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Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout

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Grosell, M., J. C. McGeer, and C. M. Wood. Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R796–R806, 2001.—Nonacclimated and Cu-acclimated rainbow trout (*Oncorhynchus mykiss*) exhibited equally rapid clearance of a single bolus of injected ^{64}Cu (3,780 nmol/kg) from the plasma (32–40 min to half-concentration). Eight hours after Cu injection, ~80% of the injected Cu was found in the liver. However, when Cu labeled with ^{64}Cu was presented intravascularly via continuous infusion at a rate of 158 nmol·kg^{-1}·h^{-1} for 72 h, Cu-acclimated fish cleared plasma Cu more effectively than nonacclimated fish. The use of chronically implanted cystic bile duct cannulas revealed a fourfold increase in hepatobiliary Cu excretion in Cu-acclimated fish during infusion, demonstrating the important homeostatic role of the liver in Cu metabolism. Extrahepatobiliary Cu excretion, likely through the gills and apparently exceeding biliary Cu excretion, was evident from appearance of ^{64}Cu in the ambient water but was not altered by Cu acclimation. Cu accumulation in white muscle also played an important a role in copper homeostasis.

^{64}Cu; copper acclimation; hepatobiliary copper excretion; plasma copper

COPPER IS A COFACTOR IN A wide range of fundamental redox reactions involving intracellular enzymes/proteins such as cytochrome oxidase, superoxide dismutase, dopamine-hydroxylase, lysyl oxidase, and extracellular ceruloplasmin (17). The redox nature of Cu is thus essential to processes such as cellular respiration, free-radical defense, and cellular iron metabolism but makes Cu a very potent toxicant. In freshwater teleost fish, Cu exerts specific inhibitory effects on branchial ionoregulation during acute exposures to water-borne Cu in the nanomolar range (see Ref. 37 for review). Not much is known about the long-term effects of sublethal Cu exposure in fish, but conditions such as hepatic cirrhosis and hemolytic anemia are often associated with excess Cu accumulation in mammals (Wilson's disease) (26).

Given that Cu is essential but yet toxic, it is not surprising that the metabolism of this metal is highly regulated. Cu metabolism has been studied extensively, especially in mammals, due to the genetically linked disorders of Cu metabolism, Wilson's disease and Menke's syndrome (see Ref. 22 for a recent review). The literature concerning Cu metabolism in lower vertebrates is sparse. However, plasma Cu levels in teleost fish appear to be tightly regulated (9–13).

In mammals, the liver is the major homeostatic organ in Cu metabolism; Cu derived from dietary uptake is very effectively taken up by the liver, where it is either incorporated into ceruloplasmin for transport to extrahepatic organs, stored in Cu-protein complexes, or excreted via the bile (see Ref. 5 for a comprehensive review). Compared with other organs, the liver of teleost fish has the highest Cu concentration ([Cu]) (1, 21, 24, 32) and the highest Cu accumulation rate during water-borne Cu exposure (10–13). Furthermore, gallbladder bile [Cu] in teleosts are generally high and increase during water-borne Cu exposure (11–13). The above observations suggest a strong homeostatic role for the liver in teleost Cu metabolism, but this role still remains to be quantified.

Cu-acclimated rainbow trout show reduced accumulation of radiolabeled ^{64}Cu in plasma compared with nonacclimated rainbow trout in situations with little or no apparent change in branchial Cu uptake. We suggested that this was due to increased hepatic clearance of the newly accumulated plasma Cu (12, 13).

In the present study, we set out to verify our hypothesis of increased hepatic clearance of new plasma Cu in Cu-acclimated fish using an approach in which the gills were bypassed by injecting or infusing the Cu directly into the vascular system through indwelling arterial catheters. The catheters also allowed for repetitive sampling of blood. The status of blood Cu was determined by analyzing plasma Cu levels, because plasma in rainbow trout accounts for >90% of whole blood Cu pool derived from recent uptake (12). Tissues were sampled to follow the fate of the injected or infused Cu from the plasma. We applied a radioisotopic method to distinguish between Cu already present in the fish and Cu derived from the injected or infused pool of Cu. To induce Cu acclimation, fish were preexposed to cold

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water-borne Cu before radioisotope experiments. The Cu-acclimation conditions were identical to those of previous studies in our laboratory (11, 12) to facilitate direct comparisons.

Gallbladder bile [Cu] are increased in fish during prolonged Cu exposure, suggesting that the hepatic excretion of Cu is stimulated in Cu-acclimated fish. To test this hypothesis, we applied a recently described cannulation technique (14) to continuously collect hepatic bile from the cystic bile duct. The applied technique yields constant hepatic bile flow with a fairly constant chemical composition for up to 108 h (14) and is thus ideally suited for these studies that were limited (due to the 12.8-h half-life of ^{64}Cu) to an initial control period and subsequent 72 h (i.e., 6 half-lives) of radioisotope exposure.

MATERIALS AND METHODS

Fish

Rainbow trout *Oncorhynchus mykiss* (weight range 160–368 g) were obtained from Humber Springs Trout Hatchery (Mono Mills, Ontario, Canada). The fish were held in 264-liter fiberglass tanks (20/tank), each supplied with a minimum of 2.5-l/min flow through of dechlorinated, aerated Hamilton City tap water [0.6 mM Na^+; 0.7 mM Cl^-; 1.0 mM Ca^{2+}; 1.9 mM HCO_3^-, pH 7.9–8.2; background [Cu] of 50 nmol = 3.2 μg/l]. Fish were allowed to acclimate to laboratory conditions for at least 7 days before experimentation. The temperature was kept at 15 ± 1°C throughout acclimation and all subsequent procedures. The fish were fed dry trout pellets (Martin's Feed Mills, Ontario, Canada) at a rate of 1% of their body mass three times per week. The Cu content of the food was 46 nmol/g (2.9 g/g dry wt).

Cu Acclimation

Two of the holding tanks, as described in *Fish*, received tap water from a head tank via a mixing chamber that was supplied with a concentrated stock of $CuCl_2 \cdot 2\ H_2O$ (analytical grade) delivered by a Mariotte bottle so as to achieve a nominal [Cu] of 315 nmol/l [20 g/l; actual measured [Cu] 358 ± 26 nmol/l (mean ± SE, n = 12)]. In the following, "Cu-acclimated fish" refers to fish exposed to these conditions for 28 days. Water flow and dosing rates were checked daily and adjusted if necessary. No mortality occurred during either holding or Cu acclimation. Water samples both from the control holding tanks and the Cu-acclimation tanks were taken for analysis of [Cu] before feeding every second day. Samples were acidified with HNO_3 [trace metal analysis grade (BDH Chemicals) was used in all procedures], and Cu was measured.

