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MECHANISMS OF GASTROINTESTINAL COPPER ABSORPTION IN THE AFRICAN WALKING CATFISH: COPPER DOSE-EFFECTS AND A NOVEL ANION-DEPENDENT PATHWAY IN THE INTESTINE

R. D. HANDY[1,*], M. M. MUSONDA[2], C. PHILLIPS[1] AND S. J. FALLA[3]

[1]*Department of Biological Sciences, The University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK,* [2]*School of Veterinary Medicine, University of Zambia, PO Box 3237G, Lusaka, Zambia* and [3]*Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

*e-mail: R.Handy@plymouth.ac.uk

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Summary

In mammals, copper (Cu) absorption occurs mostly in the small intestine, and some of the Cu transporters involved in its uptake have been characterised. In fish, however, the regions of the gut involved in Cu absorption and the membrane transport mechanisms responsible for gastrointestinal Cu uptake are unknown. Everted gut sacs and isolated perfused intestine of *Clarias gariepinus* were used to explore Cu absorption (at 22 °C). Gut sacs exposed to 100 μmol l^{-1} mucosal solution Cu ($[Cu]_m$) showed that Cu was mostly (70 %) absorbed in the middle and hind intestine. Most of the accumulated Cu was located in the mucosa. In perfused intestines, cumulative Cu absorption from the mucosal solution to the serosal perfusate was greatest at 10 μmol l^{-1} $[Cu]_m$ and decreased at higher values of $[Cu]_m$, while tissue accumulation of Cu showed a dose-dependent elevation. Absorption efficiency therefore declined with increasing Cu dose, and basolateral transport was the limiting factor in Cu uptake. Serosal applications of the P-type ATPase inhibitor vanadate (100 μmol l^{-1}) or the anion transport inhibitor DIDS (100 μmol l^{-1}) caused threefold increases in net Cu uptake (at $[Cu]_m$=10 μmol l^{-1}). The vanadate effect was explained by a reduction in transepithelial potential rather than inhibition of Cu-ATPase, but the DIDS effect was not. Transepithelial potential, water transport and tissue [Cu] were not affected by DIDS, but tissue $[K^+]$ was elevated. Removal of Cl^- simultaneously from both the mucosal and serosal solutions caused a 10-fold reduction in the rate of Cu uptake, while removal of Cl^- from the mucosal solution only completely abolished Cu absorption to the serosal perfusate. Transepithelial potential effects are discussed. We conclude that Cu absorption occurs mostly in the intestine and is normally driven by a basolateral Cu/anion symport that prefers Cl^-.

Key words: copper transport, intestine, copper absorption, Cl^- transport, anion symport, catfish, *Clarias gariepinus.*

Introduction

Freshwater teleosts have a dietary requirement for Cu of approximately 1–4 mg Cu kg^{-1} dry mass of food (Ogino and Yang, 1980; Murai et al., 1981; Knox et al., 1982, 1984; Lanno et al.,1985), but excess oral Cu is toxic (for a review, see Handy, 1996) and contributes to long-term metal contamination in wild fish populations (Dallinger and Kautzky, 1985; Farag et al., 1995). Despite these observations, very little is known about the mechanism of Cu absorption across the gut of fishes (Handy, 1996).

In mammals, Cu absorption occurs almost exclusively in the small intestine and involves several steps: (1) initial binding of Cu to the mucosa, (2) movement of Cu across the brush-border membrane into the epithelial cells, (3) intracellular trafficking of Cu to the basolateral membrane, and (4) movement of Cu from the cell into the blood. Step 1 involves electrostatic binding of metals to surface ligands on the mucosa (e.g. cadmium; Bevan and Foulkes, 1989). Step 2 may occur *via* non-energy-dependent diffusion (Linder and Hazegh-Azam, 1996), perhaps partly through Na^+ channels (Wapnir, 1991). In step 3, approximately 80 % of newly absorbed Cu binds to cytosolic metallothionein (Harrison and Dameron, 1999), to keep intracellular free [Cu] to micromolar levels or below. Step 4 must involve the active transport of Cu against the electrochemical gradient (serosa-positive, serum [Cu] approximately 1 mg l^{-1}; Linder and Hazegh-Azam, 1996) into the blood. In the intestine, this is the rate-limiting step in the transepithelial movement of heavy metals (Foulkes and McMullen, 1987; Harrison and Dameron, 1999). At normal ingested Cu loads, basolateral transport is probably mediated *via* a P-type Cu-ATPase (also called the Menkes disease protein; Harrison and Dameron, 1999), but additional diffusion or carrier-mediated systems are postulated where competition

for absorption between Cu and other transition metal ions occurs (Linder, 1991; Wapnir and Lee, 1993; Linder and Hazegh-Azam, 1996).

In fish, it is unclear which regions of the gut are important in Cu absorption, and evidence for the absorption mechanism(s) is largely circumstantial. Fish mucus binds Cu (Miller and Mackay, 1982) and so will presumably precipitate the metal on the intestinal mucosa. Metallothionein is induced in the intestine rather than stomach or oesophagus during exposure to 500 mg Cu kg^{-1} food (Handy et al., 1999), implicating the lower region of the gut in absorption. Neither the brush-border nor the basolateral transport mechanism has been characterised, although the conserved metal-binding sequence homology of the Cu-ATPase between bacteria and humans (Harris and Gitlin, 1996) suggests that a similar basolateral transporter will be present in fish intestine. The apparent competition between Cu, zinc and iron in fish nutrition (Knox et al., 1982; Baker et al., 1998) also requires that additional carrier-mediated transport is postulated for fish gut, as in the mammalian model.

The aim of this study was to elucidate the Cu transport mechanism in the gut of fish by (i) identifying the regions of the gut involved in Cu absorption using everted gut sacs, and (ii) exploring the pharmacokinetics and mechanisms of Cu absorption using an everted perfused intestine. This *in vitro* approach allows manipulation of both mucosal and serosal solutions in elucidating the transport mechanisms and avoids the rapid dynamic changes in Cu distribution that complicate *in vivo* experiments (Carbonell and Tarazona, 1994; Grosell et al., 1997). In this study, we use the African catfish *Clarias gariepinus* for several reasons: (i) the gross organisation of *Clarias gariepinus* gut is broadly similar to that of the rat, enabling some comparison between fish and mammalian models, and the application of similar perfusion techniques; (ii) the metal requirements of catfish in aquaculture are similar to those of other teleosts (Baker et al., 1997); and (iii) the species is widely distributed throughout Africa and Asia, in regions where chronic Cu pollution causes fish pathologies (e.g. Zambezi; Mwase, 1994).

Materials and methods

Stock animals

African walking catfish *Clarias gariepinus* (Burchell, 1822), weighing 404±19 g (mean ± S.E.M., N=86), were held in recirculating dechlorinated Plymouth tapwater of the following ionic composition (mmol l^{-1}): Na^{+}, 1.56±0.31; K^{+}, 0.30±0.01; Ca^{2+}, 0.13±0.001; Mg^{2+}, 1.78±0.24; total Cu <0.0004; Cl^{-}, 1.7±0.36; total ammonia, 0.012±0.009; nitrate, 0.41±0.009; nitrite, 0.0008±0.0001; pH 7.31±0.04; temperature 23±0.51 °C (means ± S.E.M., N=7). Fish were bred at the University of Plymouth (brood stock from Sparsholt College, Hampshire, UK) and were starved for 48 h prior to experimental procedures to empty the gut of food and to facilitate eversion of the gut (see below). Catfish are cannibalistic when unfed, and individual fish were isolated to a smaller tank (approximately 60 l, supplied by the recirculating water above) during the non-feeding period to protect the stock fish.

Preparation of gut sacs

Fish were killed by a blow to the head, the brain was pithed, and the mass and length of the fish were recorded. The entire gastrointestinal tract was removed from the carcass, and everted gut sacs of oesophagus, stomach and anterior, mid and hind gut were prepared from the whole gut (modified from Wilson and Wiseman, 1954). Modifications included suturing a short length (approximately 7 cm) of Portex tubing (1 mm internal diameter) to the open end of the sac to facilitate 'leak testing' and collection of the sac contents at the end of experiments. The gut saline used was modified from van der Velden et al. (1990) and contained (in mmol l^{-1}): NaCl, 117.5; KCl, 5.7; $NaHCO_3$, 25.0; $NaH_2PO_4 \cdot 2H_2O$, 1.2; $CaCl_2 \cdot 2H_2O$, 2.5; $MgSO_4$, 1.0; glucose, 5.0; mannitol, 23.0; adjusted to pH 7.4 with a few drops of 1 mol l^{-1} HCl, at 21.5±1.1 °C (mean ± S.D., N=144), gassed with 95 % O_2:5 % CO_2.

