DIETARY UPTAKE OF COPPER IN RAINBOW TROUT: A STUDY OF MECHANISMS

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DIETARY UPTAKE OF COPPER IN FRESHWATER RAINBOW TROUT(Oncorhynchus mykiss) : A STUDY OF MECHANISMS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

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- TITLE: Dietary uptake of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) A study of mechanisms.
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ABSTRACT

In aquatic environments Cu is both a vital nutrient and an important toxicant. Consequently fish require Cu as a micronutrient and can obtain this metal from either water or their diet. Inadequate intake of Cu is associated with reduced growth and development, while decreased growth rates, mortality and reduced swimming capacity have been reported in fish when Cu accumulates in excess of cellular needs. Characterization of Cu uptake is therefore critical in understanding the dynamics that govern toxicity and the risks associated with exposure to an aquatic contaminant. While mechanisms of waterborne uptake and toxicity are well understood, far less is known about gastrointestinal Cu uptake in fish. *In vivo* and *in vitro* techniques were therefore used in this study to investigate dietary Cu uptake in freshwater rainbow trout.

The mid and posterior regions of the intestine emerged as important sites for Cu absorption in trout, while the role of the stomach and anterior intestine in Cu absorption requires further investigation. The intestinal uptake route was kinetically characterized as a low affinity absorption pathway as compared to the branchial route. Cu uptake appeared to occur via a hypoxia-resistant, carrier-mediated, saturable process which could be fueled by $Cu(II)^{2+}$ at concentrations typically found in the fluid phase of chyme in the trout intestine.

Experimental manipulation of mucosal NaCl levels stimulated Cu uptake, Na_2SO_4 had an identical effect, implicating Na rather than the anion. These responses were unrelated to solvent drag, osmotic pressure or changes in TEP. The presence of excess

luminal Ag and L-histidine stimulated Cu and Na uptake indicating that a portion of Cu transport was mediated by a Na-Cu co-transport system. Partial inhibition of Cu and Na uptake by phenamil and hypercapnia stimulated Na and Cu transport suggest Cu entry could also occur via the apical Na channel. The Na-dependent mechanism thus either involves more than one component or a unique Na-Cu co-transport mechanism with these combined characteristics mediates part of Cu uptake.

Cu uptake was sensitive to pH and competed by Fe and Zn implicating DMT1 in the transport of Cu in the trout intestine. These factors had no effect on Na uptake, leading to the identification of a Na-independent mechanism for Cu uptake in the trout intestine. While the Na dependent nature of Cu uptake and Ag stimulated Cu transport argue against a role for Ctr1 in this process, Cu transport characteristics identified in this study compare well with a recently identified Cu transport in Ctr1 deficient mouse embryonic cells, indicating the existence of a similar transport mechanism in the trout intestine.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized into four chapters. Chapter 1 provides a general introduction and an outline of objectives set for this project. Chapters 2 and 3 are formatted as manuscripts to be submitted for publication with minor revisions in peer-reviewed journals. Chapter 4 provides a summary of results and conclusions from Chapters 2 and 3.

- Chapter 1: Introduction and project objectives.
- Chapter 2: Digestive processing of Cu *in vivo* and physical characterization of Cu uptake *in vitro* in freshwater rainbow trout.
- Chapter 3: Mechanism of dietary Cu uptake in rainbow trout : evidence for a Nadependent and a Na-independent pathway.
 - Chapter 4: Summary of results and conclusions.

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Chapter 1

Introduction and project objectives.

Introduction

Copper – an element essential for life

The nutritional essentiality of (copper) Cu for animals was first established when growth and hemoglobin improved after rats fed a cow's milk diet were supplemented with Cu (Hart et al., 1928). Since then Cu has been recognized as an essential trace element required for survival by all organisms from bacterial cells to humans. Cu ions undergo unique chemistry due to their ability to adopt distinct redox states, either oxidized $Cu(II)^{2+}$ or the reduced state $Cu(I)^{1+}$. Consequently, Cu ions serve as important catalytic cofactors in redox chemistry for proteins that carry out fundamental biological functions that are required for growth and development (Linder, 1991). The need for Cu, separate from Fe, in hematopoiesis was established by Hart et al., (1928). This was followed by the recognition of the fundamental need for Cu in energy metabolism (via cytochrome c oxidase) and its function in connective tissue, particularly the cross-linking of elastin and collagen by extacellular lysyl oxidase (O'Dell et al., 1961b). Many other Cu-containing enzymes were discovered, including tyrosinase, needed for melanin pigment formation, dopamine β -hydroxylase, necessary for catecholamine production, superoxide dismutase, aiding in the disposal of potentially damaging radicals produced in normal metabolic reactions, ceruloplasmin, a potential extracellular free-radical scavenger as well as a ferroxidase, in blood plasma and other extracellular fluids (Linder,1991). Given such a diverse and versatile role, the absolute requirement of Cu is easily understood.

The average intakes of Cu by human adults vary from 0.6-1.6 mg day⁻¹ (Linder, 1991). This range translates into an intake of 10 μ g (0.16nM) Kg⁻¹ day⁻¹ to 27 μ g (0.45nM) Kg⁻¹ day⁻¹. Limiting the biological availability of Cu provides further evidence of the vital nature of this element as Cu deficiency has reportedly produced more characteristics of ischemic heart disease in animals than has any other nutritional insult (Klevay, 1977). Inadequate intake of Cu is known to impede growth and development, impair immune function (Jones, 1984), reduce circulating Fe levels (Holbein, 1980), cause a significant enhancement of the acute and chronic inflammatory response (Jones, 1984) lead to loss of bone mineral density (Danks, 1988) and impaired hearing (Prohaska *et al.*, 1996). Cu deficiency is also reported to reduce Fe absorption in rats through reduced expression of duodenal Hephaestin (Hp) protein (Reeves *et al.*, 2005).

Reviewing the nutritional requirements of fish for dietborne metals, Watanabe *et al.*, (1997) established an equally important role for Cu as a micronutrient in the piscine diet. Cu is required in the diet of freshwater teleosts at concentrations of 0.8 μ g Cu g⁻¹ dry diet (Kamunde et al., 2002b) to 1.4 μ g Cu g⁻¹ dry diet (Lanno et al., 1985b). Thus a trout fed a diet of 2% its body weight would receive Cu at a dose of 16 μ g (0.26nM) Kg⁻¹ day⁻¹ to 28 μ g (0.46nM) Kg⁻¹ day⁻¹.

As in mammals, Cu deficiency has been related to growth reduction in fish fed reduced rations (Waiwood and Beamish, 1978; Collvin, 1984a). The essentiality of Cu was further established by Kamunde *et al.*, (2001) who reported reduced growth associated with reduced food conversion efficiency in Cu-deficient rainbow trout fingerlings. Ogino and Yang, (1980) similarly reported reduced growth in carp fed a Cu deficient diet. The formation of cataracts in carp has also been linked to dietary Cu deficiency (Satoh *et al.*, 1983).

Copper – the potent toxicant

While it is clear that Cu is essential, it is also a potent cytotoxin when allowed to accumulate in excess of cellular needs. Due to the special redox chemistry of cycling between the oxidized and reduced forms, similar to that of Fe, Cu readily participates in reactions that result in the production of highly reactive oxygen species (ROS) including hydroxyl radicals (Halliwell and Gutteridge, 1984). Hydroxyl radicals are believed to be responsible for devastating cellular damage that includes lipid peroxidation in membranes, direct oxidation of proteins, and cleavage of DNA and RNA molecules. In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors (e.g. Zn) from their natural ligands in key cellular signaling proteins. In humans Menkes disease is characterized by progressive neurological impairment and growth failure in infancy resulting from Cu accumulation in the intestinal epithelial cells due to the failure of Cu transport from the cytoplasm to the extracellular fluid. Wilson's disease is an autosomal recessive disorder characterized by accumulation of Cu in the liver, due to both failure of Cu incorporation into ceruloplasmin and export for biliary excretion, leading to liver cirrohsis. The Cu toxicosis condition, in either case, is caused by mutations of two closely related Cu-transporting ATPases MNK and WND, located within the membrane of cells or cell organelles in the intestine and liver respectively, and comprise proteins belonging to the P-type ATPase family of cation transporters (Bull and Cox, 1994).

In unpolluted surface waters Cu is present at concentrations of $< 1-10 \ \mu g \ L^{-1}$ (Boyle, 1979), but concentrations in the vicinity of municipal and industrial effluents, particularly from smelting, mining, refining and metal plating industries may be much higher (Hodson et al., 1979; Spry et al., 1981). In fresh water, copper is primarily viewed as an ionoregulatory toxicant, exerting substantial pathophysiologic effects at total concentrations well below 100 μ g L⁻¹ (Wood, 2001). The substantial surface area of the gills in fish serves as an interface between the environment and blood, notably for continuous diffusion of oxygen and the maintenance of ionic and acid-base balance by active transport processes (Randall et al., 1996). Metals (e.g. Cu²⁺) are readily adsorbed to or absorbed across the gill, and can cause deleterious effects in cellular structures including the epithelium and pillar cells (Laurent and Perry, 1991). At environmentally realistic concentrations, Cu inhibits branchial Na uptake (Lauren and McDonald, 1986) which is needed to counter the passive diffusive Na loss in fresh water fish (Wood, 1992). At a higher threshold, Cu also stimulates branchial Na efflux (Lauren and McDonald, 1986). Like Ag, Cu has a strong affinity for sulfhydryl groups which manifests in the well documented inhibition of Na⁺ K⁺-ATPase activity (Wood, 2001). Besides the effects on branchial Na fluxes, a reduction of growth and appetite during Cu exposure has also been reported (De Boeck et al., 1995). Waterborne Cu exposure in fish can additionally cause elevated plasma cortisol, plasma glucose and plasma ammonia levels (Lauren and McDonald, 1985). Fish and other gill breathing aquatic organisms, are therefore more likely to experience rapid changes in Cu uptake than terrestrial vertebrates, due to a potentially direct branchial uptake of Cu from the water in addition to any dietary Cu uptake.

Dietborne Cu toxicity to fish has been widely researched. The thresholds for Cu toxicity via the diet have been established at > 1 mg Cu kg⁻¹ body weight d⁻¹ for channel catfish, 1-15 mg Cu kg⁻¹ body weight d⁻¹ for Atlantic salmon, and 44 mg Cu kg⁻¹ body weight d⁻¹ for rainbow trout (Clearwater et al., 2002). Berntssen et.al., (1999a) observed increased intestinal cell proliferation, intestinal apoptosis and metallothionein levels and decreased growth rates of Atlantic salmon part fed high levels of Cu in the diet. Mortality, reduced swimming capacity, decreased growth rates, increased metallothionein levels and food refusal have also been reported in rainbow trout under similar conditions (Lanno et al., 1985b; Handy et al., 1999). Food refusal appears to be primarily associated with lipid peroxidation of the food during storage leading to decreased diet palatability. However, the metabolic cost linked with the increased energy required for Cu detoxification is associated with the reduced energy available for activity and growth. Mount et al., (1994) demonstrated that rainbow trout exposed to a Cu supplemented diet experienced approximately 30% mortality which was attributed in part to Cu leaching from the diet, resulting in toxic waterborne Cu exposure. Decline in absorption efficiency with increasing dietary Cu levels has been well documented (Turnlund et al., 1985; Clearwater *et al.*, 2000). It is likely that more than half the Cu content of the diet may remain unabsorbed under these circumstances, and in turn this may generate toxic waterborne Cu levels as faecal Cu. Thus under aquacultural operations such as sea pens or lake pens environmental contamination by dietary Cu may have an adverse effect on biodiversity and indirectly influence the health of other aquatic organisms.

Considering that Cu is both essential and toxic, organisms must implement uptake mechanisms to extract Cu from nutrients, transport Cu across biological membranes and deliver it to Cu-requiring proteins. Furthermore, precise regulatory mechanisms are required to prevent the accumulation of Cu ions to toxic levels. The importance of maintaining this critical balance is underscored by the existence of the two wellcharacterized human genetic disorders in Cu transport, Menkes and Wilson's disease (Bull and Cox, 1994). Maintaining appropriate Cu homeostasis therefore demands a critical orchestration between Cu uptake and distribution within cells, detoxification and removal. Investigation of the processes by which these steps are regulated is clearly warranted.

Mechanisms of Cu uptake :

Dietary Cu uptake in mammals

The maintenance of physiological levels of Cu is achieved in mammals almost entirely by gastrointestinal absorption and biliary excretion (Schaefer and Gatlin, 1999). Dietary Cu is absorbed in the stomach and intestine of mammals (Linder, 1991). Generally, the efficiency of Cu absorption decreases with increasing Cu intake (Turnlund *et al.*, 1985). Although extensively researched, the mechanisms involved in uptake of Cu across the apical membrane of enterocytes remain unclear. However, there is evidence that absorption of Cu across the brushborder into the enterocyte, and the subsequent transfer across the basolateral membrane into the blood occur by different mechanisms. The transfer of Cu across the apical side of the mucosal cells of the small intestine was originally thought to occur solely by diffusion as Cu^{2+} ions (Linder, 1991) or by pinocytosis as Cu-protein complexes. However, Pena et al., (1999) argued that diffusion alone would not completely account for this transfer given the refined nature of Cu uptake by unicellular organisms such as yeast. A role for a high-affinity Cu transport protein hCtr1, first discovered in yeast and now known to be present in mammalian cells has since been demonstrated (Zhou and Gitschier, 1997; Lee et al., 2000). Recent studies on human hepatocyte cells (Lee et al., 2002) indicate stimulation of Cu uptake in cells expressing transfected hCtr1. Several lines of evidence in this work including Cu uptake kinetics, localization and assembly of hCtr1 support the hypothesis that hCtr1 functions as a high affinity Cu transporter at the plasma membrane in human cells. In addition, the natural resistance associated macrophage protein (Nramp2) system, the main iron transporter in intestinal cells, may also mediate uptake of Cu through an energy independent mechanism (Rolfs and Hediger, 1999). This protein exhibits an unusually broad range of substrate specificity mediating the translocation of several divalent cations including Mn²⁺, Cu²⁺, Zn²⁺, Cd²⁺, Ni²⁺ and Pb²⁺ (Gunshin et al., 1997) and has therefore been referred to as DMT1 (divalent metal transporter) (Andrews, 1999). DMT1 has recently been designated a physiologically relevant Cu transporter (Arredondo et al., 2003) in Caco-2 cells derived from the mammalian intestine based on evidence of an intimate relationship between intestinal Cu and Fe transport. A third possibility is based on amiloride sensitivity of Cu uptake in the rat intestine (Wapnir, 1991) where a positive interaction between Cu and Na was demonstrated providing evidence for part of Cu intake via apical Na channels or Na-linked transporters.

From the lumen of intestinal enterocytes Cu is distributed to specific cellular compartments by Cu chaperones (hCox17, Hah1, CCS) for incorporation into Curequiring proteins (Pěna et al., 1999). Basolateral efflux of Cu is likely mediated by the Menkes (MNK) protein, a Cu-translocating P-type ATP-ase (Camakaris et al., 1999; Pěna et al., 1999). The MNK protein appears to be involved in both the efflux of Cu from intestinal epithelial cells and in providing Cu to secreted Cu-metalloproteins. The localization of MNK protein is regulated by Cu concentrations (Petris et al., 1996). At low cellular Cu concentrations the MNK protein is localized in the trans-golgi network (TGN) where it delivers Cu to the secretory pathway. At elevated concentrations, Cu induces the trafficking of MNK protein from TGN to the plasma membrane where it is involved in Cu efflux to protect cells from potentially toxic Cu levels. Cu-regulated trafficking of the MNK protein to the basolateral membrane of gut epithelial cells is thought to be responsible for Cu absorption into the blood. Transfer of Cu across the basolateral membrane is the rate-limiting step in intestinal Cu absorption (Linder and Hazegh-Azam, 1996). Once absorbed into blood, Cu is bound to plasma albumin and histidine (Linder, 1991) for transportation to the liver where it is incorporated to ceruloplasmin and transported via the blood to other organs (Goode et al., 1989). Excess Cu is excreted into the bile. The Wilson's protein (another P-type ATPase) mediates both these processes. While the bulk of plasma Cu is bound in ceruloplasmin, a small proportion of circulating Cu is associated with amino acids (Cousins, 1985) and transcuprien (Linder, 1991).

Branchial Cu uptake in fish

In contrast with the mammalian model in which Cu metabolism involves the regulation of uptake primarily via one (gastrointestinal) route of uptake and one-route of excretion (hepatobiliary) (Linder and Hazegh-Azam, 1996; Schaefer and Gatlin, 1999), fish possess two principal routes of Cu uptake, i.e. gut and the gills. Multiple pathways of excretion possibly exist in fish including branchial, hepatobiliary and renal routes

Mechanisms of waterborne Cu uptake in fish have been the focus of recent research (Wood, 2001; Grosell and Wood, 2002; Kamunde *et al.*, 2002a, 2003; Pyle *et al.*, 2003) and several aspects of Cu uptake via the gills have now been characterized. Available evidence indicates at least two mechanisms of Cu uptake in fish gills - the Cu-specific and the Na-sensitive pathway (Grosell and Wood, 2002). Na uptake in fresh water fish probably occurs via the proton-pump powered apical Na channel with subsequent export into blood via the basolateral Na⁺ K⁺ ATPase (Lin and Randall, 1995; Sullivan *et al.*, 1995; Bury and Wood, 1999; Wood, 2001). Evidence that Cu enters the gill cell via the apical Na channel has been provided directly from Na-competition experiments and pharmacological studies (Grosell and Wood, 2002), and indirectly from manipulation of the activity of Na uptake pathways through alterations in dietary Na (Kamunde *et al.*, 2003; Pyle *et al.*, 2003). The Cu-specific pathway is insensitive to Na-competition or to pharmacological blockade of the apical Na channel (Grosell and Wood, 2002). This

pathway is less well characterized but apical entry is likely via an apical high affinity Cu transporter, perhaps analogous or homologous to hCtr1. For both apical uptake pathways, the basolateral export is likely through a Cu-ATPase. Although not conclusive the sensitivity of branchial Cu uptake to vanadate, a blocker of P-type ATPase (Campbell *et al.*, 1999) and partial cloning of a Cu ATPase with 80% homologies to mammalian MNK (Grosell *et al.*, 2001a) are strong pointers to the existence of a Cu-ATPase in fish gills.

The specific Cu-species that traverses the branchial epithelium has not been characterized. However, the popular theory is that for both uptake pathways Cu^{2+} may be reduced to Cu^{1+} by reductases on the gill surface before uptake (Grosell and Wood, 2002) since comparable studies in other systems indicate that Cu-ATPase and hCtr1 both transport Cu^{1+} (Harris, 2001; Lee *et al.*, 2002). Surprisingly, recent evidence also indicates that the so called divalent metal transporter DMT1 may also preferentially transport $Cu(I)^{1+}$.

Dietary Cu uptake in fish

Investigating comparative aspects of waterborne *versus* dietary Cu uptake in rainbow trout Kamunde *et al.*, (2002a) demonstrated for the first time that at background Cu levels in both media, uptake of dietary Cu was more than tenfold higher than that of waterborne Cu, highlighting the importance of the gastrointestinal route relative to branchial route of Cu uptake. Specific studies on uptake mechanisms of dietary Cu in fish however have been rarely undertaken, but mechanisms of absorption similar to those in mammals are speculated. The fish stomach environment is acidic (Fange and Groves,

1979) and therefore is believed to free Cu from the food with subsequent partial gastric absorption as in mammals (Kamunde et al., 2002a). However, the major role in Cu absorption for rainbow trout was assigned to the intestine which is consistent with similar observations by Clearwater et al., (2000) for rainbow trout and Handy et al., (2000) for the African walking catfish. Intestinal uptake of Cu in fish is thought to occur via simple diffusion for apical entry and biologically mediated transport for basolateral exit (Clearwater et al., 2000; Handy et al., 2000). Nonetheless, the involvement of a putative high-affinity transporter homologous to the hCtr1 on the apical (brush border) membrane cannot be ruled out. An analogue of hCtr1 has recently been cloned from zebrafish embryos (Mackenzie et al., 2004) and while the precise location is unknown at present the brushborder could be a possible site. In addition, recent studies on interactions between dietary Cu and dietary Na in juvenile rainbow trout (Kjoss *et al.*, 2004) revealed that fish fed a high Na diet accumulated significantly higher whole body Cu levels than fish that received no additional Na, suggesting the existence of a Na-sensitive transport mechanism for Cu in the fish intestine, perhaps analogous to the Na-linked mechanism of mammals (Wapnir, 1991). Handy et al., (2000) postulated that the basolateral limiting step in Cu uptake in fish is via a Cu/anion symport and/or possibly a Cu-ATPase.

Synopsis:

It is apparent that the gastrointestinal route of Cu uptake has a major role in establishing the nutritional and toxicological status of environmental Cu. Indeed, Kamunde *et al.*, (2002b) provide evidence of the diet being the preferred source of Cu to

rainbow trout under normal dietary and waterborne conditions, contributing more than 90% of the body burden. Given this importance it is surprising that studies examining mechanisms involved in dietary Cu transport in fish are limited. The mechanistic characterization of dietary Cu uptake is therefore needed to explain the interaction of Cu with other dietary components that may influence its bioavailability/toxicity and also provide additional insight into homeostatic mechanisms operating at the gill and gut which maintain the delicate balance between Cu nutrition and toxicity.

Project objectives

The characterization of the gastrointestinal Cu absorption route was the focus of the research described in this thesis. In particular the objectives of this thesis were

- 1. To provide a relatively realistic portrayal of the natural passage of Cu along the digestive tract in rainbow trout fed a normal diet containing typical background levels of Cu. An *in vivo* disappearance test was designed which is described in Chapter 2 to assess the site of Cu absorption and the relevant dose that would be realistic for further characterization of the uptake process. This was followed by *in vitro* studies in Chapter 2 using freshly prepared intestinal sacs to gain insight into the physical characterization of Cu transport. Rate of Cu transport in different segments, transport kinetics, effects of temperature, Cu speciation and effect of O₂ depletion were specifically examined.
- 2. To ascertain the mechanistic nature of the pathway(s) involved in Cu uptake in the trout intestine using the in vitro intestinal sac technique. In Chapter 3 possible Na-

sensitive, DMT1- and Ctr1- mediated pathways were investigated with the aid of pharmacological agents and competition studies.