Experimental Design

Single Cu-bolus injection experiment. To test whether Cu acclimation increases the plasma Cu clearance, five nonexposed control fish and five Cu-acclimated fish were anesthetized with neutralized MS-222 at a final concentration of 0.1 g/l and then fitted with indwelling dorsal aortic catheters (PE-50 tubing) as described by Soivio et al. (29). For surgery on Cu-acclimated fish, water from the Cu-acclimation tanks was used to irrigate the gills thus ensuring constant exposure to Cu even during surgery. After the fish were cannulated, they were held in individual, aerated experimental chambers (volume 4 liters) served with water of the appropriate composition at a rate of at least 200 ml/min. The fish were left to recover from surgery for a minimum of 36 h under these conditions before experimentation.

An initial control blood sample (150 μl) was obtained via the dorsal aorta catheter, and the water-borne Cu exposure of the Cu-acclimated fish was terminated. The purpose of terminating the exposure at this time was to ensure that the only difference between Cu-acclimated and nonacclimated groups after this point was the preexposure/acclimation. Fish were then immediately injected with radiolabeled Cu (^{64}Cu) via the dorsal aorta catheter. The ^{64}Cu was obtained from radiation of $CuNO_3$ (solid form) in the nuclear research reactor at McMaster University. The ^{64}Cu was dissolved, after radiation, in 1.0% HNO_3. Subsequently, the redissolved ^{64}Cu was diluted in a 140 mM NaCl solution to a final concentration of 7,559 nmol (480 g)/ml (0.088 mCi/mol), and pH was adjusted to 7.8 by gradual addition of NaOH. The fish were injected with 0.5 ml/kg of this ^{64}Cu-containing NaCl solution, resulting in a dose of 3,780 nmol Cu/kg. This dose corresponds to ~1.3 times the whole body Cu pool and 12 times the plasma Cu pool in nonexposed fish.

In addition to the initial control samples, blood samples (150 μl) were obtained from all fish via the dorsal aorta catheter at 5, 10, 15, 20, 30, 40, 60, 90, 120, 240, and 480 min after ^{64}Cu injection and plasma was immediately separated by centrifugation (2 min at 14,000 *g*). To flush the catheter, a 200 μl blood sample was obtained before sampling. After each sampling, the initial 200 μl blood sample was reinjected followed by 150 μl of Cortland saline (39), replacing the volume of sampled blood, and finally the catheter was refilled with heparinized Cortland saline (100 μl) to prevent clotting. All plasma samples were analyzed for ^{64}Cu-gamma radioactivity and total [Cu].

Immediately after the 480-min blood samples, the fish were killed by an overdose of MS-222 (0.5 g/l), and liver, kidney, gill filaments, and a subsample of white muscle were obtained by dissection. For determination of background tissue [Cu], 10 nonacclimated and 10 Cu-acclimated trout were sampled directly from the holding tanks, and tissue samples were obtained as above. Tissue samples from Cu-injected fish were analyzed for ^{64}Cu-gamma radioactivity. Total [Cu] was measured in all tissue samples.

Cu-infusion experiment. The single Cu-bolus experiments revealed no effect on plasma Cu clearance. Consequently, an infusion approach was employed to mimic the constant Cu uptake during exposure to elevated ambient Cu. Nonacclimated (n = 15) and Cu-acclimated (n = 15) rainbow trout were fitted with dorsal aorta catheters and placed in individual fish chambers. An initial control blood sample (150 μl) was obtained via the dorsal aorta catheter, and the water-borne Cu exposure of the Cu-acclimated fish was terminated (for the same reason as in the single-bolus injection experiment). From a stock solution of ~105 nmol/ml ^{64}Cu (0.088 mCi/mol), Cu was infused at a rate of ~3 ml/kg via a peristaltic pump. Infusion saline was prepared for each individual fish. The ^{64}Cu concentration in the infusion saline was adjusted taking into account the body weight and pump rate to yield an infusion rate of 158 nmol (10 μg)·kg^{-1}·h^{-1} for each individual fish. This infusion rate corresponds to ~5% per hour of the whole body Cu pool or 50% per hour of the plasma pool in nonexposed fish. At 3, 6, 12, and 24 h of infusion, plasma samples were obtained as described for the single Cu-bolus injection experiment from all fish (30 in total), after which ^{64}Cu infusion was continued. Subsequently, five of the nonacclimated and five of the Cu-acclimated fish were killed by an overdose of MS-222 (0.5 g/l), and liver, kidney, gill filaments, and a subsample of white muscle

were obtained by dissection. The remaining 10 nonacclimated and 10 Cu-acclimated fish were continuously infused, and plasma samples were obtained after 36 and 48 h of infusion. After the 48-h plasma samples were obtained, five more of the nonacclimated and five more of the Cu-acclimated fish were killed and sampled as above. The remaining five nonacclimated and five Cu-acclimated fish were infused for an additional 24 h, and plasma samples were obtained after 60 and 72 h of infusion. Subsequently, these last 10 fish were killed and sampled as above. Plasma and tissue samples were analyzed for ^{64}Cu radioactivity and total [Cu].

Cu-excretion experiment. To assess hepatobiliary Cu excretion, a recently described technique (14) was employed in nonacclimated and Cu-acclimated fish during infusion and in noninfused control fish. In addition to dorsal aorta catheters, these fish were fitted with a catheter in the cystic bile duct for continuous collection of hepatic bile. Surgery was performed on a large number of fish, because the success rate for the bile duct cannulation was relatively low (<50%). In total, 17 nonacclimated fish, 14 Cu-acclimated fish, and 13 nonperfused control fish provided successful preparations. This technique facilitates collection of hepatic bile that has not been modified by the transport processes occurring across the gallbladder epithelium. A constant hepatic bile flow of ~75 $\mu l \cdot kg^{-1} \cdot h^{-1}$ is obtained with a fairly constant chemical composition, similar to that reported for other vertebrates, for a period of at least 108 h (14).

After a 24-h recovery from surgery, fish were infused with ^{64}Cu in 140 mM NaCl solution and plasma was sampled as described for the Cu-infusion experiment above. Hepatic bile was collected (using a 10-cm siphon) over a 12-h preinfusion period and in six subsequent 12-h periods during the 72-h ^{64}Cu infusion. The volume of collected bile was measured gravimetrically. The noninfused control fish were treated as above, with the exception of plasma sampling and ^{64}Cu infusion. Immediately after the 72-h plasma and bile samples, the fish were killed and sampled as above. Plasma, bile, and tissue samples were analyzed for ^{64}Cu-gamma radioactivity and total [Cu].

To assess the extrahepatobiliary Cu excretion, efflux of ^{64}Cu to the ambient water was recorded by terminating the water flow to the individual fish chambers for a 2-h period bracketing the time of blood sampling at 6, 12, 24, 48, and 72 h. A 10-ml water sample was taken from each fish chamber at the start and the end of these 2-h flux periods. Cu efflux was calculated from the total volume of the individual fish chambers, the individual fish weight, the time elapsed, and the specific activity of the plasma of each individual fish.

Analytical Techniques

^{64}Cu-gamma radioactivity in tissue samples, 100-μl plasma and bile samples, and 200-μl samples of injection stocks (single Cu-bolus injection experiment) and infusion stocks (Cu-infusion and Cu-excretion experiments) were determined using a gamma counter (MINAXI gamma Autogamma 5000 series, Canberra-Packard). The recorded counts per minute were corrected for radioactive decay by an onboard program in the counter. After tissue samples were counted, they were freeze-dried to constant weight and dry weight was determined at room temperature and humidity.

