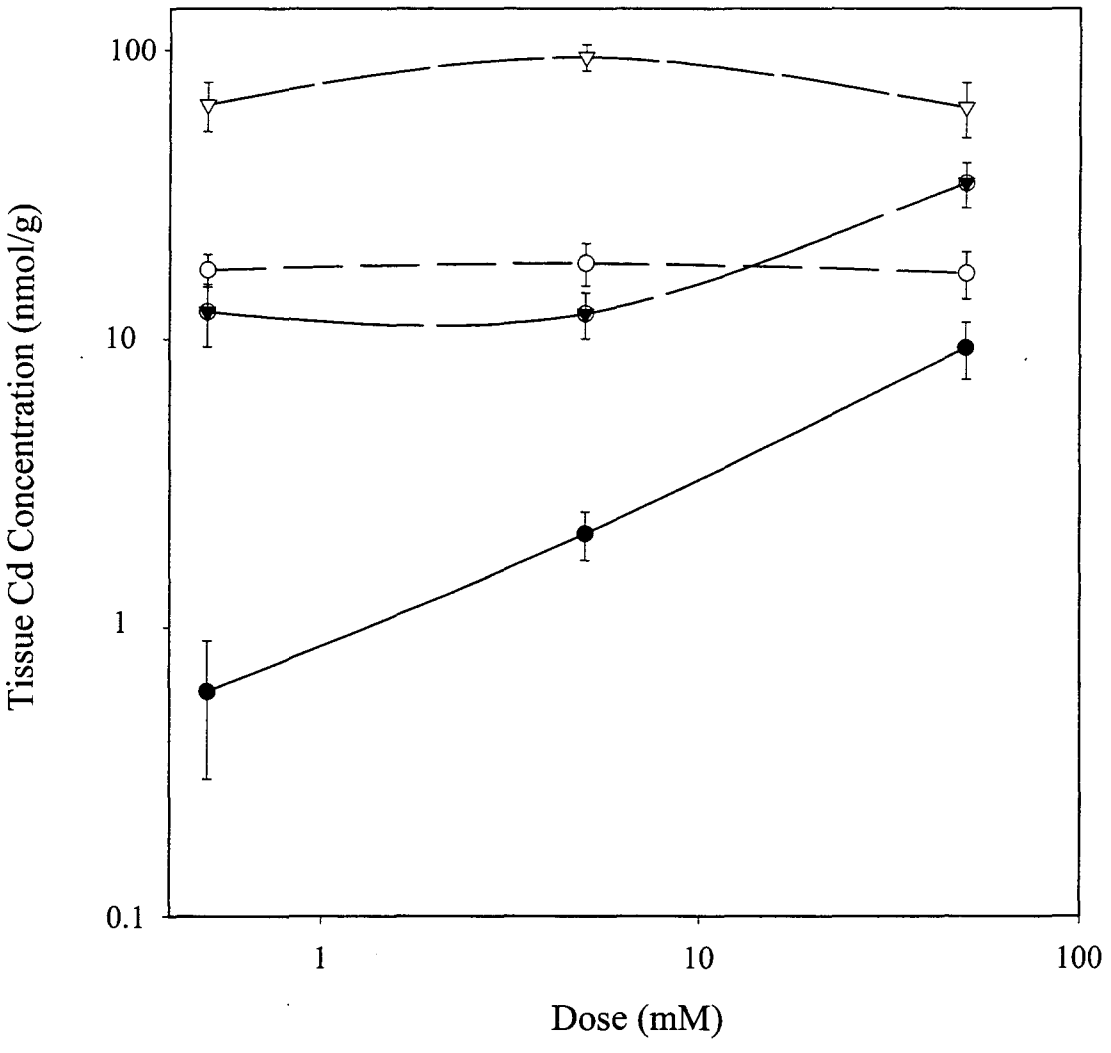

Tissue Zn Concentration (nmol/g)
100
10
1
1
10
Dose (mM)

Figure 6.

The distribution of Cd^{109} to the gastrointestinal tract tissues following exposures to 0.5, 5, and 50 mM oral doses after 168 h *in vivo*. Temperature = 15°C. Means ± SE.

--●-- Stomach.
--○-- Proximal Intestine.
--▼-- Mid-Intestine.
--▽-- Distal Intestine.


100
10
1
0.1
Tissue Cd Concentration (nmol/g)
1
10
100
Dose (mM)

cadmium was only 3% during the first 24 h of exposure (Fig. 2C). This distribution of metal occurred over most compartments and tissues within the body, but the most important for both metals were the gills, kidney, liver, plasma, and bile (Fig. 7). However, there were differences between zinc and cadmium in their distribution patterns (Fig. 7). Because of the great differences in bioavailability of the two metals, actual concentrations of Cd and Zn within tissues could not be compared. However, relative tissue burdens could be assessed to determine distinctions between the handling of each metal in the body. At the end of the fast phase, the compartments with the highest concentrations of zinc were plasma (10.8 nmol/g), gills (3.5 nmol/g), kidney (3.2 nmol/g) and liver (2.3 nmol/g). Whereas, cadmium distribution was highest in liver (0.6 nmol/g), kidney (0.6 nmol/g) and gills (0.2 nmol/g).

Although the carcass concentrations of zinc and cadmium were not elevated with comparison to the aforementioned tissues, the carcass was also considered a important tissue here since the total amount of metal bound to this compartment was significant due to the large mass of this tissue.

The remainder of the absorbed Zn and Cd was found in other tissues such as the heart, red blood cells, spleen, fat, swim bladder and gonads (Fig. 8). Levels of metal in these tissues were generally quite low, although tissue concentrations of Cd in the spleen, gonads and red blood cells were elevated at the 24 h exposure interval (Fig. 8). However, the variation amongst the individual fish was high, indicating a possibility of contamination of these tissues during dissection.

Figure 7.

The fate of Cd^{109} and Zn^{65} in the liver, kidney, gills, bile, carcass, and plasma of rainbow trout over time following exposure to 0.5 mM single oral doses. Note that Cd concentrations on the y-axis are multiplied by 10 to make trends more apparent. Temperature = 15°C. Means ± SE. n=6.

--●-- Cadmium
--○-- Zinc

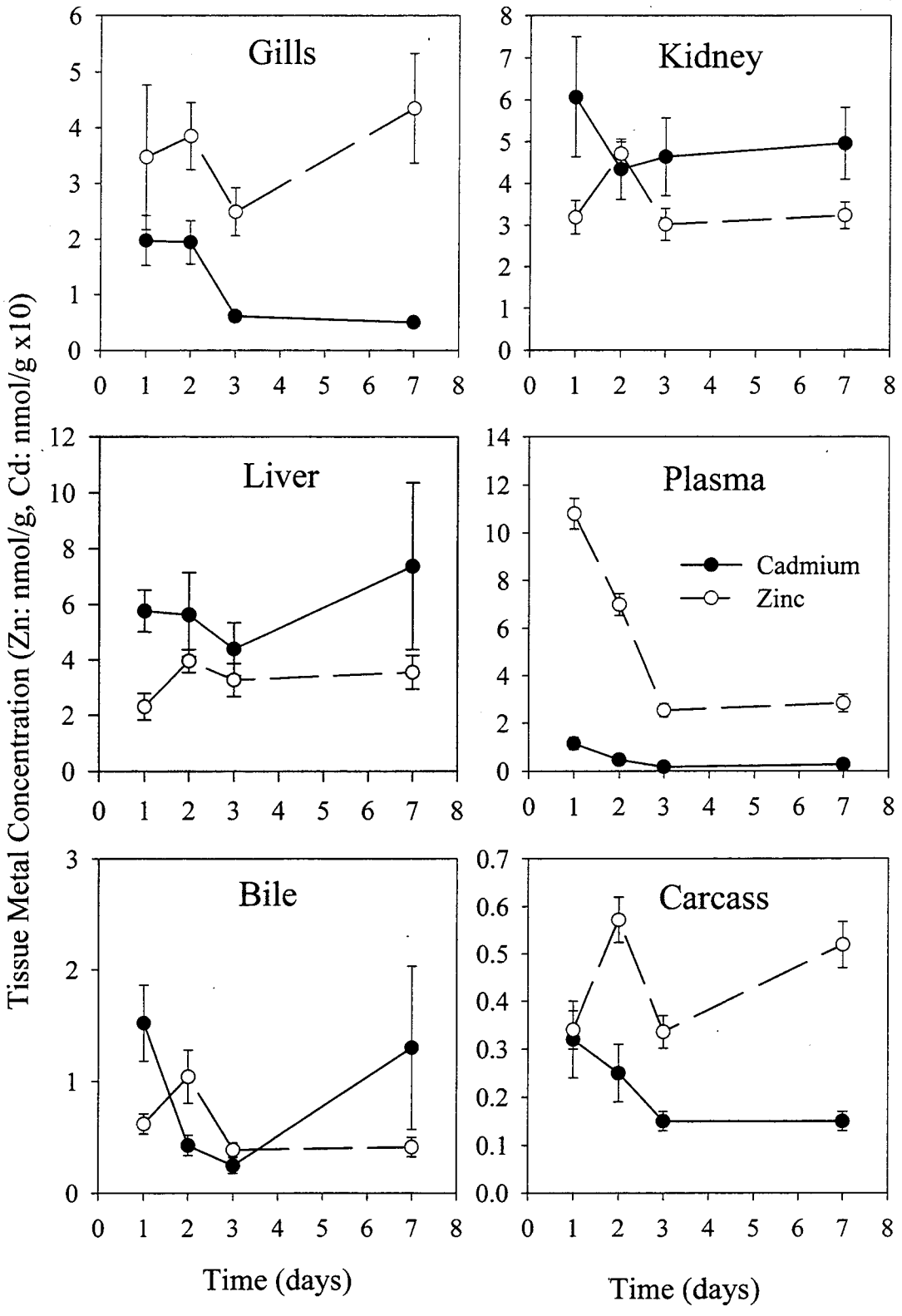
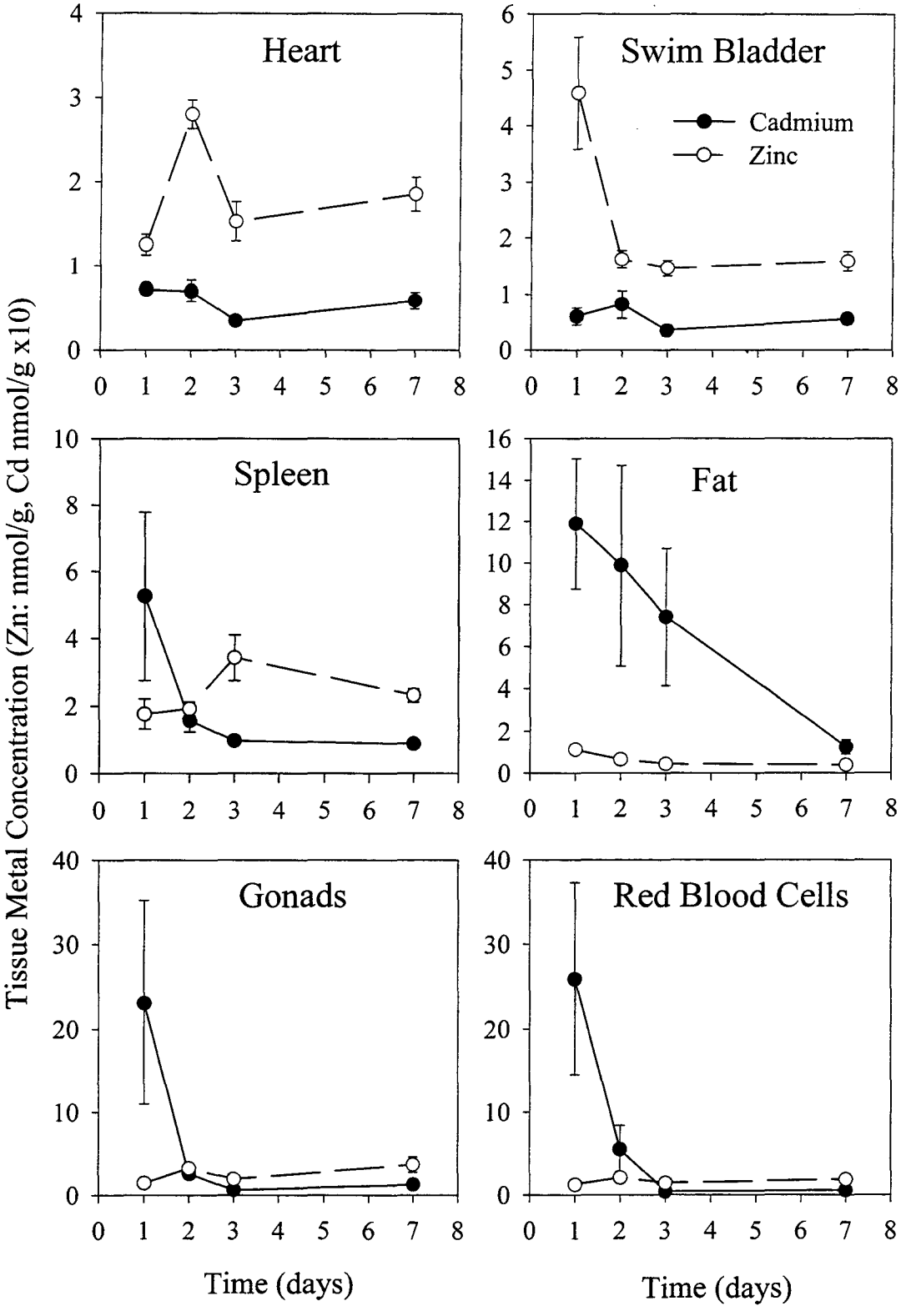