Literature Cited:

- Andrews, N.C., Fleming, M.D., Gunshin, H. (1999). Iron transport across biologic membranes. *Nutr. Rev.*, 57(4), 114-23.
- Arredondo, M., Munoz, P., Mura, C.V. and Nunez, M.T. (2003). DMT1 a physiologically relevant apical Cu¹⁺ transporter of intestinal cells. *Am. J. Phys., Cell Phys.*, 284, C1525-1530.
- Berntssen, M.H.G., Hylland, K., Wendelaar Bonga, S.E. and Maage, A. (1999a). Toxic levels of dietary copper in Atlantic salmon (*Salmo salar L.*) parr. *Aquatic Toxicology* 46, 87-99.
- Boyle, E.A., (1979). Copper in natural waters. In: J.O. Nriagu (ed.) Copper in the environment. Part I: Ecological cycling. Wiley, New York. pp. 77.
- Bull, P.C., Cox, D.W. (1994) Wilson disease and Menkes disease: new handles on heavy-metal transport. *Trends Genet*. 10, 246-252.
- Bury, N.R., Wood, C.M., (1999). Mechanisms of branchial apical silver uptake by rainbow trout is via the proton coupled Na⁺ channel. *Am.J.Physiol.*, 277, R1385-R1391.
- Camakaris, J., Vascoboinik, I. and Mercer, J.F. (1999). Molecular mechanisms of copper homeostasis. *Biochem. Biophys. Res. Commun.* 261(2), 225-32.
- Campbell, H.A., Handy R.D. and Nimmo, M. (1999). Copper uptake kinetics across the gills of rainbow trout (*Oncorhynchus mykiss*) measured using an improved isolated perfused head technique. *Aquat. Toxicol.* 46, 177-190.
- Clearwater, S.J., Farag, A.M. and Meyer, J.S. (2002). Bioavailability and toxicity of dietborne copper and zinc to fish. *Comp. Biochem. Physiol.* 132C, 269-313.
- Clearwater, S.J., Baskin, S.J. and Meyer J.S. (2000). Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* 203, 2455-2466.
- Collvin, L. (1984a). The effects of copper on maximum respiration rate and growth rate of perch, *Perca fluviatillis* L. *Water. Res.* 18,139.

- Cousins, R.J. (1985). Transport and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin. *Physiol. Rev.* 65, 238-309.
- Danks, D.M. (1988). Copper deficiency in humans. Ann. Rev. Nutr. 8, 235-257.
- De Boeck, G., De Smet, H. and Blust, R. (1995). The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, Cyprinus carpio. *Aquat. Toxicol.* 32, 127-141.
- Fange, R. and Groves, D. (1979). Digestion. In Bioenergetics and Growth, Fish Physiology vol. viii. WS Hoar, DJ Randall and JR Brett (eds.) pp. 161-260.
- Goode, C.A., Dinh, C.T., and Linder, M.C. (1989). Mechanism of Copper transport and delivery in mammals: Review and recent findings. *Adv. Exp.Med. Biol.* 258,131-144.
- Grosell, M. and Wood, C.M. (2002). Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.
- Grosell, M., Kamunde, C., Wood, C.M. and Walsh, P.J. (2001a). Copper transport across fish gills. Society for Experimental Biology, Annual Meeting, University of Kent, Canterbury, England, 2-6 April. pp. 89.
- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997). Cloning and characterization of a proton-coupled mammalian metal ion transporter. *Nature*. 388, 482-488.
- Halliwell, B., and Gutteridge, J.M.C. (1984). Oxygen toxicity, oxygen radicals, transition metals and diseases. *Biochem. J.* 219, 1-4.
- Handy, R.D., Musonda, M.M., Philips,C. and Fella, S.J. (2000). Mechanisms of gastrointestinal copper absorption in the African walking catfish:copper dose-effects and a novel anion-dependent pathway in the intestine. *J.Exp. Biol.* 203, 2365-2377.
- Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., and Musonda, M.M. (1999).
 Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (Oncorhynchus mykiss) during chronic dietary exposure to copper. *Aquat. Toxicol.* 38, 257-276.

- Harris, E.D. (2001). Copper homeostasis: the role of cellular transporters. *Nutr. Rev.* 59, 281-285.
- Hart, E.B., Steenbock, H., Waddell, J and Elvehjem, C.A. (1928). Iron in nutrition. VII. Copper as a supplement to iron for haemoglobin building in the rat. *J. Biol. Chem.* 77, 797-812.
- Hodson, P.V., Borgman, U. and Shear, H. (1979). Toxicity of copper to aquatic biota, pp.307-372. In J.O. Nriagu [ed.] Copper in the environment. Vol. II. Health effects. John Wiley and Sons. Toronto. Ont.
- Holbein, B.E. (1980). Iron-controlled infection with Neisseria meningitis in mice. Infect. Immun. 29, 886-891.
- Jones, D.G., (1984). Effects of dietary copper depletion on acute and delayed inflammatory responses in mice, *Res. Vet. Sci.* 31, 151-156.
- Kamunde, C.N., and Wood, C.M. (2003). The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss. Aquat. Toxicol.* 62, 235-254.
- Kamunde, C.N., Clayton, C. and Wood, C.M. (2002a). Waterborne versus dietary copper uptake in trout and the effects of waterborne copper acclimation. *Am. J. Physiol.* 383, R69-R78.
- Kamunde, C.N., Grosell, M., Higgs, D. and Wood, C.M. (2002b). Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J.Exp.Biol. 205, 279-290.
- Kamunde, C.N., Grosell, M., Lott, J.N.A. and Wood, C.M. (2001). Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure. Can.J. Fish.Aquat.Sci. 58, 293-305.
- Kjoss, V.A., Kamunde, C. N., Niyogi, S., Grosell, M. and Wood, C.M. (2004). Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. *J. Fish Biol.* 65, 1-17.
- Klevay, L.M. (1977). The role of copper and zinc in cholesterol metabolism, in *Advances in Nutritional Research*, Vol.1 (H.H. Draper, ed.), Plenum Press, New York, pp. 227-252.

- Lanno, R.P., Hicks, B and Hilton, J.W. (1985b). Maximum tolerable and toxic levels of dietary copper in rainbow trout (*Salmo gairdneri* Richardson) *Aquaculture* 49, 257-268.
- Laurèn, D.J. and McDonald, D.G. (1986). Influence of water hardness, pH and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, Salmo gairdneri. *Can. J. Fish. Aquat. Sci.* 43, 1488-1496.
- Laurèn, D.J. and McDonald, D.G. (1985). Effects of copper on branchial ionoregulation in rainbow trout, Salmo gairdneri Richardson: modulation by water hardness and pH. J. Comp. Physiol. 255B, 635-644.
- Laurent, P. and Perry, S.F. (1991). Environmental effects on fish gill morphology. *Physiological Zoology* 64, 4-25.
- Lee, J., Pena, M.M.O., Nose, Y. and Thiele, D.J. (2002). Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 277, 4380-4387.
- Lee, J., Prohaska, J. R. Dagenais, S.L., Glover, T.W. and Thiele, D.J. (2000). Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* 254, 87-96.
- Lin, H. and Randall, D. J. (1995). Proton pumps in fish gills. In *Cellular and Molecular Approaches to Fish Ionic Regulation*, pp 225-229. C.M.Wood and T.J.Shuttleworth (Eds). Academic Press,New York.
- Linder, M.C. and Hazegh-Azam (1996). Copper biochemistry and molecular biology. Am. J. Clin. Nutr. 63, 797S-811S.
- Linder, M.C. (1991). Biochemistry of copper. Plenum press New York. pp. 3-16.
- Mackenzie, N.C., Brito, M., Reyes, A.E. and Allende, M.L. (2004). Cloning, expression pattern and essentiality of the high-affinity copper transporter1 (ctr1) gene in zebrafish. *Gene*. 328,113-20.
- Mount, D.R., Barth, A.K.Garrison, T.D., Barten, K.A., Hockett, J.R. (1994). Dietary and waterborne exposure of rainbow trout (Oncorhynchus mykiss) to copper, cadmium, lead and zinc using a live diet. *Environ. Toxicol. Chem.* 13, 2031-2041.

- O'Dell, B.L., Hardwick, B.C., Reynolds, G., and Savage, J.E. (1961b). Connective tissue defect in the chick resulting from copper deficiency. *Proc. Soc. Exp. Biol. Med.* 108-402.
- Ogino, C. and Yang, G.-Y. (1980). Requirements of carp and rainbow trout for dietary manganese and copper. *Bull. Jap. Soc. Scient. Fish.* 46, 455-458.
- Prohaska, J.R., Hoffman, R.G. (1996). Auditory startle response is diminished in rats after recovery from perinatal copper deficiency. *J.Nutr.* 126(3), 618-627.
- Peña, M.M.O., Lee, J., and Thiele, D.J. (1999). A delicate balance : Homeostatic control of copper uptake and distribution. J. Nutr. 129, 1251-1260.
- Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J. (1996). Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J.* 15,6084-6095.
- Pyle, G.G., Kamunde, C.N., McDonald, D.G. and Wood, C.M. (2003). Dietary sodium inhibits aqueous copper uptake in rainbow trout, *Oncorhynchus mykiss. J. Exp. Biol.* 206,609-618.
- Randall, D.J., Brauner, C.J., Thurston, R.V. and Neuman, J.F. (1996). Water chemistry at the gill surfaces of fish and the uptake of xenobiotics. In *Toxicology of Aquatic Pollution- Physiological, Molecular and Cellular Approaches.* Society for Experimental biology Seminar Series 57, Taylor, E.W. (ed.), pp. 207-219. Cambridge University Press, Cambridge.
- Reeves, P.G., Demars, L.C., Johnson, W.T., Lukaski, H.C. (2005). Dietary copper deficiency reduces iron absorption and duodenal enterocyte hephaestin protein in male and female rats. J. Nutr. 135 (1), 92-8.
- Rolfs, A., and Hediger, M.A. (1999). Metal ion transporters in mammals: structure, function and pathological implications. J. Physiol. 518, 1-12.
- Satoh, S., Yamamoto, H., Takeuchi, T., Watanabe T., (1983) Effects on growth and mineral composition of rainbow trout of deletion of trace elements or magnesium from fish meal diet. Bull. *Jpn. Soc. Sci. Fish.* 49, 425-429.
- Schaefer, M. and Gatlin, J.D. (1999). Genetic disorders of membrane transport IV. Wilson's disease and Menkes disease. *Am. J. Physiol.* 276, G311-G314.

- Spry, D.J., Wood, C. M. and Hodson, P.V. (1981). The effects of environmental acid on freshwater fish with particular reference to softwater lakes in Ontarioand the modifying effects of heavy metals. A literature review. *Can. Tech. Rep. Fish. Aquat. Sci.* 999,149 pp.
- Sullivan, G.V., Fryer, J.N. and Perry, S.F. (1995). Immunolocalization of proton pump (H⁺-ATPase) in pavement cells of rainbow trout gill. *J. Exp. Biol.* 198, 2619-2629.
- Turnlund, J.R., King, J.C., Gong,B., Keyes, W.R., Michel, M.C., (1985). A stable isotope study of copper absorption in young men:effect of phytate and α-cellulose. *Am.J.Clin.Nutr.* 42,18-23.
- Waiwood, K.G. and Beamish, F.W.H. (1978). The effect of copper, hardness and pH on the growth of rainbow trout, *Salmo gairdneri*. J. Fish. Biol. 13, 591-598.
- Wapnir, R.A. (1991). Copper-sodium linkage during intestinal absorption: inhibition by amiloride. *Proc. Soc. Exper. Biol. Med.* 196, 410-414.
- Watanabe, T., Kiron, V. and Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture*. 151, 185-207.
- Wood, C.M. (2001). Toxic responses of the gill. pp 41-45 In: Schlenk, D., Benson,
 W.H. (Eds.), Target Organ Toxicity in Marine and Fresh water Teleosts,
 Vol. 1 taylor and Francis, London.
- Wood, C.M. (1992). Flux measurements as indices of H+ and metal effects on fresh water fish. *Aquat. Toxicol.* 22, 239-264.
- Zhou, B.and Gitschier, J. (1997). hCtr1: a human gene for copper uptake identified by complementation in yeast. *Proc. Natl. Acad. Sci.* USA 94, 7481-7486.

Chapter 2

Digestive processing of Cu *in vivo* and physical characterization of Cu uptake *in vitro* in freshwater rainbow trout.

Introduction

Copper (Cu) is an essential element found in all living organisms in the oxidized Cu (II)²⁺ and reduced Cu (I)¹⁺ states. Classified as a "trace metal", Cu's beneficial impact is known to occur in the micromolar range (Harris, 1991). Cu is specifically required as a catalytic cofactor in redox chemistry for proteins that carry out fundamental biological processes such as respiration, normal cell growth and development. However, Cu also participates in redox reactions that generate the hydroxyl radical, which causes considerable damage to lipids, proteins and DNA. Cu imbalances in humans lead to serious diseases such as Menkes syndrome and Wilson's disease, characterized by the inability to appropriately distribute Cu to all cells and tissues (Puig & Thiele, 2002). Consequently, there is a fine balance between Cu deficiency and surplus which organisms maintain via homeostatic control of absorption and excretion.

The physiology and toxicology of Cu in fish has been studied intensively because of its use as a prophylactic agent in fish culture and its diffuse release from industrial and domestic sources (eg. Cu plumbing) (Wood, 2001). Fish, are unique among the vertebrates, a consequence of having two routes of metal uptake, the gills and the gut. It is generally believed that fresh water teleosts have a daily dietary requirement of Cu in the region of 1.4 μ g g⁻¹dry diet (Lanno *et al.*, 1985b) or 28 ug (0.46nM) Kg⁻¹ day⁻¹. However, Kamunde et al., (2002b) showed that by considering the input from water when determining Cu requirement in fish, as little as 0.8µg g⁻¹ Cu (16 ug (0.26nM) Kg⁻¹ day⁻¹) in the diet is adequate to support normal growth in rainbow trout juveniles at normal water Cu level of 3 μ g L⁻¹. The mechanisms of waterborne Cu uptake and toxicity to fish are beginning to be well understood (Clearwater et al., 2002; Wood, 2001), however the uptake of dietborne Cu in fish is not well characterized. While several studies have indicated the diet to be the major source of Cu for fish under optimal growth conditions (Miller et al., 1993; Handy, 1996), only Kamunde et al., (2002a) have directly measured the rate of Cu uptake from food. They reported that rainbow trout fed a control diet spiked with ⁶⁴Cu took up Cu at a rate of 0.9 ng g^{-1} h⁻¹, ten times higher than the rate of waterborne Cu uptake determined in control hard water during the same study. Furthermore, few studies have assessed the mechanisms of gastrointestinal Cu uptake in fish. Handy et al., (2000) have described concentration dependent changes in basolateral Cu absorption across the catfish gut and postulated the presence of a Cu-ATPase and a Cu /anion symport, while Q₁₀ analysis (Clearwater et al., 2000) suggested that apical entry of Cu was by diffusion while basolateral exit was biologically mediated and is therefore the rate limiting step in intestinal uptake of Cu in rainbow trout. These findings correspond well to the model of Cu absorption in mammals, where an uptake phase independent of the ATP status of the cell and an energy-dependent rate-limiting transfer step have been described (Linder and Hazegh-Azam, 1996). It is also interesting that mammalian intestinal Cu uptake primarily occurs in the small intestine (Wapnir and Stiel, 1987),

whereas in fish, Cu uptake is reported to occur mainly in the mid/ posterior region of the intestinal tract (Clearwater *et al.*, 2000; Handy et.al., 2000; Kamunde *et al.*, 2002a).

The study outlined in this chapter first utilized an *in vivo* disappearance test to examine the digestive processing of Cu in intact trout fed a normal diet. Our objectives were to measure typical Cu concentrations in the chyme and to gain quantitative insight into Cu handling at various points along the digestive tract.

Having established the relevant dose and relative importance of different sites in Cu absorption, in vitro preparations of freshly prepared intestinal segments were utilized to gain insight into the physical processes governing transport kinetics. This "gut sac" technique is useful in evaluating factors contributing to ion transport. It allows manipulation of both mucosal and serosal solutions, eliminating complications encountered in vivo, arising from interactions of dietary components. It has been well documented (e.g. Barthe et al., 1999; Grosell and Jensen, 1999; Handy et al., 2000; Bury et al., 2001; Grosell et al., 2004), that gut sacs maintain tissue viability for prolonged periods and give reliable data in spite of being a closed system. The greatest advantage of this technique is its high throughput, that allows a quick analysis of transport mechanisms and provides relevant information for advanced research. Intestinal sacs were therefore employed to characterize the concentration-dependence of Cu uptake (kinetic analysis) followed by an evaluation of specific aspects of the absorptive process, notably the potential role of temperature which is an important environmental factor determining the uptake of metals by aquatic organisms. The energy-dependence of Cu uptake was also examined utilizing O₂ depletion (anoxia) as an effective inhibitory tool for oxidative phosphorylation. The aim of the study was to provide insight into the basic mechanism by which Cu is transported across the intestinal epithelium in fresh water trout.

Methods

Acclimation

Rainbow trout (*Oncorhyncus mykiss*: weight 200-300g.) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were acclimated to the laboratory in a 500 L tank supplied with aerated, flow-through, dechlorinated Hamilton tap water from Lake Ontario (Na = 0.5 -0.6 mM, Cl⁻ = 0.7 mM, Ca =1.0 mM, hardness ~ 140 ppm as CaCO₃, background Cu = < 16 pM, pH ~ 8 ; temperature =12 \pm 2°C). During the two week acclimation period, fish were fed Martin's commercial dried pellet feed (5pt) daily, at a ration of 2% wet body mass per day. The Cu content of the food was 27 µg g⁻¹ dry wt. The trout were then starved for either 7-10 days for the *in vivo* tests or for 48 h for the *in vitro* intestinal sac preparations. For each experiment trout were randomly selected from the holding tank.

In vivo – Disappearance Test

Diet Preparation:

Experimental diet was prepared using the above fish feed ground to a fine powder in a commercial blender and thoroughly mixed with equal quantity of NANOpure-II water (Sybron/Barnstead, Boston, MA, USA) using a PopeilTM automatic pasta maker (Ronco Inventions, Ilc, Catsworth,CA, USA). The mixture was manually repelleted to 5-pt. size incorporating 8.5 grade (0.400 – 0.455 mm) lead glass ballotini beads (Jencons USA Inc. Bridgeville, PA), at a fixed density (4% by dry mass of food), air-dried and refrigerated
(McCarthy *et al.*, 1992). Tests showed that the ballotini beads did not affect the palatability of the food, which was readily consumed.

Disappearance test:

To characterize the digestive processing of Cu in trout, a disappearance test was designed to follow the processing (uptake and secretion) of Cu along the digestive tract from its oral input (from food) to the anus. Handling of Cu at various points along the digestive tract was also investigated. Trout were starved for at least one week and then fed the above specially prepared food that contained lead-glass ballotini beads. The lead-glass beads act as a non-absorbable marker that can be used to quantify absorption. The percentage of absorption of a given nutrient in the meal can be calculated by comparing the nutrient:marker concentration in the chyme sample and in the test meal .

Fish were sampled at 2, 4, 8, 12, 24, 48 and 72 h after being fed. At each time point 6 fish were sacrificed by a blow to the head and weighed. The gastrointestinal tract was then tied with silk ligatures at the esophagus, stomach, anterior intestine, mid intestine and posterior intestine boundaries. Initial trials had revealed that it took approximately 8 h for the food to move from the stomach to the intestine, therefore intestinal sections were sampled only for time points 8- 72 h post feeding. The entire gut was then dissected out and x-rayed (43855 A, Single Cabinet, 110 Kv X- ray system – Faxitron X-ray Corp., IL. USA). After x-raying, the contents of each section were placed in 50 ml. Eppendorf tubes and mixed thoroughly. A sub sample of the contents was centrifuged for 5 minutes at 13000 g to obtain supernatant which was placed into pre-

weighed bullet tubes and frozen till further analysis. The remainder of the sample was oven-dried for 48 h or till completely dry to determine water content, and then digested in approximately five times volumes of 1N HNO₃ (Fisher Scientific, trace metal grade). Supernatant and digested chyme were analysed for Cu using graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA -220 with graphite tube atomizer [GTA-110], Mulgrave, Australia) following manufacturer specifications for Cu-analysis. National Research Council of Canada-certified analytical standards were run along with the samples for quality control.

The number of beads in food and in the chyme in each gut section were counted from X-ray micrographs to calculate food or chyme:bead ratio (beads g^{-1} food or chyme). Cu content was calculated as follows :

- Cu μg g⁻¹ chyme / beads g⁻¹ chyme, which provided the ratio of Cu content to that of a non-absorbed or secreted marker in the total chyme.
- Cu μg ml⁻¹ superanatant / beads ml⁻¹ supernatant, which provided the ratio of Cu content to that of a non-absorbed or secreted marker in the supernatant.
- Cu content in the solid portion of chyme was calculated by subtracting the [Cu] in supernatant from the [Cu] in the total chyme after correcting for water % of total chyme.
- % water in total chyme was calculated as : [total ml of water/total wt. of wet chyme] * 100
- Water:bead ratio was calculated as water(ml) g⁻¹ chyme / beads g⁻¹ chyme.