After we determined ^{64}Cu-gamma radioactivity and sufficient time to allow the ^{64}Cu-gamma radioactivity to decay to an undetectable level, tissue samples were digested for total Cu measurements in acid-washed glass tubes for 1 h with five volumes of 70% HNO_3 at 120°C [trace metal analytical grade HNO_3 (BDH Chemicals) was used in all procedures]. The samples were allowed to cool to room temperature, and 0.75 volumes of H_2O_2 (trace metal grade) were added. The digests were evaporated to dryness at 120°C and finally were redissolved in a known volume of 1% HNO_3.

Total [Cu] in water, injection stocks, infusion stocks, plasma, bile, and digest samples was determined by graphite furnace atomic absorption spectroscopy (AAS; Varian AA-1275 with GTA-95 atomizer) using 10- to 20-μl injection volumes, N_2 gas, and standard operating conditions as described by the manufacturer. Absorbance of the appropriately diluted unknown samples (duplicate) was related to absorbance of a series of known standard samples diluted from a certified $CuCl_2$ stock solution (Fisher Scientific).

Calculations

The sensitivity of metal analysis by radioisotopic approaches is much greater than standard spectroscopic approaches and a lot less tedious. However, when using the isotopic approach, the reported tissue metal concentrations rely on precise recordings of specific activity (radioactivity per mole of Cu) in the previous compartment, defined as the compartment from which the metal is accumulated. This is especially true for studies of essential metals like Cu and Zn, in which background levels in tissues are often high. These background levels vary and thus dilute the specific activity of the isotope to various extents during prolonged exposure to the metal.

To address this problem, the "previous compartment specific-activity approach" was earlier developed for studies of Cu uptake and metabolism in trout (12). In these analyses, the relevant compartment is the one from which a tissue accumulates Cu, i.e., liver from plasma and bile from liver. In brief, the approach assumes complete equilibrium between ^{64}Cu-labeled Cu derived from recent uptake and Cu present in the fish before the Cu exposure. This is often but not always the case (11–13). Consequently, this is used as a primary assumption, and comparisons between calculated "newly accumulated Cu" (defined as Cu accumulated during ^{64}Cu exposure) and changes in total [Cu] in the very same samples are used to verify this assumption. In situations in which this assumption is not justified, the discrepancies between the calculated newly accumulated Cu and the change in total [Cu] can provide information on the accessibility of different Cu pools to turnover within a given tissue.

The newly accumulated Cu was calculated by the following equation: a/(b/c), where a is the ^{64}Cu radioactivity in the compartment of interest, b is ^{64}Cu radioactivity in the previous compartment, and c is the total [Cu] in the previous compartment, all expressed on the same per-unit weight or volume basis. The previous compartment for the plasma was assumed to be the injection or infusion stock, the previous compartment for each of the ambient water, the gill, the liver, the kidney, and the muscle was assumed to be the plasma, and finally, the previous compartment for hepatic bile was assumed to be the liver. The plasma, rather than the gills, was chosen as the previous compartment for the ambient water, because the Cu pool in the gills is <10% of the Cu pool in the plasma and because branchial Cu was found to be highly exchangeable.

The ^{64}Cu specific activity (decay corrected) in the injection and infusion stocks was constant throughout all experiments. However, specific activity in the plasma and liver (both serving as previous compartments in the above calculation) changed during the experiments as a consequence of changes in either newly accumulated Cu or total [Cu] or simultaneous changes in both. These changes were taken

into account by using the time-integrated specific activity in these compartments wherever relevant. For all calculations, the previous compartment specific activity from each individual fish, rather than the group means, was used in the calculations.

Data Presentation and Statistical Evaluation

Data are presented as means ± SE. Total [Cu] in tissue samples from experimental nonacclimated and Cu-acclimated fish were compared with their respective controls using a two-tailed Student's *t*-test. Newly accumulated Cu levels in tissue samples from nonacclimated and Cu-acclimated fish in all experiments were compared by a two-tailed Student's *t*-test.

A two-factor ANOVA (Statgraphics for Windows version 1.4), with acclimation and time as the main variables, was used to evaluate differences between the clearance kinetics of newly accumulated Cu and total Cu in plasma of nonacclimated and Cu-acclimated fish from the single Cu-bolus experiment. The same approach was used to evaluate differences between newly accumulated Cu and total Cu in the plasma of nonacclimated and Cu-acclimated fish from the Cu-infusion and Cu-excretion experiments and also to evaluate differences between newly accumulated Cu and total Cu in hepatic bile from nonacclimated and Cu-acclimated fish from the Cu-excretion experiment. In all cases, groups were considered significantly different at $P < 0.05$.

RESULTS

Single Cu-Bolus Injection Experiments

The injection of 3,780 nmol Cu/kg resulted in an increase of plasma total [Cu] from 9.3 to 61 nmol/ml and from 5.4 to 63 nmol/ml in nonacclimated and Cu-acclimated fish, respectively (Fig. 1*A*). The corresponding plasma concentrations of newly accumulated Cu were 57 and 56 nmol/ml, respectively, immediately after injection, essentially identical to the elevation of total Cu (Fig. 1*B*). The injected Cu was cleared rapidly from the plasma both from nonacclimated and Cu-acclimated fish. Times to half concentration (determined via Sigmaplot 4.0) of newly accumulated Cu in nonacclimated and Cu-acclimated fish were 32.3 ± 1.5 and 39.7 ± 1.7 min, respectively. The corresponding times to half concentration for total plasma Cu was 56.7 ± 3.6 and 54.2 ± 2.7 min, respectively.

The liver accumulated ~80% of the injected radiolabeled Cu both in nonacclimated and Cu-acclimated fish after 8 h. Clearance of the plasma Cu was associated with ~1,000 nmol Cu/g newly accumulated Cu in the livers both of nonacclimated and Cu-acclimated fish. The mean hepatic total [Cu] in nonacclimated fish was increased from ~2,800 to 4,100 nmol/g by the Cu injection. This increase was not statistically significant but roughly corresponds to changes (1,000 nmol/g)in the newly accumulated [Cu] (Fig. 2, *A* and *B*).

The renal total [Cu] was 100 and 200 nmol/g in nonacclimated and Cu-acclimated fish, respectively, before Cu injection. Both in nonacclimated and Cu-acclimated fish, the Cu injection resulted in a significant increase in renal total [Cu] of ~100 nmol/g. The corresponding newly accumulated [Cu] exceeded the increase in total [Cu] by approximately threefold in both nonacclimated and Cu-acclimated fish (Fig. 2, *A* and *B*).