After each experiment (see Results), the gut sacs were opened, washed in deionized water to remove excess Cu, then divided into two equal parts by a longitudinal incision. One part was stripped of serosa and underlying connective tissue, while the other part was left intact. From each of these parts, two pieces were taken, one for histological examination and the other for ion analysis (Cu^{2+}, K^{+}). Histological wax sections (7 µm thick) were prepared and stained with Mallory's Trichrome as described previously (Campbell et al., 1999).

Preparation of the isolated perfused intestine

Fish were killed (as above), the entire intestinal tract (the pyloric sphincter to the rectum) was quickly removed, and the resting intestine length measured. Only preparations showing normal contractions after resting for 5–10 min in oxygenated saline and healthy gross morphology were used. Perfusions were based on the everted gut method of Ando et al. (1986) and involved mounting the everted intestine in a gently stirred gut bath containing the physiological saline described above (0.5 l) and serosally perfusing the gut with the same saline (i.e. symmetrical perfusion). The perfusate flow rate (nominally 1 ml min^{-1} *via* an Ismatec peristaltic pump) was similar to that reported for intestinal blood flow *in vivo* for fish (13.5 ml g^{-1} h^{-1} at 18 °C; Barron et al., 1987) after considering the mass range of guts used here (1.09–3.28 g wet mass) and was confirmed at the start of experiments (mean perfusate flow rate 1.02±0.02 ml g^{-1} h^{-1}, N=32; mean ± S.D.). Intestines rested on a polythethylene mesh platform (4 mm pore size) within the bath, which allowed uniform flow of the medium over the mucosal surface. The musculature of the intestine provided sufficient support to allow serosal perfusates to flow freely, so the polyester intestinal inserts described by Ando et al. (1986) were not needed. Eluted perfusate was manually collected in 10 min fractions, and the eluted volume was determined gravimetrically. The gut bath and solution reservoirs were gently gassed (95 % O_2:5 % CO_2) at room temperature (22 °C)

throughout each experiment. The entire preparation of guts routinely took less than 30 min.

The main viability criteria for perfused intestine were as follows. (i) Serosal perfusate flow. Data from preparations showing erratic, or a sudden fall in, perfusate flow were discarded. This occurred infrequently (e.g. two out of 48 perfusions). (ii) Normal peristaltic contractions. Data from guts showing marked contracture were discarded. (iii) Tissue K^+ leakage. Damaged cells will leak K^+ down the electrochemical gradient, causing a decline in tissue [K^+] as extracellular [K^+] rises. Preliminary experiments with unstripped gut segments showed that perfusate [K^+] was a function of anatomical position along the length of the gut (reflecting K^+ absorption; Loretz, 1995), but tissue [K^+] was not position-dependent and therefore provided a better overall marker of K^+ loss. Intestines that met the first two criteria did not leak K^+ (compared with unperfused tissue; results not shown). However, in some experiments, changes in tissue [K^+] were expected (e.g. with drug treatment; see Results). In these experiments, data were discarded only when low tissue [K^+] occurred with erratic changes in perfusate flow.

Perfused intestine experiments

Experiments were performed to explore the effects of changing the total Cu concentration in the mucosal solution ($[Cu]_m$) on Cu uptake, the effects of serosally applied ion-transport inhibitors and the effects of manipulating the transepithelial Cl^- gradient (see Results). In all perfused intestine experiments, Cu was added as $CuSO_4{\cdot}5H_2O$, and trials were performed daily as matched pairs (control and treatment perfusion, fish of similar body size) in identical perfusion baths. The pH and temperature of the mucosal solution were monitored at 30 min intervals using a combination pH electrode with a temperature probe. Tissues were collected at the end of each experiment for ion analysis (see below). In some additional experiments, tissue homogenates from stock fish were used to confirm drug activity (positive controls). Intestines were dissected from five starved fish (animal mass 322±73 g; mean ± S.D.) and individually homogenised in 4 ml of Tris buffer (20 mmol l^{-1} Tris-HCl, pH 7.0, plus 1 mmol l^{-1} EDTA) on ice at 10 700 revs min^{-1} (CAT-X520D homogeniser, rotor TD-17M, Bennett & Co., Weston Super Mare, UK) for six 6 s bursts with 15 s rests in ice between each homogenisation step. The resulting crude homogenate was centrifuged at 8800 ***g*** for 20 min at 4 °C (Jouan KR22i) to remove debris, and the hypotonic supernant was analysed for Na^+/K^+-ATPase activity, all according to a modification (saponin-free) of the method of Jones and Besch (1984). The protein content of the supernatant was determined by the method of Hartree (1972).

Transepithelial potential

Intestinal segments of freshly killed catfish (see above) were mounted in an aerated, perfused (1 ml min^{-1}) chamber with the mucosal surface uppermost (Simonneaux et al., 1987). Transepithelial potentials were measured using graphite reference electrodes (containing a Ag/AgCl reference encased in a KCl poymer gel; AD Instruments, Hastings, UK) connected to a two-channel amplifier (serosa-positive) with a graphical display (2e series amplifier and MacLab v3.5, AD Instruments) and corrected for voltage difference between the electrodes in the mucosal solution while recording transepithelial potential. Junction potentials were negligible. Transepithelial potentials were monitored until steady recordings occurred, usually within 20 min between each solution switch.

Trace metal analysis

Trace metal analysis followed the method of Handy et al. (1999) with minor modifications. All apparatus was acid-washed for more than 2 h in 13 % Aristar nitric acid, then triple-rinsed in deionised water. Routine water samples from stock fish tanks were analysed by flame atomic absorption spectrophotometry (F-AAS, Varian SpectraAA 600) for Na, K, Ca and Mg; detection limits were less than 0.1 mmol l^{-1} for these elements. Tissue and perfusate samples were assayed for Cu, K^+ and/or Na^+, mostly using inductively coupled plasma emission spectrophotometry (ICP-AES, Perkin-Elmer Liberty 200 AES with Varian software), but occasionally by F-AAS. All standards were matrix-matched to samples, including acidification to match either perfusates or tissue digests (see below). Detection limits for ICP-AES were (in µmol l^{-1}): Cu, 0.96; K^+, 35.5; Na^+, 14.8.

Tissues from intestinal perfusion experiments were prepared for metal analysis as follows. Segments of gut of approximately 0.5 g fresh mass were oven-dried to a constant mass, placed in 20 ml polythethylene screw-top digestion vials and digested in 5 ml of concentrated Aristar nitric acid at 70 °C for 2 h. Digests were cooled and diluted to 20 ml with deionised water. Tissue samples spiked with Cu gave a Cu recovery of 102±3.9 % (mean ± S.E.M., N=5). Tissues from the gut sac experiments were digested using a similar protocol (Handy et al., 1999). In terms of tissue metal concentrations, the ICP-AES detection limits are equivalent to 0.096 µmol g^{-1} dry mass for Cu, 3.55 µmol g^{-1} dry mass for K^+ and 1.48 µmol g^{-1} dry mass for Na^+. Tissue [Cl^-] was determined by automated titration (Corning CCM1 chloride meter).

Calculations

In this paper, nominal total Cu concentrations ([Cu]) in perfusates are described except where measured total Cu values are indicated. Within each experiment, the absolute cumulative appearance of Cu in the perfusate and cumulative perfusate flow were plotted against exposure time (for calculations, see Campbell et al., 1999). The initial (first 10 min) and overall (240 min) net Cu flux rates ($J_{net,Cu}$, in µmol g^{-1} dry tissue h^{-1}) to the serosal perfusate were calculated from the perfusate Cu content (in nmol) divided by the tissue dry mass and corrected to 1 h (similar to Campbell et al., 1999). Fluxes of Cl^- and K^+ were calculated in the same way, after correcting for the initial concentration of the ion in the perfusate. Initial and overall eluted perfusate rates (ml g^{-1} h^{-1}) were similarly calculated (Ando et al., 1986).