In vitro – Intestinal Sacs

Preparation of radioactive copper:

Dried Cu(NO₃)₂ (200 μ g) was irradiated at McMaster nuclear reactor to achieve radioactivity level of 0.6 mCi ⁶⁴Cu, (half life 12.9 h). After irradiation, the Cu(NO₃)₂ was dissolved in 0.1 mM HNO₃ (400 μ l), 0.01 mM NaHCO₃ (400 μ l) and Cortland saline (200 μ l). The resuspended Cu was then added to mucosal saline to give a final concentration of 3 μ g ml⁻¹ or 50 μ M in most trials.

Intestinal sac preparation:

Intestinal sacs were used to investigate the mechanism of Cu uptake. Fish were killed by an overdose of MS-222 (0.25 g L^{-1}) and the entire intestine was obtained by dissection. The intestine was separated from the stomach just posterior to the pyloric sphincter and from the rectum at the ilio-rectal sphincter and flushed with saline to remove food and faeces. Subsequently, sacs were prepared from the anterior, mid and posterior segments of the intestine. The regional division was made along obvious morphological differences for the posterior section, while the remaining portion between the pyloric aperture and the posterior region was split evenly into anterior and medial sections. Each segment was fitted with a short length of heat flared PE-tubing (PE 50) tied in place at the anterior end with double silk ligatures and was closed at the posterior end with double silk ligatures. The catheter served to fill and drain the sac preparation.

The sacs were filled with 1 ml of appropriate mucosal saline (Modified Cortland saline in mM: NaCl 133, KCl 5, CaCl₂·2H₂O 1, MgSO₄·7H₂O 1.9, NaHCO₃ 1.9, $NaH_2PO_4 H_2O$ 2.9, glucose 5.5, pH 7.4; Wolf, 1963) labeled with 0.04 mCi ml⁻¹ ⁶⁴Cu as Cu(NO₃)₂ or 0.04 µCi ml^{-1 22}Na as NaCl (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), the PE tubing was sealed, the sac preparation was blotted dry and mass of the preparation determined to the nearest 0.1 mg (Sartorius GMBH Gottingen; H110 ** V40, Germany). Subsequently, the sac preparation was placed in a fixed volume (12 ml) of the modified Cortland saline serving as the serosal bath and constantly aerated with 99.7% O_2 and 0.3% CO_2 (i.e. $P_{CO2} = 2.25$ torr) gas mixture. Temperature was maintained between 13-15°C. Samples of mucosal and serosal saline were collected at the start and end of the flux period which was routinely 2 h in duration and counted for 64 Cu or ²²Na activity by gamma counting. At the end of the flux period, the sac preparation was removed from the flux chamber, blotted dry and reweighed, then drained completely, cut open by a longitudinal incision and washed in saline and EDTA (1 mM, pH 7.9). The washing procedure ensured removal of loosely bound ⁶⁴Cu. The preparation was blotted dry and gently scraped to remove mucus and epithelial cells using a glass slide. The gross surface area of the intestinal tissue was determined by tracing its outline onto graph paper (Grosell and Jensen, 1999). The tissue, serosal samples, wash solutions and epithelial scrapings were counted separately for gamma activity.

Experimental series

Series 1. The time course of Cu uptake:

Isolated intestinal sacs were infused with 1 ml of ⁶⁴Cu labeled mucosal saline. Following flux periods of 1 h, 2 h and 4 h, the preparations were removed from the bathing solution and sampled as detailed above.

Series 2. Concentration - dependent Cu absorption:

Five treatment groups were employed: Cu in the mucosal saline was varied to achieve concentrations of 1, 10, 50 100 and 500 μ M. For the latter two treatments an appropriate quantity of cold Cu(NO₃)₂ solution was added to the saline in addition to ⁶⁴Cu to achieve the desired concentration.

Series 3. Effects of temperature on Cu uptake:

The temperature-dependence of Cu uptake was examined at acclimation temperature (13°C) and at 3 and 23°C. The latter two temperatures were achieved by placing the vials containing intestinal sacs and serosal medium in an ice and hot water bath respectively. This range of temperatures allowed examination of the Q_{10} effect.

Series 4. Effect of ascorbic acid on Cu ion redox state:

Preference of the transport system(s) for $Cu(II)^{2+}$ versus $Cu(I)^{1+}$ was evaluated in the presence and absence of three concentrations of a reducing agent, ascorbic acid – 100

 μ M, 500 μ M and 2.5mM. Ascorbic acid has been consistently used to assess preference of Cu(I)¹⁺ versus Cu(II)²⁺ in uptake studies (Arredondo *et al.*, 2003; Zerounian *et al.*, 2003). pH of the luminal saline was maintained between 6.0-6.5 as ascorbate reportedly shows decreasing "antioxidant efficiency" with increasing pH (Lucock *et al.*, 1995).

Series 5. Anoxia induction :

Anoxia was induced by substituting the regular O_2 (99.7%) / CO_2 (0.3%) gassing with N_2 (99.7%) / CO_2 (0.3%). As a check on the efficiency of the treatment, in this series Na uptake was measured using ²²Na in separate preparations under regular and anoxic conditions.

Analytical techniques and calculations

In each experimental study the intestinal sac preparations were blotted in a standardized fashion and weighed to the nearest 0.1mg (Sartorius GMBH Gottingen; H110 ** V40, Germany) prior to and after the flux period to allow for calculation of net fluid transport.

Net fluid transport rates were determined from the change in total mass of the sac preparation over the experimental period. This was normalized by taking into account the gross surface area of exposed epithelium and time elapsed.

Rate of fluid transport (FTR) was calculated as follows:

FTR = (IW - TW)/ISA/t

Where IW = Initial weight of sac preparation in mg.

TW = Terminal weight of sac preparation after flux in mg.

ISA = Intestinal surface area in cm²

t = Time in h

to yield a rate of net water movement from mucosal to serosal surface, expressed in $\mu l \text{ cm}^{-2} h^{-1}$.

Mucosal and serosal saline samples from the beginning and end of each flux were measured for ⁶⁴Cu and ²²Na as applicable on a Canberra-Packard Minaxi Auto Gamma counter 5000 Series (Meriden, CT, USA) with an on-board program for decay correction of ⁶⁴Cu. Samples of wash solutions, epithelial scrapings and the tissue layer from each intestinal sac were counted separately for radioactivity. The wash solutions represented loosely bound ⁶⁴Cu while epithelial scrapings accounted for a combination of mucus and surface cells. The radioactivity incorporated into the tissue layer and the serosal sample at the end of the flux period was considered as a conservative estimate of true Cu absorption (see Results).

Cu uptake rate (UR) was calculated as follows:

UR = Tissue cpm / (SA*ISA*t)

Tissue cpm represents the total ⁶⁴Cu activity of the compartment measured SA is the initial measured specific activity of the mucosal saline (cpm/ μ g) ISA is the intestinal surface area in cm² t is time in hours.

This produced a Cu accumulation rate (UR) expressed as μg cm⁻² h⁻¹.

Na uptake rate (UR) was calculated as follows:

$$UR = Tissue cpm / (SA*ISA*t)$$

Tissue cpm represents the total ²²Na activity of the compartment measured SA is the initial measured specific activity of the mucosal saline (cpm/ μ mol) ISA is the intestinal surface area in cm².

t is time in hours.

This produced a Na accumulation rate (UR) expressed as μ mol cm ⁻² h⁻¹.

The concentrations of Cu and Na in mucosal saline were measured by graphite furnace atomic absorption spectroscopy (GFAAS; Varian Spectra AA -220 with graphite tube atomizer [GTA-110], Mulgrave, Australia) and flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia) respectively for specific activity calculations again following manufacturer specifications for Cu-analysis and using approved standards as in the *in vivo* study.

Q₁₀ values were calculated using the following equation (Withers, 1992)

 $Q_{10} = (R_2 / R_1)^{[10 / (T2-T1)]}$

where R_2 and R_1 are the Cu uptake rates at the two temperatures T_2 and T_1 respectively. Q_{10} values greater than 1.0 are generally considered representative of reactions that are biologically mediated. Non-linear regression analyses of Cu uptake kinetics was performed with Sigma plot for Windows version 8.0 in order to fit the parameters of the Michaelis-Menten equation

$$J_{in} = J_{max} * K_m / [X] + K_m$$

where J_{in} is the unidirectional influx rate, [X] is the concentration of substrate, J_{max} is the maximum transport rate when the system is saturated with substrate, and K_m is the concentration of substrate which provides a J_{in} of half the J_{max} value.

A one way analysis of variance (ANOVA) was generally used to compare the groups followed by a least significant difference (LSD) test to detect difference between specific means (SPSS10 for Windows). Statistically significant differences between control and treated groups were evaluated by unpaired student's t-tests (two-tailed). Data are reported as means \pm S.E.M. (N) and differences were considered significant at p<0.05.

Results

Cu profile - in vivo, in the gastrointestinal tract:

The objectives of the *in vivo* sampling study were two-fold: (i) to determine the concentrations of Cu in the fluid phase of the intestinal contents in trout on the standard commercial diet, such that relevant mucosal fluid concentrations could be used in subsequent in vitro transport experiments; (ii) to assess the quantitative importance of the various gastro-intestinal segments in Cu uptake in vivo. The first objective was met simply by direct measurement of Cu concentrations in the supernatant (fluid phase) of the gut contents (Fig. 2-1 C), which ranged from about 0.5 to 4 μ g ml⁻¹ (i.e. 8-63 μ M). However the second goal was complex and could not be achieved simply from measurements of fluid phase (Fig. 2-1 C), solid phase (Fig. 2-1 B) and total Cu concentrations (Fig. 2-1 A) in chyme, because there were substantial changes in both water content (due to fluid secretion and absorption) and solid phase content (due to digestion and absorption) as the chyme moved along the tract. Under such conditions, a simple correction for % water (Fig. 2-2 A) is inadequate. Thus use of a non-absorbable marker (ballotini beads) was essential to provide a point of reference for both water (Fig. 2-2 B) and Cu movements (Fig. 2-3 A,B,C). Overall, the measurements indicated that the Cu-to-bead ratio in total chyme (Fig. 2-3 A) decreased significantly in the stomach at all times relative to that in the food, suggesting significant Cu absorption by the stomach wall (though see *caveat* in Discussion). Thereafter in the anterior intestine, the ratio became more variable and increased back to the level in the original food (Fig. 2-3 A),

suggesting net secretion of Cu in this section. Values then fell again in the subsequent two sections, particularly the mid-intestine, indicating the latter as an important site of net Cu absorption. Overall, the Cu-to-bead ratio decreased about 50% from food to posterior intestine, indicating that about 50% of the Cu in the food was absorbed on a net basis.

Turning now to detailed spatial and temporal patterns, there was a substantial 3.5fold increase in % water in the stomach content chyme (Fig. 2-2 A) relative to the original "dry" food (~ 15%), which gradually increased further to about 65% by 72 h. This was explained by a net addition of fluid in the stomach, as demonstrated by marked increases in the water-to-bead ratio in total chyme in this compartment. A further increase of % water to about 85% in the anterior intestine in fact reflected an approximate tripling of the water-to-bead ratio in the anterior intestine in the earlier sampling times (8-24 h) indicating a further large net secretion of fluid into this compartment. However, at later times (48-72 h), the water-to-bead ratio fell substantially in the anterior intestine, indicating significant net fluid absorption. This temporal pattern was also reflected in the mid- and posterior intestinal compartments (Fig.2-2 B), so even though % water remained relatively constant at around 80% (Fig.2-2 A), there were large movements of fluid which occurred against a background of a decreasing solid phase as nutrient digestion and absorption progressed.

Much of the changes in Cu concentrations in the chyme were explained by dilution or concentration due to these fluid movements. Thus net fluid addition in the stomach largely accounted for the 50-65% decrease in Cu concentration in the total chyme relative to its original concentration in the food (Fig.2-1 A). In the stomach

contents, approximately 16% of the total Cu was present in the supernatant initially, the remainder in the solid phase. While the Cu concentrations (Fig.2-1 B) and Cu-to-bead ratios (Fig.2-3 B) in the solid phase of stomach contents did not change over time, those in the supernatant (Fig. 2-1 C, 2-3 C) progressively decreased, supporting the interpretation of significant Cu absorption through the gastric mucosa.

In the anterior intestine, the Cu concentration in the total chyme decreased only modestly relative to that in the stomach (Fig. 2-1 A) despite the substantial fluid secretion which occurred into this compartment at 8-24 h, seen in the tripling of the water-to-bead ratio at this time (Fig.2-2 B). However, early dilution of Cu concentrations in the fluid phase was somewhat greater (Fig. 2-1 C). At later times these trends in Cu concentration reversed in both fluid phase (Fig. 2-1 C) and total chyme (Fig. 2-1 A), while Cu concentration also gradually increased in the solid phase (Fig. 2-1 B). This occurred coincident with later water removal (Fig. 2-2 B). Overall, there was clearly a net addition of Cu in the anterior intestine, seen in the increases in Cu-to-bead ratios in this compartment (Figs. 2-3 A,B,C).

In the mid and posterior intestines, progressive decreases over time in Cu concentration (Fig. 2-1 C) and Cu-to-bead ratios (Fig.2-3 C) in the fluid phases occurred against a background of simultaneous net water absorption, at least in the mid-intestine (Fig.2-3 B). Cu concentrations in the total chyme (Fig. 2-1 A) and solid phase (Fig. 2-1 B) tended to increase or remain stable, while Cu-to-bead ratios in these phases (Fig.2-3 A,B) decreased relative to the anterior intestine but were relatively stable over time. Clearly net Cu absorption occurred from the fluid phase in these segments.

Cu uptake – In Vitro

Series 1. Time course and spatial pattern of intestinal Cu uptake:

Cu uptake rates and fluid transport rates in isolated intestinal segments of rainbow trout were not significantly different in any compartment (Fig.2-4) when measured over 1 h, 2 h, or 4 h periods, suggesting that Cu and fluid uptake continued at a more or less constant rate up to 4 h. Illustrative data for the mid intestine are shown in Fig. 2-4, A -F. Most importantly, the sum of Cu uptake into the serosal fluid and muscle, chosen as a conservative index of net Cu transport, was very constant over time (Fig. 2-4 B). Not surprisingly, the "wash" compartment (Fig 2-4 D), representing Cu loosely bound to the mucosal surface exhibited a tendency (non-significant) to contribute less with longer measurement periods. On the basis of this data set, a 2 h incubation period was chosen for all further experiments.

An analysis of the relative contribution of each compartment at 2 h to total Cu accumulation (Fig. 2-5 A) revealed that 31.5% of overall Cu uptake could be attributed to the serosal compartment while 17.5% of accumulated Cu was partitioned into the muscle layer. These two compartments accounted for 49% of total Cu accumulation. The washing protocol removed 42% of total Cu (i.e. loosely bound), while the epithelium represented the smallest uptake compartment with 9% of accumulated Cu. While these data are for the mid intestine, similar values occurred in the anterior and posterior intestine respectively.

Cu uptake rate per unit surface area was approximately twice as great in the anterior intestine at 4.2 ng cm⁻² h⁻¹ relative to the mid and posterior intestine where uptake was 1.2

ng cm⁻² h⁻¹ and 2.1 ng cm⁻² h⁻¹ respectively (Fig.2-5 B). For most treatments, the responses of all three segments were similar, however, the anterior intestine preparations yielded more variable results and tended to leak from the delicate caecal extensions, hence data from the anterior intestinal preparations were not considered for further comparisons.

Series 2. Concentration-dependent Cu absorption:

Over the range of Cu concentrations tested, Cu uptake appeared to be biphasic in both mid and posterior intestine (Fig.2-6 A&B). A hyperbolic relationship was evident between 1 and 100 μ M, followed by a possible diffusive linear relationship when the concentration was increased to 500 μ M, suggesting that more than one component existed for Cu uptake in isolated intestinal segments. The saturable component was well characterized by the Michaelis Menten kinetics relationship $(r^2 = 0.96 - 0.99)$ which revealed a maximal Cu uptake rate (J_{max}) of 17 pmol cm⁻² h⁻¹ with uptake half-saturated (K_m) at a Cu concentration of 31.6 μ M in the mid intestine. The posterior intestine exhibited saturation kinetics characterized by a maximum transport capacity (J_{max}) of 41 pmol.cm⁻² h⁻¹ and an affinity of (K_m) 78.5 μ M. However, subtracting a possible diffusive component from the hyperbolic relationship considerably reduced both J_{max} and K_m in the posterior intestine to 12 pmol cm^{-2} h⁻¹ and 18.9 µM respectively, similar to the mid intestine. It was not possible to subtract a diffusive component from the data for the mid intestine. There was no significant change in TEP over the range of Cu concentrations with values fluctuating from - 0.5 mV at the lowest Cu level to 1 mV at the higher concentration in the mid intestine and from -1.3 mV to 0.5 mV in the posterior intestine.

These data facilitated selection of 50 μ M (3 μ g ml⁻¹) as the optimum range for all subsequent experiments. The selection was based on two factors – the position of the data point on the asymptote of the curves and the fact that *in vivo* data from intact trout fed a regular diet at 2 % body weight revealed the presence of approximately 0.5-4 μ g ml⁻¹ Cu in the supernatant extracted from gut contents of stomach and intestine 2 h after initial feeding (Fig. 2-1 C), suggesting this concentration to be environmentally realistic.

Series 3. Effect of temperature on Cu uptake:

Examination of temperature-dependence of Cu uptake rate, revealed a general increase in Cu uptake in the serosal and muscle compartments of the mid and posterior intestine when the incubation temperature was increased from 3 to 13°C and from 13 to 23° C (Fig. 2-7 A&B). Q_{10} values ranging from 1.5 to 3.4 in the mid intestine and 1.9 to 4.6 in the posterior intestine with respect to the serosal and muscle layer strongly suggested Cu is actively transported on an overall basis. Reducing or increasing the temperature by 10°C, however considerably reduced Cu uptake rates in the epithelial compartment of both mid and posterior intestine, Q_{10} values in the epithelial compartment were therefore considerably lower ranging from 1.3 to 0.277.

Series 4. Effect of ascorbic acid on Cu uptake:

Ascorbate had little influence on Cu uptake rates, and the small effects seen were not dose-dependent (Fig.2-8). At the highest ascorbate concentration (2.5 mM), Cu uptake was inhibited in the mid intestine. In contrast, uptake seemed slightly enhanced at 500 μ M ascorbate in the mid intestine but this did not reach significance. These results indicate that ascorbic acid has very little, if any, effect on intestinal copper absorption.

Series 5. Cu /Na uptake in response to anoxia:

Anoxia had no significant effect on the uptake of Cu in either the mid or posterior intestine (Fig. 2-9 A; B). However anoxia significantly inhibited Na uptake by 40% in the mid intestine (Fig. 2-9 A) and 20% in the posterior intestine (FIG. 2-9 B). Note that the absolute rate of Na uptake is about 5 orders of magnitude greater than Cu uptake on a molar basis.

Discussion

Site of Cu absorption

Cu-to-bead ratios in total chyme in the posterior intestine when compared to parallel values in the food indicate an apparent absorption of about 50% of the ingested Cu in the food after 72 h (Fig. 2-3A). By way of comparison, Berntssen et al., (1999b) measured an apparent Cu retention of 25% in Atlantic salmon fry reared for 3 months on a standard diet. Cu retention efficiency of 9% was reported in juvenile rainbow trout fed a 1.5% ration of standard food for 35 days (Kamunde and Wood, 2003) while Kjoss et al., (2004) observed similar values of 11-15% in trout over an exposure period of 28 days. Infusion of a single oral dose of ⁶⁴Cu in trout resulted in 12% internalization of the dietary dose after 72 h (Clearwater et al., 2000). These values are all considerably lower compared to the present study which is not surprising as the earlier measurements are based on net retention efficiency, and therefore undoubtedly underestimate absorption efficiency because they are lowered by excretory losses. However, our method of measuring disappearance of Cu from food after a single meal in a previously starved fish may have maximized absorption efficiency. Studies on nutrient bioavailability reveal that the efficiency of absorption decreases with increasing food intake (Brett and Groves, 1979) due to increases in gut evacuation time and passage of faeces. The human gastrointestinal system can reportedly absorb 30-40% of ingested Cu from a standard diet (Wapnir, 1998) but Turnlund et al., (1989) noted that the rate of Cu absorption in humans varied inversely with Cu intake and could be as low as 12% with very high Cu intakes.

An important *caveat* to our conclusion that absorption efficiency was about 50% is the possibility of Cu leaching from the food into the water in the few seconds prior to ingestion. If this had occurred, it would have caused an artificial increase in the apparent absorption of Cu. In this case, the Cu-to-bead ratio in the stomach at 2 h (Fig.2-3 A) would be a more realistic reference point for the Cu-to-bead ratio in the ingested food. Consequently, comparing the Cu-to-bead ratio in the stomach to that in the posterior intestine would indicate that only about 25% of Cu in the food is absorbed, rather than 50%.

However, if Cu is not leached from the food prior to ingestion, the considerable decline in Cu-to-bead ratio from food to stomach implicates the stomach as a major site of Cu absorption in the rainbow trout. In humans it is generally thought that the acidic environment in the stomach releases dietary Cu from food conjugates and facilitates peptic digestion (Gollan, 1975). This is followed by partial absorption in the stomach though most of the absorption occurs in the intestine. It is possible that the acidic environment of the fish stomach (Fange and Groves, 1979) may similarly solubilize the metal from the food, leading to partial absorption through the gastric epithelium as in mammals. However it appears to be controversial in mammals whether the contribution of the stomach is important relative to that of the various intestinal segments (Linder, 1991).

It is imperative therefore, that future studies measuring dietary Cu uptake consider the prospect of Cu leaching from food prior to ingestion. Simulating the mean exposure time of the feed pellets to water, and/or measurement of Cu release into the tank could fulfill this objective. It will be equally important to examine the possibility of Cu absorption through the stomach using *in vitro* techniques.