Gills
Kidney
Liver
Plasma
Bile
Carcass
Cadmium
Zinc
Tissue Metal Concentration (Zn: nmol/g, Cd: nmol/g x10)
Time (days)
Time (days)

Figure 8.

The fate of Cd^{109} and Zn^{65} in the remaining compartments of rainbow trout over time following exposure to 0.5 mM single oral doses. Note that Cd concentrations on the y-axis are multiplied by 10 to make trends more apparent. Temperature = 15°C. Means ± SE. n=6.

--●-- Cadmium
--○-- Zinc


Heart
Swim Bladder
Cadmium
Zinc
Spleen
Fat
Gonads
Red Blood Cells
Tissue Metal Concentration (Zn: nmol/g, Cd nmol/g x10)
Time (days)
Time (days)

Redistribution of Internal Metal During the Slow Phase

During the slow phase, there was no change in zinc or cadmium content in the internal compartment (Fig. 2C). However, there were changes that occurred within individual tissues (Fig. 7, 8). Plasma showed the most notable change with zinc concentration dropping from 10.8 to 2.8 nmol/g and cadmium from 1.15 to 0.03 nmol/g during the first 2 days of the slow phase (Fig. 7). Zinc content of gills, kidney, liver and bile did not change. In contrast, cadmium concentrations in the kidney and gills decreased while the liver and bile concentrations were constant (Fig. 7). Zinc concentrations in the carcass fluctuated, while cadmium concentrations dropped off in the first 3 days, then stabilized. There was also a large linear decrease in the concentration of Cd in the structural fat layer (the fat surrounding the internal organs)(Fig. 8D). This layer may also have been contaminated, however. Heart, swim bladder, spleen, gonads and red blood cells showed no change in zinc or cadmium concentration during the slow phase (Fig. 8).

Tissue Exchangeability

Since zinc concentrations were high in naive tissues, it was necessary to determine whether or not the exchange of naive zinc for newly absorbed radiolabelled zinc was a complicating factor in examining the tissue distribution and redistribution. To assess the extent of tissue exchangeability, the tissue concentrations of radiolabelled zinc after 72 h (the time to reach stabilization in most tissues) were plotted in relation to the naive tissue zinc concentrations (Fig. 9). The key tissues (liver, kidney, gills, plasma)

only exchanged 1.5-2.5% of their naive zinc. Distal intestine, proximal intestine and fat also exchanged >1% of their zinc pools. Gonads showed the lowest degree of exchangeability (0.07%), though they had the second-highest concentration of zinc in naive tissue. All other tissues exchanged between 0.1% and 1% of their pools. This plot illustrates that the tissues with the highest naive zinc concentrations were not necessarily those that exchanged or accumulated the majority of new zinc from the diet.

Though cadmium concentrations in naive tissues were low and not likely a concern for exchangeability, they were plotted in the same manner as zinc as a comparison (Fig. 10). This method allowed for the further identification of those tissues that had a greater ability to exchange or accumulate cadmium. The proximal and distal intestines and the structural fat (although this may have been contaminated) showed the greatest accumulation of cadmium with concentrations 10 fold higher than the naive values (40, 21, and 15 fold, respectively). Mid-intestine and stomach accumulated cadmium at 3 and 1.5 fold, respectively, higher concentrations than the naive cadmium levels. All other tissues accumulated less than the naive levels, although, of the remaining tissues, the tissues outlined earlier (liver, kidney, gills, plasma) accumulated the greatest proportion of cadmium.

Binding of Zinc and Cadmium to Internal Tissues

Following *in vivo* dosing, the bioavailability of zinc was 20-30%, while that of cadmium was only 1-3%. These bioavailability values remained constant with higher doses up to 25 mM for zinc and up to 50 mM for cadmium. However, at higher doses,

Figure 9.

A graphical illustration of the exchangeability of zinc tissue pools upon exposure to radiolabelled zinc in the diet. The diagonal lines indicate the relative exchangeabilities of each tissue. Those tissues to the left are more highly exchangeable and those to the right are more unexchangeable. The percentages indicate the portion of naive zinc that was exchanged for new radiolabelled zinc. 100% represents a complete turnover of the zinc pool in the compartment upon exposure.