Statistical analyses

All statistical analyses were carried out using Statgraphics 3.0 plus (at 95 % confidence limits). After checking for kurtosis, skewedness and unequal variances (Bartlett's test), data were tested by one-way analysis of variance (ANOVA) followed by Fisher's 95 % least-squares difference (LSD) multiple-range test. When ANOVA could not be applied, the non-parametric Kruskall–Wallis test was used to locate differences by individual Mann–Whitney *W*-tests or notched box and whisper plots.

In the perfused intestine experiments, temporal changes in perfusate [Cu] within treatments, treatment differences in cumulative Cu content or perfusate flow and all net flux rates were assessed using ANOVA. The possibility that the appearance of Cu in the perfusate was a passive function of perfusate outflow rate was tested by comparing the regression lines of the cumulative [Cu] perfusate with those of the cumulative perfusate outflow (Campbell et al., 1999). Treatment differences in tissue Cu or K^+ concentration were tested by ANOVA. Multivariate ANOVA was applied to explore changes in metal concentrations within different regions of the intestine (e.g. anterior, mid and hind intestine) and between treatments. The two-tailed *t*-test was used to examine some tissue differences in perfusion experiments, and a paired two-tailed *t*-test was used to analyse the transepithelial potential data.

Results

Where along the gut is Cu absorbed?

The experiments using gut sacs aimed to determine which part of the gut was responsible for Cu absorption and to indicate how much of the absorbed Cu was associated with the intestinal mucosa rather than the underlying tissue. Gut sacs were incubated in either 350 ml of control saline (no added Cu, 22±1 °C, bubbled with 95 % O_2:5 %CO_2) or exposed to an equivalent saline containing a nominal 100 μmol l^{-1} Cu (added as $CuCl_2$) for 4 h in paired experiments (one fish gut per treatment for each trial). Samples (2 ml) of the mucosal saline collected at 30 min intervals during the experiment showed that $[Cu]_m$ of controls remained below the limit of detection (<0.96 μmol l^{-1}) and that there was no statistically significant change (Kruskal–Wallis, P=0.98) in mucosal solution [K^+] ($[K^+]_m$) over 4 h (0.35±0.03 mmol l^{-1}; grand mean ± S.E.M., N=9 time points). In the Cu-treated group, mucus secretion increased, and mucus sloughed off the gut mucosa formed a blue precipitate (confirmed by protein determination), which collected at the bottom of the beaker (not on the tissue). This caused $[Cu]_m$ to decrease from the nominal 100 μmol l^{-1} to 24±6 μmol l^{-1} (mean ± S.E.M., N=8 guts) within 30 min; however, $[Cu]_m$ remained steady thereafter (at 4 h, 22±5 μmol l^{-1}, mean ± S.E.M., N=8). $[K^+]_m$ remained constant over 4 h (Kruskal–Wallis, P=0.99) at approximately 0.43±0.03 mmol l^{-1} (grand mean ± S.E.M., N=9 time points).

The tissue [Cu] of control sacs remained below the limit of detection (<0.09 μmol g^{-1} dry mass), except in the intestine. Gut segments exposed to Cu accumulated more Cu in the intestinal regions compared with the oesophagus and stomach (Table 1). The [Cu] in the gut segments decreased after stripping off the mucosa (Table 1), suggesting that most of the accumulated Cu was located in the mucosal cells. The percentage of the total tissue Cu in the mucosa for exposed guts may be estimated from the median values in Table 1; oesophagus, 39 %; stomach, 60 %; anterior intestine 30 %; mid intestine 83 %; hind intestine 70 %. Histology confirmed proper stripping of the mucosa and the integrity of intact gut segments (not shown). The mean water content (percentage moisture, N=9) of the intact gut segments of the control fish was: oesophagus, 83 %; stomach, 81 %; anterior intestine, 83 %; mid intestine, 86 %; hind intestine 88 %. Standard errors on these data were <1 %, and the mean percentage moisture content for exposed guts was identical to that of the above controls. The [Cu] profile of gut sacs is therefore not an artefact of tissue water content. The normal water content, steady tissue

Table 1. *The concentrations of Cu and K^+ in intact and stripped gut segments of catfish*

	Tissue [Cu] (μmol^{-1} g dry mass)					Tissue [K^+] (μmol^{-1} g dry mass)				
Treatment	Oesophagus	Stomach	Anterior intestine	Mid intestine	Hind intestine	Oesophagus	Stomach	Anterior intestine	Mid intestine	Hind intestine
Intact										
Control	<0.09	<0.09	0.1 (0.05)	0.1 (0.1)	0.24 (0.4)	22 (181)	38 (255)	33 (315)	29 (377)	40 (261)
Exposed	0.3 (0.4)*	0.2 (0.1)*	0.4 (0.4)*	1.1 (1.7)*	0.8 (2.7)*	44 (198)	71 (254)	71 (297)	64 (342)	77 (202)
Stripped										
Control	<0.09	<0.09	<0.09	<0.09	<0.09	38 (4.1)	64 (32)	39 (35)	49 (33)	63 (23)
Exposed	0.1 (0.1)*	0.09 (0.1)*	0.3 (0.4)*	0.2 (0.8)*	0.2 (1.3)*	39 (10)	62 (38)	39 (27)	55 (22)	33 (67)

Everted gut sacs prepared from the entire gastrointestinal tract of individual animals were incubated in saline at 22 °C for 4 h.

Control, no Cu added to the mucosal solution; Exposed, nominal 100 μmol l^{-1} Cu added to the mucosal solution ($[Cu]_m$).

Data are medians with inter-quartile ranges in parenthesis for N=6–9 experiments.

Stripped guts had the mucosal epithelia removed by dissection at the end of the experiment according to Ando and Kobayashi (1978). There are no significant differences in tissue [K^+] within each region of the gut (Kruskal–Wallis, P>0.05).

*Significantly different tissue [Cu] from that of the appropriate control for each region of the gut (Kruskal–Wallis, P<0.05).

[K^+] (Table 1) and normal histology (not shown) indicated good viability. Cu-treated intact gut segments tended to have a higher [K^+] than controls, but this was not statistically significant.

Attempts were made to collect the serosal perfusate, and volumes of 70–200 µl were obtained. Ionic analysis showed Cu absorption increasing distally into the serosal fluid; for example <0.96 µmol l^{-1} (anterior intestine fluid) and 146±87 µmol l^{-1} (hind intestine fluid) (mean ± S.E.M., N=8) in exposed sacs. The [K^+] in the sacs was typically approximately 0.5 mmol l^{-1} for both treatments, with slightly higher values of [K^+] in the anterior intestinal fluid. Transepithelial net flux rates were not calculated because of uncertainty about whether all the serosal volume had been collected.

Viability of perfused intestines

The viability of perfused intestines was generally good, as measured by tissue [K^+] and perfusate flow, although successful perfusions were more difficult to maintain at high $[Cu]_m$ (see below). Mucosal solution pH also indicated that base secretion by the intestine was normal, and independent of [Cu] (t-test, P>0.05), with an average increase in pH of 0.49±0.05 (mean ± S.E.M., N=16 experiments) from the initial pH 7.4 over 4 h.

Effect of $[Cu]_m$ on Cu uptake into the serosal perfusate

These experiments examined the effects of increasing $[Cu]_m$ on transepithelial Cu uptake. Intestines were perfused for 4 h in control conditions (no added Cu) or in the presence of 10, 50 or 100 µmol l^{-1} total Cu. The $[Cu]_m$ was monitored to confirm exposure. As in the gut sac experiments, $[Cu]_m$ in the control remained low (<0.96 µmol l^{-1}). $[Cu]_m$ values were derived from an average of nine samples collected over each experiment and were (grand mean ± S.E.M., N=5 experiments) 6.3±0.7 µmol l^{-1} at a nominal $[Cu]_m$ of 10 µmol l^{-1}, 12.6±1.7 µmol l^{-1} at a nominal $[Cu]_m$ of 50 µmol l^{-1} and 23.4±4.7 µmol l^{-1} at a nominal $[Cu]_m$ of 100 µmol l^{-1}. The loss of $[Cu]_m$ at the higher concentrations was partly explained by precipitation caused by stimulated mucus secretion.

