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Time course analysis of the mechanism by which silver inhibits active Na^+ and Cl^- uptake in gills of rainbow trout

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Morgan, Tammie P., Martin Grosell, Kathleen M. Gilmour, Richard C. Playle, and Chris M. Wood. Time course analysis of the mechanism by which silver inhibits active Na^+ and Cl^- uptake in gills of rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 287: R234–R242, 2004. First published March 11, 2004; 10.1152/ajpregu.00448.2003.—A time course analysis using ^{110m}Ag, $^{24}Na^+$, and $^{36}Cl^-$ examined gill silver accumulation and the mechanism by which waterborne silver (4.0×10^{-8} M; 4.3 μg/l) inhibits Na^+ and Cl^- uptake in gills of freshwater rainbow trout. Analyses of gill and body fluxes allowed calculation of apical uptake and basolateral export rates for silver, Na^+, and Cl^-. To avoid changes in silver bioavailability, flow-through conditions were used to limit the buildup of organic matter in the exposure water. For both Na^+ and Cl^- uptake, apical entry, rather than basolateral export, was the rate-limiting step; Na^+ and Cl^- uptake declined simultaneously and equally initially, with both uptakes reduced by ~500 $nmol \cdot g^{-1} \cdot h^{-1}$ over the 1st h of silver exposure. There was a further progressive decline in Na^+ uptake until 24 h. Carbonic anhydrase activity was inhibited by 1 h, whereas Na^+-K^+-ATPase activity was not significantly inhibited until 24 h of exposure. These results indicate that carbonic anhydrase inhibition can explain the early decline in Na^+ and Cl^- uptake, whereas the later decline is probably related to Na^+-K^+-ATPase blockade. Contrary to previous reports, gill silver accumulation increased steadily to a plateau. Despite the rapid inhibition of apical Na^+ and Cl^- uptake, apical silver uptake (and basolateral export) increased until 10 h, before decreasing thereafter. Thus silver did not inhibit its own apical uptake in the short term. These results suggest that reduced silver bioavailability is the mechanism behind the pattern of peak and decline in gill silver accumulation previously reported for static exposures to silver.

sodium-potassium-adenosinetriphosphatase; carbonic anhydrase; sodium channel; hydrogen-adenosinetriphosphatase; silver uptake

SILVER, WHEN PRESENTED AS silver nitrate ($AgNO_3$), is a potent inhibitor of active Na^+ uptake at the freshwater fish gill. In rainbow trout, complete inhibition of active Na^+ uptake occurs at an $AgNO_3$ concentration of 10^{-7} M (24), an effect that appears to be entirely attributable to the free silver ion, Ag^+ (15, 23, 35, 36). In comparison, amiloride, the most widely used inhibitor of Na^+ transport, must be presented at 10^{-4} M to cause a 75–90% inhibition (see Ref. 18 for a review). Work to date has identified inhibition of basolateral membrane Na^+-K^+-ATPase activity as the mechanism by which silver blocks Na^+ uptake in the trout gill (24). However, a recent time course study at a low level of $AgNO_3$ (2×10^{-8} M) showed strong inhibition of Na^+ uptake by silver before any effect on Na^+-K^+-ATPase activity occurred (25), suggesting an additional mechanism of Na^+ uptake blockade by silver. This work also indicated that silver accumulation at the gill is unstable over time, reaching a peak in the first few hours of exposure followed by a decline with continued silver exposure (25). The changing gill silver burden was well correlated with the degree of Na^+ uptake inhibition.

There are two possible interpretations for the pattern of gill silver accumulation. First, the pattern could be an artifact of changing silver bioavailability, a consequence of the static exposure system employed. The static system exposure is associated with accumulation of organic matter produced by the fish; as with other metals (16, 29), organic matter would be expected to strongly complex Ag^+, causing a decline in silver bioavailability (2, 7, 17, 22, 25, 27), potentially leading to changes in silver accumulation over time. Second, the pattern could be the result of a blockade of apical silver uptake, perhaps related to the early inhibition of Na^+ uptake that occurs in the absence of Na^+-K^+-ATPase inhibition.