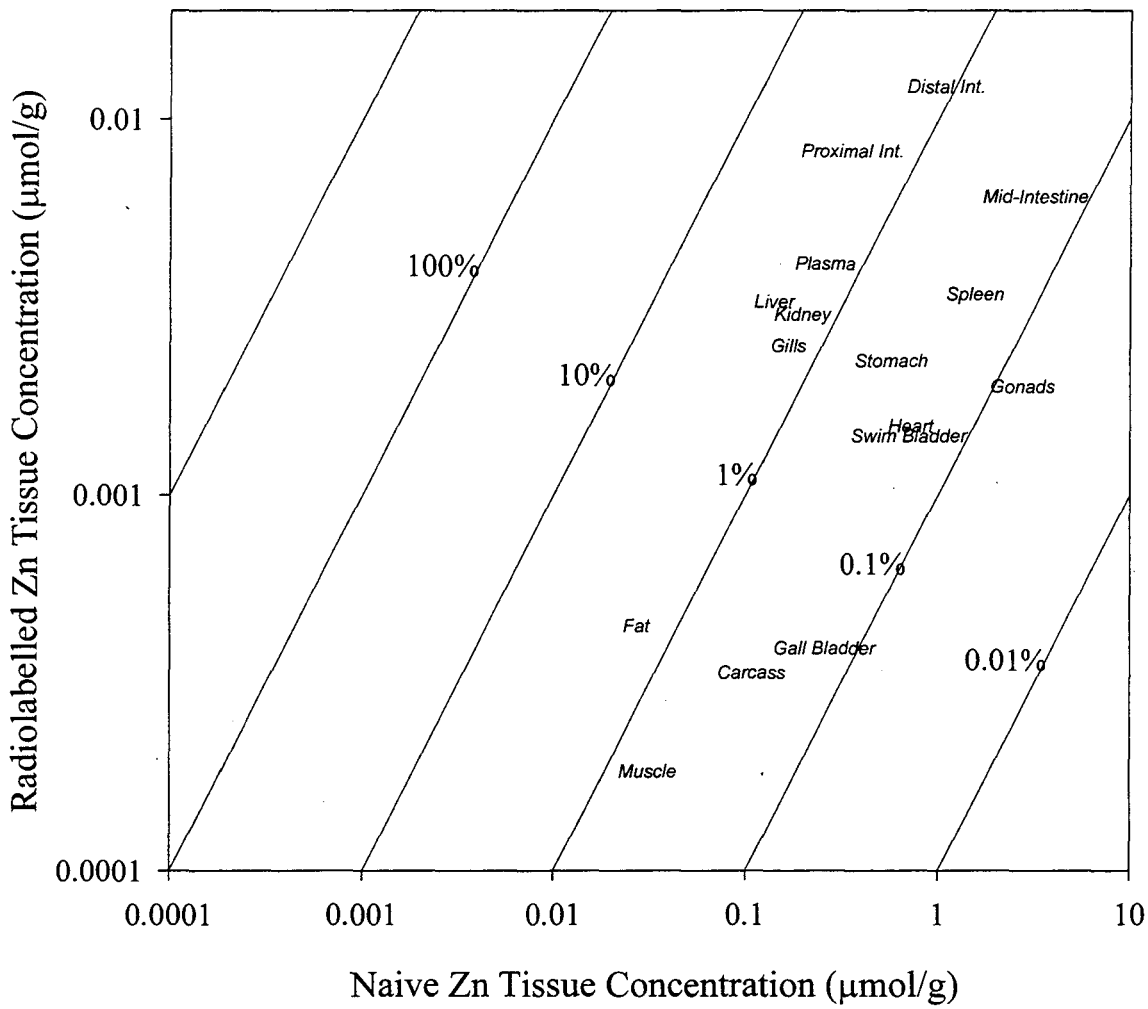
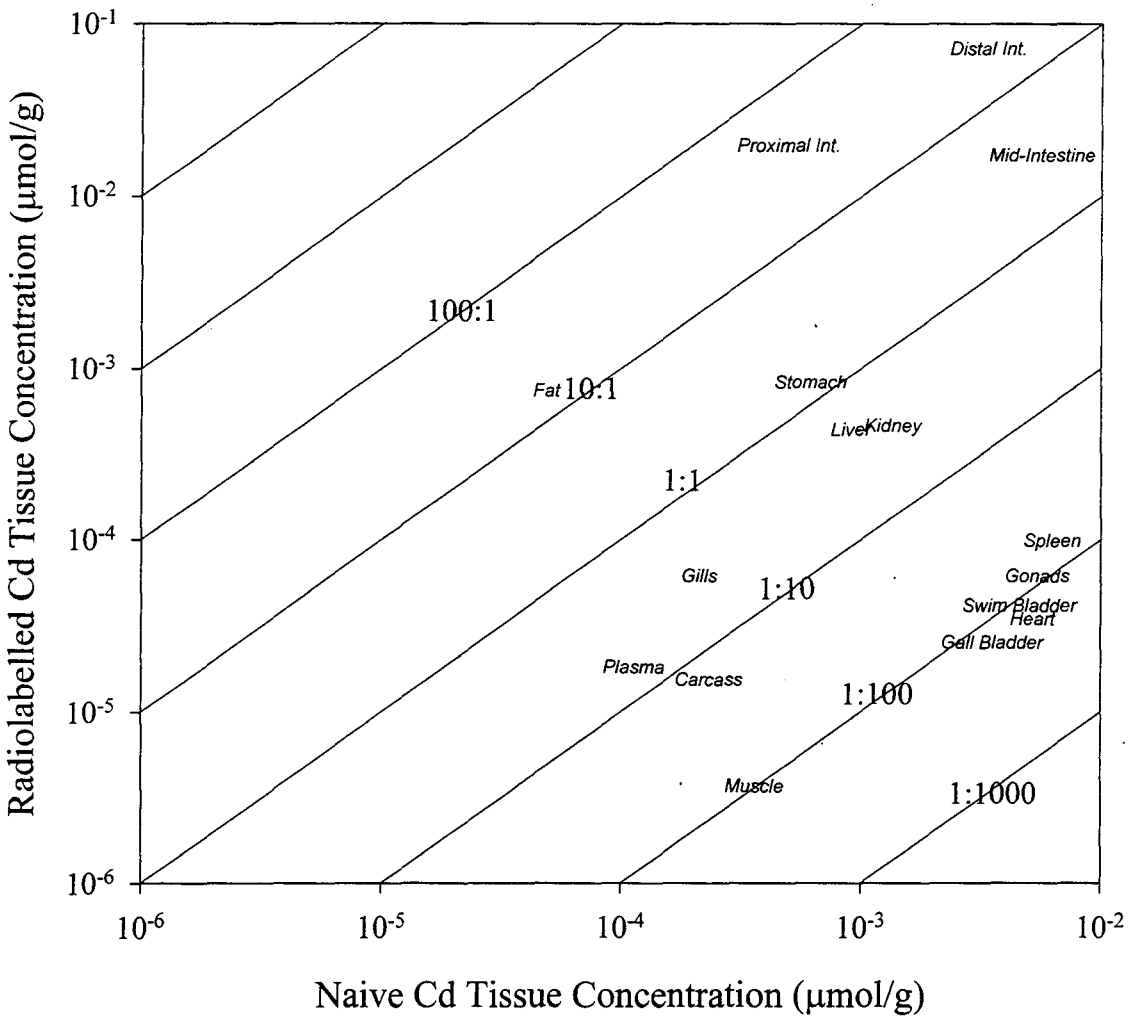

Radiolabelled Zn Tissue Concentration (µmol/g)
0.01
0.001
0.0001
0.0001
0.001
0.01
0.1
1
10
Naive Zn Tissue Concentration (µmol/g)
100%
10%
1%
0.1%
0.01%
Distal Int.
Proximal Int.
Mid-Intestine
Plasma
Liver
Kidney
Spleen
Gills
Stomach
Gonads
Heart
Swim Bladder
Fat
Gall Bladder
Carcass
Muscle

Figure 10.

A graphical illustration of the exchangeability and accumulation of cadmium in tissues upon exposure to radiolabelled cadmium in the diet. The diagonal lines indicate the relative amount of new cadmium in the tissue, with respect to the levels present in naive fish. Those tissues to the left are more highly exchangeable or accumulate cadmium and those to the right are more unexchangeable.


Radiolabelled Cd Tissue Concentration (µmol/g)
Naive Cd Tissue Concentration (µmol/g)
10-1
10-2
10-3
10-4
10-5
10-6
10-6
10-5
10-4
10-3
10-2
Distal Int.
Proximal Int.
Mid-Intestine
100:1
Fat
10:1
Stomach
Liver
Kidney
1:1
Spleen
Gills
1:10
Gonads
Swim Bladder
Heart
Gall Bladder
Plasma
Carcass
1:100
Muscle
1:1000

there were differences between the handling of zinc and cadmium in specific tissues (Fig. 11, 12). The most significant observation was that at zinc doses greater than 5 mM, the gills, liver, kidney, plasma and carcass all appeared to saturate (Fig. 11). In contrast, bile continued to concentrate zinc and, in fact, zinc concentrations in the bile rose abruptly following the saturation of internal tissues. In the case of cadmium, however, no tissues reached saturation at high oral exposures, except the gills (Fig. 12). The concentration of cadmium in the plasma also increased at high exposure concentrations.

Again, cadmium doses beyond 50 mM resulted in the death of all fish within 48 h following exposure, so it could not be determined whether or not the internal tissues could be saturated by cadmium.

Differences Between Fed and Starved Fish

Of the fish that were fed following the dose of metal, only 50% were determined to have eaten the pellet food, based on the presence of food within the GI tract and whether or not the gall bladder was empty upon dissection. These fish that had not eaten were grouped with the fish that were not fed, as the tissue metal concentrations were not statistically different for either metal. Table 3 indicates that there were also no differences between the individual tissue metal burdens of fed and starved fish for either zinc or cadmium. However, since feeding occurred during the slow phase of redistribution, it is not known whether or not there is an effect of food on the uptake of zinc or cadmium during the fast phase.

Figure 11.

Tissue distribution of Zn^{65}, 7 days following oral doses of 0.5, 5, 12.5 and 25 mM Zn at 15°C *in vivo*. Temperature = 15°C. Means ± SE.

--●-- Liver.
--○-- Bile.
--▼-- Kidney.
--▽-- Gills.
--■-- Plasma.
--□-- Carcass.

