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GASTROINTESTINAL UPTAKE AND DISTRIBUTION OF COPPER IN RAINBOW TROUT

S. J. CLEARWATER*, S. J. BASKIN, C. M. WOOD AND D. G. MCDONALD

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

*Present address: Department of Zoology and Physiology, University of Wyoming, PO Box 3166, Laramie, WY 82071, USA (e-mail: clearwat@uwyo.edu)

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Summary

A single dose of radioactive copper (^{64}Cu or new Cu) was infused into the stomach of rainbow trout (*Oncorhynchus mykiss*) to model dietary copper (Cu) uptake under conditions of a normal nutritional dose and optimum environmental temperature (16 °C, 0.117 μg Cu g^{-1} body mass). The distribution of new Cu to the gut and internal organs occurred in two phases: rapid uptake by the gut tissues (almost complete by 24 h post-infusion) followed by slower uptake by the internal organs. By 72 h, 60 % of the dose had been excreted, 19 % was still retained in the gut tissue, 10 % remained in the lumen and 12 % had been absorbed across the gut and partitioned amongst the internal organs. A reduction in water temperature of 10 °C (to 6 °C) significantly retarded components of new Cu distribution (movement of the bolus along the gut and excretion); nonetheless, by 72 h, the fraction absorbed by all the internal organs was similar to that at 16 °C. An increase in water temperature of 3 °C (to 19 °C) caused a pronounced increase in internal organ uptake by 24 h to approximately double the uptake occurring at 16 °C. The uptake of new Cu by the gut tissue had a low temperature coefficient (Q_{10}<1) consistent with simple diffusion, while the temperature coefficient for transfer of new Cu from gut tissue to the internal organs was high (Q_{10}>2), consistent with facilitated transport.

Internally, the liver and gall bladder (including bile) were the target organs for dietary Cu partitioning since they were the only organs that concentrated new Cu from the plasma. Individual tissues differed in terms of the exchange of their background Cu pools with new Cu. The background Cu in the walls of the gastrointestinal tract (excluding stomach) exchanged 45–94 % with new Cu from the gut lumen, while tissues such as the stomach, gills, kidney, carcass and fat had 5–7 % exchangeable background Cu. The liver, heart, spleen, ovary, bile and plasma had only 0.2–0.8 % exchangeable background Cu.

The gastrointestinal tissues appear to act as a homeostatic organ, regulating the absorption of nutritional (non-toxic) doses of Cu (0.117 μg g^{-1} body mass day^{-1}) by the internal organs. Within the dose range we used and at optimal temperature (16 °C), the new Cu content of the gut tissues fluctuated, but absorption of new Cu by the internal organs remained relatively constant. For example, predosing the fish with non-radioactive Cu caused new Cu absorption by the gut tissues to double and decreased new Cu excretion from 38 to 1.5 %, but had no effect on new Cu uptake by the internal organs. Feeding fish after application of the normal liquid dose of new Cu also had no effect on new Cu uptake by the internal organs, even though the presence of food in the digestive tract reduced the binding of new Cu to the gut tissues and assisted with the excretion of new Cu. The gut was therefore able to regulate new Cu internalization at this dosage.

Higher new Cu doses (10, 100 and 1000 times the normal dose), however, evoked regurgitation and increased new Cu excretion within 4 h of application but did not elevate new Cu levels in gut tissue beyond a threshold of approximately 40 μg of new Cu. Only at the highest dose (1000 times the normal dose, 192 μg g^{-1} body mass), equivalent to toxic concentrations in the daily diet (7000 μg Cu g^{-1} dry mass food), was the buffering capacity of the gut overwhelmed, resulting in an increase in internal new Cu uptake.

Key words: copper, ^{64}Cu, dietary copper, radioactive copper, copper homeostasis, gastrointestinal uptake, rainbow trout, *Oncorhynchus mykiss*, teleost.

Introduction

Copper (Cu) is a micronutrient in vertebrates, and for rainbow trout the dietary requirement is 3 μg Cu g^{-1} dry mass food (Ogino and Yang, 1980). Laboratory studies using commercial feeds with Cu added as a salt [e.g. $CuSO_4 \cdot 5H_2O$ or $Cu(NO_3)_2 \cdot 3H_2O$] have shown that dietary Cu at concentrations of and above 730 μg Cu g^{-1} dry mass causes sublethal toxicity in rainbow trout (Lanno et al., 1985b). Dietary studies have established that Cu accumulates in the

liver, kidney, blood, gills and skin of fish and is probably excreted mainly in the bile *via* liver metabolism pathways (for a review, see Handy, 1996).

The dietary uptake of Cu by fish is thought to be relatively low, unlike that in mammals, in which Cu absorption ranges from 10 to 75 % of the initial dose. Handy (1992) fed juvenile rainbow trout a diet containing 200 μg Cu g^{-1} dry mass for 32 days and calculated Cu uptake by the internal organs to be only 1.5 % (3.2 % if gut Cu content was included). Julshamn et al. (1988) calculated that Cu uptake (including that by the gut tissues) was only 1.3 % for rainbow trout fed dietary Cu at 100–900 μg Cu g^{-1} dry mass for 9 weeks.

In mammals, the mechanism of dietary Cu uptake is thought to be mainly *via* passive diffusion across the mucosal surface of the gut cell followed by active transport (possibly by a Cu-ATPase) across the basolateral membrane (Linder, 1991). Subsequently, Cu is bound to plasma albumins and transported from the gut to the liver, where it is incorporated into ceruloplasmin and then transported to other internal organs (Cousins, 1985). The mechanisms of dietary Cu absorption and transport have not yet been examined in fish, and the factors that control Cu uptake and distribution to the internal organs remain poorly understood. The majority of studies of dietary Cu uptake in fish have been long-term (e.g. 4–24 weeks), and fish are dosed repeatedly and usually with elevated levels of Cu (Lanno et al., 1985a,b, 1987; Julshamn et al., 1988; Knox et al., 1982, 1984; Miller et al., 1993; Murai et al., 1981; Handy, 1992, 1993; Lorentzen et al., 1998). Although this may mimic fish chronically feeding in a contaminated environment, individual feeding rates, and therefore Cu doses, cannot be determined accurately. Also, Cu uptake is difficult to detect against high background Cu concentrations already present in the fish. This means that we cannot accurately determine Cu uptake by individual organs, so we cannot understand exactly how fish regulate dietary Cu. Furthermore, the use of elevated concentrations of Cu may decrease Cu absorption (Lorentzen et al., 1998) because, to a certain extent, fish may be able to regulate Cu uptake and acclimate to chronically elevated diet-borne Cu. Indeed, in contaminated environments, there is very little biomagnification of Cu between fish and their prey (e.g. Dallinger and Kautzky, 1985; Clements and Rees, 1997).

The purpose of the present study was to describe Cu uptake in rainbow trout from a single dietary dose, at nutritional levels, of radioactive Cu provided *in vivo via* a surgically implanted stomach catheter. This method has several advantages over normal dietary studies since we can control the dose and chemistry of the infusate precisely, determine Cu uptake over a short time frame (72 h) and detect low levels of Cu uptake (μg) in all the internal organs. After establishing how a single dietary dose of Cu is absorbed by the gut tissues and then distributed to the internal organs, we then went on to examine the effects of temperature, dose, pre-exposure to Cu and the presence of food. Eventually, we hope to use this radioisotopic method to examine other factors that may alter dietary Cu uptake, e.g. protein complexation (Cousins, 1985; Farag et al., 2000).