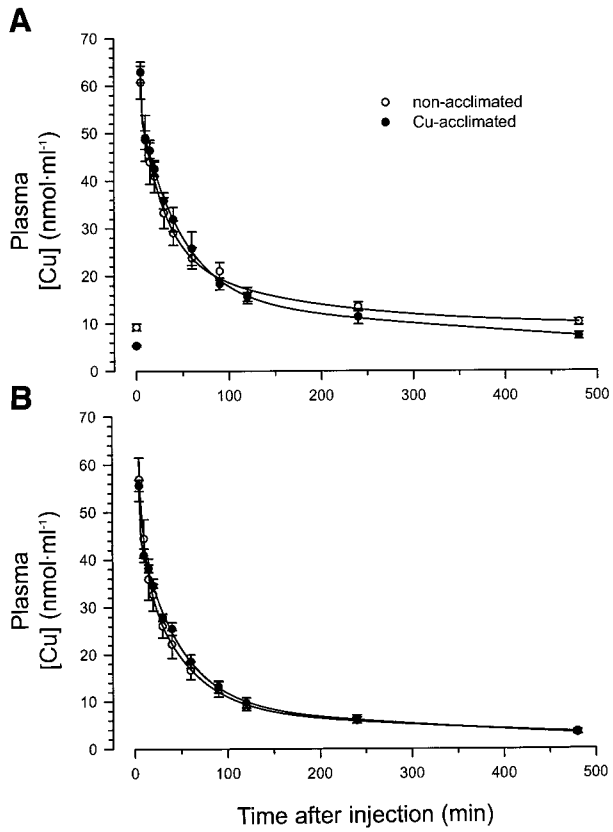

Fig. 1. *A*: total Cu concentration ([Cu]; nmol/ml). *B*: newly accumulated [Cu] (nmol/ml) in plasma samples of nonacclimated and Cu-acclimated rainbow trout in the single Cu-bolus injection experiments before and after injection with 3,780 nmol radiolabeled Cu/kg. Cu acclimation was through a 28-day exposure to 358 ± 26 nmol Cu/l before experimentation. Means ± SE (n = 5). There was no statistically significant difference between nonacclimated and Cu-acclimated fish.

Not only was there no increase in branchial total [Cu] associated with new Cu accumulation, but the branchial total [Cu] in Cu-acclimated fish was significantly reduced over the period of 8 h after injection (Fig. 2*B*). Note that at the time of injection, the waterborne Cu exposure was terminated. Branchial newly accumulated [Cu] was similar in both nonacclimated and Cu-acclimated fish (30–40 nmol/g). This newly accumulated Cu did not translate to an increased branchial total [Cu].

Notably, newly accumulated [Cu] in muscle tissue both from nonacclimated and Cu-acclimated fish were approximately one order of magnitude lower than the corresponding total [Cu]. The total [Cu] in muscle tissue was not changed by the Cu injection in either nonacclimated or Cu-acclimated fish (Fig. 2, *A* and *B*).

Cu-Infusion Experiments

Cu infusion at a rate of 158 nmol·kg^{-1}·h^{-1} resulted in an accumulation of new Cu in the plasma both in nonacclimated and Cu-acclimated fish. In both groups, infusion resulted in an immediate increase, after which

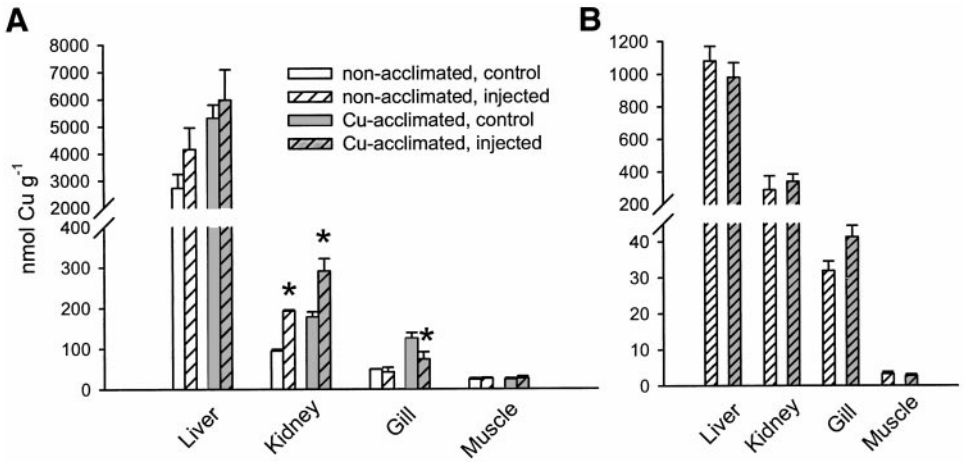

Fig. 2. *A*: total [Cu] (nmol/g dry wt) in liver, kidney, gill, and muscle of noninjected, nonexposed control fish, in Cu-acclimated, noninjected fish, and in both nonacclimated and Cu-acclimated fish 480 min after bolus injection of 3,780 nmol radiolabeled Cu/kg. *B*: newly accumulated Cu (nmol/g dry wt) in the same samples from nonacclimated and Cu-acclimated fish. Means ± SE ($n = 5$). Note the break and the different scale on the *y*-axis in both panels. *Significant difference from corresponding control values (2-tailed *t*-test, $P < 0.05$).

newly accumulated [Cu] reached a steady state of ~7 and 15 nmol/ml in Cu-acclimated and nonacclimated fish, respectively. The accumulation of new Cu in the plasma was matched by an increase in total [Cu] in the same plasma samples (Fig. 3, *A* and *B*). Total plasma [Cu] increased from initial control levels of ~8 to 15–20 and 20–25 nmol/ml in Cu-acclimated and nonacclimated fish, respectively. Both for newly accumulated and total Cu in the plasma, the overall differences between nonacclimated and Cu-acclimated fish were statistically significant ($P < 0.05$).

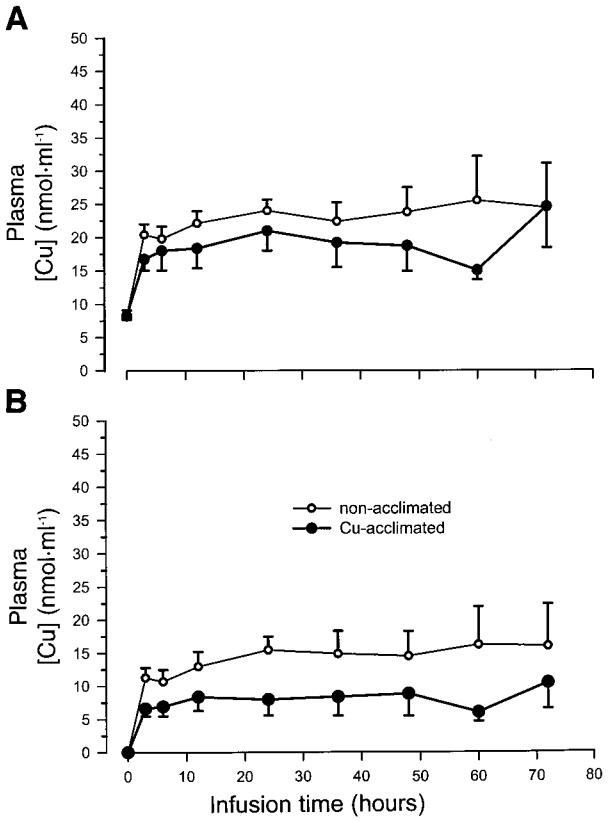

Fig. 3. *A*: total [Cu]. *B*: newly accumulated [Cu] in plasma both of nonacclimated and Cu-acclimated fish (nmol Cu/ml) before and during infusion with 158 nmol·kg^{-1}·h^{-1} radiolabeled Cu (means ± SE). Both for nonacclimated and Cu-acclimated fish, $n = 15$ for 0, 3, 6, 12, and 24 h, $n = 10$ for 36 and 48 h and $n = 5$ for 60 and 72 h. ANOVA revealed statistically significant difference ($P < 0.05$) between nonacclimated and Cu-acclimated fish both for total [Cu] and newly accumulated Cu levels ($P < 0.05$).