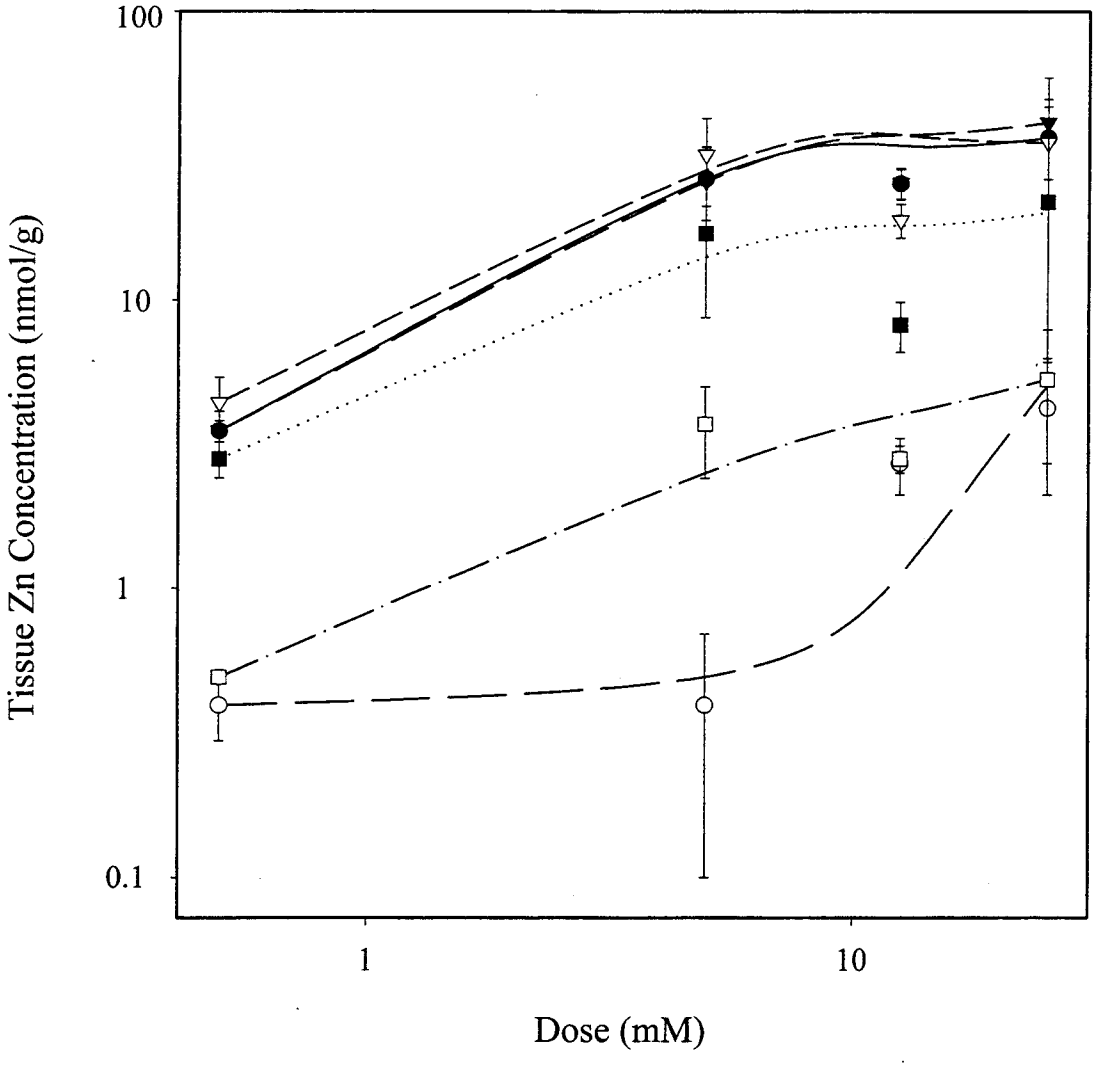

100
10
1
0.1
Tissue Zn Concentration (nmol/g)
1
10
Dose (mM)

Figure 12.

Tissue distribution of Cd^{109}, 7 days following oral doses of 0.5, 5, and 50 mM Cd at 15 °C *in vivo*. Temperature = 15 °C. Means ± SE.

--●-- Liver.
--○-- Bile.
--▼-- Kidney.
--▽-- Gills.
--■-- Plasma.
--□-- Carcass.

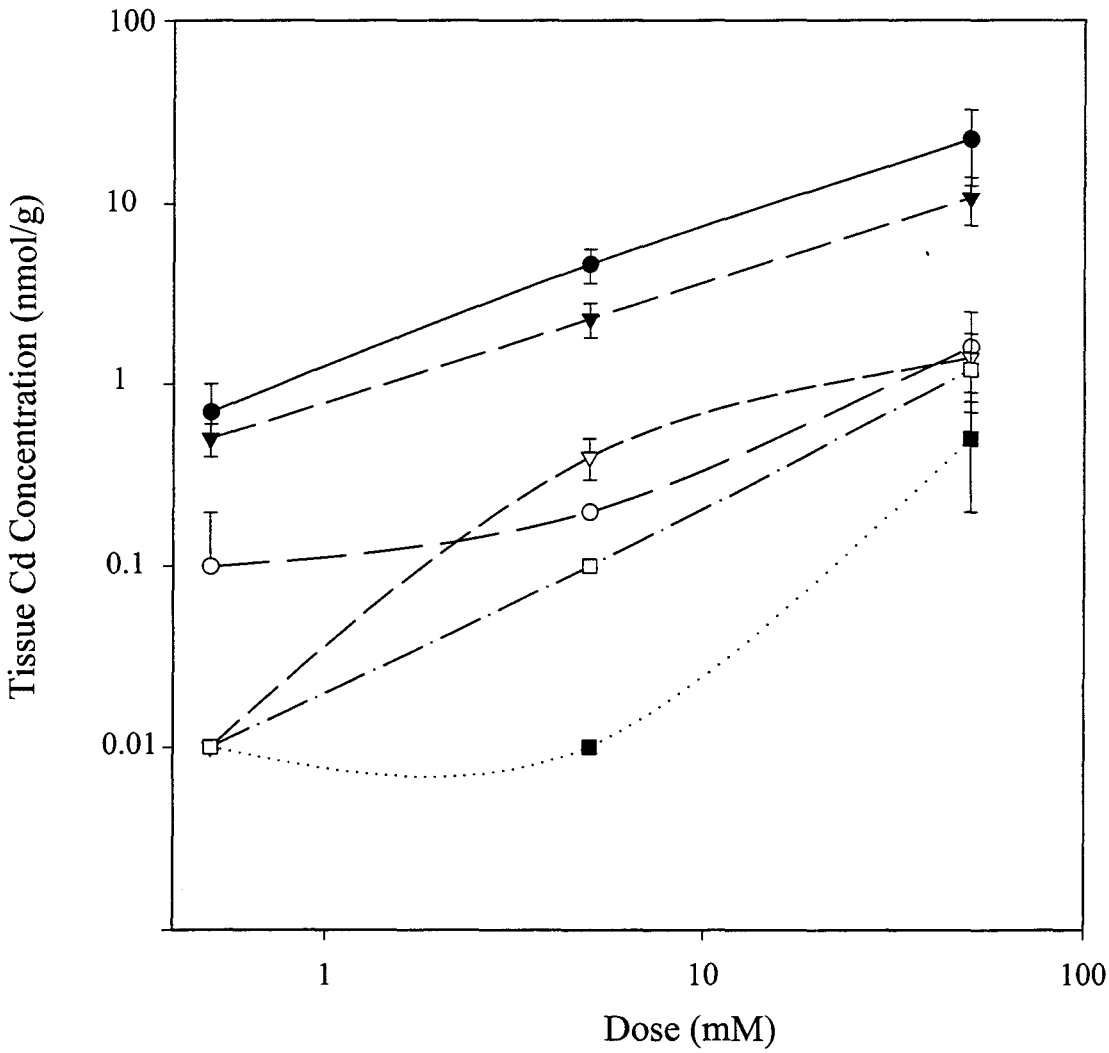

100
10
1
0.1
0.01
Tissue Cd Concentration (nmol/g)
1
10
100
Dose (mM)

Table 3.

The effects of feeding post-infusion on Zn^{65} and Cd^{109} absorption after 72 h. 0.5 mM infusion of zinc or cadmium was given prior to removal of catheters and feeding of fish. Feeding occurred during the day of infusion and two days following. Temperature = 15°C. Means ± SE. n=5-6.

Tissue	Zinc Unfed Fish (nmol/g)	Zinc Fed Fish (nmol/g)	Cadmium Unfed Fish (nmol/g)	Cadmium Fed Fish (nmol/g)
Liver	3.27 ± 0.60	3.22 ± 0.42	0.439 ± 0.096	0.463 ± 0.066
Stomach	2.27 ± 0.36	2.55 ± 0.25	0.828 ± 0.138	0.737 ± 0.311
Proximal Intestine	8.19 ± 1.66	10.71 ± 2.07	19.670 ± 4.723	12.525 ± 1.801
Mid-Intestine	6.18 ± 1.31	7.21 ± 1.05	17.294 ± 5.614	11.951 ± 2.527
Distal Intestine	12.15 ± 3.22	10.64 ± 2.68	71.245 ± 7.531	68.863 ± 18.606
Gall Bladder + Bile	0.39 ± 0.06	0.85 ± 0.36	0.025 ± 0..007	0.158 ± 0.087
Gills	2.49 ± 0.43	3.76 ± 0.52	0.061 ± 0.005	0.051 ± 0.004
Kidney	3.02 ± 0.38	3.87 ± 0.64	0.464 ± 0.093	0.480 ± 0.061
Plasma	2.53 ± 0.28	4.11 ± 1.03	0.018 ± 0.003	0.015 ± 0.005
Red Blood Cells	1.46 ± 0.14	1.99 ± 0.51	0.035 ± 0.010	0.031 ± 0.009
Muscle	0.18 ± 0.02	0.25 ± 0.04	0.004 ± 0.001	0.004 ± 0.001
Spleen	3.42 ± 0.68	3.04 ± 0.41	0.984 ± 0.011	0.107 ± 0.012
Heart	1.53 ± 0.23	1.89 ± 0.33	0.035 ± 0.006	0.030 ± 0.006
Structural Fat	0.45 ± 0.09	0.49 ± 0.08	0.741 ± 0.328	0.933 ± 0.533
Carcass	0.34 ± 0.03	0.42 ± 0.06	0.015 ± 0.002	0.015 ± 0.002
Gonads	1.94 ± 0.32	1.68 ± 0.39	0.061 ± 0.028	0.135 ± 0.087
Swim Bladder	1.44 ± 0.31	1.62 ± 0.28	0.041 ± 0.010	0.145 ± 0.060

In Vitro Metal Exposures

The Role of GI Mucus in Metal Absorption

To assess what role mucus might play in the movement and uptake of zinc and cadmium in the gut, an analysis of the metal concentration within mucus was done *in vitro*. To examine this binding, mucus was removed by gentle scraping from the mid-intestine lumen following exposure to metal. It was assumed that only mucus was removed due to the care taken in the removal process, although it is possible that other debris and some intestinal cell remnants were included in this scraping. A large amount of mucus was removed (approximately 35% of the mass of the tissue). The average mucus mass was 36.6 ± 3.9 mg in Zn treated mid-intestines and 38.3 ± 3.1 mg in those that were Cd treated. There may also have been a thin layer of mucus remaining attached to the gut tissue following this procedure, since mucus can be present in intestinal infoldings and between projections of the intestinal cells.

Following 2 h exposure to 10 mM Zn, the zinc concentration of mucus in the mid-intestine was significantly greater than the mucus concentrations of zinc in the recovered free fluid (Table 4). However, with the addition of Ca in either 1 mM or 10 mM concentrations, the mucus concentrations of zinc were no longer significantly different from the metal concentration in the free fluid. In contrast, the cadmium concentration in the mucus was not different from the concentration of cadmium within the free fluid after 2 h exposure to 10 mM Cd. The addition of Ca (either 1 or 10 mM) to the mucosal solution had no effect on the mucus concentration of Cd (Table 4).

Table 4.