We have defined ‘internalized Cu’ as the portion of the ingested dose of ionic Cu that is absorbed across the gut cells and enters the internal organs of the fish (e.g. liver, kidney and blood). Although Cu in the gut cells could also be considered ‘internalized’, we have excluded it from the internalized portion of the ingested dose because it could be excreted in the normal process of cell renewal of the digestive tract (Linder, 1991). Moreover, the gut is thought to play an important role in regulating metal uptake from the diet in fish (Harrison and Klaverkamp, 1989; Maage and Julshamn, 1993; Handy, 1993; Clements and Rees, 1997); therefore, Cu uptake by the gut tissues should be determined separately from that by the other internal organs. We considered the fate of an oral Cu dose by partitioning the Cu into four different compartments, the gut lumen, gut tissues, internalized Cu and excreted Cu.

Materials and methods

Experimental animals

Juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum) (273±9 g; mean ± S.E.M., *N*=96) were obtained from the Rainbow Springs Trout Hatchery in Thamesford, Ontario, Canada, and acclimated to laboratory conditions for at least 2 weeks prior to experimentation. Fish were held in 350 l tanks supplied with moderately hard dechlorinated Hamilton tapwater (Cu, 3 μg l^{-1}; cadmium, 0.09 μg l^{-1}; calcium, 40 mg l^{-1}; Na^+, 14 mg l^{-1}; Cl^-, 25 mg l^{-1}, hardness 140 p.p.m. as $CaCO_3$, pH 8.0, 5.5 l min^{-1}) and fed semi-moist floating pellets at a ration of 2 % body mass day^{-1} (Corey Aquaculture, five point). The food composition was as follows: 3 μg Cu g^{-1}; crude protein, 40 % (minimum); crude fat, 15 % (minimum); crude fibre, 2 % (maximum); sodium, 1 % (actual); calcium, 1 % (actual); phosphorus, 1 % (actual); vitamin A, 3600 i.u. kg^{-1} (minimum); vitamin D3, 2400 i.u. kg^{-1}; vitamin E,150 i.u. kg^{-1} (minimum). The water temperature was 6–16 °C. If experiments were performed at a water temperature different from laboratory conditions, fish were acclimated to the experimental water temperature at a rate of 3 °C day^{-1} and held at the final temperature for at least 2 days prior to surgery.

Experimental approach

Since the stomach is normally acidic in rainbow trout (Fange and Grove, 1979), we used a solution of $^{64}Cu(NO_3)_2$ in 0.1 mol l^{-1} glucose (which will not complex copper ions), for our initial studies. Except for the high dosage experiment, the Cu doses (approximately 30 μg $fish^{-1}$) were equivalent to the normal daily Cu intake of a trout consuming 3–4 % body mass day^{-1} of a diet containing 3 μg Cu g^{-1} dry mass (the minimum Cu requirement for rainbow trout) (Ogino and Yang, 1980).

Fish were anaesthetized (MS-222 0.12 g l^{-1}, $NaHCO_3$ 0.24 g l^{-1}) and placed on a surgery table, where the gills were continuously irrigated with water. An oral dosing catheter (PE-50, internal diameter 0.58 mm, external diameter 0.97 mm; 30 cm long) was then introduced to the stomach *via* the oesophagus. The catheter was held in place by two sutures in

the dorsal buccal cavity so that it terminated in the stomach. A PE-160 sleeve was surgically inserted through roof of the mouth to the dorsal body surface, and the free end of the catheter was then passed through the sleeve. This procedure was necessary to position the catheter reliably in the stomach and allow us to dose the fish with radioactive Cu while it remained relatively undisturbed. Each fish was placed in an individual, aerated 2 l Plexiglas box supplied with flowing water and allowed to recover from surgery for 3 days. On day 4, each fish was infused with a solution of radioactive Cu in 0.1 mol l^{-1} glucose *via* the catheter. At the completion of each exposure (4–72 h), the fish was killed using an overdose of anaesthetic (MS-222 1 g l^{-1}; $NaHCO_3$ 2 g l^{-1}). Blood (1.0–2.0 ml) was immediately sampled by caudal puncture, and the internal organs (swimbladder, spleen, gonads, gut, liver, gall bladder, kidney, heart, gills and fat were then removed from around the viscera) and carcass (including the head), individually weighed and stored in scintillation vials. The new Cu content of each gut section was determined by dissecting the gut into four parts (stomach, pyloric caecae, mid intestine and posterior intestine) and rinsing each part in 10 ml of deionized water, prior to storage in separate scintillation vials. Blood samples were centrifuged for 5 min at 12 000 ***g*** to separate plasma and red blood cells. All samples were measured for ^{64}Cu activity (with automatic decay correction) in a Canberra-Packard MINAXI g Auto-Gamma 5000 series Gamma counter for 5 min or until 2 % accuracy had been obtained.

Experimental series

Series 1. The effects of time and temperature on new Cu uptake

Rainbow trout were infused with a single oral dose of 30 μg of ^{64}Cu as $Cu(NO_3)_2$ (0.117±0.004 μg g^{-1}; mean ± S.E.M., N=46) in 1 ml of 0.1 mol l^{-1} glucose (pH 2.86). The fish were held at one of three experimental water temperatures (6, 16 or 19 °C). After 24, 48 or 72 h, the fish were killed, the blood was sampled, and the fish was dissected as detailed above. Fish held at 19 °C were only exposed to ^{64}Cu for 24 h, giving a total of seven experimental treatments.

Series 2. New Cu uptake and exchange with background Cu

Rainbow trout were infused as above and held in 16 °C water for 24 (N=4), 48 (N=4) or 72 h (N=4). They were then killed, the blood was sampled, and the fish was dissected as detailed above. After measurement of radioactive Cu, all organs and gut contents were analysed for total Cu content. Individual tissues were digested in 1 mol l^{-1} HNO_3 at 60 °C for 24 h, and each digest was vortexed and then centrifuged for 10 min at 10 000 ***g***. The supernatants were further diluted in 1 % HNO_3 (1–1000 times as appropriate), and the Cu content (μg g^{-1} wet mass) was determined by atomic absorption spectrophotometry.

Background Cu concentrations were determined for each internal organ, gut tissue and gut content at 24, 48 and 72 h by subtraction of ^{64}Cu (i.e. new Cu) concentrations from total Cu concentrations.

Series 3. The effects of increasing dose on new Cu uptake

Using the same *in vivo* technique as before, we exposed rainbow trout to three different doses of ^{64}Cu [approximately 300, 3000 or 30 000 μg of ^{64}Cu as $Cu(NO_3)_2$] for 4 h. The two lower doses were equivalent to the daily dose of Cu that would be received in a diet containing 75 or 800 μg Cu g^{-1} dry mass which, according to previous dietary studies, fall within the ‘no adverse effect’ and ‘sublethal toxicity’ ranges, respectively, for rainbow trout (Lanno et al., 1985b). The highest dose corresponds to the daily dose of Cu that would be received in a diet containing 7000 μg Cu g^{-1} dry mass, which would be toxic to rainbow trout (Lanno et al., 1985b; Handy, 1996).

Series 4. The effects of predosing on new Cu uptake

Rainbow trout were infused every 24 h for 5 days with 30 μg of non-radioactive Cu as $Cu(NO_3)_2$ (0.114±0.003 μg g^{-1}) in 1 ml of 0.1 mol l^{-1} glucose (pH 2.86). On day 6, the predosed fish and six naive fish were dosed with 30 μg of ^{64}Cu. Forty-eight hours after administration of the radioactive dose, the fish were killed and sampled as described above.