The Cu-infusion resulted in a gradual increase in hepatic total [Cu] in nonacclimated fish of ~1,500 nmol/g. This is in excellent agreement with the calculated newly accumulated [Cu] in the same samples. The liver of Cu-acclimated fish exhibited the same level of newly accumulated Cu, but no increase in hepatic total [Cu] was associated with this substantial Cu uptake (Fig. 4, *A* and *B*). Both for nonacclimated and Cu-acclimated fish, the increase in the hepatic concentration of newly accumulated Cu with time of infusion was statistically significant ($P < 0.05$). However, only the nonacclimated fish exhibited a statistically significant increase in total hepatic [Cu] with time of infusion ($P < 0.05$)

In agreement with generally lower total plasma [Cu] in Cu-acclimated fish compared with nonacclimated fish, the accumulation of newly accumulated Cu and the change in total [Cu] in the kidney were lower in Cu-acclimated fish than in nonacclimated fish. Parallel to the results from the single Cu-bolus injection experiments, the newly accumulated [Cu] exceeded the corresponding increase in total [Cu] in the kidney (Fig. 4, *A* and *B*). Both for nonacclimated and Cu-acclimated fish, increases both in newly accumulated and total renal Cu with time of infusion were statistically significant ($P < 0.05$).

Also parallel to the results from the single Cu-bolus injection experiments, the total branchial [Cu] in Cu-acclimated fish was reduced by >50% during the 72 h of infusion. This statistically significant decrease occurred despite a statistically significant accumulation of new Cu. In nonacclimated fish, the total [Cu] remained constant during Cu infusion, also despite statistically significant accumulation of new Cu (Fig. 4, *A* and *B*).

Levels of newly accumulated Cu in muscle tissues were similar in nonacclimated and Cu-acclimated fish. In agreement with the results from the Cu-infusion experiments, the levels of newly accumulated Cu were

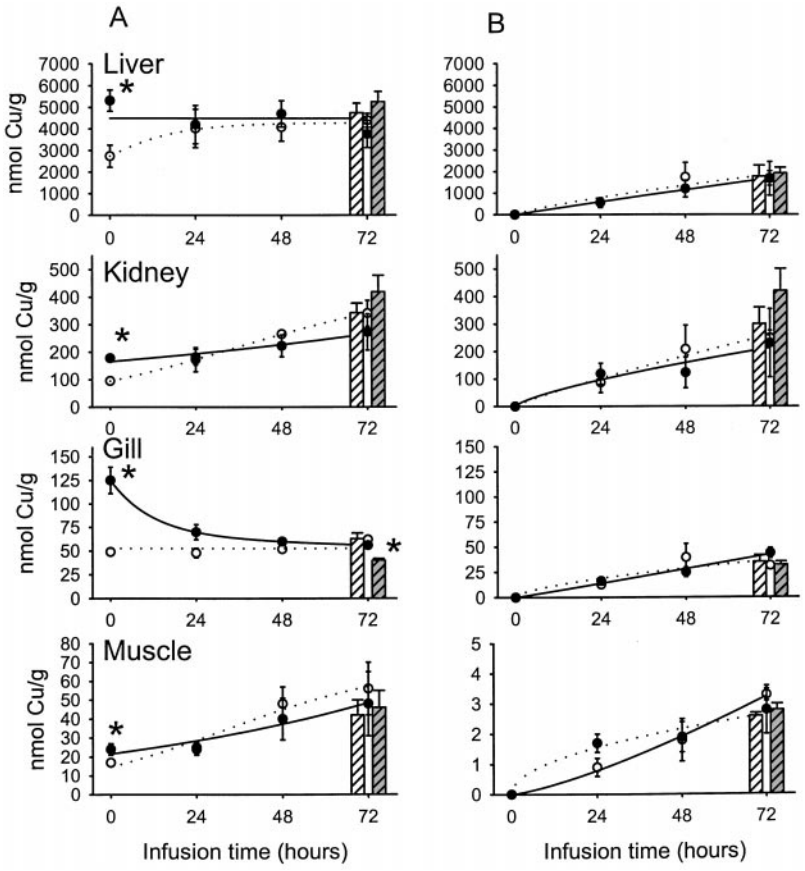

Fig. 4. *A*: total [Cu]. *B*: newly accumulated Cu in liver, kidney, gill, and white muscle (nmol/g dry wt) during and after 72 h of infusion with 158 nmol·kg^{-1}·h^{-1} radiolabeled Cu. ○, Nonacclimated fish from the Cu infusion experiments (n = 5); ●, Cu-acclimated fish from the Cu-infusion experiments (n = 5); hatched bars, nonacclimated fish from the Cu excretion experiments (n = 17); filled bars, Cu-acclimated fish from the Cu-excretion experiments (n = 14). Cu acclimation was a 28-day exposure to 358 ± 26 nmol Cu/l before experimentation (means ± SE). *Significant difference between nonacclimated and Cu-acclimated fish at the same times in the same experiments (2-tailed t-test, $P < 0.05$). The increase in newly accumulated Cu with time of Cu infusion was statistically significant in all tissues both from nonacclimated and Cu-acclimated fish (ANOVA $P < 0.05$).

only around one-tenth the corresponding increase in total [Cu] in the same samples (Fig. 4, *A* and *B*). Both for nonacclimated and Cu-acclimated fish, increases in both newly accumulated and total muscle Cu with time of infusion were statistically significant ($P < 0.05$).

Cu-Excretion Experiments

The total [Cu] in plasma showed a pattern similar to that of the Cu-infusion experiments (data not shown). Both nonacclimated and Cu-acclimated fish had an initial plasma total [Cu] of ~7 nmol/ml. The plasma total [Cu] increased gradually to a maximum of 35–40 nmol/ml in the nonacclimated fish but only to a maximum of 22–26 nmol/ml in Cu-acclimated fish. Newly accumulated Cu in the same samples exhibited a very similar pattern. As in the Cu-infusion experiments (Fig. 3), ANOVA revealed that these differences both in total Cu and newly accumulated Cu between nonacclimated and Cu-acclimated fish were statistically significant overall.