The effects of calcium competition and 2,4-DNP on the binding of Cd^{109} and Zn^{65} in mid-intestine mucus *in vitro*. Cd^{109} or Zn^{65} (10 mM) was infused with either 1 mM Ca^{+2}, 10 mM Ca^{+2}, or 1 mM DNP. Controls were infused with 10 mM metal only. Average mucus masses: Cd-38.3 ± 3.1 mg, Zn-36.6 ± 3.9 mg. Average tissue masses: Cd-111.7 ± 6.0 mg, Zn-114.7 ± 6.6 mg. Temperature = 15 °C. Means ± SE. Paired controls.
* indicates significant difference between mucus and rinse concentrations $p<0.05$.

	Treatment					
	Control	1 mM Ca^{+2}	Control	10 mM Ca^{+2}	Control	1 mM DNP
Cadmium						
in Mucus (µmol/g)	6.14 ± 0.46	6.63 ± 1.15	5.61 ± 0.78	5.59 ± 0.99	6.54 ± 1.61	5.01 ± 1.18
in Rinse (mM)	6.33 ± 0.80	5.26 ± 0.67	5.93 ± 0.69	5.81 ± 0.50	5.00 ± 0.50	5.61 ± 0.31
Zinc						
in Mucus (µmol/g)	8.15 ± 1.26 *	7.34 ± 0.96	8.11 ± 0.89 *	4.58 ± 1.33	9.75 ± 1.58 *	10.52 ± 2.89
in Rinse (mM)	5.45 ± 0.75	6.44 ± 0.88	4.25 ± 0.18	5.44 ± 0.80	5.54 ± 0.50	5.67 ± 0.28

In Vitro Gut Binding

Gut tissue concentrations of Zn and Cd and their patterns of distribution between the gut compartments were quite different *in vitro* compared to *in vivo* (Table 5). The most notable difference was that the mid-intestine bound the most zinc and cadmium *in vitro*, while the distal intestine had the highest concentrations *in vivo*. Also, *in vitro*, zinc binding to the gut tissue was 3-4 fold higher than cadmium binding, in contrast to *in vivo*, where Cd binding was higher at this concentration. After 4 hr *in vitro* exposure to 1.5 mM zinc or cadmium, the mid-intestine bound the most zinc (846 nmol/g) and cadmium (279 nmol/g), followed by the distal intestine. Zinc binding to the distal intestine was 445 nmol/g, while that of cadmium was (124 nmol/g)(Table 5). The proximal intestine bound less zinc and cadmium with tissue concentrations of only 149 nmol/g and 51 nmol/g, respectively. Stomach tissue also bound only 91 nmol/g Zn and 76 nmol/g Cd. These concentrations were much higher than *in vivo* (Table 5). Concentrations of Zn in the proximal, mid- and distal intestine *in vitro* were approximately 10, 35, and 15 fold, respectively, higher than *in vivo*. Cadmium levels in these compartments *in vitro* were also higher than *in vivo* (2, 10, and 2 fold, respectively).

These differences may be explained by the fact that the exposure conditions were different, however this is unlikely to be the only reason for the differences. Given that the dilution of metal *in vivo* may have been as much as 50% (as suggested earlier), the *in vivo* dose (originally 0.5 mM) could have been as much as 6 fold lower than that of the *in vitro* exposures (1.5 mM). This may explain differences in metal tissue concentrations, but it would also assume that the proportion change would be constant among all gut

Table 5.

A comparison of *in vitro* to *in vivo* gut binding to the stomach, proximal intestine, mid-intestine, and distal intestine. *In vitro* gut binding was achieved over a 4 h exposure period with 1.5 mM zinc or cadmium (0.5 mL) to individual gut bags. *In vivo* exposures were for 24 h to a dose of 0.5 mM zinc or cadmium. Temperature = 14-15 °C. Means ± SE.

Tissue	Exposure			
	In vitro Zn (4 h, 1.5 mM)	*In vivo* Zn (24 h, 0.5 mM)	*In vitro* Cd (4 h, 1.5 mM)	*In vivo* Cd (24 h, 0.5 mM)
Stomach (nmol/g)	91 ± 10	3.0 ± 0.4	76 ± 7	1.0 ± 0.3
Proximal Intestine (nmol/g)	149 ± 59	10.6 ± 1.3	51 ± 17	34.9 ± 1.0
Mid-Intestine (nmol/g)	846 ± 221	13.0 ± 1.9	279 ± 52	24.8 ± 1.6
Distal Intestine (nmol/g)	445 ± 77	28.6 ± 7.4	124 ± 18	40.5 ± 13.3

tissues. Clearly the metal concentrations within the mid-intestine showed the greatest difference between 4 h and 24 h for both zinc and cadmium. The stomach also showed a great decrease in metal concentration from 4 h to 24 h, although these differences were more likely due to differences in the amount of time metal spent in the lumen *in vivo*. As stated earlier, it was assumed that the stomach passed the infused metal very rapidly to the intestine. Thus a 4 h exposure to metal *in vitro* could exaggerate the binding of metal to the stomach.

Transepithelial Uptake of Zinc and Cadmium

In vitro results (Fig. 13, 14) indicated that the greatest transepithelial uptake of both Zn and Cd occurred in the mid-intestine at a rate of 1.27 nmol/g/min (r^2=0.975) for Zn and 1.45 nmol/g/min (r^2=0.962) for Cd. The distal intestine had transepithelial uptake rates of 0.50 nmol/g/min (r^2=0.999) for Zn and 0.35 nmol/g/min (r^2=0.989) for Cd. The proximal intestine showed very little capability to transfer either zinc or cadmium with uptake rates of only 0.21 nmol/g/min (r^2=0.973) for Zn and 0.10 nmol/g/min (r^2=0.965) for Cd. Neither metal was taken up to any extent by the stomach.

Uptake across the brush border membrane was illustrated by the binding of metal to the tissue in Figure 15A. Results indicated that uptake across the brush border was a saturable process for both zinc and cadmium. However, zinc uptake was saturated at a much higher tissue concentration (2.5 μmol/g), than cadmium (1 μmol/g). The concentration that caused saturation was 10 mM for both metals. Figure 15B illustrates that transepithelial uptake of both zinc and cadmium was also saturable. Zinc (1.14 ±

Figure 13.

In vitro uptake rates for 1.5 mM Zn^{65} over 4 h flux period for the different segments of the gastrointestinal tract of rainbow trout in oxygenated Cortland's saline. Means ± SE. Temperature = 15 °C.

--●-- Stomach, n=10.
--○-- Proximal Intestine, n=4.
--▼-- Mid-Intestine, n=7.
--▽-- Distal Intestine, n=8.

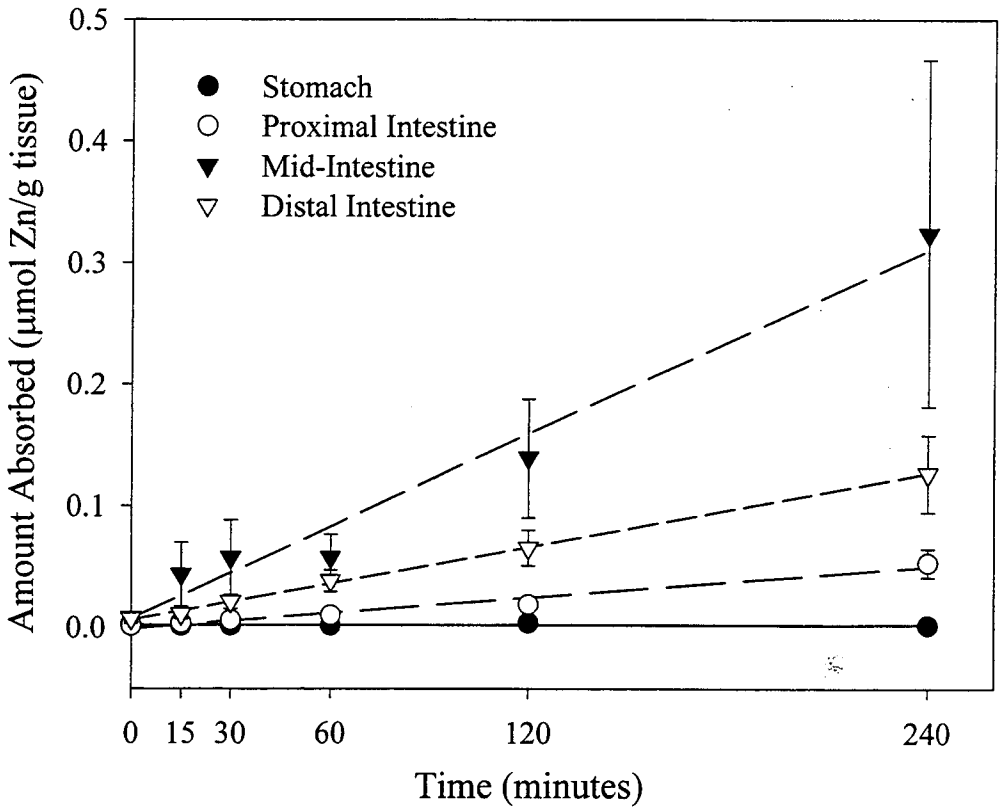
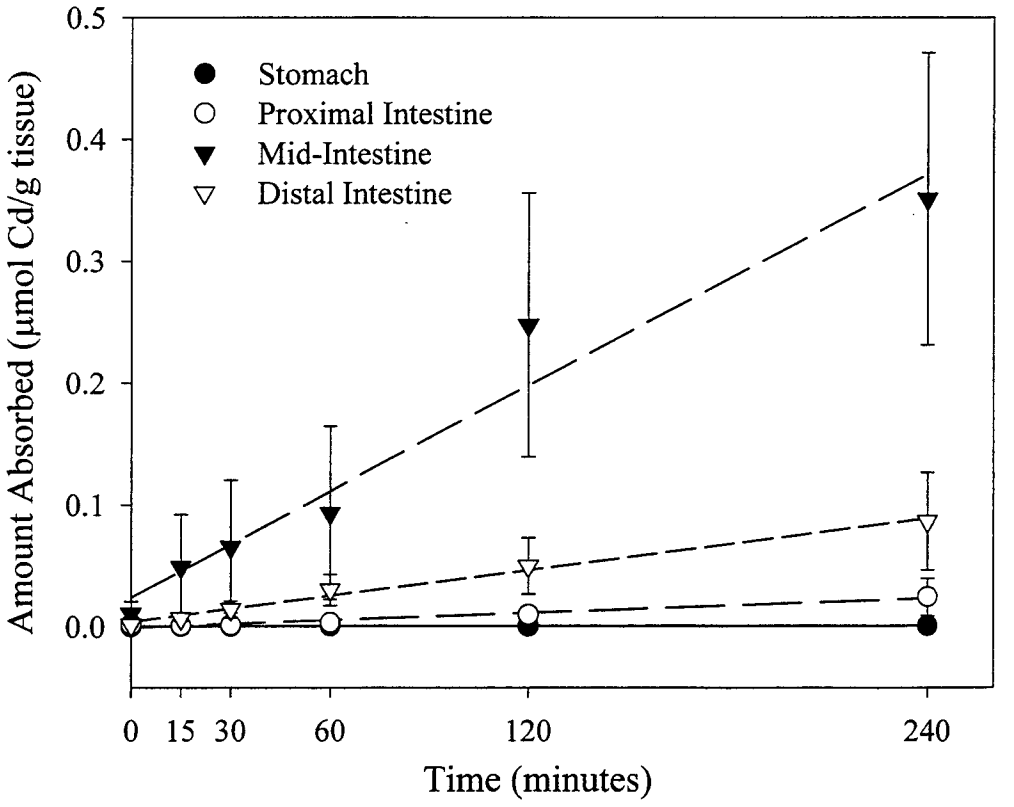