The appearance of Cu in the serosal perfusate followed both time- and treatment-dependent trends (example experiment, Fig. 1A). The cumulative Cu content of the perfusate followed a linear trend over time, except in the controls where, in three out of five preparations, the Cu content approached steady state

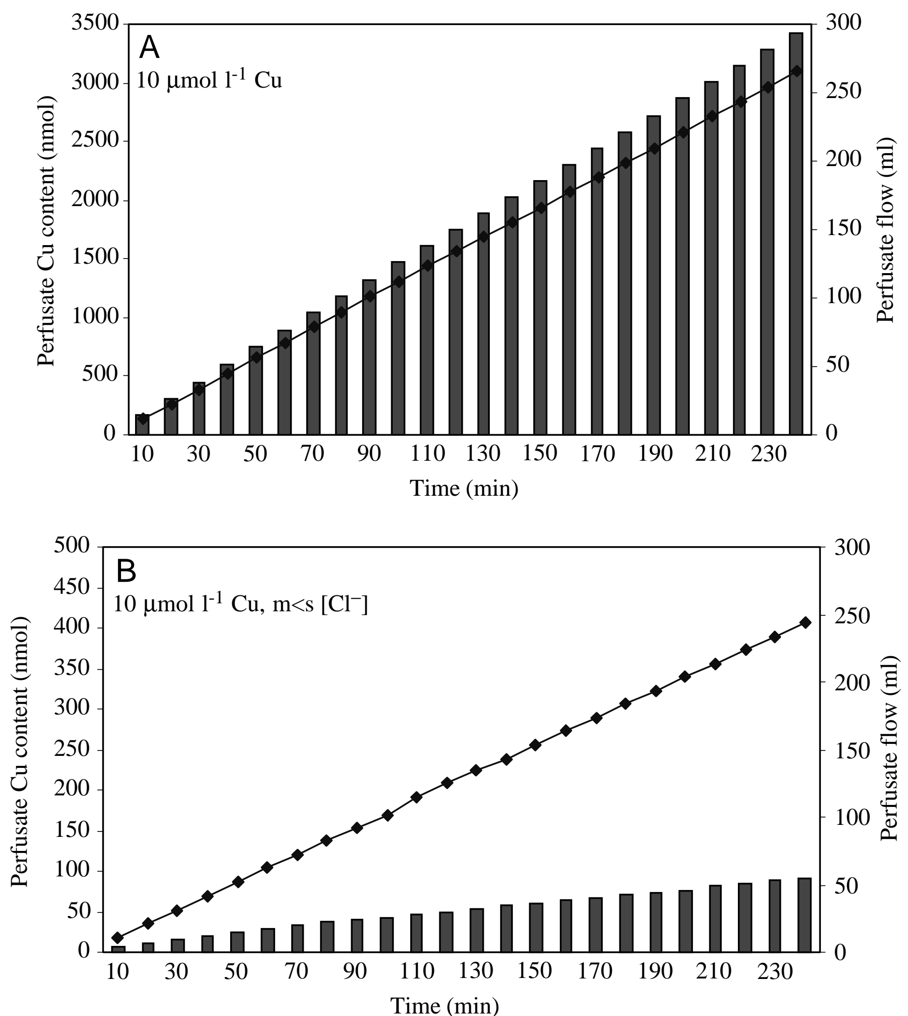

Fig. 1. The appearance of Cu in 10 min fractions of the serosal perfusate during exposure of everted perfused whole intestine to 10 µmol l^{-1} Cu in the mucosal solution ($[Cu]_m$). Data are plotted as the cumulative Cu content of the serosal perfusate (nmol, filled columns) and cumulative perfusate flow (ml, diamonds) over time (min). Graphs are representative examples of individual perfusions (from 5–6 perfusions) to illustrate the sensitivity and temporal resolution of the technique within a trial. (A) Salines contained normal [Cl^-]; (B) Cl^- was removed from the mucosal (m) solution and replaced by gluconate (an impermeable anion). The serosal (s) solution [Cl^-] is 140 mmol l^{-1}. Fish masses are (A) 291 g and (B) 301 g. Note the differences in the left-hand y-axis scale in A and B.

at the end of the experiment. In the controls, the increase in serosal solution Cu concentration reflects excretion of endogenous Cu from the tissues, rather than net movement from the mucosal solution. The time courses for perfusate Cu content changes compared with cumulative perfusate flow were different, suggesting that Cu uptake is not occurring by passive solvent drag. Curve fits for both the Cu and perfusate flow data were good (r^2 values typically 0.99), and the slopes of the curve fits for Cu were between three and 10 times greater (P<0.05) than those for perfusate flow. The cumulative Cu content of the serosal perfusate from intestines exposed to 10 μmol l^{-1} Cu was greater than that of controls at the end of the experiment (Mann–Whitney W-test, P<0.05), while the slope for the 50 μmol l^{-1} treatment tended to be similar and that for the 100 μmol l^{-1} treatment lower than that of the controls. The latter perfusions were difficult to maintain for the entire 4 h. In the 50 μmol l^{-1} Cu group, one out of five perfusions was stopped after 3 h because of variability in perfusate flow. However, this variability in perfusate flow was small (<10 % of total flow), occurred only transiently, between 70 and 90 min into the perfusion, and then recovered. It was not observed in other successful preparations. In the 100 μmol l^{-1} group, three out of six perfusions were stopped in the last 20 min of the planned 4 h perfusion to avoid steady deterioration, and another (discarded) perfusion was stopped within the first hour of the experiment. These effects were not caused by muscle contracture, since all intestines appeared normal, but changes in water permeability may have occurred (Table 2).

Cu uptake rates were calculated from the time-dependent changes in perfusate Cu content, and changes in water transport were calculated from the time-dependent changes in perfusate flow (Table 2). The isolated intestine showed a net uptake of Cu from the mucosal medium in all treatments, broadly following the dose-dependent trends described above. The similarity between initial Cu uptake rates and overall rates indicates that saturation of the serosal solution with Cu did not occur over 4 h. Changes in net water flux across the intestine were not the same as those for Cu transport. Controls initially showed a slight net water uptake, which gradually becomes a net efflux over the entire experiment. The addition of 10 μmol l^{-1} Cu tended to prevent this steady net efflux, so that net uptake of water to the serosal solution occurred. Higher $[Cu]_m$, however, has a tendency to cause large and variable net losses of water from the serosal perfusate (Table 2).

If the data in Table 2 are expressed per kilogram of fish, the intestinal contribution to whole-body Cu uptake is approximately 0.4–2.2 μmol kg^{-1} h^{-1}, and overall water fluxes are –48 to 12 ml kg^{-1} h^{-1}, depending on Cu treatment.

Table 2. *Net flux rates for Cu and water across the perfused catfish intestine*

Mucosal [Cu] (μmol l^{-1})	Net Cu flux, $J_{net,Cu}$ (μmol g^{-1} h^{-1})		Net water flux, J_{net,H_2O} (ml g^{-1} h^{-1})	
	Initial rate	Overall rate	Initial rate	Overall rate
0 (control)	0.8±0.7	1.4±0.5	7.8±10.9	–30.7±24.7
10	2.0±0.8	2.7±0.4	33.6±7.9	12.4±15.7
50	1.9±1.3	2.2±1.1	–47.3±42.9	–92.9±43.3
100	0.7±0.4	0.8±0.3	–40.4±21.1	–40.4±14.0

Everted intestines were serosally perfused for 4 h at 22 °C in the presence or absence of Cu in the mucosal solution.

Control, no Cu added to the mucosal solution. Exposed treatments ranged from 10 to 100 μmol l^{-1} Cu added to the mucosal solution ($[Cu]_m$).

Values are means ± S.E.M. (N=4–6) expressed per gram dry mass of intestine.

Negative values indicate a net loss from the serosal solution.

Initial and overall rates are calculated from cumulative perfusate data at 10 min and 4 h, respectively.

There were no statistical differences within columns (ANOVA, P>0.05).

Effect of $[Cu]_m$ on Cu accumulation by the perfused intestine

These data are from the tissues of the perfusion experiment described above. The [Cu] in the intestinal tissue reflected the exposure concentration, with the highest levels in the intestines exposed to 100 μmol l^{-1} $[Cu]_m$ (Fig. 2). Values increased from 0.6±0.2 μmol Cu g^{-1} dry mass in the controls to 4.5±1.4 μmol Cu g^{-1} dry mass (means ± S.E.M., N=6) in the 100 μmol l^{-1} $[Cu]_m$ group. The increases in tissue [Cu] were statistically higher than in the controls (P<0.05), except in the 10 μmol l^{-1} $[Cu]_m$ group. A comparison of [Cu] in the anterior, middle and hind regions of intestines from each treatment revealed no significant differences along the length of the intestine, although plots from individual experiments suggested a tendency for Cu to accumulate more in the hind regions during exposure (not shown).