Tissue total [Cu] and newly accumulated Cu levels in nonacclimated and Cu-acclimated fish of the Cu-excretion experiments did not differ (Fig. 4). These data were in good agreement with corresponding values from the 72-h sampling point from the Cu-infusion experiments (Fig. 4).

The total bile [Cu] in Cu-acclimated fish increased throughout the entire infusion period from an initial control value of ~10 nmol/ml to a maximum of 45 nmol/ml after 108 h of infusion. The corresponding total biliary Cu-excretion rates increased approximately fourfold from 0.75 to 3.38 nmol·kg^{-1}·h^{-1} (Fig. 5*A*). This increase was significant overall. In comparison, the total [Cu] in hepatic bile of noninfused control fish was lower and remained constant at 10–12 nmol/ml (0.75–0.90 nmol·kg^{-1}·h^{-1}) throughout the experimental period. In the nonacclimated but infused fish, total [Cu] in hepatic bile and thus biliary Cu excretion was very similar to noninfused control fish (Fig. 5*A*).

Generally, the calculated rates of newly accumulated Cu in hepatic bile from both nonacclimated and Cu-acclimated fish were in good agreement with the measured total [Cu] in the same samples. On infusion, the newly accumulated [Cu] in nonacclimated fish increased significantly within the first 12 h and remained constant at 10–14 nmol/ml (0.75–1.05 nmol·kg^{-1}·h^{-1}) throughout the remaining infusion period. In comparison, the corresponding values from Cu-acclimated fish reached a maximum of 34 nmol/ml (2.55 nmol·kg^{-1}·h^{-1}) at the end of the experiment (Fig. 5*B*). Overall, the difference between nonacclimated and Cu-acclimated fish was statistically significant ($P < 0.05$).

On the basis of the appearance of ^{64}Cu radioactivity in the ambient water during experiments in which the hepatic bile was collected, the extrahepatobiliary excretion of newly accumulated Cu was similar both in

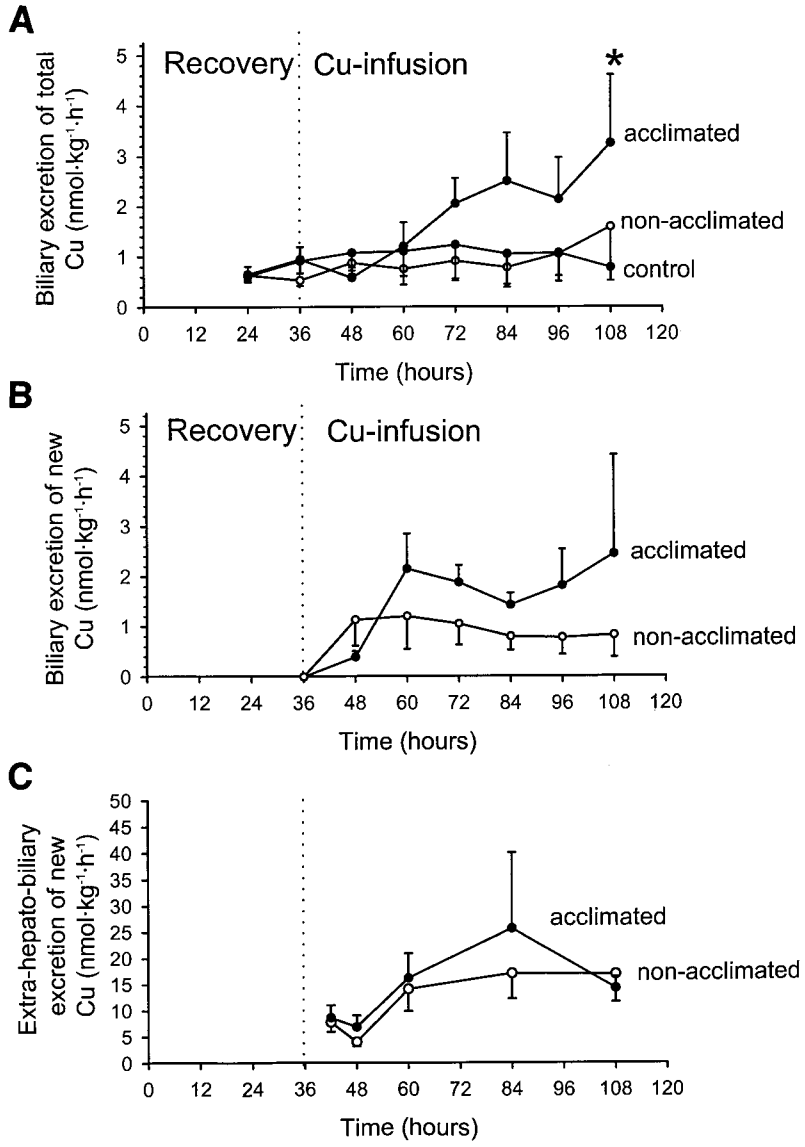

Fig. 5. *A*: biliary excretion of total Cu. *B*: newly accumulated Cu (nmol·kg^{-1}·h^{-1}) before and during Cu infusion (158 nmol·kg^{-1}·h^{-1} radiolabeled Cu) in nonacclimated and Cu-acclimated fish as well as biliary excretion of total Cu (nmol·kg^{-1}·h^{-1}) of noninfused control fish. For noninfused control fish, n = 9–13. For nonacclimated fish, n = 7–17. For Cu-acclimated fish, n = 5–14. *C*: extrahepatobiliary Cu efflux (nmol·kg^{-1}·h^{-1}) of newly accumulated Cu in nonacclimated (n = 6)) and Cu-acclimated fish (n = 7; means ± SE). ANOVA revealed statistically significant differences ($P < 0.05$) between nonacclimated and Cu-acclimated fish both for total [Cu] and newly accumulated biliary Cu levels. *Significant difference from corresponding control values (2-tailed t-test, $P < 0.05$). There was no statistically significant difference between extrahepatobiliary Cu efflux in nonacclimated and Cu-acclimated fish.

nonacclimated and Cu-acclimated fish (Fig. 5*C*). The extrahepatobiliary Cu excretion increased gradually to reach a level of ~15 nmol·kg^{-1}·h^{-1} after 24 h both in nonacclimated and Cu-acclimated fish.

DISCUSSION

Clearance of Plasma Cu

The rapid clearance of Cu from the plasma after a one-dose injection and the subsequent reestablished control plasma levels demonstrate tight and effective regulation of plasma Cu levels in both nonacclimated and Cu-acclimated fish. Hepatic Cu uptake from the plasma was the dominant homeostatic mechanism. Eight hours after the one-dose Cu injection, ~80% of the injected dose was recovered in the hepatic tissue. In contrast, the results from fish that received Cu via a continuous infusion demonstrate that acclimation to water-borne Cu exposure involved stimulated clearance of plasma Cu. As outlined below, the liver again appeared to play an important role in this elevated clearance via stimulated hepatobiliary excretion.