Using the in vitro data of Fig. 2-5B for rates of Cu transport across the various intestinal segments, together with representative measurements of total intestinal surface area in a 250 g trout, we estimate that unidirectional ⁶⁴Cu uptake rates measured *in vitro* summed along the entire intestinal tract would amount to approximately 740 ng kg⁻¹ h⁻¹ (Table 2-1). In comparison whole body unidirectional Cu uptake rates in vivo during a 48 h dietary ⁶⁴Cu exposure in juvenile rainbow trout were approximately 900 ng kg⁻¹ h⁻¹ (Kamunde et al., 2002a). This suggests that the intestine can account for most of the dietary Cu absorption in the rainbow trout, leaving only a small contribution from the stomach. Further examination of the spatial distribution of Cu in vitro reveals a 5-10 fold higher Cu uptake rate in the anterior intestine compared to the mid and posterior regions (Fig. 2-5B) which is consistent with evidence from Clearwater et al., (2000) suggesting that $\sim 80\%$ of an absorbed ⁶⁴Cu dose originally infused into the stomach of adult rainbow trout is found in the anterior intestinal tissue, 20% in the mid- and posterior intestinal tissue and <1% in the stomach tissue after 72 h. In juvenile rainbow trout, Kamunde and Wood, (2003) reported 50% of net Cu accumulation in the anterior intestine compared to 20% in the posterior and 10% in the mid intestine. It appears from the above that both in vitro unidirectional Cu uptake as seen in the present study, and net Cu flux observed in earlier studies (Kamunde and Wood, 2003) is considerably higher in the anterior intestine.

Against this consistent background localizing the bulk of *unidirectional* Cu uptake to the anterior intestine, it is therefore very interesting that the *in vivo* "disappearance test" using the Cu-to-bead ratio analysis clearly demonstrated that the anterior intestine is not a site of net Cu uptake, but rather a site of net Cu addition to the chyme. This is demonstrated by substantial increases in the Cu-to-bead ratios in this compartment relative to the preceding compartment the stomach (Fig.2-3A). The obvious conclusion is that although unidirectional uptake of Cu is high in this region, there is an even higher efflux component which significantly elevates the Cu-to-bead ratio in the chyme. Notably, this is also a site of substantial fluid addition to the chyme, as indicated by the 3fold increase in the water-to-bead ratio in this segment (Fig.2-2B). Biliary, pancreatic and intestinal wall secretions may all play a role in this region. Indeed the presence of high levels of free amino-acids and small peptides with Cu-binding properties in bile and pancreatic secretions has been well documented in mammals (Gollan and Deller, 1973; DiSilvestro et al., 1983). Several studies have identified the presence of high affinity Cubinding macromolecules in mammalian bile (Owen, 1964b; Frommer, 1971; Gollan, 1975) that are responsible for the poor absorption of biliary Cu, thereby ensuring that it stays in the tract. In adult trout which had been starved, Grosell et al., (2001b) measured a mean bile flow of 75 μ l kg⁻¹ h⁻¹ and calculated hepatobiliary Cu excretion rates of 0.75 nmol kg⁻¹ h⁻¹ - 1.6 nmol kg⁻¹ h⁻¹ which can account for an addition of approximately 40 ng kg⁻¹ h⁻¹ of Cu to the anterior intestinal chyme. Biliary secretions are greatly stimulated by food intake (Linder, 1991) thereby likely increasing the release of bile-borne Cu into the intestine many fold above this value after a single meal, as in the present study. Additional investigation of the factors responsible for the net efflux of Cu into the anterior intestinal contents, such as cell sloughing, pancreatic secretions and the changing flow and composition of bile after feeding is clearly warranted.

In contrast to the anterior intestine, the present in vivo data clearly indicated a decline in the Cu-to-bead ratios in the mid and posterior intestinal regions relative to the anterior intestine, with progressive absorption from the fluid phase in these compartments (Fig.2-3). Substantial unidirectional Cu uptake was also measured in these regions in vitro (Fig.2-5) suggesting an important role for the mid and posterior intestine in Cu uptake. Indeed, since the Cu-to-bead ratios in total chyme of the anterior intestine are the same as those in the original food (Fig. 2-3A), it could be argued that all *net* Cu absorption occurs in the mid and posterior intestine in vivo. In an in vivo study in trout using radioactive ⁶⁴Cu, Clearwater *et al.*, (2000), demonstrated ~ 20% was absorbed by the mid and posterior intestinal tissues, and <1% was found in or on the stomach after a 72 h exposure. A detailed in vitro study of gastrointestinal tissues of the African walking catfish (Clarias gariepinus) indicated that, similar to rainbow trout, net Cu uptake was greater in the middle and posterior intestine rather than in the esophagus stomach or anterior intestine (Handy et al., 2000). An earlier study using oral Cu exposure in trout (Handy et al., 1999) was in accord with these results as increased metallothionein induction in response to elevated diet-borne Cu occurred in the intestine rather than the stomach or esophagus. Kamunde et al., (2002a), suggest that the posterior intestine is the most active site for unidirectional Cu uptake in juvenile rainbow trout, followed in decreasing order by the pyloric cecae + anterior intestine, mid intestine and stomach.

Characterization of Cu uptake in the trout intestine

The unidirectional uptake of Cu in the trout intestine measured as a function of Cu concentration can be described by two mechanisms. The differential dose-response relationship observed (Fig.2-6) indicates a saturable component at low Cu concentrations (1-100 μ M), suggesting a carrier-mediated process. This component was superceded by a possible linear diffusive pathway at Cu concentrations of 500 μ M. Similar Cu uptake characteristics have been determined for intestinal Cu transport in mammals and one other fish species.

Bronner and Yost, (1985) and Wapnir and Steil, (1987) described Cu uptake as arising from a combination of transport via a saturable, carrier-mediated process plus a non-mediated diffusive component for mouse duodenum and rat jejunum respectively. Bronner and Yost, (1985) interpreted their data to conclude that at low Cu concentrations, almost all the Cu is absorbed by the saturable carrier, while at higher concentrations increasing amounts appear to be absorbed by diffusion. Cu uptake via a saturable carrier has been described more recently in polarized Caco-2 cell monolayers, derived from mammalian intestine (Arredondo *et al.*, 2000; Zerounian *et al.*, 2003), in mouse embryonic cells (Lee *et al.*, 2002) and in the African walking catfish (Handy *et al.*, 2000).

Additional evidence for the presence of a carrier-mediated component to Cu uptake can be derived from the significant increase in Cu uptake with increase in temperature. According to the Arrehenius equation, Q_{10} values measure the increase in a reaction velocity when standardized to a ten degree increase in temperature. Q_{10} values greater than 1.5 are generally considered to represent a biologically mediated process (usually one assisted by an enzymatic reaction and/or transporter), while a Q_{10} below 1.5 usually indicates a process dependent on the physicochemical properties of the reaction constituents (Hoar, 1983). In the present study, Q₁₀ values approaching or exceeding 2.0 for Cu uptake into the serosal and muscle compartments (Fig.2-7- i.e. basolaterally effluxed Cu) in both the mid and posterior intestine suggest that the basolateral transport of Cu is biologically mediated. Q₁₀ values lower than 1.5 measured from the epithelial compartment indicate a non-mediated apical route of Cu uptake. Possibly, the latter may become the rate-limiting process for transport across the entire mucosal epithelium at higher Cu concentrations where the diffusive mechanism appears to predominate (Fig.2-6). Indeed this observation is in accord with an earlier study *in vivo* in rainbow trout (Clearwater *et al.*, 2000). This investigation, using a similar Q_{10} analysis, concluded that intestinal uptake of Cu probably occurred via simple diffusion across the apical membrane while a biologically mediated process was responsible for basolateral exit. Intestinal Fe uptake in the European flounder was also reported to involve a carriermediated process in addition to simple diffusion (Bury et al., 2001).

The affinity of the saturable Cu uptake component determined in the present study for the mid and posterior intestine respectively ($K_m = 31.6 \ \mu M$ in mid intestine, 78 μM , or 18.9 μM with correction for linear component in posterior intestine; Fig.2-6) was in the range of Cu concentrations normally found in the fluid phase of the chyme *in vivo* (8-63 μM ; Fig.2-1C). These values are similar to that ($K_m = 21 \ \mu M$) obtained in the rat intestine (Wapnir and Steil, 1987) and comparable to the affinity of mouse embryonic cells for Ctr1-independent Cu transport, reported to be ~ 10 μM (Lee *et al.*, 2002). K_m's between 11-15 μ M have also been measured for rat hepatocytes in the absence or presence of histidine (Darwish *et al.*, 1984).

Whether the saturable process discerned for Cu uptake in the trout intestine was governed by a single transporter is debatable as Cu may be transported by more than one transport system, with different affinities and capacities. Several potential transporters are expressed by mammalian enterocytes (Linder, 1991) though conclusive evidence as to which ones are functionally important *in vivo* is still lacking. Currently, carrier-mediated Cu uptake is believed to be facilitated by the high affinity Ctr1 and/or the divalent metal transporter 1 (DMT1) (Rolfs and Hediger, 2001) in the mammalian intestine.

Interestingly, branchial Cu uptake in fresh water rainbow trout similarly reveals saturation kinetics at low Cu concentrations for both the Na-sensitive and insensitive components and a linear relationship when Cu concentrations were raised (Grosell and Wood, 2002). However, the branchial affinity for Cu uptake in trout as reported by Grosell and Wood, (2002) was approximately three orders of magnitude lower with K_m =7.1 nM – 9.6 nM for the Na-sensitive and insensitive components respectively. Branchial transporters have to work at the much lower Cu levels (nM) which are normally present in water while the trout intestine is a much lower affinity absorptive pathway that functions at the much higher Cu levels (μ M) which are normally present in the fluid phase of the chyme (Fig.2-1C). Despite the lower affinity, a maximum transport capacity of about 11 pmol g⁻¹ h⁻¹ was calculated on a whole body basis for the trout intestine which is comparable to maximum branchial Cu uptake ($J_{max} = 3.5 \text{ pmol.g}^{-1} \text{ h}^{-1}$ for "Na-insensitive" Cu uptake and 21.2 pmol.g⁻¹ h⁻¹ for "Na-sensitive Cu uptake") (Grosell and

Wood, 2002). Kamunde *et al.*, (2002a) have assigned a greater role for the dietary route of Cu uptake in juvenile rainbow trout as uptake rates of dietary Cu were > 10-fold higher than uptake rates of waterborne Cu. At least in part, this discrepancy may reflect the fact that Kamunde *et al.*, (2002a) worked at background Cu concentrations rather than saturating Cu concentrations of the two transport epithelia. This discrepancy could also be a result of differences between kinetics governing transport in intact animals *versus* a closed *in vitro* system.

Ascorbic acid was used to reduce $Cu(II)^{2+}$ to $Cu(I)^{1+}$ in the mucosal solution to test which form of Cu was the preferred form for transport (c.f. Arredondo et al., 2003; Zerounian et al., 2003). However there was negligible effect of ascorbic acid on Cu uptake rates by the trout intestine over a large range of concentrations tested. This result suggests, either that the valence of Cu present does not matter or more likely that sufficient quantities of endogenous reductase are already present on the intestinal epithelium. Therefore exposure to additional levels of a reducing agent would not be expected to augment Cu uptake. The latter implies that $Cu(I)^{1+}$ is the transported form. Indeed the presence of endogenous plasma membrane reductases capable of reducing Cu has been reported in mammalian brush border membranes (Knopfel and Solioz, 2002). Bell et al., (2002) have noted the presence of relatively high concentrations of glutathione disulphide and suggest that $Cu(II)^{2+}$ entering the cell would be immediately reduced by glutathione to $Cu(I)^{1+}$. In either case, the results indicate that Cu uptake rates are not influenced by change in the redox state of the Cu ion in bulk solution, an important consideration for the design of future *in vitro* experiments intended to analyze the mechanism of Cu transport (Chapter 3).

Our findings are contrary to those from Arredondo et al., (2003) in Caco-2 cells (derived from mammalian intestine) where the presence of ascorbate in the medium was considered necessary to obtain significant apical Cu uptake but correspond completely with data from Zerounian et al., (2003) in Caco-2 cell monolayers showing no significant effect of a range of ascorbic acid concentrations on Cu uptake. An earlier study using intestinal segments in rats (Van Campen and Gross, 1968) reported a 37% reduction in absorption of an intraduodenal dose of Cu (1 μ g = 16 μ moles total; volume dilution not reported) in the presence of 2.5 mg (16 µmoles total; volume dilution not reported) of ascorbate. A similar small inhibition of Cu uptake was observed at the mid intestine with $50 \,\mu\text{M}$ Cu and 2.5 mM ascorbate in the present study (Fig. 2-8A). Possibly this could be a non-specific effect of the high ascorbate concentration used since using a similar approach as Van Campen and Gross, (1968), Zerounian et al., (2003; citing unpublished observations from S. Allerton and M.C. Linder) found no effect of 1 mg (6 µmoles total; volume dilution not reported) ascorbate on the rate of appearance of ⁶⁴Cu in blood and organs after 1 h. Lanno et al., (1985a) examined the effect of adding different amounts of ascorbic acid (0-10,000 mg kg⁻¹) to a diet containing excessive Cu concentrations (800 mg kg⁻¹) known to cause a decrease in growth rate of juvenile rainbow trout. Their results indicated that ascorbic acid had no measurable effect on Cu uptake or metabolism. Using Cu balance studies in rats on high ascorbate diets, Johnson and Murphy, (1988) also did not find an effect on apparent Cu absorption. We therefore conclude that for future experiments measuring Cu uptake rates in the trout intestine (Chapter 3) Cu $(II)^{2+}$ can be the species employed in the luminal medium as the redox state of the Cu ion had little effect on Cu uptake rates.

In cases of severe O_2 limitation, most cells and tissues cannot continue to meet the energy demands of active ion transporting systems and therefore reduce or shut down these processes via inhibition of Na⁺K⁺ATPase activities and/or ion channel "arrest"(Hochachka, 2001). Anoxia can therefore be used as an effective inhibitory tool to characterize ion transport via energy driven pathways. In the present study, the anoxia was nominal (gassing with 99.7% N₂, 0.3% CO₂) and is probably best considered extreme hypoxia. Our observation that this treatment significantly inhibited Na uptake (Fig.2-9) in the trout intestine conforms with earlier studies citing inhibition of active intestinal transport of Na in the jejunum of rat exposed to extreme hypoxia (Lifshitz *et al.*, 1986). Nevertheless, 60-80% of Na uptake continued in the present study (Fig.2-9) suggesting that transport processes in teleost gut may be quite resistant to low oxygen.

In contrast, Cu uptake in the trout intestine did not show any significant change in response to anoxia. Few studies have examined the effect of O_2 depletion on Cu uptake rates in vertebrates. A possible explanation for the above response could be that ATP generated from anaerobic glycolysis was sufficient to maintain Cu transport as the energy demand for Cu ATPase activity may be far less compared to Na⁺/K⁺-ATPase, which is considered to be the cell's dominant energy utilizer. Indeed, Hogstrand *et al.*, (2002) reported an unexpected and marked increase rather than inhibition in Ag influx and accumulation in response to cyanide treatment in the intestine of the European flounder

(*Platichthys flesus*). In the same species Lennard and Huddart, (1992) demonstrated that, while hypoxia caused a reduction in contractile force and membrane potential of the heart, no significant changes in mechanical activity or membrane potential occurred in gut tissues. Intestinal tissues may be well adapted to function under hypoxic or anoxic conditions.

In conclusion, our results confirm the mid and posterior regions of the intestine as important sites of Cu absorption, suggest that the anterior intestinal region plays a complex role in bidirectional Cu transport and indicate a need for further investigation into the possible role of the stomach in Cu absorption in the fresh water rainbow trout. As well, we provide evidence that Cu uptake occurs via a hypoxia-resistant, carrier-mediated, saturable process which can be fueled by $Cu(II)^{2+}$ at concentrations which are typical of those in fluid phase of the chyme *in vivo* in the trout intestine. The study opens an opportunity to investigate the nature and identity of the specific carriers and /or ion-channels involved in this process and gain further insight into factors governing intestinal uptake of Cu in rainbow trout.

Literature Cited:

- Arredondo, M., Munoz, P., Mura, C.V. and Nunez, M.T. (2003). DMT1 a physiologically relevant apical Cu¹⁺ transporter of intestinal cells. *Am. J. Phys.*, 284, C1525-C1530.
- Arredondo, M., Uauy, R.and Gonzalez, M. (2000). Regulation in copper uptake and transport in intestinal cell monolayers by acute and chronic copper exposure. *Biochim Biophys. Acta.* 1474,169-176.
- Barthe, L., Woodley, J. and Houin, G. (1999). Gastrointestinal absorption of drugs: methods and studies. J. Fundam.Clin.Pharmacol. 13, 154-168.
- Bell, R.A., Ogden, N. and Kramer, J.R. (2002). The biotic ligand model and a cellular approach to class B metal aquatic toxicity. *Comp. Biochem. Physiol.* Part C 133, 175-188.
- Berntssen, M.H.G., Lundebye, A.K. and Maage, A. (1999b). Effects of elevated dietary copper concentrations on growth, feed utilization and nutritional status of Atlantic salmon (Salmo salar L.) fry. *Aquaculture* 17, 167-181.
- Brett, J.R. and Groves, T.D.D. (1979). Physiological Energetics. In *Bioenergetics* and Growth, Fish Physiology vol.viii. WS Hoar, DJ Randall and JR Brett (eds.), pp. 280-344. Academic Press, New York, San Francisco, London.
- Bronner, F., and Yost, J.H. (1985). Saturable and nonsaturable copper and calcium transport in mouse duodenum. *Am.J.Physiol.* 249, G 108-112.
- Bury, N.R., Grosell, M., Wood, C.M., Hogstrand, C., Wilson, R.W., Rankin, J.C., Busk, M., Lecklin, T. and Jensen, F.B. (2001). Intestinal iron uptake in the European flounder (Platichthys flesus). J.Exp. Biol. 204, 3779-3787.
- Clearwater, S.J., Farag, A.M. and Meyer, J.S. (2002). Bioavailability and toxicity of dietborne copper and zinc to fish. *Comp. Biochem. Physiol.* 132C, 269-313.
- Clearwater, S.J., Baskin, S.J. and Meyer J.S. (2000). Gastrointestinal uptake and distribution of copper in rainbow trout. *J. Exp. Biol.* 203, 2455-2466.

- Darwish, H.M., Cheney, J.C., Schmitt, R.C. and Ettinger, M.J., (1984). Mobilization of Cu(II) from plasma components and mechanisms of hepatic copper transport. *Am. J. Physiol.* 246, G 72-G 79.
- DiSilvestro, R.A., Cousins, R.J. (1983). Physiological ligands for copper and zinc. Annu. Rev. Nutr. 3, 261-88.
- Fange, R. and Groves, D. (1979). Digestion. In *Bioenergetics and Growth, Fish Physiology* vol. viii. WS Hoar, DJ Randall and JR Brett (eds.) pp. 161-260.
- Frommer, D.J., (1971). The binding of copper by bile and serum. *Clin. Sci.* 41, 485-493.
- Gollan, J.L., 1975. Studies on the nature of complexes formed by copper with human alimentary secretions and their influence on copper absorption in the rat. *Clin. Sci. Mol. Med.* 49, 237-245.
- Gollan, J.L., and Deller, D.J. (1973). Studies on the nature and excretion of biliary copper in man. *Clin.Sci.* 44, 9-15.
- Grosell, M., Wood, C.M., Wilson, R.W., Bury, N.R., Hogstrand, C., Rankin, C., Jensen, F.B., (2004). Bicarbonate secretion plays a role in chloride and water absorption in the European flounder intestine. Am. J. Physiol. Regul Integr Comp Physiol. [Epub ahead of print]
- Grosell, M., and Wood, C.M. (2002). Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.
- Grosell, M., McGeer, J.C. and Wood, C.M. (2001b). Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. *Am. J. Physiol.* 280, R796-R806.
- Grosell, M., and Jensen, F.B. (1999). No₂⁻ uptake and HCO₃⁻ excretion in the intestine of the European flounder (*Platichthys flesus*). J. Exp. Biol. 202, 2103-2110.
- Handy, R.D., Musonda, M.M., Philips, C. and Fella, S.J. (2000). Mechanisms of gastrointestinal copper absorption in the African walking catfish:copper dose-effects and a novel anion-dependent pathway in the intestine. *J.Exp.Biol.* 203, 2365-2377.
- Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., and Musonda, M.M. (1999). Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout

(Oncorhynchus mykiss) during chronic dietary exposure to copper. *Aquat. Toxicol.* 38, 257-276.

- Handy, R.D. (1996). Dietary exposure to toxic metals in fish. In *Toxicology of Aquatic pollution. Physiological, Cellular and Molecular Approaches.* EW Taylor (ed.) pp. 29-60. Cambridge University Press.
- Harris, E.D. (1991). Copper transport: an overview. Pro. Soc. Exp. Med. 192, 130-140.
- Hoar, W.S. (1983). General and Comparative Physiology. Third edition. Englewood Cliffs, NJ:Prentice-Hall, Inc. pp.851.
- Hochachka, P.W., Lutz, P.L. (2001). Mechanism, origin and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol.* Part B 130, 435-459.
- Hogstrand, C., Wood, C.M., Bury, N.R., Wilson, R.W., Rankin, J.C. and Grosell, M. (2002). Binding and movement of silver in the intestinal epithelium of a marine teleost fish, the European flounder (Platichthys flesus). *Comp. Biochem. Physiol.* Part C 133, 125-135.
- Johnson, M.A. and Murphy, C.L. (1988). Adverse effects of high dietary iron and ascorbic acid on copper status in copper deficient and copper-adequate rats. *Am. J. Clin. Nutr.* 47, 96-101.
- Kamunde, C.N., and Wood, C.M. (2003). The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss. Aquat. Toxicol.* 62, 235-254.
- Kamunde, C.N., Clayton, C. and Wood, C.M. (2002a). Waterborne versus dietary copper uptake in trout and the effects of waterborne copper acclimation. *Ann. J. Physiol.* 383, R69-R78.
- Kamunde, C.N., Grosell, M., Higgs, D. and Wood, C.M. (2002b). Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *J.Exp.Biol.* 205, 279-290.
- Kjoss, V.A., Kamunde, C. N., Niyogi, S., Grosell, M. and Wood, C.M. (2005). Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. J. Fish Biol. 66, 468-484.