The overall changes in tissue [Cu] occurred while tissue [K$^+$] remained constant between treatments

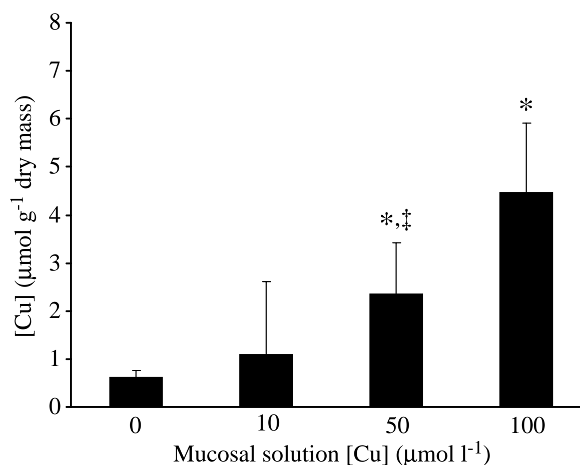

Fig. 2. Copper concentrations in everted perfused whole intestine tissue (μg g^{-1} dry mass) after a 4 h exposure to 0 (control; no added Cu), 10, 50 or 100 μmol l^{-1} Cu in the mucosal solution ($[Cu]_m$). Values are means + S.E.M. (N=5 or 6 perfusions). An asterisk indicates a significant difference (Kruskall–Wallis test, P>0.05) from the control value. A double dagger indicates a significant difference from the preceding $[Cu]_m$. Salines contain normal [Cl$^-$] (140 mmol l^{-1}).

(Kruskall–Wallis, P>0.05). Tissue [K$^+$] was (means ± S.E.M., N=5 or 6) 196.2±23.5 μmol g^{-1} dry mass for controls, and 183.3±6.7 μmol g^{-1} dry mass, 168.8±5.1 μmol g^{-1} dry mass and 172.6±5.8 μmol g^{-1} dry mass for the 10, 50 and 100 μmol l^{-1} [Cu]$_m$ groups, respectively.

Does the P-type ATPase inhibitor sodium vanadate prevent Cu uptake?

These experiments explored the effects the addition of sodium vanadate, a P-type ATPase inhibitor (Cantley et al., 1978), to the serosal solution, which might be expected to inhibit putative basolateral Cu-ATPase and, thus, intestinal Cu absorption. The effects of vanadate were explored at 10 μmol l^{-1} [Cu]$_m$, a concentration that would normally stimulate Cu uptake to the serosal perfusate (Table 2). Two series of experiments were performed. The first series explored the effects of 50 μmol l^{-1} vanadate over 1 h, and the second series examined the effects of 100 μmol l^{-1} vanadate over 4 h. The application of 50 μmol l^{-1} vanadate had no effect on perfusate Cu content, and no differences in either Cu or water fluxes across the intestine were observed. However, when the vanadate dose was increased to 100 μmol l^{-1}, a significant increase (t-test, P<0.05) in cumulative perfusate Cu content compared with the control occurred within 1 h (not shown). This was reflected in a two- to threefold increase in the net Cu uptake compared with controls, without any significant effects on water transport within the first hour (Table 3). These changes in Cu uptake rate occurred without changes in tissue Cu or K$^+$ concentration (Table 4), suggesting movement of Cu through the intestinal tissue rather than retention of Cu in the tissue. Vanadate tended to decrease the level of NaCl in the tissue (Table 4). Positive controls using tissue homogenates confirmed the potency of the vanadate used. Ouabain-inhibitable Na$^+$/K$^+$-ATPase activity was 6.49±2.6 μmol P$_i$ mg^{-1} h^{-1} in the absence and 1.3±0.75 μmol P$_i$ mg^{-1} h^{-1} in the presence of 50 μmol l^{-1} vanadate (80 % inhibition; means ± S.E.M.; N=3).

Table 3. *Effect of vanadate or DIDS on Cu and water fluxes across the perfused catfish intestine*

Drug concentration	Net Cu flux, $J_{net,Cu}$ (μmol g^{-1} h^{-1})		Net water flux, J_{net,H_2O} (ml g^{-1} h^{-1})	
	Initial rate	Overall rate	Initial rate	Overall rate
Vanadate (μmol l^{-1})				
0 (control)§	1.1±0.6	1.0±0.4	−33.8±43.1	−5.3±11.8
50§	1.5±0.4	1.0±0.2	−8.7±14.3	−30.1±27.2
100§	3.9±0.5*,‡	3.1±0.8*,‡	−25.9±15.6	−32.7±17.7
DIDS (mmol l^{-1})				
0 (control)	2.0±0.8	2.7±0.4	33.6±7.9	12.4±15.7
0.1	7.1±1.5*	8.0±1.4*	34.5±17.5	0.4±0.04

Everted intestines were serosally perfused for up to 4 h at 22 °C in the presence of Cu in the mucosal solution.

The mucosal solution [Cu] was 10 μmol l^{-1} ([Cu]$_m$) in all treatments; control, no added drug. Drugs were added to the serosal perfusate only.

Values are means ± S.E.M., expressed per gram dry mass of intestine.

§Initial and overall rates are calculated from cumulative perfusate data at 10 min and 1 h, respectively (N=6–7, except 100 μmol l^{-1} vanadate, when N=3).

Values for the DIDS experiments were calculated at 10 min and 4 h, respectively (N=8).

*Significant difference from appropriate control or ‡ from the 50 μmol l^{-1} vanadate value (ANOVA, P<0.05) within columns.

Negative values indicate a net loss from the serosal solution.

Does the anion transport inhibitor DIDS alter Cu uptake?

These experiments explored the effects of the stilbene

Table 4. *The effects of vanadate or DIDS on Cu, K$^+$, Na$^+$ and Cl$^-$ concentrations in perfused catfish intestine*

Treatment/[drug] (μmol l^{-1})	Tissue [Cu] (μmol g^{-1} dry mass)	Tissue [K$^+$] (μmol g^{-1} dry mass)	Tissue [Na$^+$] (μmol g^{-1} dry mass)	Tissue [Cl$^-$] (μmol g^{-1} dry mass)
Vanadate series 1 (1 h)				
0 (control)	0.3±0.1	187.2±10.9	ND	ND
50	0.3±0.1	178.7±5.9	ND	ND
Vanadate series 2 (4 h)				
0 (control)	1.1±1.5	183.3±6.7	452.2‡, 499.4‡	380.0‡, 386.4‡
100§	0.8±0.2	180.5±12.7	335.9±36.6	251.6±18.7
DIDS				
100	0.8±0.4	458.8±69.1*	527.3±70.2	314.0±48.2

Everted intestines were serosally perfused for up to 4 h at 22 °C in the presence of Cu in the mucosal solution.

The mucosal solution [Cu] was 10 μmol l^{-1} ([Cu]$_m$) in all treatments; control, no added drug. Drugs were added to the serosal perfusate only.

Values are means ± S.E.M. (N=7–10, or N=4§), expressed per gram dry mass of intestine.

There were no significant differences within columns compared with respective controls (ANOVA, P>0.05).

Note the vanadate experiments were conducted for both 1 h and 4 h, respectively.

*See text. ‡The individual values from two experiments. ND, no data.

derivative 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), an anion transport inhibitor, on Cu absorption. Pathways that are DIDS-sensitive and anion-dependent play a part in the absorption of some divalent metal ions (see Discussion), and these experiments explore whether this is also true for Cu. Intestines were perfused serosally with 0.1 mmol l^{-1} DIDS for 4 h in the presence of a $[Cu]_m$ of 10 µmol l^{-1}. The application of DIDS produced linear cumulative increases in perfusate Cu content and flow, like the linear response observed in the 10 µmol l^{-1} Cu experiments that served as controls here (e.g. Fig. 1A). However, the magnitude of the change in Cu content was much greater in the presence of DIDS. This was reflected in a roughly threefold increase in Cu uptake compared with the control, whilst no significant changes in water transport occurred (Table 3). The increase in Cu uptake was also larger than that observed in the vanadate experiments. The changes in Cu flux rate occurred without changes in tissue [Cu], but with a 2.5-fold increase in tissue $[K^+]$ (Table 4). Tissue NaCl appeared to be constant (Table 4).