Hepatic Role in Cu Homeostasis

The majority of the injected or infused Cu was recovered in hepatic tissues both of nonacclimated and Cu-acclimated fish, demonstrating the homeostatic role of the liver in teleost fish. In nonacclimated fish, the level of newly accumulated Cu was matched by the increase in total [Cu] in the same samples. This is in agreement with our previous reports of newly accumulated Cu (11–13) and demonstrates that the assumptions underlying the calculations of newly accumulated Cu (see MATERIALS AND METHODS) are justified for hepatic tissue both in situations in which Cu is derived from water-borne Cu uptake and in situations in which Cu is presented as an intravascular injection or infusion. There is also evidence for two separate pools of Cu in fish blood plasma. The success of the previous compart-

ment specific activity approach shows that the Cu from both pools is equally available for hepatic uptake and accumulation.

Newly accumulated hepatic Cu in Cu-acclimated fish was similar to nonacclimated fish but was not associated with a corresponding increase in total [Cu]. This means that the Cu taken up by the liver as indicated by appearance of ^{64}Cu radioactivity must have been balanced by Cu leaving the liver. This elimination of hepatic Cu can either be via the plasma to extrahepatic tissues or via excretion through the bile.

Plasma Cu Pools

From mammals, it is known that plasma Cu generally comprises two distinct pools. One pool is tightly bound to ceruloplasmin, a plasma protein of ~134 kDa (5, 16, 22). A second Cu pool is associated with albumin and amino acids and is believed to be derived from recent uptake (from the gut in mammals) (5, 8, 23, 35). Cu-containing ceruloplasmin is synthesized in the mammalian liver and is released to the blood to provide Cu to extrahepatic tissues (see Ref. 22 for a comprehensive review). Ceruloplasmin is also present in teleosts (4, 25, 28, 33) and is thus likely to play a similar role. Cu derived from recent uptake in the European eel was associated with a 70-kDa plasma protein and also with low molecular weight substances, presumably, albumin and amino acids, respectively (9). It thus appears that the presence of two distinct plasma Cu pools applies in teleost fish as it does in mammals.

Renal Cu Accumulation and Uptake

The presence of a two-pool system in blood plasma is further supported by our previous analysis (13) of kidney function in Cu-acclimated trout, in which we show clear renal discrimination between the clearance of the total plasma Cu pool and the newly accumulated plasma Cu pool. In the present study, the total [Cu] in the kidney was increased by injection and infusion both in nonacclimated and Cu-acclimated fish. However, newly accumulated renal Cu was not balanced by corresponding increases in total Cu. This suggests that the newly accumulated Cu pool, presumably associated with albumin and amino acids, was more available for renal Cu accumulation than the total Cu pool, which was presumably associated with ceruloplasmin.

Cu Uptake and Accumulation in Muscle Tissue

Total [Cu] and levels of newly accumulated Cu in the muscle, representative of the majority of the body, were very low compared with any of the other investigated tissues. Interestingly, the total [Cu] in muscle tissue of infused fish increased in both nonacclimated and Cu-acclimated fish at a rate ~10-fold greater than the uptake rate of newly accumulated Cu in the same samples. This clearly indicates that the newly accumulated Cu, presumably bound to albumin and amino acids, is much less available for uptake by the muscle tissue than the total Cu pool, much of which presumably is bound to ceruloplasmin.

The liver accumulates the injected or infused Cu very specifically and this, at least in part, protects other tissues from excessive Cu accumulation (see *Hepatic Role in Cu Homeostasis*). The increase in total [Cu] in muscle tissue (which cannot be explained by newly accumulated Cu) indicates that Cu from a pool other than the newly accumulated Cu pool is mobilized and made available for uptake by the muscle tissue during Cu infusion. Notably the increase in total [Cu] in muscle tissue was only observed in the prolonged infusion studies and not in the 8-h-long injection study (Fig. 4 vs. Fig. 2). This shows that some time is required for the above-mentioned mobilization of Cu available for uptake by the muscle.

One possible explanation for this observation is the release of hepatic, ceruloplasmin-bound Cu to the circulatory system in response to Cu infusion, thereby making more ceruloplasmin-bound Cu available for accumulation by the muscle tissue. In turn, this would make more hepatic Cu-storage sites available in a situation in which clearance of newly accumulated plasma Cu is required. This interpretation is in accord with most mammalian studies. In mammals, it is generally believed that Cu derived from a recent uptake is taken up by the liver, later incorporated into ceruloplasmin, and then released back into the circulatory system where the presence of ceruloplasmin-bound Cu facilitates Cu uptake by other tissues (22). This interpretation is also supported by our previous observations (12) of an increase in plasma total [Cu] in excess of what could be explained from the recorded levels of newly accumulated Cu during water-borne Cu exposure. A recent study, however, showed no Cu deficiency in human patients suffering from aceruloplasminemia (18). Whereas it appears that ceruloplasmin facilitates Cu uptake by some extrahepatic tissues such as the muscle in the present study, Cu uptake sufficient to avoid deficiency may still occur in the absence of ceruloplasmin.

The increase in total [Cu] of ~20 nmol/g (dry wt) in muscle tissue of both nonacclimated and Cu-acclimated fish over the 72 h of Cu infusion is low compared with the liver. However, due to the large mass of muscle tissue (50% of the body wt), it amounts to ~2,500 nmol/kg, which is considerable compared with the 11,400 nmol/kg infused over the 72 h of experimentation. Thus the muscle mass can be considered as a secondary buffer compartment for overall Cu homeostasis.

Branchial Cu Uptake and Accumulation.

Consistent with previous reports (11, 12), water-borne Cu exposure increased branchial total [Cu] more than twofold. Although Cu injection and infusion caused significant levels of newly accumulated branchial Cu both in nonacclimated and Cu-acclimated fish, there was no corresponding increase in total [Cu] of the gill once the water-borne Cu exposure was stopped. In contrast, Cu-acclimated fish exhibited rapid loss of branchial total Cu despite ongoing new

accumulation of injected or infused Cu. Because the only difference between the nonacclimated and Cu-acclimated fish was the water-borne preexposure to Cu, the reduced branchial [Cu] in the Cu-acclimated fish can only be explained by the termination of the water-borne exposure just before Cu injection or infusion.

The rapid clearance of branchial Cu suggests the involvement of a carrier in branchial Cu elimination. It is uncertain whether the Cu cleared from the gills moved across the basolateral membrane into the plasma for subsequent clearance by the liver or moved across the apical membrane to the ambient Cu-free water. Recently, Campbell et al. (3) reported carrier-mediated Cu transport from water to blood across the gills of rainbow trout using a perfused-head preparation. This elegant study identifies the basolateral membrane as the rate-limiting step in branchial Cu uptake, which is in close agreement with our previous findings for both European eel and rainbow trout (11, 12). On the basis of the K_m value and vanadate sensitivity, Campbell and co-workers suggest that the carrier is similar to the Cpx-type ATPase (Cu-ATPase) involved in Cu transport (3). Although these findings are not conclusive (due to the lack of specificity of vanadate), the presence of a Cu-ATPase in teleost fish is probable given the highly conserved gene coding for this enzyme in organisms ranging from yeast and bacteria to humans (31). A recent study of ours reported vanadate-sensitive, Mg^{2+}- and ATP-dependent transport of silver (Ag) across the basolateral membrane of rainbow trout gills, strongly suggesting the involvement of a P-type ATPase (2). Because the bacterial Cu-ATPase has been reported to use both Cu and Ag as a substrate (30), this result further supports the suggested presence of a Cu-ATPase in the gills of rainbow trout.