Series 5. The effects of the presence of food on new Cu uptake

Eleven fish were infused with a single oral dose of 30 μg of ^{64}Cu as $Cu(NO_3)_2$ (0.109±0.006 μg g^{-1}) in 1 ml of 0.1 mol l^{-1} glucose (pH 2.86), as in previous experiments. After 2 h, the fish were transferred from the individual boxes to one of two large holding tanks (75 l) supplied with running water (16 °C) and aeration. The stomach catheters were removed during the transfer, and the fins were clipped for later identification. Four hours after transfer to the large holding tanks, the fish in one tank were offered pelleted feed, and 5 h after transfer the remaining food was netted out of the tank. Forty-eight hours after the infusion of the radioactive Cu, the fish were killed and sampled as described above.

The transfer of the fish from their individual boxes, removal of the the catheter and marking were achieved in less than 20 s per fish. One person (wearing surgical gloves to minimize epidermal injury to the fish) held the unanaesthetized fish, while a second person snipped the sutures holding the catheter, removed it and then clipped the fins. Despite this handling, 4 h later, six out of eight fish offered food ate spontaneously. Assuming that these fish ate 1 % of their body mass in the 1 h that the pellets were available to them, they would have consumed approximately 4.5 g of food containing 13 μg of non-radioactive Cu (pellet Cu content is 3 μg g^{-1} dry mass).

Preparation of radioactive Cu

Dried $Cu(NO_3)_2$ (500 μg or 750 μg) was irradiated by the McMaster Nuclear Reactor (^{64}Cu, half-life 12.9 h) to achieve a predetermined level of radioactivity (usually 19 MBq). After irradiation, the $Cu(NO_3)_2$ was dissolved in 0.1 mol l^{-1} HNO_3 (usually 400 μl) then 0.01 mol l^{-1} $NaHCO_3$ (usually 400 μl). The resuspended Cu was then added to 0.1 mol l^{-1} glucose to give a final concentration of 30 μg Cu μl^{-1}.

Data analysis

Calculation of newly accumulated Cu

Newly accumulated Cu (^{64}Cu or new Cu) (Cu_{new}; μg new Cu g^{-1} wet mass) in the organs of the fish was calculated according to the following equation:

$$Cu_{new} = \frac{R}{M \times SA}, \quad (1)$$

where R (cts min^{-1}) is the radioactivity of the tissue corrected for background and quench, M is tissue wet mass (g) and SA is the specific activity (cts min^{-1} μg^{-1} Cu) of the radioactive Cu and glucose solution determined from three (200 or 500 μl) samples counted with the tissue samples. In this way, uptake of the radioactive dose from the gut can be calculated independent of non-radioactive Cu already present in the organs of the fish. Internalized Cu was calculated by addition of new Cu found in all the internal organs, carcass, plasma and red blood cells. New Cu excretion was calculated by subtracting internalized new Cu and new Cu found in the gut tissues and gut lumen from the initial dose.

Q_{10} calculations

Q_{10} values were calculated from the data at 6, 16 and 19 °C at different time periods using the equation:

$$Q_{10} = (R_2/R_1)^{[10/(T_2-T_1)]}, \quad (2)$$

where R_2 and R_1 are the new Cu uptake rates (μg g^{-1} h^{-1}) at the two temperatures T_2 and T_1 respectively.

Statistical analyses

The statistical package JMP (version 3.2.4., SAS Institute Inc.) was used to compare new Cu concentrations using one-way analyses of variance (ANOVAs) and Tukey's test for *a posteriori* comparisons of significantly different means (P<0.05). The assumptions of ANOVA (normal distribution of the data and homogeneity of the variances) were examined for each data set and, if necessary, data were subjected to logarithmic, square root or arcsine transformations to achieve normal distributions. Data are presented as means ± S.E.M. and are presented graphically as mass (μg) to show the relative importance of each compartment in the uptake of the total dose.

Results

Movement of new Cu along the gut at 16 °C

By 24 h post-infusion, most of the new Cu (99 %, 29.7 μg) had moved past the stomach and had been distributed along the rest of the gut. When both tissues and luminal content are considered, 44 % (13.1 μg) was found in the pyloric caecae, 12 % in the mid intestine and 23 % in the posterior intestine (Fig. 1A,B). In the pyloric caecae, the new Cu was distributed approximately equally between luminal fluid and tissue, whereas in the mid and posterior intestine most of the new Cu was luminal (Fig. 1C).

Over the next 48 h, the new Cu moved further down the gut, with tissue levels tending to decline in the pyloric caecae and mid intestine and tissue levels tending to increase in the posterior intestine. Most of the change, however, was an overall significant reduction in luminal new Cu contents of all gut tissues (Fig. 1A). The steady decrease in luminal new Cu mirrors new Cu excretion, which was between 15 and 24 % day^{-1} (Fig. 1A).

Only a small fraction of the new Cu dose (6 % or 1.8 μg) was internalized across the gut tissues by 24 h, increasing to

Table 1. *New Cu content in fish held at 6, 16 or 19 °C after 24, 48 or 72 h exposure to a dietary dose of Cu*

	24 h			48 h		72 h	
	6 °C	16 °C	19 °C	6 °C	16 °C	6 °C	16 °C
Internal organs (μg kg^{-1})	3±0[a]	7±1[b]	13±2[b]	7±1	8±1	9±1	16±3
Gut tissues (μg kg^{-1})	1199±291	1043±125	760±55	1319±125	804±232	1199±206	910±204
Gut lumen (μg)	12±4	14±1	12±1	12±1	9±2	10±1[a]	3±1[b]
Excreted* (μg)	4±5	4±2	6±1	0±0[a]	12±3[b]	3±2[c]	18±2[d]
Liver (μg kg^{-1})	92±7[a]	256±12[a,b]	441±67[b]	253±27[c]	556±87[d]	386±55[e]	667±81[f]
Gall bladder and bile (μg kg^{-1})	19±10	82±27	129±79	101±28	251±102	123±38	332±131
Kidney (μg kg^{-1})	12±2	21±2	34±4	25±3	25±1	34±4	45±9
Fat (μg kg^{-1})	3±1[a]	21±7[a]	162±58[b]	11±4	20±9	27±11	24±6
Gill (μg kg^{-1})	4±1[a]	8±1[b]	31±16[b]	9±1	8±1	10±1	17±3
Carcass (μg kg^{-1})	1±0	2±1	2±0	2±1	2±0	3±1	6±2
Red blood cells (μg l^{-1})	2±0.9[a]	1±0.2[a]	6±0.3[b]	6±2.5	1±0.4	5±3.2	20±8.9
Plasma (μg l^{-1})	9±1[a]	17±3[a,b]	60±8[b]	20±3	13±2	18±4	40±12
Sample size	4	7	4	9	6	8	8

Letters indicate Tukey's grouping of significantly different means within time.

See Fig. 1 for significantly different means within temperature across time.

*Excretion of new Cu (^{64}Cu) was calculated by subtracting internalized new Cu and new Cu found in the gut tissues and gut lumen from the initial dose.

Values are means ± S.E.M. and are mostly presented as μg kg^{-1} wet mass.

12 % (3.5 µg) by 72 h, despite the fact that the gut tissue readily absorbed new Cu from the gut lumen (Fig. 1A).

Movement of new Cu along the gut at 6 °C

Movement of new Cu along the gut was significantly depressed at 6 °C in comparison with warmer water temperatures. By 48 h post-infusion, luminal and tissue new Cu levels in the stomach in fish held at 6 °C were still higher than in fish held at 16 °C; similarly, by 72 h, there was more new Cu in the pyloric caecae tissues and less in the posterior intestine tissues of the fish held at the lower water temperature (Fig. 1B,C). Furthermore, luminal contents did not decrease significantly over time and excretion was only 0.2–13 % day^{-1} (Fig. 1A,C).