- Knopfel, M. and Solioz, M. (2002). Characterization of a cytochrome b(558) ferric/cupric reductase from rabbit duodenal brush border membranes. Biochem. Biophys .Res. Comm. 291(2), 220-5.
- Lanno, R. P., Slinger, S.J. and Hilton, J.W. (1985a). Effect of ascorbic acid on dietary copper toxicity in rainbow trout. (*Salmo gairdneri* Richardson). *Aquaculture*. 49, 269-287.
- Lanno, R.P., Hicks, B and Hilton, J.W. (1985b). Maximum tolerable and toxic levels of dietary copper in rainbow trout (*Salmo gairdneri* Richardson) *Aquaculture* 49, 257-268.
- Lee, J., Pena, M.M.O., Nose, Y. and Thiele, D.J. (2002). Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 277,4380-4387.
- Lennard, R., Huddart, H.(1992). Hypoxia-induced changes in electrophysiological responses and associated calcium movements of flounder (*Platichthys flesus*) heart and gut. Comp. Biochem. Physiol. Comp. Physiol. 101(4), 717-21.
- Lifshitz, F., Wapnir, R.A., Teichberg, S. (1986). Alterations in jejunal transport and (Na+-K+)-ATPase in an experimental model of hypoxia in rats. *Proc Soc Exp Biol Med.* 181(1), 87-97.
- Linder, M.C. and Hazegh-Azam (1996). Copper biochemistry and molecular biology. Am. J. Clin. Nutr. 63, 797S-811S.
- Linder, M.C. (1991). Biochemistry of copper. Plenum press New York. pp.135-139.
- Lucock, M.D., Priestnall, M., Daskalakis, I., Schorah, C.J., Wild, J. and Levene, M.I. (1995). Nonenzymatic degradation and salvage of dietary folate: Physicochemical factors likely to influence bioavilability. *Biochem.and Mol. Med.* 55, 43-53.
- McCarthy, I.D., Carter, C.G. and Houlihan, D.F. (1992). The effect of feeding hierarchy on individual variability in daily feeding in rainbow trout, *Oncorhynchus mykiss. J. Fish Biol.* 41, 257-263.
- Miller, P.A., Lanno, R.P., McMaster, M.E. and Dixon, D.G. (1993). Relative contributions of dietary and waterborne copper to tissue copper burdens and waterborne-copper tolerance in rainbow trout. (Oncorhynchus mykiss). Can. J. Fish. Aquat. Sci. 50, 1683-1689.

- Owen, C.A., Jr., (1964b). Absorption and excretion of ⁶⁴Cu- labeled copper by the rat. *Am.J.Physiol.* 207, 1203-1206.
- Puig, S., Thiele, D.J. (2002). Molecular mechanisms of copper uptake and distribution. *Curr Opin Chem Biol.* 6(2), 171-80.
- Rolfs, A.and Hediger, M.A. (2001).Intestinal metal ion absorption: an update. Curr. Opin. Gastroenterol. 17(2), 177-183.
- Turnlund, J.R., Keyes, W.R., Anderson, H.L., Acord, L.L. (1989). Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope ⁶⁵Cu. *Am. J. Clin. Nutr.* 49, 870-8.
- Van Campen, D.R. and Gross, E. (1968). Influence of ascorbic acid on the absorption of copper by rats. J. Nutr. 95, 617-622.
- Wapnir, R.A. (1998). Copper absorption and bioavailabiliy. Am. J. Clin. Nutr. 67, (suppl.) 1054S-1068S.
- Wapnir, R.A. and Steil L. (1987). Intestinal absorption of copper:effect of sodium. *Proc.Soc.Exp.Biol. Med.* 185, 277-282.
- Withers, P.C. (1992). *Comparative Animal Physiology*. Orlando, Saunders College Publishing.
- Wolf, K. (1963). Physiological salines for freshwater teleosts. Progr. Fish Cultur 25, 135-140.
- Wood, C.M. (2001). Toxic responses of the gill. pp 41-45 In: Schlenk, D., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Fresh water Teleosts, Vol. 1 taylor and Francis, London.
- Zerounian, N.R., Redekosky, C., Malpe, R. and Linder, M.C. (2003). Regulation of copper absorption by copper availability in the Caco-2 cell intestinal model. Am. J. Physiol. 284, G739-G747.

Figure Legends:

Fig.2-1. Cu concentration in digestive tract contents of each section. (A) Cu concentration in total chyme. (B). Cu concentration in solid phase. (C) Cu concentration in fluid phase. Values are means \pm SEM (n = 6). Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters show significant differences between different time points. Statistical significance with respect to the previous compartment was tested using paired t-tests. Asterisks denote significant decrease from previous compartment at same time point.



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Fig. 2-2. (A) % water in total chyme along the digestive tract (B) Water : bead ratio in total chyme. Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters show significant differences between different time points. Statistical significance with respect to the previous compartment was tested using paired t-tests. Asterisks denote significant decrease from previous compartment at same time point and squares represent significant increase from previous compartment at same time point.


Fig. 2-3. Cu:bead ratio in digestive tract contents of each section. (A) Cu:bead ratio in total chyme. (B). Cu:bead ratio in solid phase.(C) Cu:bead ratio in fluid phase. Values are means \pm SEM (n = 6). Statistical significance within the same compartment was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters show significant differences between different time points. Statistical significance with respect to the previous compartment was tested using paired t-tests. Asterisks denote significant decrease from previous compartment at same time point and squares represent significant increase from previous compartment at same time point.



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Fig.2-4. Time course of Cu accumulation in the isolated mid intestinal segment of rainbow trout, exposed to 50 μ M Cu. Values are means \pm SEM (n = 5 at each time point).



Fig.2-5 A. Relative Cu accumulation, expressed as a proportion of total accumulation in serosal, muscle, rinse and epithelial compartments of trout mid intestinal segment over a 2 h measurement period. (B) Cu uptake rates *in vitro* in anterior, mid and posterior sections of the intestine, using isolated intestinal segments of rainbow trout. Values are means \pm SEM (n = 5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test; Means labeled with different letters are significantly different (p<0.05).





Fig. 2-6. Cu uptake kinetics in isolated intestinal segments (A- mid intestine; B-posterior intestine) from rainbow trout exposed to five different Cu concentrations at 1 μ M, 10 μ M, 50 μ M, 100 μ M & 500 μ M. Means <u>+</u> SEM (n=3 per treatment).



MID INTESTINE



в

POSTERIOR INTESTINE



Fig. 2-7. *In vitro* Cu uptake rate in isolated intestinal segments (A- mid intestine; B – posterior intestine) of rainbow trout at 3°C, 13°C and 23°C. Values are means \pm SEM Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (p<0.05).



Fig. 2-8. Effect of ascorbate on Cu uptake rates (A- mid intestine; B-posterior intestine) Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using one way ANOVA. Means labeled with different letters are significantly different (p<0.05).



Fig. 2-9. Cu and Na uptake rates in isolated intestinal segments (A- mid intestine; B – posterior intestine) from rainbow trout exposed to anoxia. Values are means \pm SEM (n=5 per treatment).Statistical significance was tested using unpaired t-tests (2-tailed). Means labeled with asterisks are significantly different (p<0.05).



Table 2-1. Unidirectional Cu uptake rate in intestinal segments of rainbow trout

Table 2-1.

Region	Unidirectional Cu uptake rate ng cm ⁻² h ⁻¹	Surface area cm ⁻²	Unidirectional Cu uptake rate ng h ⁻¹
Anterior intestine	4.4	30	132
Mid Intestine	1.6	11	17.6
Posterior intestine	2.2	16	35.2
Total intestinal Cu uptake rate for a 250 g fish =			184.8 ng h ⁻¹ or

739.2 ng kg⁻¹ h⁻¹

Chapter 3

Mechanism of dietary Cu uptake in rainbow trout : evidence for a Na- dependent and a Na-independent pathway.

Introduction

Dietary (copper) Cu intake by humans is thought to be in the region of 0.6-1.6 mg day⁻¹, or about 0.4-0.9 ng g⁻¹ h⁻¹, (Linder and Hazegh-Azam, 1996). In trout, Kamunde *et al.*, (2002 a) directly measured a very similar value (~ 0.9 ng g⁻¹ h⁻¹) for dietary Cu uptake. In humans, Cu is reported to be taken up from the diet in the upper small intestine (duodenum and early jejunum) and is rapidly dispersed to the intracellular Cu chaperone proteins prior to efflux from the enterocyte through a mechanism probably involving the Menkes ATPase (Snarp, 2003). However, despite the essentiality of Cu, the mechanisms involved in its uptake across the apical membrane of enterocytes remain unclear.

Investigating the role of Na with respect to Cu transport in the lumen of jejunal and ileal segments, in the perfused rat intestine, Wapnir and Stiel, (1987) demonstrated an association between Cu and Na in the luminal phase of Cu absorption. Further investigation of this association using an *in situ* perfusion procedure in the rat intestine revealed an inhibition of Cu uptake in the presence of amiloride, an inhibitor of Na channels and some Na-linked transporters (Wapnir, 1991), providing evidence for Cu entry via a Na-linked mechanism, such as an apical Na channel.

Two other possible candidate proteins responsible for the absorption of dietary Cu in mammals have emerged; the divalent metal transporter 1 (DMT 1) and Cu transporter 1 (Ctr1). When expressed in Xenopus oocytes, DMT1 was shown to be capable of transporting a range of divalent metals including Fe^{2+} , Zn^{2+} , Cu^{2+} , and Mn^{2+} (Gunshin et al., 1997). Ctr1 has also been shown to have Cu transport activity in transfected cell lines, displaying a substrate preference for Cu (I) i.e. Cu¹⁺ (Lee et al., 2002). However, mammalian Ctr1 mRNA expression is not regulated following dietary Cu restriction (Lee et al., 2000) in human Caco-2 cells or following exposure to high Cu conditions (unpublished data cited by Sharp, 2003). This is in contrast to yeast Ctr1, which is transcriptionally regulated by Cu availability (Dancis *et al.*, 1994). Alternatively Sharp, (2003) has reported that high Cu levels could modify the expression of DMT1 in Caco-2 cells and suggest that DMT1 and not Ctr1 acts as the major intestinal Cu transporter. Arredondo et al., (2003) have recently demonstrated an association between Cu and Fe transport and suggest that, rather surprisingly DMT1 preferentially transports monovalent Cu (I) (i.e. Cu^{1+}) relative to Cu (II) (i.e. Cu²⁺) in cultured Caco -2 cells.

Several transport proteins involved in cellular Cu uptake have thus been identified in mammals; in contrast, very little is known about the mechanisms of Cu metabolism in lower vertebrates, including fish. Fish resemble higher vertebrates at the organ level with respect to Cu homeostasis which entails regulated uptake, distribution and excretion (Grosell *et al.*, 1998 a,b; Kamunde *et al.*, 2001). However, fish possess two potential routes of uptake from the environment, from the water via the gills and from the diet via the digestive tract. Recent studies investigating interactions between branchial and

gastrointestinal uptake of Cu (Kamunde *et al.*, (2001, 2002 a,b) provide evidence that under normal levels of Cu in the water and food, rainbow trout sources over 80% of its Cu from food. However, while the mechanisms of waterborne Cu uptake have been the focus of most current research, mechanisms of dietary Cu uptake in fish have received little attention.

Several aspects of the mechanisms of waterborne Cu uptake in fish have now been characterized. Two pathways of branchial Cu uptake have been identified, namely the Cu-specific and Na-linked pathways. Evidence that Cu enters the gill cell via a Na- channel has been provided directly by Na-competition experiments and by pharmacological studies (Grosell and Wood, 2002). Kamunde and Wood, (2003) and Pyle *et al.*, (2003) have demonstrated that increased dietary Na intake resulted in reduced branchial uptake of Na, accompanied by a reduction in branchial Cu uptake. Together these studies provide considerable evidence for a Na-associated Cu transport mechanism in the fish gills.

The Cu-specific pathway is insensitive to Na-competition or to pharmacological blockade of the apical Na-channel in the gills (Grosell and Wood, 2002). This pathway is believed to involve the high affinity Cu transporter (Ctr) analogous to hCtr1 (Lee *et al.*, 2000). An orthologue of hCtr1 has recently been cloned in zebrafish embryos (Mackenzie *et al.*, 2004) and while its precise location is yet to be identified, the gill and intestine appear to be prime candidates. Vanadate (P-type ATPase blocker) sensitivity of branchial Cu uptake (Campbell *et al.*, 1999) and partial cloning of a Cu-ATPase (Grosell *et al.*, 2001a) provide evidence for basolateral export of Cu via a Cu ATPase in the fish gills.

Available literature therefore suggests certain similarities between mammalian gut and fish gill Cu transport. However despite these recent gill findings, and the knowledge that diet is the dominant route of Cu uptake, transport mechanisms for Cu in the fish gut remain poorly understood. In the African walking catfish, removal of Na from the luminal saline tends to slow Cu absorption in the intestine (Handy *et al.*, 2002) reinforcing evidence for a shared route of uptake of these nutrients.

Isolated intestinal sacs in rainbow trout were effectively used in the preceding study (Chapter 2) to investigate the basic mechanisms of Cu uptake. In agreement with earlier studies using a similar technique (Barthe *et al.*, 1999; Grosell and Jensen, 1999; Handy *et al.*, 2000; Bury *et al.*, 2001; Grosell *et al.*, 2004), the intestinal sacs maintained viability for the 4 h duration of the flux period with mid- and posterior intestinal preparations yielding consistent results. The study demonstrated that uptake of Cu appeared to be biologically mediated in these sections and occurred by a saturable process. The involvement of a specific carrier or a channel-mediated transport mechanism was indicated.

The main aim of the present study was therefore a mechanistic characterization of the pathway(s) involved in Cu uptake in the mid- and posterior intestine of the rainbow trout using the *in vitro* gut sac technique. Possible Na-sensitive Cu uptake was assessed by manipulation of Na levels in the gut lumen and use of relevant pharmacological agents that are known inhibitors of Na transport. The role of amino acids was investigated using L- and D-histidine and P_{CO2} elevation at constant pH was used to investigate the possible contribution of a proton pump. The potential of DMT1 and Ctr1 to mediate Cu transport

was investigated through examination of pH sensitivity, known to stimulate both transport proteins and the possibility of inhibition of Cu uptake in the presence of 10-fold excess of other divalent metals. Transepithelial potential (TEP) in these experiments was measured to determine whether any of the observed effects on Cu transport were mediated by changes in the voltage gradient.

Methods

Acclimation

Rainbow trout (*Oncorhyncus mykiss*: weight 250-300g.) were obtained from Humber Springs Trout Hatchery (Orangeville, ON., Canada) and acclimated as described in Chapter 2 to dechlorinated Hamilton tap water from Lake Ontario (Na = 0.5 -0.6 mM, Cl = 0.7 mM, Ca = 1.0 mM, hardness ~ 140 ppm as CaCO₃, background Cu =< 16 pM, pH ~8 ; temperature =12 ± 2°C). During the two week acclimation period, fish were fed Martin's commercial dried pellet feed (5-pt, Cu content = 27 µg g⁻¹ dry wt.) daily, at a ration of 2% wet body mass per day. Prior to a sampling day, fish were not fed for 48 h to clear the gut of ingesta. For each experiment trout were randomly selected from the holding tank.

In vitro – Intestinal sacs

Intestinal sacs were used to analyse the mechanism of Cu uptake, which was evaluated at a single concentration of 50 μ M (3 μ g ml⁻¹) ⁶⁴Cu as Cu(NO₃)₂ chosen on the basis of the *in vivo* sampling and results of Chapter 2. Trout were euthanised by an overdose of MS-222 (0.25 g L⁻¹), the intestine was excised and placed in ice cold saline. Intestinal sacs were made from mid and posterior intestine segments according to protocol from Chapter 2. In brief, the posterior end of each intestinal segment was tightly sealed by double silk ligatures, while at the anterior end a small PE-50 catheter was secured in place, allowing administration of experimental solutions. The resulting

intestinal sacs were filled with 1 ml of mucosal saline containing radiolabeled ⁶⁴Cu 0.04 mCi ml⁻¹ as Cu(NO₃)₂ (Nuclear Research Reactor McMaster University) or 0.04 μ Ci ml⁻¹ ²²Na as NaCl-Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) in a modified Cortland saline (in mM: NaCl 133, KCl 5, CaCl₂·2H₂O 1, MgSO₄·7H₂O 1.9, NaHCO₃ 1.9, NaH₂PO₄· H₂O 2.9, glucose 5.5, pH 7.4; Wolf, 1963). The catheter was heat-sealed and the preparation blotted dry, weighed (to the nearest 0.1 mg) and immersed in a serosal bath containing 12 ml of the modified Cortland saline. During the flux period, the serosal saline was aerated with 99.7% O_2 and 0.3% CO_2 (i.e. $P_{CO2} = 2.25$ torr) gas mixture, temperature 13-15° C. Following a 2 h flux period, intestinal sacs were drained, reweighed, thoroughly rinsed (with 1 mM EDTA in saline) and the mucosal surface scraped with a glass microscope slide. The intestinal surface area was determined using the method of Grosell and Jensen, (1999). Samples of scrapings (epithelium), intestinal tissue remaining after scraping (muscle layer), the serosal flux bath (serosal layer) and the wash solutions were counted for ⁶⁴Cu or ²²Na activity in a Canberra-Packard Minaxi Auto Gamma 5000 Series (Meriden, CT, USA) Auto Gamma counter with an on-board program for decay correction of ⁶⁴Cu.

TEP Measurements

For selected treatments, transepithelial potential measurements were performed on sac preparations of mid and posterior intestine bathed with mucosal saline on the mucosal surface and serosal saline on the serosal surface. TEP was measured using agar/salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes to a Radiometer PHM 82 standard pH meter (Radiometer; Copenhagen). All TEP values are expressed with mucosal reference at 0 mV, while the sac preparation was exposed to mucosal and serosal salines of appropriate composition. Tip potential was routinely less than 1 mV, and the electrodes were checked for symmetry. The mucosal side was accessed via the cannulation catheter and the serosal side via the outside bathing solution. Triplicate measurements over a 5-minute period were averaged.

Experimental Series

Series 1. Effect of Na on Cu uptake:

To examine the effect of increasing Na concentration on Cu uptake, five treatment groups were employed. NaCl levels in mucosal saline were altered to achieve Na concentrations of 3 mM, 35 mM, 70 mM, 140 mM and 280 mM while serosal saline remained at 140 mM. Osmolality was measured using a 5100C vapor pressure osmometer (Wescor Inc. Utah, USA). At control levels of 140 mM Na, measured osmolality of the modified Cortland saline was 280 mOsm. Thus osmolality of mucosal saline at 3, 35 and 70 mM Na was raised from 37, 80 and 187 mOsm respectively to 280 mOsm with 125 – 260 mM mannitol. This ensured that there was no osmotic gradient. For the 280 mM Na treatment, measured osmolality of the mucosal saline was 494 mOsm so the osmolality of serosal saline was raised to this value with 231 mM mannitol. Following a 2 h flux period, intestinal sacs were sampled as per the protocol described.

Series 2. Role of solvent drag in Cu absorption:

To evaluate whether solvent drag contributed to Cu uptake, osmolality of mucosal saline as compared to serosal was increased by 300 mOsm using 330 mM mannitol. This created a gradient for inhibition/reversal of mucosal to serosal fluid transport. Gut sacs were infused with this osmotically elevated mucosal saline spiked with ⁶⁴Cu and following a 2 h flux period, sampled as described previously.

Series 3. Osmotic pressure

Observations of stimulated Cu uptake in the presence of elevated mucosal Na raised the possibility that elevated osmolality, rather than elevated NaCl could be responsible (even in the absence of a solvent drag effect). To test this possibility, osmolality of the mucosal saline was raised to 494 mOsm to mimic osmolality at 280 mM Na using mannitol, while Na concentration in mucosal saline was maintained at control levels of 140 mM. Osmolality of the serosal saline was raised accordingly to be iso osmotic with mucosal saline.

Series 4. Effect of Cl substitution by SO₄ on Cu uptake:

To evaluate whether observed response to NaCl manipulation in Series 3 was related to changes in Cl rather than in Na concentration, the Cortland saline was modified to ensure substitution of all Cl ions by SO₄ ions. The following saline was used – (in mM) Na₂SO₄, 133; K₂SO₄, 5; CaSO₄, 1; MgSO₄·7H₂O, 1.9; NaHCO₃, 1.9; NaH₂PO₄·H₂O, 2.9; glucose, 5.5; pH 7.4. Three treatment groups were employed by altering Na₂SO₄

concentration in the mucosal saline to obtain 70 mM, 140 mM and 280 mM Na. In all cases salines were osmotically compensated with mannitol as in Series 3 to ensure the same osmolality of mucosal and serosal saline.

Series 5. Effect of phenamil:

To test the possible involvement of the apical Na channel in gut Cu and Na uptake, phenamil (RBI. Sigma-Aldrich Canada), an amiloride analogue that is an irreversible Na channel inhibitor (Garvin *et al.*, 1985; Kleyman and Cragoe, 1988) was employed at a concentration of 100 μ M, dissolved in 0.1% DMSO (Caledon lab. On. Canada). Gut sections of control and treatment groups were incubated in 0.1% DMSO and 100 μ M phenamil + 0.1% DMSO (both in mucosal saline) respectively for 1 h. After this preincubation period, control and phenamil-treated gut sections were thoroughly rinsed in saline to remove traces of DMSO and phenamil. This procedure eliminated any potential problem with phenamil-⁶⁴Cu complex formation possibly rendering Cu unavailable for uptake. Gut sacs were then infused with appropriate mucosal saline to separately measure Cu and Na uptake.