Biliary Cu Excretion

Acclimation to water-borne Cu exposure increased hepatobiliary Cu excretion during Cu infusion (Fig. 5). The calculated newly accumulated Cu in hepatic bile was in very good agreement with the actual measured total [Cu] in both nonacclimated and Cu-acclimated fish. This is consistent with previous reports on newly accumulated and total Cu in rainbow trout gallbladder bile (12, 13). However, the observed [Cu] in hepatic bile (10–50 nmol/ml) in the present study was substantially lower than our previous measurements for gallbladder bile in nonacclimated and Cu-acclimated rainbow trout (160–330 nmol/ml). On a relative basis, this difference is similar to differences between bile acid concentrations in hepatic bile (15–50 mM) versus gallbladder bile (100–360 mM) and highlights the role of the gallbladder epithelium in concentrating certain components in bile between meals by means of water and salt extraction (14). This confounding factor means that for meaningful evaluation of hepatobiliary excretion rates (of not only Cu, but any xenobiotic or waste product), measurements of hepatic bile flow and corresponding concentrations of substances are necessary, as in the present study.

No differences in hepatic bile flow were observed among the noninfused control fish, nonacclimated perfused fish, and Cu-acclimated perfused fish. The overall mean bile flow was 75 $\mu l \cdot kg^{-1} \cdot h^{-1}$, as previously reported by us for nonexposed fish (14). On the basis of the hepatic bile flow and hepatic bile total [Cu], hepatobiliary Cu excretion rates were calculated to be 0.75 $nmol \cdot kg^{-1} \cdot h^{-1}$ in noninfused fish, up to 1.6 $nmol \cdot kg^{-1} \cdot h^{-1}$ in nonacclimated infused fish, and up to 3.4 $nmol \cdot kg^{-1} \cdot h^{-1}$ in Cu-acclimated infused fish after 72 h of Cu infusion. These values are low compared with the whole body Cu infusion rate of 158 $nmol \cdot kg^{-1} \cdot h^{-1}$ and are also lower than the calculated extrahepatobiliary Cu excretion of ~15 $nmol \cdot kg^{-1} \cdot h^{-1}$ both in nonacclimated and Cu-acclimated fish (Fig. 5*C*).

However, it should be noted that the Cu infusion rate applied in the present investigation was very high compared with Cu uptake rates in fish exposed to environmentally realistic [Cu]. This is evident from the fact that plasma [Cu] remained elevated by at least threefold throughout the entire infusion period as opposed to an initial 50% increase and rapid recovery in rainbow trout exposed to environmentally realistic water-borne Cu levels (12). The homeostatic role of the hepatobiliary system in the present study appears to be exceeded by the infused Cu dose. Indeed the biliary excretion rate was only ~0.5% and 1.4% of the Cu-infusion rate in nonacclimated and Cu-acclimated fish, respectively. Note, however, that the plasma Cu pool is <10% of the whole body pool. Although the hepatobiliary excretion rates were low compared with the infusion rate, the two- to threefold difference between nonacclimated and Cu-acclimated fish may have been responsible for a substantial proportion of the difference in plasma Cu levels.

Extra Hepatobiliary Cu Excretion

Renal Cu excretion in rainbow trout is very low (13), and because the branchial surface area comprises >50% of the total body surface area in trout (Ref. 36 vs. Ref. 38) and there is intimate contact between the blood and the ambient water at the branchial epithelium, the extrahepatobiliary Cu excretion can likely be attributed to branchial Cu excretion. The recorded extrahepatobiliary Cu excretion rate (Fig. 5*C*) did not differ between nonacclimated and Cu-acclimated fish, suggesting no involvement of elevated branchial Cu excretion in Cu acclimation. The extrahepatobiliary excretion rate did, however, exceed the hepatobiliary Cu-excretion rate substantially, even in Cu-acclimated fish, suggesting that branchial Cu excretion may be of importance in whole body homeostasis.

The calculated extrahepatobiliary excretion rates were based on appearance of ^{64}Cu in the ambient water. Because changes in total [Cu] in the same water samples were below the resolution of our analytical procedures, these calculations of whole body homeosta-

sis unfortunately cannot be validated. However, in the blood plasma, the newly accumulated Cu is more loosely bound to plasma proteins than Cu present in the plasma before Cu infusion. Furthermore, the plasma [Cu] was highly elevated during the infusion studies, thus creating a high plasma-to-water [Cu] gradient. Consequently, the calculated extrahepatobiliary Cu-excretion rates could well be overestimating the net branchial Cu-excretion rates in noninfused fish.

Perspectives

Because plasma proteins and amino acids do not appear in bile (7), Cu must enter the bile via hepatocyte-transcellular transport mechanisms in which Cu is dissociated from the protein. This theory is supported by observations that ^{64}Cu of European eel is associated mainly with a 70-kDa protein (presumably albumin) in blood plasma but exclusively with low-molecular weight substances in gallbladder bile of the same animals (9). Hepatobiliary Cu excretion thus involves at least two steps: *1*) transport from the plasma across the basolateral membrane of the hepatocyte and *2*) transport from the hepatocyte across the apical membrane (canalicular membrane) into the hepatic bile in the bile canaliculi. The specificity and rate of Cu clearance by the liver suggests the involvement of a Cu carrier in the basolateral membrane of the rainbow trout hepatocyte. Whereas evidence exists for a basolateral membrane Cu carrier in mammalian livers (7, 34), this remains to be investigated in lower vertebrates including teleost fish. The liver [Cu] in the nonacclimated fish reached a maximum value after 24 h of Cu infusion, whereas the biliary [Cu] in the same animals did not increase until 72 h. Even in the Cu-acclimated fish, the hepatic bile total [Cu] did not increase substantially until after 36 h of infusion. These observations are consistent with the presence of one or more carrier processes in the canalicular membrane that are stimulated by Cu acclimation.

From mammalian studies, at least two specific carriers involved in Cu excretion, both located in the canalicular membrane, have been documented. First, the Cu-ATPase, described above, isolated from mammalian hepatocyte canalicular membranes exhibits active Cu transport in isolated membrane vesicles (6). Second, a mammalian canalicular multiorganic anion transporter (cMOAT) contributes significantly to biliary Cu excretion (19). In addition, lysosomal excretion of Cu into hepatic bile in mammals has also been documented (15). Whereas some evidence exists for possible lysosomal Cu excretion into bile in teleost fish (20, 27), nothing is known about the involvement of the above-mentioned carriers in teleost biliary Cu excretion, thereby offering an exciting area for further research.

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