After 24 h, only a small fraction of the new Cu dose (2 %) was internalized at these low water temperatures; however, by 72 h, the amount absorbed (8 %) was only slightly lower (no statistically significant difference) than that absorbed at 16 °C (12 %) despite the strikingly different pattern of new Cu movement along the gut (Fig. 1A).

New Cu uptake in fish held at 19 °C for 24 h

Increasing the water temperature to 19 °C resulted in even greater absorption of new Cu by the internal organs after 24 h (i.e. 12 % uptake compared with only 6 % uptake at 16 °C and 2 % uptake at 6 °C) (Fig. 1A), especially by the liver, gills, fat, plasma and red blood cells (Table 1). But, by 24 h, the 3 °C increase in water temperature had not significantly altered new Cu uptake by the gut tissue or movement of new Cu along the gut lumen (Fig. 1A,C). Approximately 21 % of the new Cu dose was excreted by 24 h at 19 °C compared with 15 % at 16 °C and 13 % at 6 °C (not statistically different) (Fig. 1A).

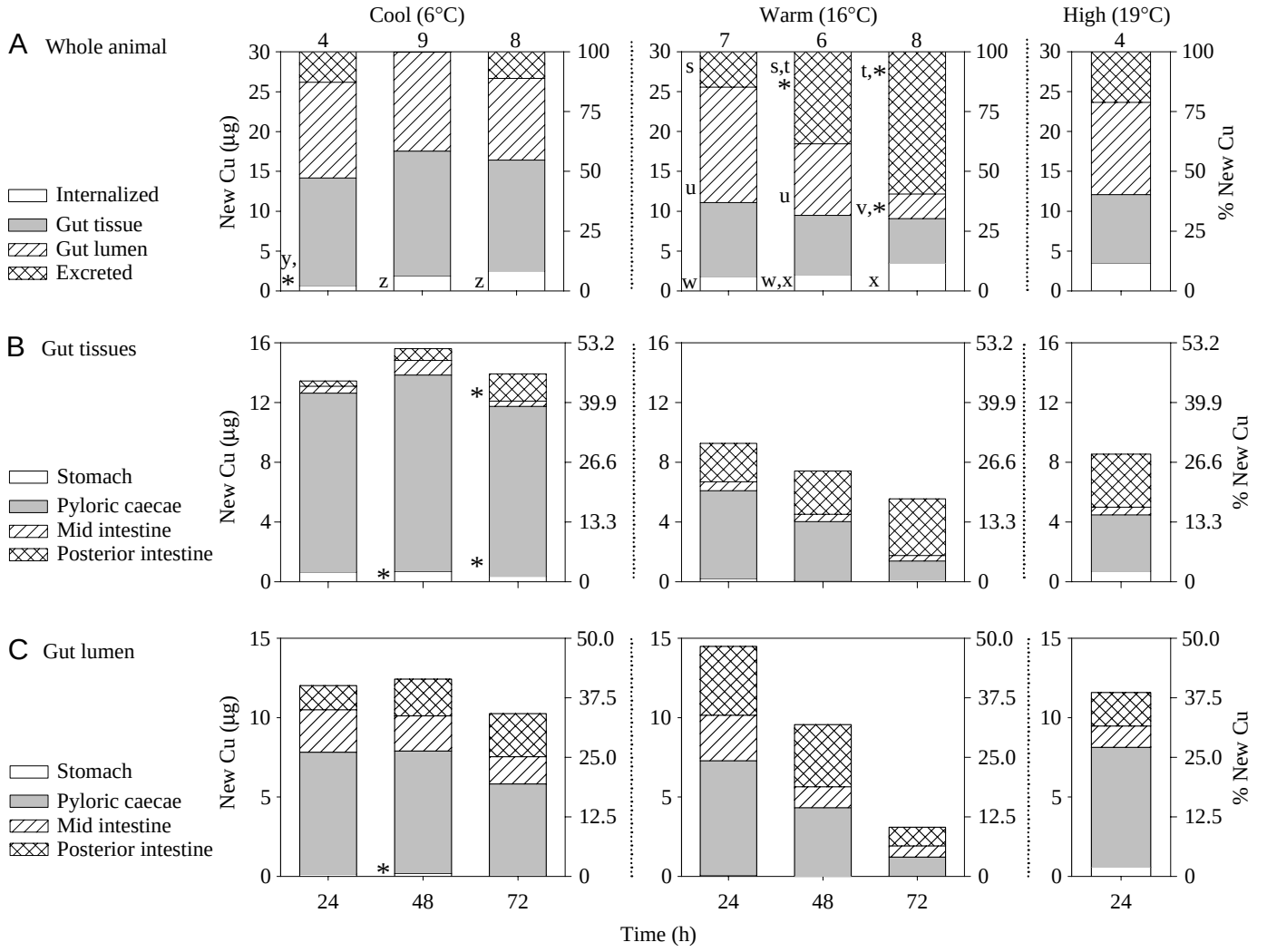

Fig. 1. (A) Distribution of a 30 µg dose of ^{64}Cu (new Cu) in the whole animal (internalized; see data analysis section, gut tissues, gut lumen or excreted), (B) amongst the gut tissues and (C) in different parts of the gut lumen after 24, 48 and 72 h at 6, 16 or 19 °C. Sample sizes are indicated above the columns. An asterisk indicates significantly different (P<0.05) means between temperatures at the same time (see Table 1 for more details). Letters indicate significantly different means between times at the same temperature. See Table 1 for concentration data and standard errors.

Table 2. *Q_{10} values calculated from new Cu uptake rates between different compartments measured at different times and temperatures*

From	To	Temperature (°C)	Time (h)	Q_{10}
Gut lumen	Gut tissue	6–16	24	0.87
		16–19	24	0.35
		6–19	24	0.70
		6–16	48	0.61
		6–16	72	0.76
Gut lumen	Internal organs	6–16	24	2.33
		16–19	24	6.91
		6–19	24	3.00
		6–16	48	0.88
		6–16	72	0.56

Q_{10} data

Q_{10} values measure the increase in a reaction velocity caused by a 10 °C increase in temperature (Hoar, 1983). A Q_{10} greater than 2 indicates an enzymatic process, while a Q_{10} below 1.5 usually indicates a purely physical process such as diffusion (Hoar, 1983). Q_{10} values from our temperature experiments suggest that gut tissue uptake of new Cu is *via* simple diffusion; however, in the first 24 h, uptake into the internal organs is probably a biologically mediated process, but later occurs mainly *via* non-mediated transport (Table 2).

Internal distribution of new Cu

Only the liver and gall bladder concentrated new Cu compared with the plasma; the remaining organs contained new Cu concentrations in equilibrium with the plasma or had significantly lower concentrations (the gills and carcass) (Fig. 2). By 72 h, 42 % of the internalized new Cu was partitioned into the liver and 30 % was in the carcass. The carcass makes up 84 % of the body mass, so it could still account for a large fraction of the internalized new Cu even though it did not concentrate new Cu from the plasma. The other organs with significant amounts of new Cu after 72 h are (in rank order according to the percentage of internalized fraction, not concentration), plasma (14.5 %), fat (2.7 %), gills (2.7 %), red blood cells (2.5 %), gall bladder (and bile) (2.1 %), kidney (2.0 %) and other organs (1.8 %).