0.5
0.4
0.3
0.2
0.1
0.0
Amount Absorbed (µmol Zn/g tissue)
Stomach
Proximal Intestine
Mid-Intestine
Distal Intestine
0
15
30
60
120
240
Time (minutes)

Figure 14.

In vitro uptake rates for 1.5 mM Cd^{109} over 4 h flux period for the different segments of the gastrointestinal tract of rainbow trout in oxygenated Cortland's saline. Means ± SE. Temperature = 15 °C.

--●-- Stomach, n=6.
--○-- Proximal Intestine, n=6.
--▼-- Mid-Intestine, n=4.
--▽-- Distal Intestine, n=5.


0.5
0.4
0.3
0.2
0.1
0.0
Amount Absorbed (µmol Cd/g tissue)
Stomach
Proximal Intestine
Mid-Intestine
Distal Intestine
0
15
30
60
120
240
Time (minutes)

0.37 nmol/mm^2/min) and cadmium (1.16 ± 0.07 nmol/mm^2/min) uptake saturated at similar rates, however, saturation of zinc transport did not occur until the exposure concentration was beyond 15 mM, while cadmium saturation occurred at 10 mM exposures.

The addition of either 1 mM $CaCl_2$ or 10 mM $CaCl_2$ to 10 mM Zn in the mid-intestine gut bag had no significant effect on the transfer of zinc across the brush border membrane (Table 6). Transepithelial uptake of Zn was also unaffected by 1 mM $CaCl_2$, although transepithelial transport was increased nearly 3 fold in the presence of 10 mM $CaCl_2$. Calcium had no effect on the transepithelial uptake of Cd at either 1 mM or at 10 mM $CaCl_2$. Brush border uptake of Cd was also unaffected at both calcium concentrations as indicated by tissue cadmium concentrations.

It was observed that the yellow 2,4-dinitrophenol (DNP) solution was able to cross intestinal membranes successfully as it was infused into the mucosal solution and appeared in the serosal bath. The presence of 1 mM DNP in the mucosal solution did not alter the brush border uptake of either zinc or cadmium (Table 6). However, the transepithelial transport rate of zinc was reduced from 1.4 to 0.8 nmol/mm^2/min in the presence of DNP. The rate of cadmium transepithelial uptake was decreased 5 fold from 1.52 to 0.30 nmol/mm^2/min.

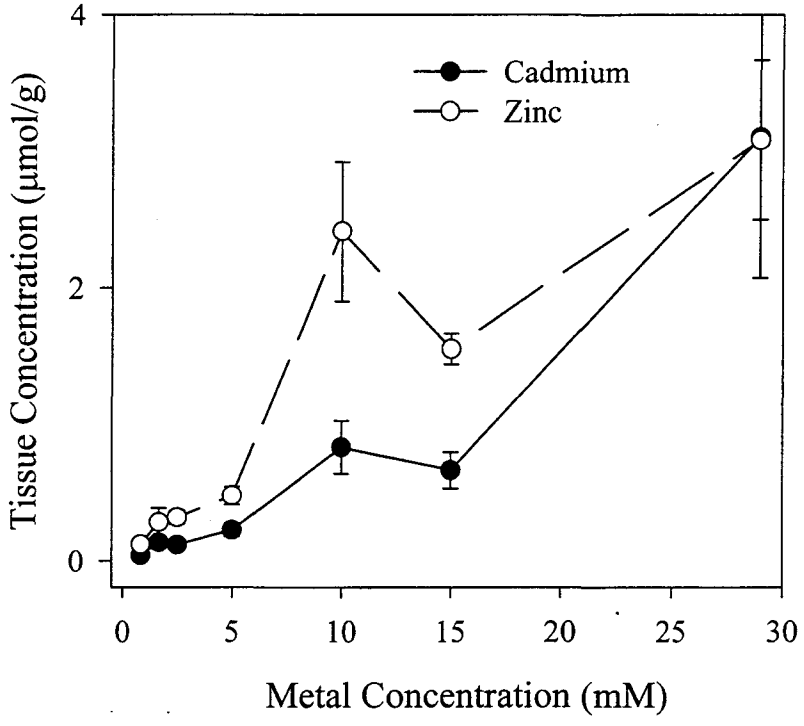
Figure 15.

A. A comparison of the mid-intestine tissue concentrations following 2 h *in vitro* exposure to concentrations of Cd^{109} and Zn^{65} ranging from 0.83 to 28 mM. Temperature = 15 °C. Means ± SE.

B. *In vitro* transepithelial uptake rates of Cd^{109} and Zn^{65} in the mid-intestine at the same exposure concentrations as above. Temperature = 15 °C. Means ± SE.

--●-- Cadmium
--O-- Zinc

A.


Cadmium
Zinc
Tissue Concentration (µmol/g)
Metal Concentration (mM)
0
2
4
0
5
10
15
20
25
30

B.

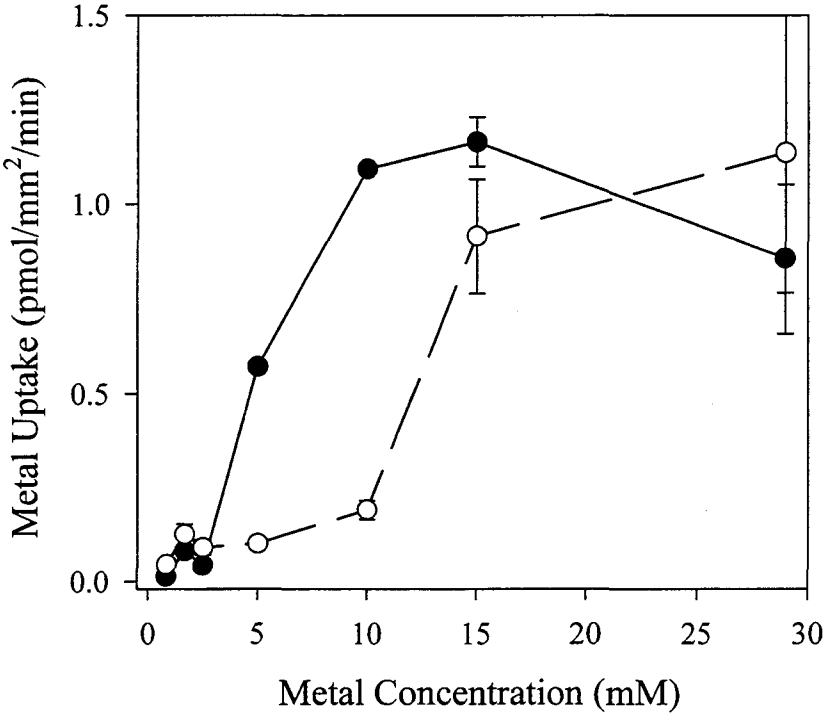

Metal Uptake (pmol/mm^2/min)
Metal Concentration (mM)
0.0
0.5
1.0
1.5
0
5
10
15
20
25
30

Table 6.

The effects of calcium competition and 2,4-DNP on uptake by the mid-intestine and the transepithelial transport of Cd^{109} and Zn^{65} *in vitro*. Uptake into the mid-intestine (brush border membrane transport) is shown as tissue concentration of metal. Cd^{109} or Zn^{65} (10 mM) was infused with either 1 mM Ca^{+2}, 10 mM Ca^{+2}, or 1 mM DNP. Controls were infused with 10 mM metal only. Temperature = 15 °C. Means ± SE. Paired controls. * indicates significant difference between control and treatment transport rates $p<0.05$.

	Treatment					
	Control	1 mM Ca^{+2}	Control	10 mM Ca^{+2}	Control	1 mM DNP
Cadmium						
in Tissue (μmol/g)	0.88 ± 0.18	1.05 ± 0.45	0.57 ± 0.14	1.03 ± 0.25	1.38 ± 0.55	1.13 ± 0.33
Transport ($pmol/mm^2/min$)	2.26 ± 0.16	1.71 ± 0.21	1.04 ± 0.18	1.12 ± 0.21	1.52 ± 0.12 *	0.30 ± 0.05
Zinc						
in Tissue (μmol/g)	0.76 ± 0.19	1.23 ± 0.46	0.89 ± 0.46	1.27 ± 0.60	0.83 ± 0.31	1.16 ± 0.20
Transport ($pmol/mm^2/min$)	2.86 ± 0.51	2.26 ± 0.39	1.09 ± 0.34	2.81 ± 0.84	1.40 ± 0.31	0.83 ± 0.11

DISCUSSION

In this thesis I have identified a number of aspects of the dietary uptake of zinc and cadmium and their internal distribution that were previously unknown in any fish species. These findings pertain to the following issues: the rate of movement of metals along the gut and the role of mucus in this movement, regional differences in the binding of metals to gut tissues, the relationship between gut metal binding and the transfer of metals to internal tissues, the specific mechanisms of gut binding and transepithelial transfer, and the specific tissues to which metals distribute to upon initial absorption and subsequent redistribution. In the discussion that follows, I propose descriptive models for each of these issues. In some instances, the behavior of Zn and Cd have been found to be quite similar while in others they are quite different. The differences undoubtedly reflect the differences in chemistry, toxicity and essentiality between the two metals. However, the similarities are perhaps more important, as they may well indicate metal handling properties in fish that are generic; i.e. properties that may well apply to a host of other metals such as Cu, Pb or Ag.