Does manipulation of mucosal and serosal solution Cl^- concentration alter Cu uptake?

Experiments to explore the Cl^--dependence of Cu uptake involved two types of 4 h perfusion: (i) nominally Cl^--free, in which Cl^- was removed from both mucosal and serosal media; (b) Cl^- present in the serosal solution only. In all the experiments, Cl^- was replaced by gluconate (an impermeable anion). The $[Cu]_m$ was 10 µmol l^{-1}, except in the parallel equivalent control (no added Cu).

Transepithelial Cu absorption was dramatically reduced when Cl^- was removed from the bathing medium (*t*-test, $P<0.01$). Removal of Cl^- from both the mucosal and serosal solutions caused at least a 10-fold reduction in the appearance of Cu in the serosal perfusate during exposure to 10 µmol l^{-1} $[Cu]_m$. Similar but less marked declines were also observed in controls without Cu. In the complete absence of Cl^-, Cu uptake into the serosal compartment was linear and generally only double the rate of eluted perfusate flow. However, this cannot be explained by solvent drag since Cu and water net fluxes are in opposite directions (Table 5). There were no statistical differences (*t*-test, $P>0.05$) in [Cu] of the whole intestine between the experiments with normal Cl^- and those with modified Cl^-, despite the decline in transepithelial Cu uptake in the latter (Fig. 2; Table 6). This suggests that the effect of Cl^- on Cu absorption is not a simple blockade of basolateral Cu uptake, but also a decline in net Cu transfer across the mucosal membrane. Whole-intestine $[K^+]$ was always lower in the normal-$[Cl^-]$ experiment than in those with Cl^- manipulation (*t*-test, $P<0.05$). Thus, removal of Cl^- elevates intestinal tissue $[K^+]$, regardless of Cu exposure.

Transfer of Cu to the serosal perfusate was almost completely abolished when Cl^- was removed from the mucosal solution compartment only (Cl^- gradient mucosal<serosal), while eluted perfusate flow remained normal (e.g. Fig. 1B). This reduction was statistically significant compared with either the normal-$[Cl^-]$ experiments or experiments in which Cl^- was absent from both sides of the preparation (*t*-test, $P<0.05$). These changes were reflected in the calculated flux rates (Table 5).

Intestinal tissue was divided into anterior, middle and hind regions at the end of the experiments and analysed for ionic composition. Whole intestine [Cu] was statistically lower when the Cl^- gradient was mucosal<serosal compared with Cl^--free on both sides (Table 6). Intestinal tissue $[Cl^-]$ followed a

Table 5. *Effect of manipulating external $[Cl^-]$ on net flux rates for Cu, K^+, Cl^- and water in perfused intestines*

		Cl^- removed from both mucosal and serosal solutions		Cl^- removed from mucosal solution (Cl^- gradient m<s)	
Variable	Treatment	Initial rate	Overall rate	Initial rate	Overall rate
$J_{net,Cu}$ (µmol g^{-1} h^{-1})	Control	0.6±0.1	1.2±0.6	0.3±0.1	0.3±0.1
	Exposed	0.5±0.2*	0.9±0.4*	0.1±0.0*,‡	0.2±0.0*,‡
$J_{net,K}$ (mmol g^{-1} h^{-1})	Control	−0.54±0.25	−0.58±0.28	0.05±0.06	0.08±0.04
	Exposed	−0.35±0.19	−0.46±0.23	−0.02±0.03	−0.02±0.04
$J_{net,Cl}$ (mmol g^{-1} h^{-1})	Control	0.7±0.1	1.4±0.5	−28.4±11.0	−0.4±0.1
	Exposed	0.8±0.1	0.8±0.1	−14.5±3.2‡	−0.4±0.1‡
J_{net,H_2O} (ml g^{-1} h^{-1})	Control	−427.0±309.0	−213.0±101.5	−87.7±50.9	−224.0±175.3
	Exposed	−226.0±93.0	−231.0±89.8	−81.3±26.3	−88.9±41.5

Everted intestines were serosally perfused for 4 h at 22 °C in the presence or absence of Cu in the mucosal solution. Control, no Cu added to the mucosal solution. Exposed, 10 µmol l^{-1} Cu added to the mucosal solution.

Values are means ± S.E.M. (N=5 perfusions per treatment).

*Significantly different ($P<0.05$, *t*-test) from appropriate normal $[Cl^-]$ experiments in Table 2.

‡Significantly different ($P<0.05$, *t*-test) from the equivalent value from Cl^--free perfusions on both sides of the intestine.

There were no significant differences within columns for any variable.

Negative values indicate a net loss from the serosal solution.

m, mucosal; s, serosal.

similar trend, but there were no significant differences in tissue [Na^+] or [K^+] between the Cl^- manipulation experiments (Table 6). There were no regional differences in Cu, Na^+, K^+ or Cl^- composition along the length of the gut in controls, but there were some changes during Cu exposure that were related to the transepithelial Cl^- gradient (Table 6). For example, [Cl^-] was particularly low in the anterior and middle regions of the intestine, while [K^+] was higher in the posterior region when Cl^- was removed from the mucosal solution compared with removal of Cl^- from both solutions. Cu tended to dehydrate the middle and hind region of the intestine, but only when Cl^- was absent from both mucosal and serosal solutions (Table 6).

Do the experimental manipulations alter transepithelial potential?

These experiments were performed to aid interpretation of the perfused intestine experiments, since some of the manipulations above might alter membrane potential and therefore the electrochemical gradient for Cu. A first series (normal [Cl^-]) started with normal saline (no added Cu) on both sides, then explored the effects of Cu (as sulphate) in the mucosal solution, followed by either serosally applied vanadate or DIDS for 1 h (Cu still present in the mucosal solution). The transepithelial potential of the intestine after resting in normal saline was 1.35±1.00 mV (mean ± S.E.M., N=16) and did not change significantly (t-test, P>0.05) in the presence of 10 μmol l^{-1} $[Cu]_m$ (1.13±0.33 mV, N=11), even when followed by 0.1 mmol l^{-1} serosal DIDS (1.13±0.28 mV, N=5), but was significantly reduced (t-test, P<0.05) by 0.1 mmol l^{-1} serosal vanadate (0.42±0.51 mV, N=6). Additional control experiments in which either DIDS or vanadate was added to the mucosal solution had no effect on transepithelial potential, indicating that neither drug could leak to the sensitive serosal side within 1 h.

A second series explored the effects of removal of Cl^-. Asymmetrical removal of Cl^- from only the mucosal solution significantly decreased transepithelial potential to −6.73±0.37 mV (N=5) compared with the normal saline control. Although the transepithelial potential partially recovered when Cl^- was also removed from the serosal solution (both sides Cl^--free; −1.03±0.48 mV; N=5), it still remained below the initial control value after 1 h (t-test, P<0.05).

Discussion

Where along the gut is Cu absorbed?

The gut sac experiments suggest that Cu is mostly absorbed in the distal regions of the intestine in control guts (Table 1). This was confirmed during exposure to 100 μmol l^{-1} Cu, where the largest increases occurred in the middle and hind intestine,

Table 6. *Ionic composition of perfused whole intestines after manipulation of external [Cl^-]*

Variable	Treatment	Cl^- removed from both mucosal and serosal solutions	Cl^- removed from mucosal solution (Cl^- gradient m<s)
[Cu] (μmol g^{-1} dry mass)	Control	17.6±8.4	0.8±0.7
	Exposed	11.6±4.2	0.4±0.2[a,*]
[Na^+] (μmol g^{-1} dry mass)	Control	455.5±36.6	546.8±52.0
	Exposed	354.1±71.4	487.5±23.8
[K^+] (μmol g^{-1} dry mass)	Control	414.3±21.0	461.6±25.6[b]
	Exposed	409.8±43.7	471.2±16.2[b]
[Cl^-] (μmol g^{-1} dry mass)	Control	156.9±71.2	21.5±54.0
	Exposed	88.1±26.2	<0.3[c,*]
Water (%)	Control	83.6±0.4	84.7±1.4
	Exposed	81.0±0.4[d,‡]	85.1±0.7[d,*]

Everted intestines were serosally perfused for 4 h at 22 °C. Intestines were divided in to anterior, middle and hind regions for metal analysis after each perfusion to elucidate regional differences in tissue ion concentration. Only a few regional differences were observed, so the data (mean ± S.E.M., N=5 perfusions per treatment) are expressed for the whole intestine by summation of ion contents in each region of the intestine.