Factors influencing the distribution of new Cu: exchange with background Cu?

In a separate experiment, we examined whether the background concentration of Cu in each tissue influenced the distribution of new Cu over 72 h. Background Cu concentrations were highest in the liver (66±12 to 105±16 μg Cu g^{-1} wet mass) and ranged between 0.10±0.03 and 2.89±0.6 μg Cu g^{-1} wet mass in the other internal organs and gut tissues (Fig. 3). There was very little Cu in the lumen of the gut that could not be accounted for by the addition of

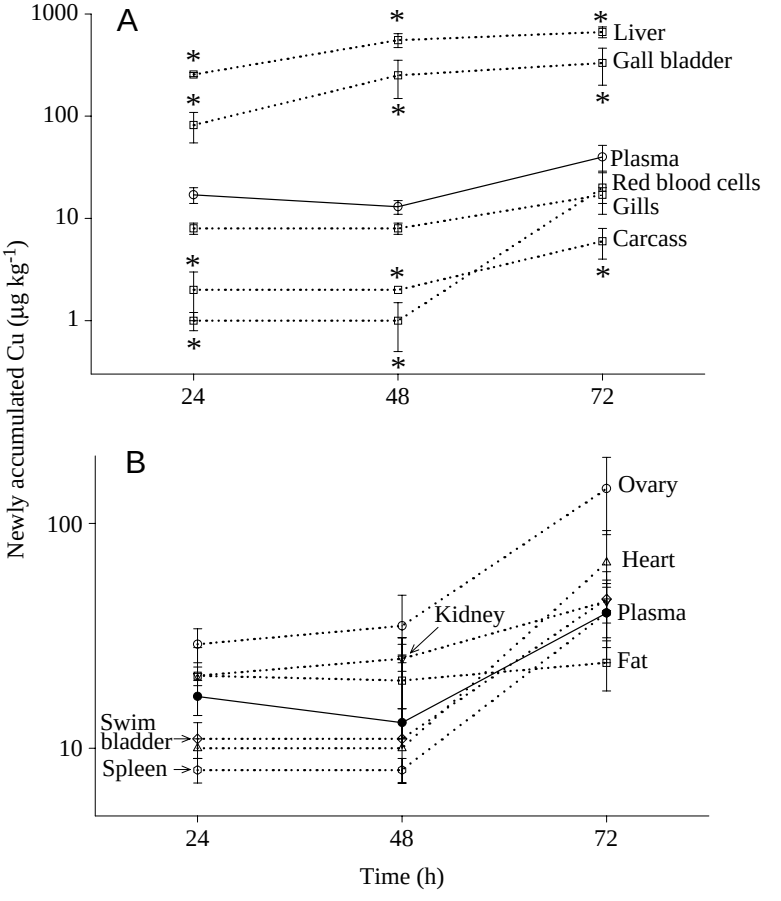

Fig. 2. The change in ^{64}Cu (new Cu) concentration in internal organs and tissues over time at 16 °C compared with new Cu concentrations in the plasma (solid line). (A) Liver and gall bladder concentrated new Cu compared with the plasma, while the red blood cells, gills and carcass (including the head) appear to be actively excluding new Cu. (B) Results for the other internal organs, which are in equilibrium with the plasma. An asterisk indicates a value significantly different (P<0.05) from that of the plasma within a time. Values are means ± S.E.M., N=6–8.

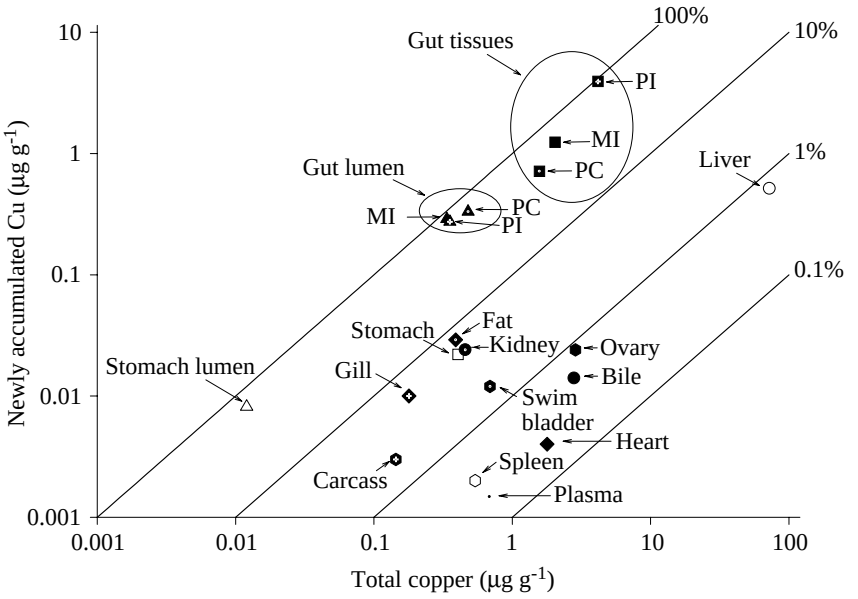

Fig. 3. The relationship between background copper concentration (μg g^{-1} wet mass) and the absorption of ^{64}Cu (new Cu) (μg g^{-1} wet mass) 72 h after infusion. The lines represent different ratios of [new Cu]:[background Cu]. PC, pyloric caecae; MI, mid intestine; PI, posterior intestine.

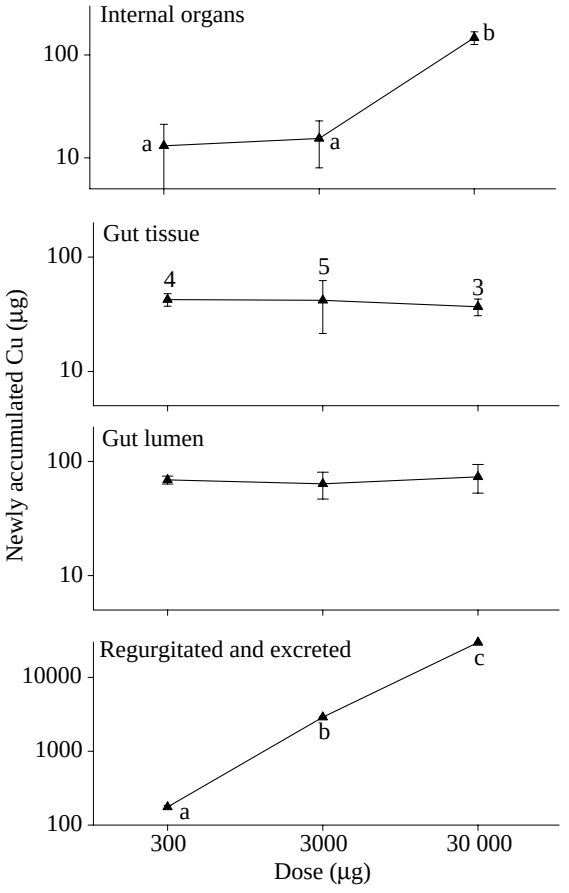

Fig. 4. Excreted ^{64}Cu (new Cu), luminal new Cu and new Cu content of either the internal organs or gut tissues after exposure to 300, 3000 or 30 000 μg of copper in 0.1 mol l^{-1} glucose for 4 h at 16 °C. Sample sizes are indicated on the graph. Letters show Tukey's grouping of significantly different (P<0.05) means. Values are means ± S.E.M.

radioactive Cu. Background Cu concentrations in the stomach lumen were initially 0.18±0.07 μg Cu g^{-1} wet mass, then decreased (not significantly) to 0±0 μg Cu g^{-1} wet mass at 48 and 72 h, suggesting that gastric secretions may have contributed background Cu in the first 24 h of digestion. The other sections of the gut lumen (pyloric caecae, mid intestine and posterior intestine) did not show any pattern of change in background Cu concentrations over time with values ranging between 0.02±0.02 and 1.20±1.10 μg Cu g^{-1} wet mass. The kidney was the only internal organ that showed a significant change in background Cu content, increasing from 0.25±0.03 μg Cu g^{-1} wet mass at 24 h to 0.41±0.04 μg Cu g^{-1} wet mass at 48 h and remaining high at 72 h (0.43±0.05 μg Cu g^{-1} wet mass).