The Role of Mucus in Metal Movement Along the GI Tract

There is evidence from our *in vitro* work that the layer of mucus within the gut is able to bind both Zn and Cd, but does not have a high capacity for Cd as suggested by other authors (Noël-Lambot, 1981). In a review of the mammalian literature (see Powell

et al, 1999) it was suggested that mucus binds to metals and only those that are easily absorbed by intestinal cells are able to move across the mucus layer, while those that are toxic are bound more strongly. This suggestion is also supported by our *in vitro* work that showed displacement of zinc from the mucus in the presence of calcium, but no displacement of cadmium under the same conditions. Epithelial mucus of rainbow trout has been shown by Pärt and Lock (1983) to have a much higher affinity for Cd than Ca with dissociation constants (K_d) of 0.95 and 15 μM, respectively. Powell *et al* (1999) rank the mucus affinity for Zn to be higher than Ca in mammalian intestines, but Cd is not ranked. From our work, it is suggested that the rank order of affinities is Cd >Zn >Ca in rainbow trout intestinal mucus. Since the gut of freshwater rainbow trout has been shown to contain 2 mM Ca^{+2} in the luminal contents under starved conditions (Shehadeh and Gordon, 1969), competition for binding sites within the mucus *in vivo* may be very important in determining the amount of zinc and cadmium bound to mucus.

Even if metals are not concentrated in the mucus layer, mucus serves to bind metals and slow their movement along the gut due to its negative charges and viscosity. Guth and Engelhardt (1989) have shown that mucus in the gut of mammals is 50% less permeable to Na^+ and K^+ ions than a saline solution. This is likely true for metals and other cations also. Given that cadmium is retained in the lumen to a greater extent than zinc, it is likely that differences in the binding affinities to the mucus layer affect the rate of movement of ions within the layer.

There are other possible explanations for the differences in the rates of Cd and Zn movement along the gut lumen. Cadmium has been shown to cause an increase in mucus

cell activity in the intestine of fish (Gardner and Yevich, 1970; Crespo *et al*, 1986), which may account for the increased amount of bound Cd in the GI tract *in vivo*. This was not measured, however. If more mucus is produced in the presence of Cd, it is reasonable to assume that more Cd would be retained in the lumen as a consequence. *In vitro* results in the present study indicate that there was no difference in mucus mass between Zn and Cd at 10 mM metal concentrations in the mid-intestine. It is possible, however, that these concentrations elicited the maximal mucus production, since these concentrations approached tissue uptake and transepithelial transport saturation levels also.

There may also be differences in the nature of the mucus between different segments of the GI tract as suggested by Noël-Lambot (1981) in the eel (*Anguilla anguilla*). The distal intestine lumen was the region where Cd was bound most strongly in the intestine. This may be related to differences in mucus composition within the gut. Noël-Lambot (1981) showed that Cd and Ca concentrations in the distal mucus were approximately 4 and 9 times greater, respectively, than in the anterior gut mucus, however he did not measure differences in the composition of mucus in the two compartments.

The presence of Cd in the gut lumen after 7 days could also be attributed to excretion of the metal by the intestinal cells. Several authors have shown this to be an important means of ridding the body of excess metals in fish (Haesloop and Schirmer, 1985; Harrison and Klaverkamp, 1987) and mammals (Andersen, 1989). However, *in*

vivo gut tissue metal burdens are constant over the period from 3-7 days in the present study, indicating that this is likely not the case.

There is some evidence from the present study that would indicate that fish intestinal mucus is able to bind intestinal metals as shown by other authors (Noël-Lambot, 1981). Whether mucus is able to concentrate metals in the intestine is still unclear but it appears that binding of metals impedes their transit down the intestine. More work clearly needs to be done in this area to determine the relative binding affinities of different metals and ions within the GI tract of fish *in vivo* and to examine the role of mucus in protection against toxic metals in fish.

Regional Binding of Cd and Zn Along the GI Tract

The binding of metals to the gastrointestinal tissue may serve as a means of sequestering metal so that it may be excreted or sloughed off during renewal of the gut mucosa layer. However, the uptake of metals by the gut tissue is also an intermediary step in their transepithelial transport. Therefore, the tissue that binds the most metal may be the tissue with the greatest capacity to transport the metal across the gut, or it may be the tissue with the greatest role in sequestering metals to protect the organism.

In vivo results have shown that in comparison to the 3 intestinal compartments, the stomach only binds a relatively small amount of cadmium or zinc under low metal exposure concentrations. There is however, *in vivo* evidence, that the stomach can bind both Cd and Zn when exposed to higher concentrations or longer retention times in the lumen. It is not known why this occurs but it may be a result of cell damage incurred by

high levels of soluble metal ions in the lumen. At all concentrations, the stomach was able to bind more Zn than Cd.

In vitro, the mid-intestine bound the most metal but there was a very large difference to the tissue metal concentration *in vivo*. It could be hypothesized based on the high binding to the mid-intestine *in vitro* and much lower binding over a longer exposure *in vivo*, that binding within the mid-intestine occurs very rapidly and that binding is labile. The presence of a labile zinc or cadmium pool suggests that there is binding to a large number of transport proteins and smaller number of storage proteins. There is a much smaller difference between *in vitro* and *in vivo* concentrations of zinc and cadmium in the proximal and distal intestines. Based on these findings, it is more likely that within these tissues, there is more binding to non-labile storage proteins and less to transport proteins. *In vivo*, exposure to high doses of metal also resulted in the distal intestine binding the most metal. This is further evidence that the distal intestine may bind metals more strongly and thus contain more storage proteins. The intestinal tissues may therefore be ranked in order from the highest to the lowest ratio of storage proteins to transport proteins as follows: distal > proximal > mid-intestine.

Comparison of Zinc and Cadmium Binding to the Gut

Saturation of the gut compartments occurred at much lower tissue concentrations of cadmium than zinc. These differences can likely be attributed to a different complement of binding proteins for each metal within the intestinal cells. Metals can bind strongly to a number of proteins and amino acids within tissues, with a preference

for those that contain sulfhydryl groups (Konovalov, 1994). The most important of these binding proteins may be metallothionein, which is a low molecular weight protein containing approximately 30% cysteine groups (Konovalov, 1994, Ley *et al*, 1983). The high number of cysteine groups gives metallothionein a very high binding capacity for metals (Ley *et al*, 1983). Since there are high levels of metallothionein (MT) in the intestine (Hodson, 1988; Shears and Fletcher, 1984), it would be expected that much of the binding of metals within these cells could be attributed to binding to this protein. However, the differences in binding capacity of the intestinal tissues in the present study cannot be explained based solely on the presence of MT in the intestinal cells, due to the fact that Cd has a higher affinity for MT than Zn (Eaton, 1985). This would likely result in the displacement of native zinc from MT upon exposure to cadmium. The reason for the higher binding capacity of the intestinal tissue for zinc in rainbow trout may be related to the findings of Shears and Fletcher (1979) who demonstrated that there was more than one binding system for zinc in the cytosol of intestinal cells in winter flounder and that these systems had a high capacity to bind zinc. Zinc could also not be displaced from these binding sites by a number of different metals including cadmium (Shears and Fletcher, 1979). This suggests that there may be more binding sites within the intestinal cytosol for essential metals such as zinc, and as a result, a higher binding capacity for zinc as compared to cadmium in rainbow trout.

Regional Absorption of Cd and Zn Along the GI Tract

The rapid absorption of zinc and cadmium over the first 24 h of *in vivo* exposure suggests that, since only the stomach lumen was emptied during that time, the stomach was the major absorptive tissue in the gastrointestinal tract for metals. However, *in vitro* results did not support this. In fact, the transport of Cd and Zn into the serosal bath via the stomach was non-existent. Shears and Fletcher (1983) have also shown that the stomach plays a very small role in the absorption of zinc in winter flounder. This is also true in rats (Van Campen and Mitchell, 1965).

Therefore, the absorption of metals must occur in the intestine. It was shown that during the fast phase of distribution, there was movement of metals down the entire length of the intestine. It is not known exactly how quickly this absorption occurred since the first sampling period was at 24 h. This fast phase was also the most important for metal uptake into the body.

One detailed study of rainbow trout intestinal uptake has shown the proximal intestine to be the most important site for nutrient uptake (Buddington and Diamond, 1987). Shears and Fletcher (1983) also found that the pyloric region was the greatest site of uptake of zinc in the winter flounder (*Pseudopleuronectes americanus*). It has a very large surface area due to the pyloric caecae (Buddington and Diamond, 1987) and there is no evidence of differences in the histology of the proximal intestine and the mid-intestine, as shown by electron microscopy (Ezeasor and Stokoe, 1981). Based on these two findings alone, one could assume the proximal intestine to be the most important surface for absorption not only of nutrients, but metals also. However, my *in vitro*

findings contradict that view, suggesting that the mid-intestine is the most important tissue for the transfer of both Zn and Cd to the body on a per weight basis, followed by the distal intestine and finally the proximal intestine.

The decreases in tissue concentration of metal from 4 h *in vitro* to 24 h *in vivo* indicated that following a rapid uptake of metal by the gut tissues there was release into the bloodstream, and this release was greatest in the mid-intestine. There is also evidence that amino acids (Marcotte and De La Noüe, 1984) and glucose (Stokes and Fromm, 1964) are taken up most efficiently from the mid-intestine of rainbow trout. It is suggested here, then, that uptake of metals in rainbow trout is not necessarily related to the surface area of the intestinal tissue, but rather to regional specializations such as types and numbers of transporters, or leakiness of cell membranes. Other authors also suggest that regionalization of carriers and cell types for metal uptake exists within the intestine of fish (Handy,1999) and mammals (Davies, 1980; Tacnet *et al*, 1990; Kowarski *et al*, 1974).