Series 6. Influence of CO₂:

In these experiments, the serosal saline was gassed with 0.3% CO₂ ($P_{CO2} = 2.25$ torr) in O₂ (control), 1% CO₂ (7.5 torr) in O₂ and 3% CO₂ (22.5 torr) in O₂ delivered from a Wösthoff gas mixing pump (Bochum, Germany). The goal was to use elevated P_{CO2} to drive any proton pump which might be present, and to assess its possible role in Cu and

Na uptake. Mucosal and serosal saline were pre- equilibrated with these gases prior to the 2 h flux period and appropriate gassing of serosal saline was continued throughout the experiment. The pH of the saline was kept at 7.4 using the Henderson-Hasselbalch equation

 $pH = pK + log[HCO_3] / (\alpha CO_2) (P_{CO2})$ to calculate the necessary NaHCO₃ concentration required to maintain this pH. Na levels in the saline were accordingly adjusted by reducing NaCl to accommodate the additional NaHCO₃, and the pH was checked to ensure that it remained at 7.40 ± 0.05.

Series 7. L & D-histidine mediated Cu and Na uptake:

To test the possible effects of amino acids, 10 mM solutions of either L - or D - histidine (Sigma Aldrich) were added to the mucosal saline. L- histidine is known to be the major Cu(II)-binding amino acid in human serum and the complex is considered to be the physiologically important transport form of Cu(II), which may occur either via ligand exchange or mediated via a histidine transporter on the brush border membrane (Lau & Sarkar, 1971). Osmolality of the serosal saline was raised using mannitol to accommodate addition of 10 mM histidine on the mucosal side. Solutions were made fresh on the day of experiment.

Series 8. Ag competition study:

500 μ M AgNO₃ (Sigma Aldrich), 10- fold excess in relation to Cu, was added to mucosal saline. Ag is known to enter via Na channels in the trout gill (Bury & Wood, 1999) but to augment Na transport in other systems (Klyce and Marshall, 1982; Rangachari and Matthews, 1985). Cl salts in the saline were substituted with SO₄ salts as in Series 4 to avoid precipitation of Ag. The stock solution of Ag(NO₃)₂ was prepared fresh and stored in the dark.

Series 9. Effect of pH on Cu uptake:

To assess the effect of pH on Cu uptake, the pH of mucosal saline was adjusted to 6.0, 7.4 and 8.0 using 1N H_2SO_4 and 2N KOH respectively. The saline was buffered with addition of 10 mM MES (Sigma-Aldrich). MES belongs to a range of "better buffers", tertiary amines which do not complex metals (Kandegedara and Rorabacher, 1999).The pH of serosal saline was maintained at the optimal 7.4 range.

Series10. Cation competition:

 $500 \mu M ZnSO_4$ or Fe(NO₃)₃, 10-fold excess in relation to Cu was added to mucosal saline, Zn and Fe are known to be transported by DMT1 (Gunshin *et al.*, 1997). Cl salts in the saline were substituted with SO₄ salts to avoid precipitation of the cations (in mM) Na₂SO₄, 133; K₂SO₄, 5; CaSO₄, 1; MgSO₄·7H₂O, 1.9; NaHCO₃, 1.9; NaH₂PO₄·H₂O, 2.9; glucose, 5.5; pH 7.4.

Analytical techniques and calculations

In each experimental study the intestinal sac preparations were blotted and weighed to the nearest 0.1 mg (Sartorius GMBH Gottingen; H110 ** V40; Germany) prior to and after the 2 h flux to allow for calculation of net fluid transport.

Net fluid transport rates were determined from the difference in total mass of the sac preparation over the experimental period. This was normalized by taking into account the gross surface area of exposed epithelium and time elapsed.

Rate of fluid transport (FTR) was calculated as follows :

FTR = (IW - TW)/ISA/t

Where IW = Initial weight of sac preparation in mg

TW = Terminal weight of sac preparation after flux in mg

ISA = Intestinal surface area in cm²

t= Time in h

to yield a rate of net water movement from mucosal to serosal surface, expressed in $\mu l \text{ cm}^{-2} h^{-1}$

Mucosal and serosal saline samples from the beginning and end of each flux were measured for⁶⁴Cu and ²²Na as applicable on a Canberra-Packard Minaxi Auto Gamma counter 5000 Series (Meriden, CT, USA) with an on-board program for decay correction of ⁶⁴Cu. As described in Chapter 2, since the wash solutions represented loosely bound ⁶⁴Cu and epithelial scrapings were a combination of mucus and surface cells, the radioactivity incorporated into the tissue layer and the serosal sample at the end of the flux period was considered as a conservative estimate of true Cu absorption (see Results). Cu uptake rate (UR) was calculated as follows:

UR = Tissue cpm / (SA*ISA*t)

Tissue cpm represents the total ⁶⁴Cu activity of the compartment measured SA is the initial measured specific activity of the mucosal saline (cpm/ μ g) ISA is the intestinal surface area in cm² t is time in hours

This produced a Cu accumulation rate (UR) expressed as μg cm $^{-2}$ h⁻¹.

Na uptake rate (UR) was calculated as follows:

$$UR = Tissue cpm / (SA*ISA*t)$$

Tissue cpm represents the total ²²Na activity of the compartment measured SA is the initial measured specific activity of the mucosal saline (cpm/ μ mol) ISA is the intestinal surface area in cm² t is time in hours

This produced a Na accumulation rate (UR) expressed as μ mol cm⁻² h⁻¹.

The concentrations of Cu and Na in mucosal saline were measured by graphite furnace atomic absorption spectroscopy (GFAAS; Varian SpectrAA -220 with graphite tube atomiser[GTA-110],Mulgrave,Australia) and flame atomic absorption spectroscopy (FAAS; Varian SpectrAA – 220 FS, Mulgrave, Australia) respectively for initial specific activity calculations following manufacturer specifications for Cu-analysis. National Research Council of Canada-certified analytical standards run at the same time were within the specified range.

The geochemical equilibrium modeling program MINEQL+ (Version 4.01; Environmental Research Software) was used to determine the effect of pH on Cu (II) speciation.

Statistical analyses

A one way analysis of variance (ANOVA) was generally used to compare the groups. Differences between specific means were then compared with a least significant difference (LSD) test (SPSS10 for Windows). Statistically significant differences between control and treated groups were evaluated by unpaired student's t-tests (two-tailed). Data are reported as means \pm S.E.M. and differences were considered significant at p<0.05.

Results

Series 1. Effect of Na gradients on Cu uptake:

Fig.3-1(A;B) illustrates Cu uptake rate in isolated intestinal segments from rainbow trout in response to manipulation of mucosal NaCl levels over the range 3-280 mM. A concentration-dependent increase in the rate of Cu uptake is observed with Cu absorption increasing significantly from 0.49 ng cm⁻² h⁻¹ at 3-35 mM Na to 1.5 ng cm⁻² h⁻¹ at 140 mM Na and 4.8 ng cm⁻² h⁻¹ at 280 mM Na in the mid intestine. Cu uptake in the posterior intestine was more or less uniform at the lower Na concentrations but dramatically increased from 1.2 ng cm⁻² h⁻¹ at 140 mM Na to 3.5 ng cm⁻² h⁻¹ at 280 mM Na. This approximately 2-fold increase in Cu uptake. Serosal transepithelial potential (TEP) measured against a mucosal reference set at 0 mV was -6 mV at 3 mM Na in the mid intestine (Fig. 3-1 C). Increasing Na concentration resulted in a progressively less negative TEP with values of +1.2 mV at 280 mM Na. The trend was similar in the posterior intestine (Fig. 3-1 D).

Series 2. Effect of solvent drag on Cu uptake:

Cu uptake occurred simultaneously with considerable fluid transport and net water flux was elevated 2-5 fold with increasing mucosal Na concentration in both mid and posterior intestine (Fig.3-2 A;B). In view of the increased fluid uptake accompanying increased mucosal Na concentration and increased Cu transport in Series 1 (Fig 3-1), this series evaluated whether solvent drag was responsible for the increased Cu uptake. However, reversing fluid transport by manipulation of the osmotic gradient had no effect on Cu transport rate in either segment (Fig.3-3 A; B), indicating that solvent drag was not involved.

Series 3. Influence of osmotic pressure:

Increasing the osmolality of the mucosal fluid to 495 mOsm with mannitol (serosal fluid set to the same level) to mimic the 280 mM Na level had no significant effect on Cu uptake in either mid or posterior intestine (Fig.3-4 A ; B), eliminating the possible role of osmotic pressure in stimulating Cu uptake.

Series 4. Possible Cl dependency of Cu uptake:

Replacing all Cl with SO₄ confirmed the Na-dependent pattern of Cu uptake in Fig.3-1 (A; B). A 2-fold increase in Cu uptake rate with increasing Na concentration from 70 to 280 mM (as Na₂SO₄) was observed in the mid and posterior intestine (Fig.3-5 A;B) similar to that seen with Na present as NaCl (Fig. 3-1) suggesting Cu uptake to be sensitive to Na and insensitive to Cl. As with NaCl manipulation, TEP exhibited an increasing serosal side positive trend with the increase in Na concentration (Fig.3-5 C; D).

Series 5. Effect of phenamil on Cu and Na uptake:

Mid and posterior intestinal segments exposed to 100 μ M phenamil exhibited an approximate 35% and 20% decrease in Cu and Na uptake respectively (Fig.3-6 A;B) compared to drug-free, DMSO solvent controls. Pretreatment with 100 μ M phenamil did not significantly influence TEP which remained unchanged at 1 mV and 0 mV in the mid and posterior segments respectively (data not shown).

Series 6. Influence of elevated CO₂:

Inducing hypercapnia by changing gassing of serosal saline from 0.3% CO₂ to 1% and 3% CO₂ (at constant pH = 7.4) caused a general trend of elevated Cu uptake which was significant (Fig.3-7 A;B) at the highest CO₂ concentration in both the mid and posterior intestine (3-fold increase). Na uptake was also elevated in both segments by about 50%, which was significant at 1% CO₂, with no further rise at 3% CO₂ (Fig.3-7 C & D). Hypercapnia did not induce any significant change in TEP which remained stable at 1 mV and 0 mV in the mid and posterior segments respectively (data not shown).

Series 7. L & D-histidine mediated Cu /Na uptake:

The presence of 10 mM L- histidine significantly increased both Cu and Na uptake. Cu uptake rate exhibited a 3 fold increase in the mid intestine while Na uptake showed a significant 40% increase. Similar trends were seen in the posterior intestine with Cu uptake increasing 2 fold and uptake of Na by about 50% (Fig.3-8 A; B).

Exposure to 10 mM D-histidine however had no significant effect on the uptake of either Cu or Na when compared to controls (Fig.3-8 C; D).

Series 8. Effect of Ag on Cu and Na uptake:

The presence of 500 μ M AgNO₃ in the mucosal saline significantly increased transepithelial uptake of Cu 3 fold in the mid intestine and doubled Cu uptake rate in the posterior intestine. Similarly, Na uptake rate increased by a significant margin of 50% in the mid intestine (Fig.3-9 A;B).Transepithelial potential increased significantly from + 0.75 mV in control mid intestine preparations and - 0.25 mV in posterior intestine sections to approximately + 2 mV in both sections of Ag treated groups, displaying a strong serosal side positive trend (Fig.3-9 C; D).

Series 9. Influence of luminal pH on Cu and Na uptake:

Cu uptake in rainbow trout intestine appeared to be sensitive to variation in luminal pH. There was no significant difference in Cu uptake when pH of mucosal saline was lowered from 7.4 to 6.0. However, Cu uptake declined by approximately 50% in the mid intestine when pH of mucosal saline was raised to 8.0 (Fig. 3-10 A; B). Similar trends of significantly lower rates of Cu uptake were observed in the posterior intestine at pH 8.0. The geochemical equilibrium modeling program MINEQL+ (Version 4.01; Environmental Research Software) was used to determine the effect of pH gradients on Cu (II) speciation (Fig.3-11). About 60% of the total concentration of Cu was present as Cu^{2+} at pH 7.4, increasing to 90% at pH 6.0. However a steady decrease in % total
concentration of Cu^{2+} was evident as pH increased particularly beyond 7.4, and only 18% of the total concentration of Cu remained as Cu^{2+} at pH 8.0. The decline in Cu^{2+} correlated with a steep increase in the % total concentration of CuCO₃.

Na transport and speciation on the other hand were unaffected with change in pH as uptake of Na was not significantly different between segments maintained at a mucosal pH of 6.0, 7.4 or 8.0 (Fig.3-10 A & B). Na⁺ accounted for 99.5% and Na₂SO₄ for 0.5% of total Na (Fig. 3-11 B). Variation of pH did not influence TEP significantly which remained unchanged at -1 mV and -0.5 mV in the mid and posterior intestine respectively (data not shown).

Series 10. Cation competition

The presence of 500 μ M ZnSO₄ in the mucosal saline significantly decreased transepithelial uptake of Cu by 75% in the mid intestine and 40% in the posterior intestine (Fig. 3-12 A; B). Similarly, Cu uptake rate significantly declined by 80% in the mid intestine when exposed to 500 μ M Fe(NO₃)₃ while a modest inhibition of 20% (non-significant) was seen in the posterior intestine (Fig. 3-12 C ; D). There was no effect of either cation on Na uptake rates in the mid or posterior intestine. Transepithelial potential increased marginally from + 0.75 mV in control mid intestine preparations to 1.2 mV on exposure to Zn and from - 0.25 mV to + 0.5 mV in the posterior intestine. This modest serosal side positive trend was not significant. There were no changes in TEP upon exposure to Fe (data not shown).

Discussion

Our data provide evidence for two possible pathways of intestinal Cu uptake in rainbow trout. Based on observations of a greater transport of Cu in the presence of Na, one of these uptake pathways appears to be Na-dependent. A second mechanism of Cu uptake apparently involves a specific metal transporter with features similar to a recently characterized Cu transporter from Ctr1-deficient embryonic cells in mammals (Lee *et al.*, 2002) and clearly accounts for a portion of Cu transport. Evidence that this pathway of Cu uptake was not dependent on Na is based on the sensitivity of Cu uptake to pH and competition with other divalent cations, these factors having no effect on Na transport. While referring to these mechanisms as Na-dependent and Na-independent Cu uptake we take into account the diverse responses of Cu transport to various treatments and suggest that within each category, the existence of more than one component of Cu uptake is possible.

Na-dependent Cu uptake

A notable finding of this study was the observation of a Na concentrationdependent increase in Cu uptake (Fig.3-1) which provides compelling evidence that Na may be involved in the uptake of Cu in trout intestine. Na-sensitive Cu transport has been earlier described in rainbow trout gills (Grosell and Wood, 2002) however the mechanism at the gills where branchial Cu uptake was reduced with increasing ambient Na concentration is diametrically different from our observation of stimulated Cu uptake across the intestinal epithelium.

Under normal physiological Na levels and symmetrical conditions the measured TEP for Cu uptake was about -1.5 mV. The teleost intestine in general exhibits a serosanegative transepithelial potential of a few mV when bathed in symmetrical saline solutions, unlike the serosa-positive mammalian intestine (Loretz, 1995). The observed serosa-negative potential in the teleost intestine is established as a result of excess net Cl absorption over Na absorption reflecting differential permeability of this leaky epithelium to the two ions (Field *et al.*, 1978; Loretz, 1983). It therefore follows that variation of Na levels in the mucosal saline leading to assymetrical conditions would influence TEP, thereby affecting ion transport. However, serosal transepithelial potential (TEP) was progressively less negative with increasing Na levels (Fig. 3-1, C; D), suggesting that stimulation of Cu uptake was not related to changes in TEP as the trend towards a serosa-positive electrical gradient with increasing luminal Na would have provided progressively less assistance for the transport of positively charged Cu ions.

Increasing the osmolality of the luminal fluid to mimic the highest Na concentration had no significant effect on Cu uptake (Fig.3-4) eliminating osmotic pressure as an explanation. Furthermore, the contribution of solvent drag and the nature of the accompanying anion are unimportant (see below). Thus the interaction of Na with Cu transport appears to be direct rather than indirect. In accord with the present results, Nadependent stimulation of Cu uptake has also been demonstrated in the jejunum and ileum of rat (Wapnir and Steil, 1987), though its exact mechanism remains unclear. In trout the intestinal Na-Cu interaction appears to be important *in vivo* as well as *in vitro*. Very recently Kjoss *et al.*, (2004) found that juvenile rainbow trout that received the highest dietary Na along with high dietary Cu showed the highest Cu retention and the highest whole-body Cu at the end of the exposure period, a result which was considered to reflect a positive interaction between Cu and Na transport in the gastrointestinal tract.

Enhanced Cu uptake paralleled a simultaneous and dramatic increase in fluid transport (Fig. 3-2), raising the possibility that the former was a solvent drag effect. However, since the increase in Cu uptake rate was essentially unaffected even under conditions of osmotically manipulated reversal in net water flux (Fig. 3-3), solvent drag was not associated with the Na-stimulated transport of Cu. Handy *et al.*, (2000) excluded the effect of solvent drag on Cu uptake in the intestine of the African walking catfish as absolute rates of Cu transfer into the serosal perfusate were higher than those for water, and Cu uptake to the serosal solution occurred when the net water flux was in the opposite direction. Wapnir and Steil, (1987) have shown that Na-associated Cu absorption in the rat intestine was not related to water fluxes as maximum Cu absorption corresponded with minimum net water absorption.

When Cl was replaced with SO₄ an identical response of Na-dependent stimulation in Cu uptake was observed at the highest Na concentration (Fig. 3-5) which was independent from changes in TEP, suggesting Cu uptake to be insensitive to Cl and that the nature of the accompanying anion is unimportant. These results are contrary to observations of Handy *et al.*, (2000) in the African walking catfish who reported a 10-fold reduction in the rate of Cu uptake when Cl was removed simultaneously from both mucosal and serosal solutions in the African walking catfish. The authors related this decrease in Cu uptake to an inhibition of Cu/anion symport resulting from the blockade of DIDS-sensitive basolateral Cl transporters. However, Wapnir, (1991) did not find any significant effect of furosemide on Cu absorption in the rat intestine, a drug which inhibits an electroneutral Na-K-2 Cl co-transporter, suggesting that the latter is not involved in Cu passage across the jejunal apical membrane in these animals.

The partial inhibition of both Cu and Na uptake with 100 µM phenamil, an amiloride analogue (Fig. 3-6) suggests that these two nutrients share a common apical uptake mechanism and implicates the apical Na channel in the process. Wapnir, (1991) provided evidence linking the transport of Cu to the apical Na channel in the rat intestine, specifically a considerable reduction in Cu absorption with 1 mM amiloride. This observation, together with earlier findings that the presence of luminal Na stimulated Cu transport (Wapnir and Steil, 1987) led to the proposal of Na-associated Cu transport (Wapnir, 1991). The results from the present study with trout intestinal sacs also correspond with a reported inhibition of Cu uptake in rainbow trout gills exposed to 100 µM phenamil (Grosell and Wood, 2002). However, Grosell and Wood, (2002) reported a discrepancy between the inhibitory effect of phenamil on Na and Cu uptake, phenamil inhibiting Na uptake more effectively than Cu uptake. This is not the case in the trout intestine where phenamil equally inhibited both Na and Cu uptake. When taken together with our finding of stimulated Cu uptake with increasing Na levels in the trout intestine in contrast to inhibition of Cu uptake by increasing Na levels at the trout gills, this clearly

indicates that Na-dependent Cu uptake in the trout intestine occurs via a mechanism different from that found in the gills.

Another piece of evidence suggesting some sort of linkage between Na and Cu transport in the trout intestine was the significant increases in both Na and Cu transport observed under induced hypercapnia (Fig. 3-7). Several lines of evidence from previous studies in isolated frog skin and urinary bladder of the water turtle (Harvey, 1992; Ehrenfeld and Garcia-Romeu, 1977; Schwartz and Steinmetz, 1977) indicate that active H⁺ excretion is dependent on availability of CO₂ and the uptake of Na. The former provides the source of protons from the catalysed hydration of CO₂ and the latter provides the electrically balancing positive charge via entry through apical Na channels which are electrically coupled to the proton pumps. Examining factors regulating proton transport in the urinary epithelia of water turtle Al-Awqati et al., (1983a) found that one of the mechanisms by which CO₂ causes the change in H⁺ transport is by rapid increases in the number of these proton pumps. A similar CO₂-stimulated increase in H⁺ extrusion and perhaps also in the number of functional proton pumps, can explain the increased uptake of Na under induced hypercapnia in the trout intestine. If Cu can also move through the Na channel in the trout intestine as indicated by the phenamil results (Fig. 3-6) then the elevated uptake of Cu and Na (Fig.3-7) would occur via a common mechanism.

Additional evidence of linkage between Na and Cu transport in the trout intestine was provided by the marked effect of Ag in stimulating both Cu and Na uptake (Fig.3-9). The result was surprising inasmuch as Ag is known to inhibit Na uptake at the gills in rainbow trout (Morgan *et al.*, 1997) and *vice versa* (Bury and Wood, 1999) but again reinforces the differences between the Na-Cu interactions at the two epithelia. The presence of Ag in the lumen resulted in a significantly more positive serosal TEP (Fig.3-9) indicating that the stimulated transport was unrelated to the change in voltage as it occurred against the electrical gradient as with the stimulation of Cu transport by increasing luminal Na (Fig.3-1). This suggests that Ag activates some sort of dual Na-Cu transport mechanism in the trout intestine. In support of this suggestion are several reports wherein Ag appears to increase the apical conductance to Na in a variety of epithelia such as the rat ileum (Clarkson and Toole, 1964), toad skin (Gerencser *et al.*, 1977), toad bladder (Walser, 1970) and frog skin (Curran, 1972; Li and DeSouza, 1977) with the increased entry of Na contributing to the current generated.