Next, we considered the affect background Cu might have on partitioning of new Cu. We have only presented data from 72 h because the ratios of [new Cu]:[background Cu] in the different organs remained relatively constant over time. To begin with, we assumed that new Cu distributed to tissues strictly according to the background Cu level in that tissue. If this were the case, the ratio of [new Cu] to [total Cu] would be constant for all tissues. Using the liver at 72 h as an index tissue, this ratio would be approximately 1:100, meaning that approximately 1 % of background hepatic Cu was readily exchangeable. The gill, kidney, carcass, swim bladder and fat had higher ratios, indicating that 2–7 % of their background Cu was readily exchangeable, while the heart, spleen, gall bladder plus bile, ovaries and plasma had less than 0.8 % exchangeable Cu. The stomach tissues had only approximately 5 % exchangeable background Cu, unlike the pyloric caecae, mid intestine and posterior intestine, which had 45 %, 61 % and 94 % exchangeable Cu, respectively.

The effects of increasing dose on new Cu uptake

After 4 h, only 40 %, 4 % and 1 % of the initial infused dose was retrieved from the fish dosed with 300, 3000 and 30 000 μg

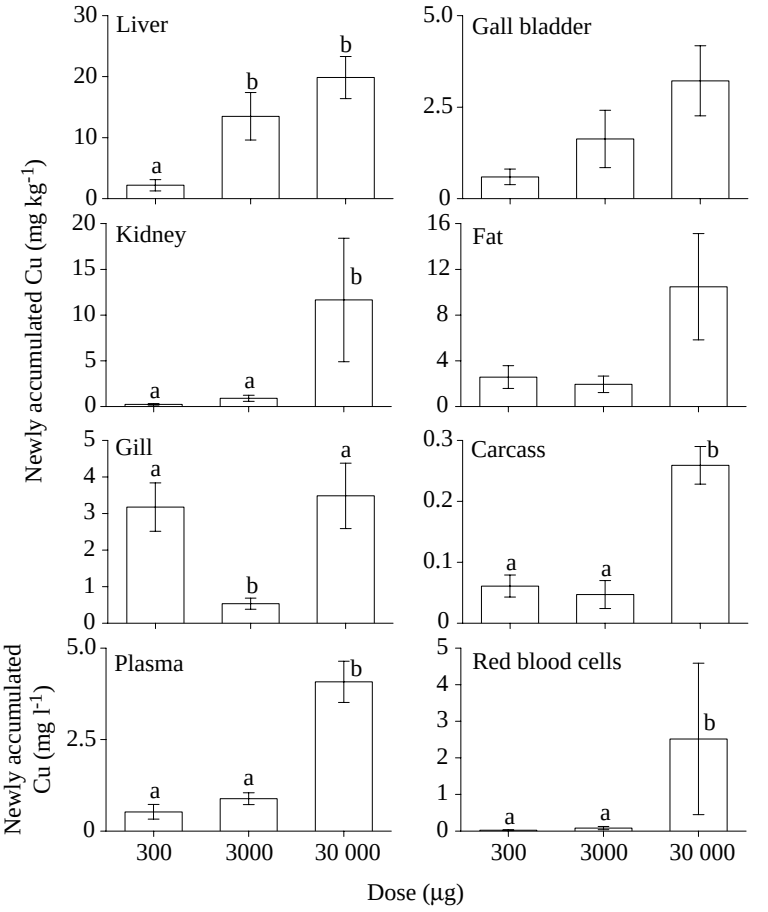

Fig. 5. ^{64}Cu (new Cu) content of the carcass, mesenteric fat, plasma, red blood cells and selected internal organs after exposure to 300, 3000 or 30 000 µg of copper in 0.1 mol l^{-1} glucose for 4 h at 16 °C. Sample sizes are the same as in Fig. 4. Letters show Tukey's grouping of significantly different (P<0.05) means. The new Cu content of the kidney, carcass and blood components at the three different doses is representative of the profile of uptake in the heart, ovary, spleen and swimbladder. Values are means ± S.E.M.

of new Cu, respectively, indicating that the fish had regurgitated much of the infusate (Fig. 4). However, at least a portion of the new Cu was excreted in the normal manner because, in all the fish, some of the infused radioactive Cu was found in the posterior intestinal lumen and tissues. The new Cu content of the gut tissues and the gut lumen was remarkably constant (approximately 40 µg), suggesting that, once a threshold of new Cu concentration had been reached, the remaining new Cu was excreted, regurgitated or taken up by the internal organs.

In general, the new Cu content of the internal organs remained relatively low in the fish exposed to either a non-toxic or a sublethal dose of new Cu and increased significantly in the fish exposed to a (theoretically) toxic oral dose of new Cu (Fig. 5). The exceptions to this trend were the liver and gall bladder, which showed a more gradual increase in new Cu uptake over the dose range, and the gills, which showed variable new Cu uptake, probably due to contamination by waterborne radioactive Cu (regurgitated).

The effects of predosing on new Cu uptake

Predosing the fish with non-radioactive Cu decreased new Cu absorption by the liver, but had no effect on new Cu uptake by any of the other internal organs (Table 3). Predosing decreased new Cu excretion and significantly increased new Cu uptake by the posterior intestinal tissues but did not change

Table 3. *New Cu (µg) found in each compartment after exposure to 30 µg of new Cu in 0.1 mol l^{-1} glucose for 48 h at 16 °C*

Experiment	Compartment	Single dose	Predosed
Predosing	Internal organs	2.08±0.16	2.11±0.25
	Gut tissues	7.42±1.68	14.41±1.51*
	Gut lumen	10.04±1.83	13.03±1.20
	Excreted	11.54±2.85	0.44±1.56*
	Sample size	6	9
Feeding		Not fed	Fed
	Internal organs	2.45±0.38	1.65±0.31
	Gut tissues	10.11±1.35	3.59±1.52*
	Gut lumen	10.35±2.12	4.49±1.46
	Excreted	7.09±3.44	20.28±1.93*
	Sample size	5	6

Values are means ± S.E.M.

Predosing experiment: fish were either exposed to a single dose of radioactive Cu (single dose) or predosed daily for 5 days with 30 µg of non-radioactive Cu prior to ^{64}Cu exposure.

Feeding experiment: fed fish were allowed to feed after administration of the radioactive copper (fed), and the remainder either did not feed or were not offered food (not fed). See text for further details.

Asterisks indicate significantly different (P<0.05) means between treatments.

uptake by other sections of the gut. When added separately, significantly larger amounts of new Cu were found in the lumen of the stomach and posterior intestine of predosed fish (data not shown).

The effects of the presence of food on new Cu uptake

The presence of food in the digestive tract 6 h after infusion of the radioactive dose had no significant effect on new Cu uptake by the internal organs (Table 3) even though it increased new Cu excretion from 24 to 68 % by 48 h. Fed fish tended to have less new Cu in the gut lumen ($P<0.057$) and the gut tissues ($P<0.03$), especially the posterior intestine ($P<0.01$). The exception was the stomach lumen, which contained slightly (but not statistically significant) higher new Cu concentrations in the fed fish (data not shown).