Ion concentrations are in μmol g^{-1} dry mass of tissue. Moisture content is percentage of fresh mass.

Exposed, 10 μmol l^{-1} [Cu] in the mucosal solution; control, no Cu added to the mucosal solution.

*Significantly different (P<0.05, t-test) from Cl^--free perfusions on both sides of the intestine within a treatment (rows).

‡Significantly different (P<0.05, t-test) from the control value within a column.

[a]Decrease in whole intestine [Cu] due to significantly less Cu in all regions of the intestine.

[b]The hind intestine only showed significant increases in [K^+] (+10 % control; +12 % exposed) when Cl^- was removed from the mucosal solution compared with Cl^- removal from both solutions.

[c]Difference arising from Cl^- depletion from all intestinal regions, but most severe loss in anterior and middle regions (<0.3 μmol g^{-1} dry mass).

[d]Differences arising from changes in water content of the middle and hind intestine only.

m, mucosal; s, serosal.

accounting for 70 % of the Cu accumulation in the entire gut. Most of this Cu was located in the mucosa rather than the underlying muscle (Table 1), as it is in mice (Yoshimura, 1994). In our study, the mucosal Cu was detected largely inside the mucosal cells and was not surface-bound. Tissues were rinsed in deionised water and, in preliminary rapid 'solution dipping' experiments (see Foulkes, 1988), we were unable to detect changes in surface-bound Cu by ICP-AES (data not shown). Intestinal absorption of Cu is also implicated in trout, in which metallothionein induction occurs in the intestine rather than the stomach or oesophagus during oral Cu exposure (Handy et al., 1999). In rats, the intestine absorbs 1.6 times more Cu than the stomach (van Campen and Mitchell, 1965). Thus, the intestine seems to be the main site of Cu absorption in both fish and mammalian gut. The normal water content of the gut (81–88 %) compared with other preparations (e.g. eel intestine 86–88 %; Ando and Kobayashi, 1978), its normal histology and the absence of any K^+ leakage indicated good viability of the gut sacs.

Dose-dependent intestinal Cu absorption and accumulation

The first series of intestinal perfusions explored the effects of increasing $[Cu]_m$ on transepithelial Cu absorption and Cu accumulation in the intestinal tissue. Absorption of Cu into the serosal perfusate was highest when $[Cu]_m$ was 10 μmol l^{-1} and decreased at higher exposure concentrations (Table 2), while Cu accumulation in the tissue showed a dose-dependent increase (Fig. 2). This suggests that the intestinal mucosa is able to accumulate Cu, but not to transfer it into the blood at high exposure doses. Copper accumulation in (or on) the mucosa therefore gives a misleading picture of systemic bioavailability, and absorption efficiency should be used instead. Decreasing absorption efficiencies of 68 %, 6 % and 3 % are obtained for 10, 50 and 100 μmol l^{-1} $[Cu]_m$ respectively (estimated from bath Cu content and cumulative uptake). Absorption efficiency also declines with increasing oral dosage in mammals (Linder, 1991; Buckley, 1996). In humans, the optimal absorption efficiency is approximately 55 % (normal daily intake approximately 1 mg Cu; Buckley, 1996), and it appears that catfish might achieve a similar level. The function of a decline in absorption efficiency at high doses may be to protect the animal from absorbing excess Cu.

The mechanism of Cu absorption

Cu uptake by the perfused intestine is best explained by carrier-mediated active transport for several reasons. (i) Solvent drag is excluded because the absolute rates of Cu transfer into the serosal perfusate are higher than those for water, and Cu uptake to the serosal solution occurs when the net water flux is in the opposite direction (Table 2). (ii) Decreasing net Cu uptake at higher Cu doses indicates a carrier-mediated mechanism. The continued increase in tissue [Cu] (Fig. 2) with increasing [Cu] in the mucosal solution, coupled with a decline in the net uptake to the serosal solution, suggests the Cu-dependent inhibition of a basolateral Cu transporter while Cu transfer across the mucosal membrane into the intestinal cells continues. The basolateral step is, therefore, the rate-limiting factor in Cu absorption, as for other toxic metals (Foulkes and McMullen, 1987; Foulkes, 1988). (iii) The effects of serosal applications of transport inhibitors and manipulation of bath $[Cl^-]$ imply energy-dependent carrier-mediated transport into the blood (see below).

The free $[Cu^{2+}]$ has not been measured in fish intestinal cells, although sequestration by metallothionein (Handy et al., 1999) suggests that it will be less than 1 μmol l^{-1}, as in mammals (Linder, 1991). Transport across the basolateral membrane into the blood will therefore be against the electrochemical gradient (equilibrium potential −16 mV; filterable serum [Cu] of 0.3 μmol l^{-1}; Bettger et al., 1987; membrane potential −70 mV). In our experiments, vanadate (a P-type ATPase inhibitor; Cantley et al., 1978) was applied to the serosal solution to inhibit basolateral Cu uptake, because this uptake involves a P-type Cu-ATPase in mammalian intestinal cells (Harrison and Dameron, 1999), and Cu uptake in trout gills is completely blocked by 50 μmol l^{-1} serosal vanadate (Campbell et al., 1999). However, 50 μmol l^{-1} serosal vanadate over 1 h had no effect on Cu uptake in African catfish, whilst 100 μmol l^{-1} serosal vanadate within 1 h caused a threefold increase in Cu uptake, rather than inhibition (Table 3). This effect was evident whether data were calculated on the basis of either initial or overall Cu uptake, indicating a sustained effect, and it occurred without significant changes in net water flux (Table 3) or tissue [Cu]. Thus, vanadate seems to stimulate a net movement of Cu through the tissue rather than retention of Cu in the mucosa. This effect of vanadate is best explained by changes in transepithelial potential rather than by inhibition of Cu-ATPase. The exact location of Cu-ATPase in intestinal cells is disputed, but current evidence now suggests that it is located on the Golgi network where Cu uptake into secretory vesicles occurs. These vesicles then migrate and fuse with the basolateral membrane to effect net Cu transport out of the cell (Harrison and Dameron, 1999). This might explain why vanadate did not inhibit Cu uptake, since vanadate might not have access to the active Cu-ATPase. Vanadate does not leak through/across catfish intestine quickly (transepithelial potentials did not change in control experiments with luminal vanadate). Most intracellular vanadium is chelated in the cytoplasm of fish intestinal cells, and only 2 % of the vanadium would be in the membrane fractions (Edel and Sabbioni, 1993) where the Cu-ATPase is expected to occur. Low potency of the vanadate used in these experiments is excluded since positive controls of intestinal homogenates showed 80 % inhibition of the Na^+/K^+-ATPase. However, vanadate did cause a reduction in transepithelial potential (a change of −0.93 mV). Using thermodynamic calculations detailed by Loretz (1995), this voltage change represents a change in current flow of 0.12 nequiv cm^2 s^{-1} (assuming a resistance of 88 Ω cm^2) compared with a change in Cu flux of 0.11 nequiv cm^2 s^{-1} (calculated from Table 3). Thus, a vanadate-dependent fall in transepithelial potential alone can theoretically explain a reduction of the

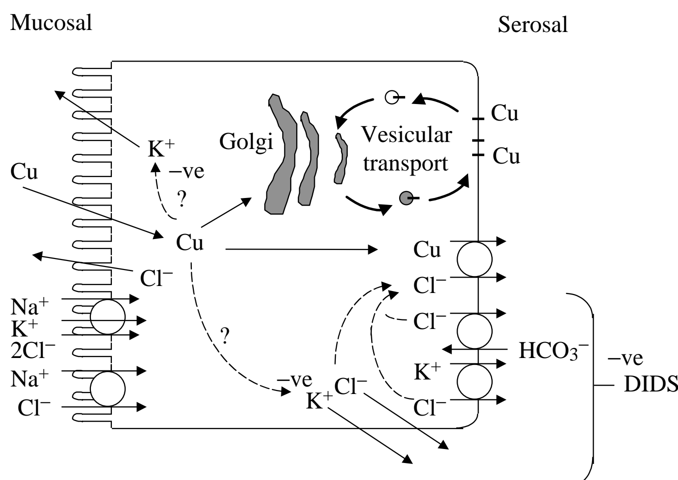

Fig. 3. Proposed working model of Cu absorption in the catfish intestine. Cu normally enters the cell through apical cation channels and leaves the cell basolaterally, either *via* vesicular Cu-ATPase activity or by Cu/anion symport. Intracellular [Cl^-] is maintained slightly above equilibrium by apical Na^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport, and Cl^- normally exits the cell through the voltage-dependent basolateral Cl^- channel, by K^+/Cl^- symport and by Cl^-/HCO_3^- antiport. Blockade of these normal basolateral routes of Cl^- efflux with DIDS causes intracellular concentrations of both K^+ and Cl^- to rise. In the presence of Cu, the Cl^- is quickly eliminated from the cell to the blood *via* Cu/anion symport, and so no net change in tissue Cl^- concentration is observed while Cu uptake to the blood increases. Cu itself or activation of Cu oxidases may block K^+ channels, preventing K^+ efflux so that tissue [K^+] rises dramatically. The stoichiometry of the Cu/anion symport is uncertain, but the preferred anion is probably Cl^-. Putative basolateral Cu/iron and Cu/zinc antiports are excluded from the diagram for clarity. −ve, negative.

electrochemical gradient that enables greater Cu uptake into the blood.