Examining the effects of Ag on ion transport and permeability of the rabbit corneal epithelium, Klyce and Marshall, (1982) found that Ag increases the conductance of the apical membrane of the corneal epithelium to cations, primarily Na. This effect was attributed to a rapid stimulation of cation conductance in the transcellular pathway and a slower non-selective increase in the permeability of the paracellular pathway. While Klyce and Marshall, (1982) described an apparent lack of amiloride-sensitive Na conductance in the corneal epithelium of rabbit, Curran, (1972) reported that the Ag induced Na conductance was independent of the amiloride sensitive Na channel which is normally present in frog skin. Consistent with these data Li and de Souza, (1976) reported reduced amiloride sensitivity of overall Na transport across the frog skin after treatment with Ag. Collectively these observations argue against a Na-channel/H⁺-ATPase or Na/H⁺

exchange system and point towards an alternate pathway possibly involving a facilitated Na-Cu co-transport system.

The presence of L- histidine in the transport medium promoted Cu and Na uptake in the trout intestine (Fig.3-8). It is well known that several amino acids are transported into cells against large concentration gradients and the mechanism for a good proportion of amino acids is entirely by a co-transport involving Na. Specifically Curran et al., (1967) established a positive relationship between unidirectional influxes of Na and amino acids across the mucosal border of rabbit ileum and provided evidence that interactions between Na and amino acid transport depend in part on a common entry mechanism at the mucosal border of the intestine. Results obtained using the brush-border membrane vesicle technique demonstrate that in fish the energy source for amino acid transport is also the Na gradient (Ferraris and Ahearn, 1984; Balocco et al., 1993; Storelli et al., 1986; Vilella et al., 1989). In Dicentrarchus labrax, a marine fish cultivated in the Mediterranean, neutral amino acids with linear side chains were reported to be transported via saturable Na-dependent routes (Boge et al., 1985). In Xenopus laevis oocytes expressing the neutral and basic amino acid transporter (NBAT) Ahmed et al., (1997) showed that transport of histidine at physiological pH (7.5) was via a Nadependent mechanism with a stoichiometry of 1:1 (histidine:Na). Based on this evidence it is reasonable to propose that the stimulation in Na uptake (Fig. 3-8) was likely mediated by a L-histidine transporter.

The preference of Cu for amino acid residues bearing N or S ligands and the formation of Cu(II)-S-His homo-polynuclear clusters (Bell et al., 2002) has been

documented. Bingham and McArdle, (1994) demonstrated that L- histidine enhances the uptake of Cu in mammalian hepatic cells. Similar results have been obtained in placental cells (Mas and Sarkar, 1992). In vitro studies have demonstrated that L-histidine can facilitate Cu uptake in mammalian brain (Hartter and Barnea, 1988) by a high-affinity process analogous to that of neutral amino acids. Histidine forms a ternary complex with albumin- $Cu(II)^{2+}$ and induces the protein to release Cu as a histidine- $Cu(II)^{2+}$ complex a process that modulates the availability of Cu to the cell. The formation of the Cu(II)²⁺-Lhistidine complex enhances the cellular uptake of Cu as the complex produced from weak intramolecular H-bonding interactions is considered to be particularly favourable for the transport of Cu in the hydrophobic environment of biological membranes. Although a major effort has been made to study the Cu(II)²⁺-L-histidine system, the exact mechanism by which the complex provides Cu to crucial enzymes remains unclear (Deschamps et al., 2004). L-histidine clearly allows ready access of Cu to the transport mechanism but evidence that the histidine ligand itself penetrates the cell is debated (McArdle et al., 1990). L-histidine may stimulate Cu uptake either by facilitating the release of Cu to the transport moiety or the enhanced transport may be mediated specifically by a histidine transporter.

In the present study L- and D-steroisomers were used in an attempt to resolve the precise mechanism involved in L-histidine stimulated Cu uptake. The rationale for this was that mammalian intestinal histidine transport is far greater for L- than for D-histidine (Gibson and Wiseman, 1951). In contrast to L-histidine, D- histidine did not stimulate either Cu or Na uptake (Fig.3-8), the data therefore support a stereospecific action of

histidine on Cu uptake. Based on this evidence of a stereoselective response to histidine for stimulated Cu uptake and a parallel L-histidine-dependent Na uptake, it is likely that the stimulatory effect was a consequence of Cu-Na co-transport via a histidine transporter located on the apical membrane of the trout intestine.

Overall, our data clearly implicate a Na-dependent mechanism in mediating at least a portion of Cu transport across the trout intestine, but there is a dichotomy in the results. On the one hand, phenamil partially inhibits both Na and Cu transport and hypercapnia stimulates both Na and Cu transport, results which are best explained by both Cu and Na entering through an apical Na-channel as in the trout gill (Grosell and Wood, 2002). On the other hand, elevated luminal Na stimulates rather than competitively inhibits Cu transport and the presence of Ag and of L-histidine stimulate both Na and Cu transport, results which are best explained by some sort of Na-Cu co-transport system. The answer may lie in the presence of more than one Na-dependent mechanism or alternatively in the presence of a unique Na-Cu co-transport mechanism with these unusual combined characteristics. For example, with respect to the phenamil effect amiloride and its analogues have been reported to inhibit a wide range of Na transport mechanisms in addition to the apical Na-channel notably the Na^+/H^+ and Na^+/Ca^+ exchangers, Na⁺/K⁺. ATPase, Na-glucose and Na-alanine co-transporters (Kleyman and Cragoe, 1988). The possibility that phenamil could have blocked one of these cotransport mechanisms cannot therefore be excluded.

Na-independent Cu uptake

Metal metabolism is known to be greatly influenced by medium pH (Campbell and Stokes, 1985). In addition two of the possible candidate proteins responsible for the absorption of dietary Cu in mammals are recognized to be pH sensitive. Ctr1 is stimulated by low extracellular pH and the transport of metal ions by all the members of the NRAMP family, which includes DMT1 is driven by protons (Nelson, 1999). The functional characteristics of DMT1 determined by Gunshin *et al.*, (1997) suggest that the transport of metal ions is coupled to the co-transport of H⁺ down its electrochemical gradient which ensures uptake of divalent cations, even at trace amounts. In the present study we assessed the pH sensitivity of Cu uptake to ascertain if either of these transporters are involved in the intestinal transport of Cu in trout.

An effect of pH on Cu uptake was not evident when pH was lowered to 6.0, however a marked decrease in Cu uptake was observed when pH was raised to 8.0 (Fig. 3-10). These effects were specific to Cu and not Na transport suggesting the presence of a non Na-dependent pathway for Cu uptake that demonstrates characteristics of DMT1 or Ctr1 mediated transport. However, the pH effect upon intestinal Cu uptake in rainbow trout could also be explained by altered Cu speciation, related to the decline in % total concentration of $Cu(II)^{2+}$ as it is replaced mainly by $CuCO_3$ at high pH (Fig. 3-11). If the latter were the case, it might suggest that Cu transport is dependent on the presence of $Cu(II)^{2+}$ as the primary substrate and thereby allude to a role for DMT1 in Cu transport according to the traditional view that DMT1 preferentially transports the divalent forms

of metals (Gunshin et al., 1997). However Arredondo et al., (2003) recently demonstrated that DMT1 preferentially transports $Cu(I)^{1+}$ over divalent $Cu(II)^{2+}$ in cultured human intestinal Caco-2 cells. Further complicating interpretation is the fact that treatment with ascorbate had no effect on Cu uptake (Chapter 2) indicating either that the valence of Cu present is not critical or that sufficient quantities of endogenous reductase (ER) are present on the intestinal epithelium of trout. The presence of endogenous plasma membrane reductases capable of reducing Cu has been reported in mammalian brush border membranes (Knopfel and Solioz, 2002). If this were the case the ER would reduce $Cu(II)^{2+}$ to monovalent $Cu(I)^{1+}$ which would be taken up more readily by Ctr1 (Sharp, 2003). However Ctr1 is inhibited rather than stimulated by Ag^+ in contrast to our data (Lee et al., 2002), a detail that moderates its likely contribution in Cu uptake. Furthermore, although both Ctr1 and DMT1 are expressed in mammalian enterocytes, higher levels of Ctr1 are expressed in other tissues and conclusive evidence for the localization of the protein to the apical membrane has been provided only for DMT1 (Andrews, 2000; Trinder et al., 2000). A major role for Ctr1 in cellular Cu uptake has been established only in the brain and its functional relevance in other tissues such as the intestine remains to be defined (Sharp, 2003). Indeed, mammalian Ctr1 mRNA expression is not regulated following dietary Cu restriction (Lee et al., 2000) or exposure to high Cu conditions (unpublished data cited by Sharp, 2003).

A number of different trace metals are absorbed from the diet and it has been suggested that the divalent metal transporter (DMT1) represents a common uptake pathway for these important micronutrients in mammals (Yamaji *et al.*, 2001). At the gene level this transporter has been identified in fish (Donovan *et al.*, 2002; Dorschner and Phillips, 1999) and Bury and Grosell, (2003) have recently implicated it in the branchial uptake of iron in the zebrafish. We investigated the possible influence of Fe and Zn on Cu uptake to determine if these micronutrients share a common uptake path across the intestinal epithelium in rainbow trout.

Results of these studies reveal significant inhibition of Cu uptake in the presence of 10-fold greater Fe (500 μ M Fe(NO₃)₃) or Zn (500 μ M ZnSO₄). The response was more pronounced for both treatments in the mid intestine where a maximum inhibition of approximately 80% was observed (Fig. 3-12). Na uptake rate remained unchanged in the presence of either cation providing additional evidence for the presence of a non Nadependent pathway for Cu transport, via a specific metal ion transporter.

The inverse relationship of Cu uptake to luminal Fe content suggests a role for DMT1 in the uptake of Cu in trout intestine. DMT1 has also been implicated in the transport of Cu in human intestinal Caco-2 cells (Arredondo *et al.*, 2003). Evaluating Fe competition for Cu transport Arredondo *et al.*, (2003) observed significant concentration-dependent inhibition in Cu uptake with Fe²⁺. 80% and 90% inhibition of Fe uptake was also evident at a Cu : Fe ratio of 10:1 and 100 : 1 respectively. In the same study an approximate 50% inhibition in Cu uptake was reported in cells transfected with DMT1 antisense oligionucleotides, suggesting that at least 50% of Cu transport is mediated by DMT1. Tennant *et al.*, (2002) provide additional evidence that these metals may utilize the same apical transporter, demonstrating that a 100-fold excess of Cu reduces Fe uptake by 50% in Caco-2 TC7 cells. These cells when cultured in high Cu medium also showed

decreased expression of DMT1 protein and mRNA, similar to the down-regulation of intestinal Cu uptake observed *in vivo* (Turnlund *et al.*, 1989) following exposure to high dietary Cu levels, giving further credence to a role for DMT1 in Cu absorption. The presence of a regulated divalent Fe transport process has been suggested in the intestine of the marine flounder as increased $Fe(II)^{2+}$ bioavailability and an up-regulation of ironbinding proteins was observed in response to haemoglobin depletion (Bury *et al.*, 2001). The preferential transport of $Fe(II)^{2+}$ relative to $Fe(III)^{3+}$, the dependence of Fe uptake on the proton gradient and identification of the zebrafish ortholog of DMT1 (Donovan *et al.*, 2002) suggest that Fe is most likely transported via DMT1 (Bury and Grosell, 2003). Taken together the present evidence of Cu-Fe interactions in the trout intestine and the identification of DMT1 in the zebrafish argue that Cu uptake in the trout intestine occurs in part via DMT1.

The comparable inhibition of Cu uptake in the trout intestine by 10-fold excess of Zn (Fig. 3-12) is also in accord with the involvement of DMT1 (Gunshin *et al.*, 1997), though other explanations are possible, such as Zn-Cu competition for binding-sites on intestinal metallothionein which may play a role in Cu uptake (Fischer *et al.*, 1981). In mammals, several investigators have reported inhibition of Cu absorption when dietary or intra-luminal Zn:Cu ratios were from 500:1 to 1000:1 (Oestreicher and Cousins, 1984; Evans *et al.*, 1970; Van Campen, 1969). Wapnir and Lee, (1993) reported a reduced capacity of the small intestine to take up Cu from the intestinal lumen in rats fed five times their normal recommended dietary Zn level. Condomina *et al.*, (2002) observed a concentration-dependent inhibition of Zn uptake in the presence of Cu in the rat intestinal

segments *in vitro*. In rainbow trout Glover and Hogstrand, (2003) reported significant inhibition of intestinal Zn absorption in the presence of equimolar Cu levels. While several studies have been designed to elucidate the mechanism of this interaction, the consensus seemed to implicate metal binding protein metallothionein (MT). Given the greater affinity of Cu to bind to MT (Hogstrand and Wood, 1996) a competitive interaction between Zn and Cu for MT was believed to influence the absorption of either metal (Shears and Fletcher, 1983). However, Masters *et al.*, (1994) and Reeves, (1998) reported reduction of Cu uptake with high dietary Zn in mice lacking the MT gene, suggesting that MT induction was not entirely responsible for reduced Cu absorption. Decreased cellular uptake of Zn in response to Fe treatment (Talkvist *et al.*, 2000) in human intestinal Caco-2 cells when considered against similar responses of Cu to elevated Fe levels (Arredondo *et al.*, 2003) suggest that these metals may utilize the same apical transporter.

Conclusion

At least two types of transporters emerge as obvious contenders for the intestinal uptake of Cu in rainbow trout, one Na-dependent, the other metal specific. Based on our evidence that treatments which accelerate Na transport also accelerate Cu uptake, while treatments which depress Na transport inhibit Cu uptake, we propose a novel Nadependent mechanism of Cu uptake, possibly involving a Na-Cu co-transport on the amino acid transporter or a unique Na-Cu co-transporter on the brush border membrane of trout intestine running off the Na gradient. A contribution from Cu uptake via an apical Na channel is also possible. Based on pH sensitivity and inhibition of Cu uptake with Fe and Zn DMT1 appears as the other likely candidate, although a role for Ctr1 cannot be excluded. Interestingly another transporter has been identified in Ctr1 deficient mouse embryonic cells (Lee *et al.*, 2002), that transports Cu in the absence of Ctr1 with Km ~ 10 μ M, in a saturable, time, temperature and pH dependent manner. The transport is competed by Zn, but function is not affected by Ag or ascorbate. It is possible that such a transporter exists in the trout intestine as the present study has reported almost identical Cu transport characteristics.

Literature Cited:

- Ahmed, A., Yao, P.C., Brant, A.M., Peter, G.J. and Harper, A.A. (1997). Electrogenic L-histidine transport in neutral and basic amino acid transporter (NBAT)expressing *Xenopus laevis* oocytes. J. Biol. Chem. Vol. 272 (1), 125-130.
- Al-Awqati, Q., Gluck, S., Reeves, W., Cannon, C. (1983a). Regulation of proton transport in urinary epithelia. *J Exp Biol.* 106,135-41.
- Andrews, N.C. (2000). Iron homeostasis: insights from genetics and animal models. *Nat Rev Genet*. (3), 208-17.
- Arredondo, M., Munoz, P., Mura, C.V. and Nunez, M.T. (2003). DMT1 a physiologically relevant apical Cu¹⁺ transporter of intestinal cells. *Am. J. Phys.*, 284, C1525-C1530.
- Balacco, C., Bogè, G., Roche, H. (1993). Neutral amino acid transport by marine fish intestine. Role of the chain. J. Comp. Physiol. 163B, 340-347.
- Barthe, L., Woodley, J. and Houin, G. (1999). Gastrointestinal absorption of drugs: methods and studies. J. Fundam.Clin.Pharmacol. 13, 154-168.
- Bell, R.A., Ogden, N. and Kramer, J.R. (2002). The biotic ligand model and a cellular approach to class B metal aquatic toxicity. *Comp. Biochem. Physiol.* Part C 133, 175-188.
- Bingham, M.J. and McArdle, H.J. (1994). Hepatology. 20, 1024.
- Bogè, G., Roche, H.and Pèrès, G. (1985). Role of chloride ions in glycine transport in a sea fish the bass (*Dicentrarchus labrax*). *Biochim. Biophys. Acta.* 193, 228-230.
- Bury. N.R. and Grosell, M. (2003). Waterborne iron acquisition by a fresh water teleost fish, zebrafish *Danio rerio. J. Exp. Biol.* 206, 1529-1535.
- Bury, N.R., Grosell, M., Wood, C.M., Hogstrand, C., Wilson, R.W., Rankin, J.C., Busk, M., Lecklin, T. and Jensen, F.B. (2001). Intestinal iron uptake in the European flounder (*Platichthys flesus*). J.Exp. Biol. 204, 3779-3787.

- Bury, N.R., Wood, C.M., (1999). Mechanisms of branchial apical silver uptake by rainbow trout is via the proton coupled Na⁺ channel. *Am.J.Physiol.*, 277, R1385-R1391.
- Campbell, H.A., Handy R.D. and Nimmo, M. (1999). Copper uptake kinetics across the gills of rainbow trout (*Oncorhynchus mykiss*) measured using an improved isolated perfused head technique. *Aquat. Toxicol.* 46, 177-190.
- Campbell, P.G.C., and Stokes, P.M. (1985). Acidification and toxicity of metals to aquatic biota. *Can. J. Fish. Aquat. Sci.* 42, 2034-2049.
- Clarkson, T.W. and Toole, S.R. (1964). Measurement of short-circuit current and ion-transport across the ileum. Am. J. Physiol. 206, 658-668.
- Condomina, J., Zoronza, S.T., Granero, L. and Polache, A. (2002). Kinetics of Zn transport *in vitro* in rat small intestine and colon: interaction with copper. *Eur. J. Pharm. Sci.* 16(4-5), 289-95.
- Curran, P.F. (1972). Effect of silver ion on permeability properties of frog skin. Biochim. Biophys. Acta. 288, 90-97.
- Curran PF, Schultz SG, Chez RA, Fuisz RE. (1967). Kinetic relations of the Naamino acid interaction at the mucosal border of intestine. J. Gen. Physiol. 50(5),1261-86.
- Dancis A, Haile D, Yuan DS, Klausner RD. (1994). The Saccharomyces cerevisiae copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. J. Biol Chem. 269(41), 25660-7.
- Deschamps, P., Kulkarni, P.P., Gautam-Basak, M., Sarkar, B. (2004). The saga of copper(II)-L-histidine. *Coord. Chem. Rev.* (In Press).
- Donovan, A., Brownlie, A., Dorschner, M.O., Zhou, Y., Pratt, S.J., Paw, B.H., Phillips, R.B., Thisse, C., Thisse, B., Zon, L.I. (2002). The zebrafish mutant gene chardonnay (cdy) encodes divalent metal transporter 1 (DMT1). *Blood.* 100(13), 4655-9.
- Dorschner, M.O. and Phillips, R.B. (1999). Comparative analysis of two Nramp loci from rainbow trout. *DNA Cell Biol*. 18, 573-583.

- Ehrenfeld, J., and Garcia-Romeu, F. (1977). Active hydrogen excrtion and sodium absorption through isolated frog skin. *Am.J. Physiol.* 233(1), F46-F54.
- Evans, G.W., Majors, P.F. and Cornatzer, W.E. (1970). Mechanism for cadmium and zinc antagonism of copper metabolism. *Biochem. Biophys. Res. Commun.* 40, 1142-1148.
- Ferraris, R.P. and Ahearn, G.A. (1984). Sugar and amino acids transport in fish intestine. *Comp. Biochem. Physiol.* 77A, 397-413.
- Field, M., Karnaky, K.J., Jr., Smith, P.L., Bolton, J.E., and Kinter, W.B. (1978). Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus*: I. Functional and structural properties of cellular and paracellular pathways for Na and Cl. J. Membr. Biol. 41, 265-293.
- Fischer, P.W.F., Giroux, A. and L'Abbe. (1981). The effect of dietary zinc on intestinal copper absorption. Am. J. Clin. Nutr. 34, 1663-1675.
- Garvin, J.L., Simon,S.A., Cragoe, E.J. and Mandel, L.J. (1985). Phenamil: an irreversible inhibitor of sodium channels in the toad urinary bladder. J. Membr. Biol. 87, 45-54.
- Gerencser, G.A., Corvette, K.M., Loo, S.Y. and Hong, S.K. (1977). Effect of silver chloride on the short-circuit current across the isolated toad skin. *Life Sci.* 20, 1883-1890.
- Gibson, Q.H. and Wiseman, G. (1951). Selective absorption of stereoisomers of amino acids from loops of the small intestine of the rat. *Biochem. J.* 48, 426-429.
- Glover, C.N. and Hogstrand, C.(2003). Effects of dissolved metals and other hydrominerals on *in vivo* intestinal zinc uptake in fresh water rainbow trout. *Aquat.Toxicol.* 62, 281-293.
- Grosell, M., Wood, C.M., Wilson, R.W., Bury, N.R., Hogstrand, C., Rankin, C., Jensen, F.B., (2004). Bicarbonate secretion plays a role in chloride and water absorption in the European flounder intestine. *Am J Physiol Regul Integr Comp Physiol.* [Epub ahead of print]
- Grosell, M., and Wood, C.M. (2002). Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.