Discussion

The dynamics of new Cu uptake by the intestinal tissue

Our radioisotopic method has shown that, at 16 °C, gut tissues of rainbow trout rapidly absorb approximately one-third of a nutritional dose of Cu (no increase after 24 h), then slowly release it to the plasma for uptake by the internal organs of the fish. In other words, the intestinal tissue is providing a reservoir of new Cu for the internal organs, at least in the first 3 days after exposure to a nutritional dose of the metal. The response of the gut to higher doses of Cu supports the role of the gut tissue as a buffer for the internal organs. As the new Cu dose increased from 10 to 1000 times the control dose, the new Cu content of both the gut and the internal organs remained relatively constant until the new Cu dose reached a 'theoretically toxic' level (Lanno et al., 1985b; Handy, 1996), when the new Cu absorbed by the internal organs increased markedly. The highest Cu dose probably overwhelmed the gut's capacity to limit new Cu uptake by the internal organs. Maage and Julshamn (1993) suggested that in Atlantic salmon (*Salmo salar*) the gut has a similar role, buffering the internal organs from both high and low zinc concentrations.

Studies in mammals have shown that transport of Cu from the gut lumen into the gut tissue and from the gut tissue across the basolateral membrane of the enterocytes are separate processes (Crampton et al., 1965; Linder, 1991). The rate-limiting step is thought to be transport of Cu across the basolateral membrane into the plasma. In the current study, the Q_{10} for new Cu uptake into the gut tissue was less than 1 in all comparisons, indicating that this portion of the mechanism is probably simple diffusion (Hoar, 1983). In contrast, the Q_{10} for new Cu uptake from the intestinal lumen to the internal organs in the first 24 h was greater than 2.0, indicating that in rainbow trout at least part of the process of new Cu uptake into the internal organs (probably transfer across the basolateral membrane of the enterocytes) is *via* a biologically mediated mechanism, rather than *via* simple diffusion (Hoar, 1983). Biologically mediated transport might initially be stimulated by the presence of infusate (or food) in the stomach or upper intestine, thus explaining the time-dependence of the mechanism.

The effects of pre-exposing the gut to Cu

In our previous experiments, the fish were not fed for 4 days prior to receiving a dose of radioactive Cu. Copper deficiency in mammals is thought to cause up-regulation of intestinal Cu absorption (Linder, 1991), so we were concerned that our experimental protocol was artificially elevating new Cu uptake in our experimental fish. Our pre-dosing experiment examined how a dosing regime of frequent dietary Cu exposure (mimicking daily feeding) would affect the absorption of a single dose of radioactive Cu. We expected the gut to absorb less radioactive Cu after pre-exposure to daily doses ($30\,\mu g\,day^{-1}\,fish^{-1}$) of non-radioactive Cu equivalent to that required in a rainbow trout diet (approximately $3\,\mu g\,Cu\,g^{-1}$ dry mass) (Ogino and Yang, 1980). Contrary to our expectations, pre-exposing fish to Cu increased the capacity of gut tissues to bind and sequester new Cu but did not increase uptake by the internal organs.

The fact that Cu uptake by the internal organs did not change with increased Cu exposure suggests that, at the dosage level we used, the gut can effectively regulate new Cu internalization under both a pre-exposure and a single-dose regime. The data also suggest that 4 days without feeding have not caused significant Cu deficiency in our experimental fish. The increased new Cu uptake by the gut tissues may have been caused by metallothionein induction, even though the pre-exposure doses were in the 'dietary range'. Metallothionein is a metal-binding protein thought to function in the detoxification and storage of dietary metals in enterocytes and hepatocytes (Olsson et al., 1997). In rats, Cu bound to metallothionien may be stored until the enterocytes are sloughed off into the gut lumen and excreted in the normal process of gut cell renewal (Owen, 1965; Linder, 1991). Alternatively, pre-exposure of the gut to non-radioactive Cu may have increased the pool of readily exchangeable Cu in the gut tissues, thereby increasing the uptake of new Cu.

The effects of temperature and gut motility on new Cu uptake

Increased water temperature increases gut motility in rainbow trout (Fange and Grove, 1979). We were curious whether this would be the case for a liquid dose of new Cu and whether decreased residence time in the gut would limit absorption of new Cu. In fact, new Cu excretion was markedly increased by a 10 °C increase in water temperature from 6 °C, but by 72 h after the intial exposure this had very little effect on absorption of new Cu by the internal organs. We were only able to examine new Cu uptake at 19 °C for 24 h. These data suggest that higher water temperatures, outside the optimal range for a cool-water species such as rainbow trout, may increase new Cu absorption by the internal organs. However, absorption of new Cu by 24 h was no higher than that achieved by 72 h at lower water temperatures, so it may also mean that new Cu uptake to a constant level (i.e. approximately 12 % of the initial dose) was simply more rapid at higher water temperatures.

Internalization of new Cu

In this study, at least 12 % of a single dietary dose of new Cu was internalized after 72 h at 16 °C. More Cu might have been absorbed by the internal organs given sufficient time for the new Cu to be completely absorbed from the gut and gut tissues. (Because ^{64}Cu has a half life of 12.9 h, we were limited to measuring ^{64}Cu uptake for 72 h). Previous estimates of dietary Cu internalization by rainbow trout were only 1.5 %, but were obtained in feeding studies using elevated Cu concentrations in the diet (Julshamn et al., 1988; Handy, 1996). In fish (Lorentzen et al., 1998) and rats (Farrer and Mistilis, 1967; Marceau et al., 1970), Cu absorption decreases as dietary Cu concentration increases. Also, Julshamn et al. (1988) and Handy (1996) were only able to detect net Cu accumulation in excess of the normal balance between Cu uptake and excretion.

Factors that may alter dietary Cu uptake

Because the gut tissues exchange so rapidly with new Cu and accumulate 5–12 times as much Cu as the internal organs, transfer from the gut tissues to the internal organs is probably the rate-limiting step in dietary Cu uptake. In theory, then, the factors that alter the transfer of Cu from the gut tissues to the internal organs will be those that change Cu internalization most effectively. For example, the presence of food in the digestive tract had no affect on internalization of new Cu; although food decreased the new Cu found in the gut tissues, there was still enough new Cu to supply the internal organs. Higher water temperatures tended to increase new Cu internalization, probably by stimulating Cu transport across the basolateral membrane of the gut tissue to the internal organs.

Control of new Cu uptake and partitioning amongst internal organs

The background Cu in the gut tissues, not including the stomach, was highly exchangeable with new Cu from the gut lumen; this fits with the absorptive role of this section of the intestine. The stomach, in contrast, had only 5 % exchangeable background Cu; in mammals, the stomach absorbs relatively little Cu or other nutrients and functions mainly in digestion (Linder, 1991). In the remaining internal organs and fluids, the ratio of new:background Cu was highly variable, so background Cu did not appear to have a strong influence on partitioning of new Cu.