Transepithelial transport of Cd and Zn

Results from the present *in vitro* work suggest a model that may help to fill some voids in the current knowledge of Zn and Cd intestinal uptake mechanisms in fish.

The information from this study leads us to believe that intestinal uptake of Zn and Cd fits into a 3-compartment model (1, lumen; 2, cell; 3, plasma) where the 3 compartments are separated by 2 membrane systems (brush border and basolateral).

Metal concentrations within each compartment are very different. A concentration gradient exists in which compartment $1 > 2 >> 3$.

The first compartment is the lumen in which the liquid contents (including soluble metal ions) appear to equilibrate with the mucus layer in the gut. This mucus layer may help to present metal ions to the intestinal layer for absorption or it may bind strongly to toxic metals and prevent their uptake by the intestinal cells. The role of mucus in metal absorption is not entirely clear as discussed earlier.

The first stage of intestinal absorption is the movement across the brush border membrane (BBM) of the intestinal cells. There was a very rapid uptake of both metals across the brush border membrane of the intestinal cells. Uptake by the tissue increased as exposure concentrations were raised until apparent saturation, however, luminal metal concentrations were 5-10 fold higher than those found in the tissue, implying that uptake by the tissue was selectively limited by the properties of the brush border membrane. The selectivity of the membrane appeared to favour zinc transport, as much more zinc entered the cells as compared to cadmium. It is not known what the mechanism of this selectivity is but it is implied that the carriers or other uptake mechanisms have a higher affinity, or at least higher capacity, for the essential metal, zinc, as compared to the non-essential metal, cadmium.

Within the intestinal cells, both metals were likely bound to transport and/or storage proteins as discussed earlier. A knowledge of the ratio of binding to these proteins may help to distinguish the differences between essential and non-essential metal uptake.

Given the slow rates of transepithelial transport demonstrated in these experiments, it is likely that either movement of the metal within the cytosol or transport across the basolateral membrane is the limiting rate. Shears and Fletcher (1983) also suggest that there is a very rapid transport of zinc into the intestinal cells and a much slower release into the body in winter flounder.

There is saturation of the basolateral transport mechanism for both Cd and Zn. This would suggest that the transport of metal across this membrane is either carrier-mediated or active transport. With the use of 2,4-dinitrophenol (DNP), which is a potent uncoupler of oxidative phosphorylation (Goodman Gilman *et al*, 1985), it was shown that transepithelial transport of Cd and Zn could be decreased significantly. Tissue accumulation of each metal was unchanged, suggesting that this blockage of transport occurred at the basolateral membrane. It would appear that Cd transport across the basolateral membrane was almost entirely ATP-driven given the amount of inhibition shown. Zinc, on the other hand, may have an active component and an ATP independent mechanism of transport, since only half of Zn transport could be blocked using 2,4-DNP.

It is possible, however, that neither metal was actively transported but that their transport relied on ionic or electrical gradients maintained by other ATP pumps such as Na^+/K^+ ATPase, as suggested by Raffaniello *et al* (1992) using mammalian Caco-2 cells. By inhibiting these pumps, it would appear that the transport mechanisms for Zn and Cd were ATP-driven, when in fact, they may not be at all.

The pathways described above may be unique for Cd and Zn, but it is very unlikely that intestinal cells would have transport mechanisms for Cd transport given its

toxic and non-essential qualities. Since Ca^{+2} transport in fish is thought to involve low affinity carrier mediated processes at the BBM and ATP reliant transport at the basolateral membrane (Flik and Verbost, 1993; Klaren *et al*, 1993), movement of Zn and Cd by this pathway cannot be ruled out from the above observations. In fact, Shoenmakers *et al* (1992) have suggested that Cd may be transported by a Na^{+}/Ca^{+2} exchanger in basolateral membranes of tilapia intestinal cells. However, our *in vitro* results showed that neither 1 mM nor 10 mM Ca in the lumen had an effect on Cd uptake into the tissue, or on transepithelial transport. Zinc transport was also unaffected by Ca at these concentrations. This suggests that Zn and Cd were not utilizing Ca transport mechanisms in the gut of rainbow trout. Therefore, other mechanisms for their transport must exist in this species.

Differences in the bioavailability of zinc and cadmium appear to be due to regulation of uptake by the intestinal cells at the brush border membrane, as there were distinct differences in the tissue concentrations of the two metals following *in vitro* exposure. There did not appear to be any control of cadmium uptake at the basolateral membrane, as both metals saturated at the same rate of uptake. Thus the prevention of cadmium entry into intestinal cells may be an important means of controlling Cd toxicity.

Distribution and Redistribution of Cd and Zn in the Body

Following a single oral dose, one of the most important differences between dietary Zn and Cd was the bioavailability of the two metals (20-30% vs. 1-3%, respectively). Chronic dietary studies have indicated that the bioavailabilities of zinc

(36%; Hardy and Shearer, 1985) and cadmium (1%; Harrison and Klaverkamp, 1989; 2%; Handy, 1992) are comparable to those found in this acute uptake study.

A strong relationship between naive tissue concentrations of zinc and cadmium would suggest that upon exposure to each metal in the diet, there would be similarities in the pattern of distribution of newly absorbed zinc and cadmium within the body. This was true for certain tissues, such as the three intestinal compartments (high tissue metal levels) and muscle, carcass and gall bladder (low tissue levels). However, these tissues showed some deviation. These differences may be related to differences in the storage or excretion of the two metals, as discussed later.

The tissues determined in this acute dietary study to be important agree with those determined using long term contaminated feeding methods. Chronic feeding studies have shown that the main tissues for zinc accumulation are ranked in order from gut>liver>gill>kidney (Hardy *et al*, 1987). Studies on the accumulation of cadmium by tissues in rainbow trout (Harrison and Klaverkamp, 1989; Handy, 1992, 1993) all show the gut to be most important followed by gills, kidney and liver, although all indicate differences in the rank order of these latter three tissues. These differences may be related to the concentration of cadmium in the diet, the size of the fish, or even the length of the study. The fact that the key tissues are the same for both chronic and acute exposures suggests that there is very little redistribution of metals within the body over time. Metal concentrations within these tissues may increase, however, with chronic exposure or higher doses.

In fact, the major difference between cadmium and zinc in the internal tissues was that at higher doses, Zn concentrations in the tissues reached saturation, whereas Cd concentrations increased linearly. The reason for this is unknown but could be related to the essential role of zinc in the body. Since zinc is an essential micronutrient, it is very likely that regulatory controls exist to protect tissues from toxicity and to allow movement of zinc to other tissues that are in need. In contrast, cadmium levels in tissues are likely not controlled due to the lack of essentiality of this metal. This results in increasingly high levels of cadmium in internal tissues, with toxic effects. Other studies using rats have reported the same phenomena where, upon saturation of the gut tissues with Cd, there were further increases in internal tissue burden of cadmium with increased oral doses (Lehman and Klaassen, 1986; Moore *et al*, 1973; Goon and Klaassen, 1989).

Excretion of Zinc and Cadmium

There were no major trends in zinc tissue concentration over time to implicate specific organs as excretory tissues. However, given that zinc bioavailability is similar at all doses, and that tissue levels saturated, the remainder of the absorbed zinc dose must be excreted. Since bile concentrations of zinc rose rapidly at high doses, it is suggested that this is the means by which zinc is excreted from the body. Gills have been demonstrated to be important excretory mechanisms for Zn in fish (Hardy *et al*, 1987), but the role of bile has yet to be explored by other authors.

It can be suggested that cadmium excretion occurs via the gills, since gill cadmium levels dropped over time. Handy (1996) also suggests that the gills may be an

excretory organ for cadmium in fish. High levels in the gall bladder of eels fed high cadmium diets indicate that bile may also be an important excretion site (Haesloop and Schirmer, 1985). Cd levels in the bile are higher after 7 days in the present study, suggesting that this may also be an excretory mechanism that is more slowly activated than gill excretion.

Exchangeable Zinc Pools

One of the major differences between zinc and cadmium is that there is a large amount of zinc present in naive fish tissues, whereas cadmium concentrations are very small. Thus it is likely that the presence of radiolabelled zinc in tissues may simply be a result of turnover and exchange of the native zinc within that tissue. Cadmium radioactivity in tissues, however, is likely to be newly accumulated since the levels in the naive tissues are very low in comparison. Examination of the exchangeable fraction of zinc following exposure to dietary doses (0.5 mM) of the metal, revealed that there was very little exchange of zinc within any tissue over 72 h (<2.5%). It is clear that the most exchangeable tissues were the distal and proximal intestines and the liver, kidney, gills, and plasma). Differences in exchangeability are likely related to the relative amounts of transport protein within each compartment, since zinc in storage proteins and enzymes is probably more strongly bound. Thus, newly absorbed zinc at low levels in the diet is likely bound to labile proteins, but at higher levels may displace other bound metal in storage proteins. These findings suggest that, with further experimentation, we could define the ratio of labile and non-labile binding to zinc within particular tissues.

Methodological Issues

Tissue viability is a concern whenever using an *in vitro* method. However, other authors have determined that similar gut sacs to those utilized in the present experiments are viable for periods greater than 4 hrs (Ingham and Arme, 1977; Stokes and Fromm, 1964; House and Green, 1965), so we are confident that the viability of our gut tissues was not a problem, given that the majority of exposures were only 2 h, and none exceeded 4 h. Our uptake rates were also constant over the 4 hr incubation periods, leading us to believe that the tissues were still alive. Another problem with the *in vitro* gut bag method is that it lacks some of the control systems that are present in live animals eg. circulating transport proteins in the plasma, peristaltic movement. It is thought, however, that the absence of these systems had little impact on the outcome of the present experiments.

One of the major issues with the *in vivo* method used here is whether or not uptake from a liquid dose simulates that of normal feeding conditions. However, it was clearly shown that feeding fish following a dose of metal did not result in any differences in the uptake and distribution of either zinc or cadmium. Much of this feeding occurred during the slow phase of distribution, however, and because of this, alterations in the uptake of the *in vivo* dose may not have been seen since most uptake and distribution was shown to occur during the fast phase. In order to solve this issue, it is necessary that solid food be given in some manner during the fast phase of absorption.

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