DIDS-sensitive and anion-dependent uptake of divalent cations has been reported in renal cells (Cd; Endo et al., 1998), red blood cells (Zn, Cu; Alda and Garay, 1989, 1990), cardiac myocytes (Mg^{2+}; Ödblom and Handy, 1999) and intestine (Mg^{2+}; Bijvelds et al., 1996). However, the existence of such a pathway for Cu has not been previously investigated in the intestinal tissue of vertebrates and, to our knowledge, has not been reported for any epithelia in fish. In our experiments, the serosal application of $0.1\,\mathrm{mmol\,l^{-1}}$ DIDS caused a sustained net increase in Cu uptake into the serosal perfusate and elevation of tissue K^+ levels (Tables 3, 4). In addition, removal of Cl^- from the solutions largely abolished Cu absorption (compare Tables 2 and 5). These observations support the idea of a DIDS-sensitive anion-dependent mechanism for Cu uptake in the intestine, which we argue could be a basolateral Cu/anion symport.

DIDS inhibits a variety of other ion-transport systems including Cl^- channels (Bicho et al., 1999), Cl^-/HCO_3^- antiport (Ando, 1990), Na^+/HCO_3^- cotransport (Calonge and Ilundáin, 1998) and perhaps lactate transport (Faelli et al., 1998), but has no overall effect on transepithelial potential in our experiments. In our working hypothesis (Fig. 3), Cu normally enters the cell through apical cation channels and may leave the cell basolaterally, either *via* vesicular Cu-ATPase activity or by Cu/anion symport. Intracellular [Cl^-] is maintained slightly above equilibrium at approximately $30\,\mathrm{mmol\,l^{-1}}$ as a result of the activity of apical Na^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporters (Loretz, 1995). Cl^- would normally exit the cell passively *via* the voltage-dependent basolateral Cl^- channel and K^+/Cl^- symport (Loretz, 1995; Bicho et al., 1999) and on the Cl^-/HCO_3^- antiport during base secretion (Ando, 1990). Serosal DIDS blocks these basolateral transporters, causing both intracellular K^+ and Cl^- concentrations to rise. In the presence of Cu, the Cl^- is quickly eliminated from the cell into the blood *via* Cu/anion symport, and so no net change in tissue Cl^- concentration is observed while Cu efflux from the cell to the blood (net uptake) increases (Tables 3, 4). Cu itself or activation of Cu oxidases may block K^+ channels (Wu et al., 1996), preventing K^+ efflux so that tissue [K^+] rises dramatically (Table 4).

Wapnir (1991) performed some similar experiments on *in situ* perfused rat intestine, which focused on events at the mucosal membrane, and found that simultaneous removal of Na^+ and Cl^- from the lumen slowed the disappearance of Cu from the mucosal solution. This NaCl-dependent element was approximately 15 % of the overall Cu flux across the mucosal membrane, unlike in the catfish where 93 % of the Cu flux is dependent on the mucosal solution [Cl^-], suggesting important species differences in Cu uptake at the mucosal membrane. Wapnir (1991) argues against Cu uptake on the mucosally located $Na^+/K^+/2Cl^-$ transporter in favour of Cu entry through an amiloride-sensitive Na^+ channel. The latter is problematic since we now know that Cu^{2+} decreases the amiloride-sensitivity of Na^+ channels (Flonta et al., 1998). The small Na^+ effect on Cu transport in rat intestine (Wapnir, 1991) may only apply in conditions of Na^+ deficiency, since elevated dietary [Na^+] has no effect on Cu transport (Wapnir and Lee, 1993). In catfish, the absence of obvious changes in contractility (pH-induced; Underhay and Burka, 1997), acidosis of the medium or depletion of tissue [Na^+] exclude Cu entry *via* the mucosal Na^+/H^+ exchanger. The entry of Cu into the cell *via* a proton-coupled metal-ion transporter (DCT1, renamed DMT1; Gunshin et al., 1997) is unlikely under normal physiological conditions when DMT1 is weakly expressed (rats, Trinder et al., 2000) and requires luminal acidosis to operate (not observed with catfish). However, Cu transfer across the mucosal membrane as diffusible electroneutral complexes with Cl^- (Bogdnova et al., 1999) or through relatively non-selective divalent cation channels is possible.

In solutions without Cl^-, the net flux of Cl^- is small (Table 5) and tissue [Cl^-] is low (compare Table 4 and 6); thus, Cu uptake *via* the Cu/anion symport is reduced and net Cu uptake slows (compare Tables 2 and 5). Residual Cu transport following Cl^- removal from both solutions is only

12 % of the original activity, indicating that the anion-dependent pathway is the most important mechanism for Cu absorption. In addition, this pathway normally relies on Cl^- derived from the gut lumen, since removal of Cl^- from only the mucosal solution completely abolishes Cu absorption (Table 5). The latter treatment also reduces tissue [Cl^-] much more than when Cl^- is removed from both solutions (Table 6) and lowers the transepithelial potential. This effect might be explained by the voltage characteristics of Cl^- channels, since depletion of mucosal solution Cl^- would decrease intracellular [Cl^-], causing a depolarisation of the basolateral membrane and outward rectification of Cl^- channels (Bicho et al., 1999). Similarly, depolarisation would slow apical entry of Cu and reduce tissue [Cu] (Table 6). This suggested link between Cl^- channel characteristics and Cu metabolism is strongly supported by the clear allosteric effects of voltage-dependent Cl^- channels on intracellular Cu loading (Davis-Kaplan et al., 1998). Voltage-sensitivity of basolateral Cu transport cannot be excluded since removal of Cl^- reduces the transepithelial potential. Alternatively, voltage effects on intracellular Cl^- may indirectly alter Cu speciation (e.g. the formation of Cu chloride species) and so affect transport *via* the Cu/anion symport. Endo et al. (1998) applied similar speciation arguments to anion-dependent absorption of Cd. Regional differences in tissue [Cl^-] and [K^+] occur along the length of the intestine with asymmetrical Cl^- solutions (Table 6). The significance of these observations are unclear, but they suggest the presence of Cu-sensitive Cl^- efflux pathways in the anterior and middle intestine, and a greater abundance of Cl^--dependent K^+ transport in the hind intestine.

In conclusion, the African walking catfish *Clarias gariepinus* absorbs Cu mostly (70 %) in the middle and hind intestine, rather than in the stomach or oesophagus. Absorption efficiency declines with increasing Cu dose, while simultaneous Cu accumulation in the tissue indicates that basolateral transport is the limiting factor in Cu uptake to the blood. Under normal conditions, basolateral Cu uptake is mostly *via* a novel Cu/anion symport that prefers Cl^- and is probably stimulated by indirect effects of DIDS on tissue KCl levels. A contribution from a basolateral Cu-ATPase is not excluded, but is difficult to demonstrate given the changes in transepithelial potential in the presence of vanadate. It remains to be established whether such anion-dependent mechanisms exist in the gut of higher vertebrates and whether the system will operate with other anions, e.g. bicarbonate. Further evidence for Cl^--dependent Cu transport might be obtained by simultaneously measuring Cl^- and Cu conductance in voltage-clamped enterocytes.

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