Apical silver uptake by rainbow trout gill cells occurs by the same mechanism as for Na^+, via the proton pump-coupled Na^+ channel (4). Protons are extruded by an H^+-ATPase located on the apical membrane, generating an electrochemical gradient for transport of Na^+ and Ag^+ into the cell through an amiloride-sensitive apical channel (28). Protons are supplied to the H^+-ATPase through the catalyzed hydration of CO_2 by carbonic anhydrase (CA). Inhibition of any of these components of apical Na^+ uptake (i.e., the Na^+ channel, the H^+ pump, or CA) by silver could explain the pattern of gill silver accumulation. In particular, inhibition of CA by silver would be expected to decrease the intracellular supply of H^+ and HCO_3^-, inhibiting both apical Na^+ and Ag^+ uptake, and also apical Cl^- uptake by the decreased availability of HCO_3^- for Cl^-/HCO_3^- exchange (see Ref. 18). Together with constant silver export across the basolateral membrane (which is active and occurs by an as of yet unidentified ATPase; see Ref. 3), CA inhibition would lead to a peak and decline in gill silver accumulation. Inhibition of CA could block apical Na^+ uptake before any effect on Na^+-K^+-ATPase activity, providing an additional mechanism of Na^+ uptake blockade by silver. Silver inhibits gill CA in vitro and in vivo, although the degree of inhibition appears to be much less than that of Na^+-K^+-ATPase (6, 24).

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The objective of the present study was to distinguish between the two possible interpretations for the pattern of gill silver accumulation, i.e., changing bioavailability resulting from organic matter production by the fish vs. inhibition of an apical entry process by Ag^+ that could be responsible for both the early inhibition of Na^+ uptake and of Ag^+ uptake itself. Our particular focus within the latter was on the potential inhibition of CA. Our approach was to carry out a detailed time course analysis of the sequence of branchial events during continuous exposure of juvenile rainbow trout to a low level (nominally 3.3×10^{-8} M) of $AgNO_3$ under flow-through experimental conditions. Such conditions should limit changes in silver bioavailability because organic matter produced by the fish would be continually washed away, whereas silver would be continually renewed by the inflowing water. Under these conditions, detailed time point sampling and radiotracer techniques were employed to monitor changes in gill and body silver accumulation, gill and body Na^+ and Cl^- uptake, and gill Na^+-K^+-ATPase and CA activity. Separate analysis of gill and body (whole body minus gill) fluxes allowed calculation of apical uptake and basolateral export rates for silver, Na^+, and Cl^-.

MATERIALS AND METHODS

Experimental animals and acclimation. Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 0.5–3 g were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were held in a 200-liter polyethylene tank initially supplied with flowing, aerated, dechlorinated Hamilton city tap water [approximate ionic composition in mM: 0.5 [Na^+], 0.7 [Cl^-], 1.0 [Ca^{2+}], 0.2 [Mg^{2+}], and 0.05 [K^+], where brackets denote concentration; pH 7.8–8.0, total organic carbon (TOC) ~3 mg C/l, hardness ~140 mg/l as $CaCO_3$ and temperature 14°C]. All fish were then gradually acclimated to synthetic soft water over a 2-wk period (generated by reverse osmosis of Hamilton city dechlorinated tap water; Culligan Aqua-Cleer Reverse Osmosis System, Toronto, ON, Canada). During this period, the ratio of soft water to tap water that the fish received was increased until the desired ionic composition was achieved. Fish were maintained in this soft water [approximate ionic composition in mM: 0.05 [Na^+], 0.05 [Cl^-], 0.05 [Ca^{2+}], 0.02 [Mg^{2+}], and 0.02 [K^+]; pH 7.0, TOC ~0.7 mg C/l, hardness ~10 mg/l as $CaCO_3$ and temperature 16 ± 3°C] for at least an additional 2 wk before experimentation. During soft water acclimation, fish were fed to satiation one time daily with commercial trout pellets (Martin Mills, Tavistock, ON, Canada). Feeding was suspended for 1 day before and during the experiment to minimize silver binding to organic matter in uneaten food and waste products during the exposure period.