The liver and gall bladder were target organs for new Cu partitioning since they were the only organs that concentrated new Cu from the plasma. All the other organs and tissues either remained in equilibrium with the plasma or had lower new Cu concentrations than the plasma. In previous studies, trout fed contaminated food also accumulated a high proportion (11–37 %) of the body burden in the liver (Julshamn et al., 1988; Handy 1992), and total hepatic Cu concentrations increased with high dietary Cu concentrations (Knox et al., 1982, 1984; Lanno et al., 1985a,b, 1987; Julshamn et al., 1988; Gatlin et al., 1989; Handy, 1993; Miller et al., 1993). In the present study, trout accumulated 42 % of the total internalized new Cu in the liver, even though less than 1 % of the hepatic background Cu was exchangeable. In general, changes in hepatic new Cu concentrations reflected changes in overall internalization of the new Cu dose. These data suggest that partitioning of Cu in fish may be similar to that in mammals. In mammals, Cu absorbed by the gut is released into the plasma, transported as a complex with albumin and absorbed by the liver. In the liver, Cu is incorporated into ceruloplasmin, then released to the plasma again to be transported to other internal organs (Owen, 1965; Harris, 1991).

Biliary Cu concentrations are measured less frequently in fish than hepatic Cu concentrations. In our study, accumulation of new Cu in the bile (0.08–0.32 $\mu g\,ml^{-1}$) was less than in rainbow trout exposed to 20 $\mu g\,l^{-1}$ waterborne radioactive Cu (3–5 $\mu g\,ml^{-1}$) (Grosell et al., 1997), which is not surprising given the higher uptake and toxicity of waterborne Cu. It is difficult to compare the data, however, because new Cu uptake was calculated differently in the two studies. Grosell et al. (1997) used liver specific activity rather than the specific activity of the waterborne Cu to calculate biliary uptake. We used the specific activity of the new Cu infused into the stomach to calculate biliary uptake, rather than liver specific activity. Biliary excretion is an important part of Cu homeostasis in fish since Cu-rich granules form around the hepatic canaliculi of trout exposed to high Cu concentrations in the diet (Lanno et al., 1987). Biliary Cu concentrations increase with high Cu concentrations in the sediment (Andreasson and Dave, 1995), with elevated dietary Cu concentrations (Knox et al., 1984) and after acclimation to waterborne Cu (Grosell et al., 1998a,b).

In contrast to the liver and gall bladder, renal new Cu concentrations were not significantly higher than plasma concentrations throughout our 72 h experiment, suggesting that the kidney was not important in the distribution of new Cu. Other feeding studies show similar trends: although some dietary Cu accumulates in the kidney, it accounts for only a small proportion of the body burden (Lanno et al., 1985a,b; Handy, 1992, 1993). In rainbow trout exposed to waterborne Cu, urinary Cu excretion is also of minor importance compared with biliary excretion (Grosell et al., 1998b). In the present study, the kidney was the only organ with a significant change in background Cu over time; new Cu content in the kidney did not change significantly over the same period. These data imply that the specific activity of new Cu in the plasma decreased. If this were the case, however, we would have observed increases in the background Cu content of other internal organs. So, if the specific activity of the plasma remained the same, perhaps the kidney excreted new Cu more rapidly than background Cu. This mechanism is possible since the kidneys of rainbow trout exposed to waterborne Cu (20 $\mu g\,l^{-1}$) excreted newly accumulated radioactive Cu more readily than Cu present before the waterborne exposure (Grosell et al., 1998b).

The carcass (which included the head) contained the lowest new Cu concentration of all the internal organs and tissues. Despite this, because of its large mass, it accounted for 18–30 % of the internal new Cu burden. The Cu concentrations

in muscle tissues of rainbow trout fed 10 000 p.p.m. Cu were also the lowest of all the internal organs (Handy, 1993) and changed little with increases in dietary Cu in Atlantic salmon (Lorentzen et al., 1998). Similarly, the concentration factor for the whole body of rainbow trout fed 3.5–1000 μg Cu g^{-1} dry mass was much lower than for liver (Julshamn et al., 1988).

Elevation of branchial Cu content has been observed in rainbow trout fed Cu-contaminated food (Handy, 1992; Miller et al., 1993), but our study shows that, over 3 days, a single low dose of dietary Cu was not concentrated in the gills. The erythrocytes also did not concentrate new Cu in comparison with the plasma, but after 72 h may have started to take up increased amounts of new Cu. Copper concentrations did not change in the erythrocytes of channel catfish fed Cu-contaminated food even though hepatic Cu concentrations increased (Murai et al., 1981).

Mesenteric fat (removed from around the viscera), the swim bladder, the spleen and the heart contained new Cu concentrations that were in equilibrium with the new Cu content of the plasma. To our knowledge, no other study has measured Cu uptake by the heart, gonads, fat, swim bladder or spleen after dietary Cu exposure.

Excretion of new Cu and the effects of the presence of food

Our method of dosing the fish with new Cu in a liquid form may have decreased both gut motility and subsequent excretion of new Cu, because gut motility and excretion rates are stimulated by the physical presence of food (Fange and Grove, 1979). We tested this idea in our feeding experiment and showed that the presence of food in the digestive tract within 6 h of receiving a dose of new Cu did indeed decrease the amount of new Cu found in the gut tissue and lumen. The fed fish excreted 68 % of their initial dose after 48 h of exposure, compared with 24 % excreted by the unfed fish. Gut clearance rates (i.e. 100 % excretion) for trout fed 'normal food' are approximately 40 h at 15 °C (Fange and Grove, 1979), so the excretion rates of the fed fish in our experiment were much closer to normal than those of the unfed fish. As well as stimulating gut motility, the physical presence of food may have increased sloughing of the gut tissue as it moved down the digestive tract, thereby increasing the excretion of new Cu. Another explanation for increased new Cu excretion is that the fed fish ingested more Cu (contained in the pellets) than the non-feeding fish; extra non-radioactive Cu in the intestine could displace radioactive Cu in the gut tissues and increase excretion of radioactive Cu. If the gut tissues of the fed fish absorbed just 50 % of the Cu present in the pellets, the extra Cu could be sufficient to produce the results we observed. However, the results of the high dose experiment suggest that the gut tissues can rapidly absorb up to 40 μg of new Cu. So, at the doses we used in the feeding experiment (30 μg of radioactive Cu plus approximately 13 μg of non-radioactive Cu in pellets), non-radioactive Cu would be unlikely to displace all the radioactive Cu in the gut tissues.

Yet another factor that might have decreased the uptake of new Cu was the presence of ligands in the food (e.g. fibre) binding new Cu and making it unavailable for absorption into the gut cells (Cousins, 1985; Watanabe et al., 1997). However, decreased amounts of new Cu were found in the gut lumen, which contained food throughout its length.

It is interesting that the gall bladders of the fed and unfed fish had similar new Cu concentrations despite the fact that the gall bladders of the fed fish were virtually empty. Rainbow trout excrete Cu in the bile (Andreasson and Dave, 1995), so biliary excretion by the fed fish could have contributed to increased new Cu excretion and possibly decreased new Cu uptake into the gut tissue.

Since food was not added to the gut until 6 h after the infusion of radioactive Cu, the fate of newly accumulated Cu in our experiment may still be somewhat different from that of Cu in solid food. Nonetheless, the effect of the presence of food on the uptake of Cu should be considered in any application of our model since it may strongly influence the ability of the gut to excrete excess Cu.

In summary, our radioisotopic method has enabled us to begin modelling the dynamics of gastrointestinal Cu uptake in rainbow trout at normal dietary concentrations. Transfer from the gut tissues to the internal organs is the rate-limiting step in dietary Cu uptake, and factors that influence this process will alter Cu internalization most effectively. Our dosing technique could easily be modified to examine the effects of providing Cu in the diet in the presence of different ligands (e.g. amino acids, phytate) and in the presence of different dietary components (e.g. other metals) that may increase or decrease Cu uptake (Cousins, 1985).

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