- Grosell, M., Kamunde, C., Wood, C.M. and Walsh, P.J. (2001a). Copper transport across fish gills. Society for Experimental Biology, Annual Meeting, University of Kent, Canterbury, England, 2-6 April. pp. 89.
- Grosell, M., and Jensen, F.B. (1999). No₂⁻ uptake and HCO₃⁻ excretion in the intestine of the European flounder (*Platichthys flesus*). J. Exp. Biol. 202, 2103-2110.
- Grosell, M., Hansen, H.J.M. and Rosenkilde, P. (1998a). Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne copper exposure. *Comp. Biochem. Physiol.* 120C, 295-305.
- Grosell, M.H., Hogstrand, C. and Wood, C.M. (1998b). Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non-acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 40, 275-291.
- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997). Cloning and characterization of a proton-coupled mammalian metal ion transporter. *Nature*. 388, 482-488.
- Handy, R.D., Eddy, F.B. and Baines, H. (2002). Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. *Biochim. Biophys. Acta.* 1566, 104-115.
- Handy, R.D., Musonda, M.M., Philips,C. and Fella, S.J. (2000). Mechanisms of gastrointestinal copper absorption in the African walking catfish:copper dose-effects and a novel anion-dependent pathway in the intestine. *J.Exp.Biol.* 203, 2365-2377.
- Hartter, D.E., and Barnea, A. (1988). Brain tissue accumulates 67copper by two ligand-dependent saturable processes. A high affinity, low capacity and a low affinity, high capacity process. *J Biol Chem.* 263(2),799-805.
- Harvey, B.J. (1992). Energization of sodium absorption by the H(+)-ATPase pump in mitochondria-rich cells of frog skin. *J Exp Biol.* 172, 289-309
- Hogstrand, C. and Wood, C.M. (1996). The physiology and toxicology of zinc in fish. In *Toxicology of Aquatic Pollution* (ed. E.W. Taylor), pp.61-84. Cambridge: Cambridge University Press.

- Kamunde, C.N., and Wood, C.M. (2003). The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss. Aquat. Toxicol.* 62, 235-254.
- Kamunde, C.N., Clayton, C. and Wood, C.M. (2002a). Waterborne versus dietary copper uptake in trout and the effects of waterborne copper acclimation. *Am. J. Physiol.* 383, R69-R78.
- Kamunde, C.N., Grosell, M., Higgs, D. and Wood, C.M. (2002b). Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J.Exp.Biol. 205,279-290.
- Kamunde, C.N., Grosell, M., Lott, J.N.A. and Wood, C.M. (2001). Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure. Can.J. Fish.Aquat.Sci. 58, 293-305.
- Kandegedara, A. and Rorabacher, D.B. (1999). Noncomplexing tertiary amines as "Better" buffers covering the range of pH 3-11. Temperature dependence of their acid dissociation constants. *Anal.Chem.* 71, 3140-3144.
- Kjoss, V.A., Kamunde, C. N., Niyogi, S., Grosell, M. and Wood, C.M. (2005). Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. J. Fish Biol. 66, 468-484.
- Kleyman, T.R. and Cragoe, E.J. Jr. (1988). Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. 105, 1-21.
- Klyce, S.D. and Marshall, W.S. (1982). Effects of Ag⁺ on ion transport by the corneal epithelium of the rabbit. J. Membr. Biol. 66, 133-144.
- Knopfel, M. and Solioz, M. (2002). Characterization of a cytochrome b(558) ferric/cupric reductase from rabbit duodenal brush border membranes. *Biochem. Biophys .Res. Comm.* 291(2), 220-5.
- Lau, S.J. and Sarkar, B. (1971). Ternary coordination complex between human serum albumin, Cu(II) and L-histidine. J. Biol. Chem. Vol.246(19), 5938-5943.
- Lee, J., Pena, M.M.O., Nose, Y. and Thiele, D.J. (2002). Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 277, 4380-4387.

- Lee, J., Prohaska, J. R. Dagenais, S.L., Glover, T.W. and Thiele, D.J. (2000). Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* 254, 87-96.
- Li, J.H. and DeSouza, R.C. (1977). Effects of Ag⁺ on frog skin: interaction with oxytocin, amiloride and ouabain. *Experientia* 33/4, 433-436.
- Linder, M.C. and Hazegh-Azam (1996). Copper biochemistry and molecular biology. Am. J. Clin. Nutr. 63, 797S-811S.
- Loretz, C.A. (1995). Electrophysiology of ion transport in the teleost intestinal cells. In Cellular and Molecular Approaches to Fish Ionic Regulation, Fish Physiology Eds. C.M. Wood and T.J. Shuttleworth.
- Loretz, C.A., Freel, R.W., AND Bern, H.A. (1983). Specificity of response of intestinal ion transport systems to a pair of natural peptide hormone analogs: Somatostatin and urotensin II Gen. Comp. Endocrinol. 52, 198-206.
- Mackenzie, N.C., Brito, M., Reyes, A.E. and Allende, M.L. (2004). Cloning, expression pattern and essentiality of the high-affinity copper transporter1 (ctr1) gene in zebrafish. *Gene*. 328,113-20.
- Mas, A. and Sarkar, B. (1992). Uptake of 67Cu by isolated human trophoblast cells. *Biochim. Biophys. Acta*.1135, 123-128.
- Masters, B.A., Kelly, E.J., Quaife, C.J., Brinster, R.L. and Palmiter, R.D. (1994). Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc. Natl.Acad. Sci.* U.S.A.91, 584-588.
- McArdle, H.J., Gross, S.M., Danks, D.M. and Wedd. A.G. (1990). Role of albumin's copper binding by mouse hepatocytes. *Am. J. Physiol.* 258, G988-G991,
- Morgan, I.J., Henry, R.P. AND Wood, C.M. (1997). The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na+ and Cl- transport. *Aquat. Toxicol.* 38, 145-163.
- Nelson, N. (1999). Metal-ion transporters and homeostasis. *EMBO J.* 18, 4361-4371.
- Ostreicher, P. and Cousins, R.J. (1984). Copper and Zinc absorption in the rat: Mechanism of mutual antagonism. J. Nutr. 115, 159-166.

- Pyle, G.G., Kamunde, C.N., McDonald, D.G. and Wood, C.M. (2003). Dietary sodium inhibits aqueous copper uptake in rainbow trout, *Oncorhynchus mykiss. J. Exp. Biol.* 206, 609-618.
- Rangachari, P.K. and Matthews, J. (1985). Effect of Ag⁺ on isolated bullfrog gastric mucosa. *Am. J. Physiol.* 248, G443-G449.
- Reeves, P.G. (1998). Copper metabolism in metallothionein- null mice fed a highzinc diet. J. Nutr. Biochem. 9, 598-601.
- Schwartz, J.H. and Steinmetz, P.R. (1977). Metabolic energy and P_{CO2} as determinants of H+ secretion by turtle urinary bladder. *Am.J. Physiol.* 233(2), F145-F149.
- Sharp. P.A. (2003). Ctr1 and its role in body copper homeostasis. Int. J. Biochem. Cell Biol. 35, 288-291.
- Shears, M.A. and Fletcher, G.L. (1983). Regulation of Zn2+ uptake from the gastrointestinal tract of a marine telest, the winter flounder (*Pseudopleuronectes americanus*). Can. J. Fish Aquat. Sci. 40.(Suppl. 2),197-205.
- Storelli, C., Vilella, S. and Cassano, G. (1986). Na+ dependent D-glucose and Lalanine transport in eel-intestinal brush-border membrane vesicles. Am. J. Physiol. 251, R463-469.
- Talkvist, J., Bowlus, C.L. and Lönnerdal, B. (2000). Functional and molecular responses of human intestinal Caco-2 cells to iron treatment. *Am. J. Clin. Nutr.* 72, 770-5.
- Tennant, J., Stansfield, M., Yamaji, S., Srai, S.K. and Sharp, P.A. (2002). Effects of copper on the expression of metal transporters in human intestinal Caco-2 cells. *FEBS Letters* 527, 239-244.
- Trinder, D., Oates, P.S., Thomas, C. Sadleir, J and Morgan, E.H. (2000). Localization of divalent metal transporter1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. *Gut* 46, 270-276.
- Turnlund, J.R., Keyes, W.R., Anderson, H.L., Acord, L.L. (1989). Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope ⁶⁵Cu. *Am. J. Clin. Nutr.* 49, 870-8.

- Van Campen, D.R. (1969). Copper interference with the intestinal absorption of zinc-65 by rat. J. Nutr. 97, 104-108.
- Vilella S, Cassano G, Storelli C. (1989). How many Na+-dependent carriers for Lalanine and L-proline in the eel intestine? Studies with brush-border membrane vesicles. *Biochim Biophys Acta*. 984(2),188-92.
- Walser, M. (1970). Calcium transport in toad bladder: permeability to calcium ions. Am. J. Physiol. 218, 582-589.
- Wapnir, R.A., and Lee, S.Y. (1993). Dietary regulation of copper absorption and storage in rats: Effects of sodium, zinc, and histidine-zinc. J. Amer. Coll. Nutr. Vol. 12, No. 6. 714-719.
- Wapnir, R.A. (1991). Copper-sodium linkage during intestinal absorption: inhibition by amiloride. *Proc. Soc. Exper. Biol. Med.* 196, 410-414.
- Wapnir, R.A. and Steil, L.(1987) Intestinal absorption of copper: effect of sodium. *Proc. Soc. Exp. Biol. Med.* 185, 277-282.
- Wolf, K. (1963). Physiological salines for freshwater teleosts. Progr. Fish Cultur 25, 135-140.
- Yamaji, S., Tennant, J., Tandy, S., Williams, M., Srai, S.K. AND Sharp, P.A. (2001). Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells. FEBS Letters. 507, 137-141.

Figure Legends:

Fig. 3-1. Effect of increasing luminal Na (as NaCl) on Cu uptake rate (A- mid intestine; B- posterior intestine) and transepithelial potential (C-mid intestine; D-posterior intestine) in isolated intestinal segments from rainbow trout. Plotted values represent means \pm SEM (n=5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (p<0.05).



Fig. 3-2. Effect of increasing luminal Na (as NaCl) on fluid transport rate in isolated intestinal segments (A- mid intestine ; B- posterior intestine) from rainbow trout. Plotted values represent means \pm SEM (n=5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (p<0.05).





Fig. 3-3. An analysis of the possible relationship between solvent drag and Cu uptake rate in isolated intestinal segments (A- mid intestine; B- posterior intestine) from rainbow trout. Cu uptake rate measured in the presence and absence of net water efflux. Values represent means \pm SEM (n=5 per treatment). Statistical significance was tested using unpaired t-test (2-tailed). Means labeled with asterisks are significantly different (p<0.05).



Fig.3-4. Cu uptake in isolated intestinal segments (A- mid intestine ; B- posterior intestine) from rainbow trout as a function of serosal and mucosal osmolality varied with mannitol. Values represent means \pm SEM (n=5 per treatment).





Posterior Intestine



Fig. 3-5. Effect of increasing luminal Na as (Na₂SO₄) on Cu uptake rate (A- mid intestine; B- posterior intestine) and transepithelial potential (C -mid intestine; D-posterior intestine) in isolated intestinal segments from rainbow trout. Plotted values represent means \pm SEM (n=5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (p<0.05).



Fig 3-6. Effect of pre incubating isolated intestinal segments (A- mid intestine; Bposterior intestine) from rainbow trout in 100 μ M phenamil (an irreversible Na-channel blocker) on Cu and Na uptake. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using unpaired t- test (2 – tailed). Means labeled with asterisks are significantly different (p<0.05).






Fig. 3-7. Cu and Na uptake rates in isolated intestinal segments from rainbow trout bathed in solution gassed with 0.3, 1 and 3.0 % CO₂ in O₂. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using one way ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (p<0.05).



Fig. 3-8. Influence of 10 mM L- histidine (A- mid intestine; B- posterior intestine) and 10 mM D- histidine (C- mid intestine & D- posterior intestine) upon Cu and Na uptake in isolated intestinal segments from rainbow trout. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using unpaired t- test (2- tailed). Means labeled with asterisks are significantly different (p<0.05).



control 10 mM L - histidine



control 10 mM D-His Fig. 3-9. Influence of luminal AgNO₃ at 500 μ M on Cu and Na uptake (A- mid intestine; B- posterior intestine) and TEP (C- mid intestine; D- posterior intestine) in isolated intestinal segments from rainbow trout. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using unpaired t-test (2-tailed). Means labeled with asterisks are significantly different (p<0.05). A Mid Intestine

B Posterior Intestine





Fig. 3-10. Influence of luminal pH buffered with 10 mM MES on Cu and Na uptake in isolated intestinal segments (A- mid intestine; B- posterior intestine) from rainbow trout. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using one way ANOVA. Means labeled with different letters are significantly different (p<0.05).



Fig. 3-11. Cu (A) and Na (B) speciation as a function of variable mucosal pH, derived from the geochemical speciation program MINEQL + (Version 4.01; Environmental Research Software).



Fig. 3-12. Influence of luminal ZnSO₄ (A- mid intestine; B- posterior intestine) and Fe(NO₃)₃ (C- mid intestine; D- posterior intestine) at 500 μ M on Cu and Na uptake in isolated intestinal segments from rainbow trout. Values are means \pm SEM (n=5 per treatment). Statistical significance was tested using unpaired t-test (2-tailed). Means labeled with asterisks are significantly different (p<0.05).



Chapter 4:

Summary of results and conclusions

Characterization of intestinal Cu uptake in the rainbow trout:

Absorption efficiency of dietborne Cu ranged between 25-50% which was comparable to reported absorption efficiency in humans (Wapnir, 1998) and other fish species (Berntssen *et al.*, 1999). The mid and posterior intestine emerged as important sites of net Cu absorption. Unidirectional Cu uptake appeared to be many-fold higher in the anterior intestine *in vitro* but this appeared to be superceded by an even greater efflux component *in vivo* attributing a complex role of bidirectional Cu transport to this region. The data presented here also suggest that substantial Cu absorption occurred in the stomach *in vivo*. It remains to be determined however whether this was an artifact resulting from Cu leaching into the water prior to ingestion, since a comparison of unidirectional whole body Cu uptake rates reported *in vivo* (Kamunde *et al.*, 2002a) with *in vitro* measurements of unidirectional Cu uptake along the entire intestinal tract in the present study reveals that the intestine can account for most of the dietary Cu absorption in rainbow trout, leaving only a small contribution from the stomach.

The basic mechanisms of intestinal Cu uptake appear to be conserved between fish and mammals. Two components of uptake were determined. A saturable and therefore carrier-mediated pathway at lower Cu concentrations was superceded by a possible diffusive pathway at higher Cu concentrations. Q_{10} analysis of Cu uptake provided further evidence that basolateral transport of Cu was biologically mediated while apical uptake probably occurred via simple diffusion.

The affinity for Cu uptake was lower in the trout intestine (i.e. higher K_m) when compared to the gills but nevertheless within the range of affinity constants derived from investigations of mammalian intestinal uptake. Uptake of Cu was hypoxia-resistant and fueled by Cu(II)²⁺ as substrate, with endogenous reductases in the intestinal epithelium possibly reducing Cu to Cu(I)¹⁺ to facilitate the uptake process. The relative affinity of the gill and intestine for Cu together with differential effects of Na (inhibition at gill, stimulation at gut) strongly suggest the mechanism(s) of uptake differ considerably between these two routes.

Mechanisms of Cu uptake in the trout intestine:

The present study describes two pathways of Cu uptake in the trout intestine, one Na-dependent and the other metal specific. These mechanisms may be the same as in mammals. An amiloride-sensitive Na pathway for Cu uptake has been reported in the rat intestine (Wapnir, 1991) while dietary Cu uptake via metal specific transporters in mammals has been the focus of most recent research (Lee *et al.*, 2002; Sharp, 2003; Arredondo *et al.*, 2003; Zerounian et, al, 2003). Fig. 4.1 summarizes the various mechanisms that may contribute to intestinal Cu uptake in *O. mykiss* based on the results of the present study.

In response to treatments that augment Na transport, Cu uptake increased leading to the proposal of a novel Na-dependent mechanism of Cu uptake involving a Na-Cu cotransport. The facilitation of Cu uptake by L-histidine but not D-histidine provides evidence that the amino acid transporter could have mediated this process. L-histidine associated Cu uptake appears to be a phylogenetically conserved process with several instances of L-histidine facilitated Cu uptake being reported in mammals (Bingham and McArdle, 1994; Hartter and Barnea, 1988). Alternatively, the possibility of a unique Na-Cu co-transport mechanism located on the brush-border, fueled by the Na gradient is an interesting prospect which definitely requires further investigation. Small proportions of both Cu and Na uptake were inhibited with phenamil, an amiloride analogue and Nachannel blocker, while exposure to hypercapnia stimulated the uptake of Cu and Na. These responses indicate a part of Cu uptake could occur via the apical Na-channel. However, the possibility that phenamil could have blocked a Na-nutrient co-transport mechanism which also transports Cu cannot be excluded.

The data presented in this study relate the inhibition of Cu uptake at high pH to the decline in concentration of Cu(II)²⁺. Taken together with the contention that endogenous reductases located on the brush border possibly reduce Cu(II)²⁺ required as the substrate to Cu(I)¹⁺, thereby facilitating Cu uptake, the evidence compares well with DMT1 being recognized as a physiologically relevant Cu(I)¹⁺ transporter in mammals (Arredondo *et al.*, 2003). Additional support for DMT1 mediated Cu uptake comes from the substantial inhibition of Cu uptake with 10-fold excess of Fe and Zn. While DMT1 has been characterized as the mammalian Fe transporter (Andrews, 2000), uptake of Zn and other divalent metal ions via this pathway has also been established (Gunshin *et al.*, 1997). Although a role for Ctr1 in Cu uptake cannot be excluded, the Na-dependent nature of Cu

uptake in the trout intestine and the fact that Ag stimulates Cu uptake make this possibility less likely as Ctr1 is inhibited rather than stimulated by Ag (Lee *et al.*, 2002) in contrast to our data. The characteristics of Cu uptake defined in the present study are commensurate with features of the recently characterized Cu transporter in mouse embryonic cells deficient in Ctr1 (Lee *et al.*, 2002).

Relevance of research:

The results described here show that certain manipulations *in vitro* stimulated quantitative Cu transport while others caused inhibition. For example, greater Cu uptake was found to occur with increasing Na levels and addition of L-histidine to the luminal saline. Potentially, adding Na and/or L-histidine to the diet could improve absorptive efficiency and enhance fish growth and productivity, addition of excess Cu to diets would be less desirable owing to its potentially toxic effects and regulatory limitations. Indeed the facilitated transport of Cu in the presence of excess L-histidine has found application for the use of Cu(II)-L-histidine complex as a therapeutic agent to treat Menkes disease (Deschamps *et al.*, 2004). Menkes disease is characterized by extremely low intestinal Cu absorption in humans.

Identification of intestinal uptake pathways for transition elements like Cu is vital for understanding the factors governing their assimilation, metabolism and homeostasis. Given the essential and at the same time toxic nature of Cu, such information that outlines factors affecting dietary Cu bioavailability could be useful in the prediction of Cu toxicity. The physiological characterization of dietary Cu uptake therefore provides knowledge on bioavailability and homeostatic physiology which determine the toxic effects of Cu. Incorporation of such factors into models developed for environmental regulation of Cu has important implications for environmental protection. For example one significance of these findings from a nutritional and environmental risk assessment perspective must lie in the indication that dietary Na excess or deficiency may be significant risk factors in Cu homeostasis.

Figure 4.1 Schematic diagram of a conceptual model for transcellular Cu uptake pathways in the trout intestine.

The pathways of transcellular Cu uptake in part are linked to Na, possibly involving Cu co-transport with Na on the amino acid transporter. Alternatively, the presence of a unique transporter in the trout intestine is hypothesized which runs off a Na gradient and co-transports Cu. Phenamil sensitivity of Cu uptake appears to indicate Cu entry via the apical Na channel, however it is also possible that phenamil could have inhibited a Na-Cu –nutrient co-transport mechanism.

A second pathway of Cu uptake is independent of Na effects, sensitive to pH and competed by Fe and Zn implicating DMT1 in the process. It is likely that external $Cu(II)^{2+}$ is reduced to $Cu(I)^{1+}$ by an endogenous reductase. It also remains possible that $Cu(I)^{1+}$ could enter via a Ctr1 like transporter.

Abbreviations : ATP – Adenosine 5' triphosphate; AAT – Amino acid transporter; CR - Copper Reductase; Ctr1-Copper transporter 1; DMT1- Divalent metal transporter 1 ENaC- Epithelial Na Channel



Literature Cited:

- Andrews, N.C. (2000). Iron homeostasis: insights from genetics and animal models. *Nat. Rev. Genet.*(3), 208-17.
- Arredondo, M., Munoz, P., Mura, C.V. and Nunez, M.T. (2003). DMT1 a physiologically relevant apical Cu¹⁺ transporter of intestinal cells. *Am. J. Phys.*, 284, C1525-C1530.
- Berntssen, M.H.G., Lundebye, A.K. and Maage, A. (1999b). Effects of elevated dietary copper concentrations on growth, feed utilization and nutritional status of Atlantic salmon (*Salmo salar L.*) fry. *Aquaculture* 17, 167-181.
- Bingham, M.J. and McArdle, H.J. (1994). Hepatology. 20, 1024.
- Deschamps, P., Kulkarni, P.P., Gautam-Basak, M., Sarkar, B. (2004). The saga of copper(II)-L-histidine. *Coord. Chem. Rev.* (In Press).
- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997). Cloning and characterization of a proton-coupled mammalian metal ion transporter. *Nature*. 388, 482-488.
- Hartter, D.E., and Barnea, A. (1988). Brain tissue accumulates 67copper by two ligand-dependent saturable processes. A high affinity, low capacity and a low affinity, high capacity process. J. Biol. Chem. 263(2), 799-805.
- Kamunde, C.N., Grosell, M., Higgs, D. and Wood, C.M. (2002b). Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *J.Exp.Biol.* 205,279-290.
- Lee, J., Pena, M.M.O., Nose, Y. and Thiele, D.J. (2002). Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 277, 4380-4387.
- Sharp. P.A. (2003). Ctr1 and its role in body copper homeostasis. Int. J. Biochem. Cell' Biol. 35, 288-291.
- Wapnir, R.A. (1998). Copper absorption and bioavailabiliy. Am. J. Clin. Nutr. 67, (suppl.) 1054S-1068S.

- Wapnir, R.A. (1991). Copper-sodium linkage during intestinal absorption: inhibition by amiloride. *Proc. Soc. Exper. Biol. Med.* 196, 410-414.
- Zerounian, N.R., Redekosky, C., Malpe, R. and Linder, M.C. (2003). Regulation of copper absorption by copper availability in the Caco-2 cell intestinal model. *Am. J. Physiol.* 284, G739-G747.