Experimental design. After acclimation, 160 fish were transferred to an 80-liter polyethylene tank containing 20 liters of aerated synthetic soft water (composition as above). The tank was supplied with a continuous flow-through of soft water at a rate of 130 ml/min. Before the addition of fish to the tank, the walls of the tank were preequilibrated with ^{110m}Ag-labeled $AgNO_3$ to ensure saturation of silver binding sites on the tank walls, and then the water in the tank was spiked with ^{110m}Ag-labeled $AgNO_3$ [33 μCi; specific activity (SA) 0.26 μCi/μg Ag; RISOE Nuclear Research Reactor, Roskilde, Denmark] from a light-shielded stock bottle to bring the silver immediately up to the exposure concentration. The addition of silver gave a total silver concentration of 3.3×10^{-8} M (3.6 μg/l) in the exposure water at the start of the experiment. To maintain the exposure concentration during the experiment, ^{110m}Ag-labeled $AgNO_3$ was added directly to the exposure water from the stock bottle by a peristaltic pump. Vigorous aeration of the exposure water ensured thorough mixing.

Before silver exposure (initial time point on Figs. 1–6) and at 0.5 h, every hour from 1 to 8 h, and at 12, 18, and 24 h of silver exposure, eight fish were sampled for gill and body silver accumulation, via ^{110m}Ag, and gill and body Na^+ and Cl^- uptake, via ^{24}Na and ^{36}Cl (from which whole body Na^+ and Cl^- uptake was calculated; see below for methods of uptake measurements). Care was taken to remove all gill tissue except the pseudobranch, which was left behind. The body was that portion of the rainbow trout remaining after the gills were excised. An additional eight fish were sampled before silver exposure (initial time point on Figs. 1–6), every hour from 1 to 5 h, and at 8, 12, and 24 h of silver exposure for determination of gill Na^+-K^+-ATPase and gill CA activity. All sampled fish (including non-silver-exposed controls) were washed in a concentrated solution containing $AgNO_3$ (9.4 mg/l) and NaCl (2.9 g/l) to remove any loosely bound radioisotopes by displacement. A test with non-silver-exposed trout was performed to evaluate whether the concentrated silver solution itself had any effects on the Na^+-K^+-ATPase or CA activities of the gills. One group ($n = 6$) was subjected to the high silver wash, whereas the other group ($n = 6$) was not washed. The results demonstrated that the washing procedure had no effect on the enzyme activities.

However, once it had been established that the experimental treatment itself did not cause an inhibition of gill CA activity, it was necessary to test whether the effect of silver was on gill CA or on the CA in the erythrocytes trapped in the gills. Because of the small size (0.5–3.0 g) of the fish used in the original study, it was not possible to perfuse the gills free of erythrocytes for the separate analysis of CA activity. However, to address this issue, we employed larger trout (~40 g), treated identically and exposed to the same level of waterborne silver for 1 h, corresponding to the time of almost maximal inhibition of CA activity in the original data set (see RESULTS and Fig. 3*B*). Another group of fish was exposed to control conditions. In both groups of fish, blood samples were taken by caudal puncture and spun down (10,000 *g* for 2 min) to provide an erythrocyte pellet that was immediately frozen in liquid nitrogen and stored at −80°C. The ventral aorta was then cannulated, and the gills were perfused free of erythrocytes using ~30 ml Cortland saline heparanized at 20 IU/ml. The gills were frozen immediately in liquid nitrogen and stored at −80°C. Gills and blood were then assayed separately for CA activity.

In the standard protocol of the original study, after being washed, the fish were killed by an overdose of MS-222 (1 g/l) and blotted dry. The gills were obtained by dissection (nonperfused gills were used in the original study) and either counted for radioactivity for analysis of silver concentration and whole body ion uptake or frozen immediately in liquid nitrogen and stored at −80°C for later analysis of Na^+-K^+-ATPase and CA activity. The bodies were counted for radioactivity for analysis of silver concentration and whole body ion uptake. After radioactivity counting, gills and bodies were weighed.

At each time point, 2 × 5-ml nonfiltered and 2 × 5-ml filtered (Acrodisc polyethersulfone 0.45-μm syringe filters; Pall Gelman Laboratory, Ann Arbor, MI) water samples were taken and counted for ^{110m}Ag radioactivity for analysis of the total and dissolved silver concentration, respectively. The nonfiltered samples were also analyzed for chloride and TOC concentration. Samples (3 × 5 ml) were taken from the silver stock bottle and were acidified with HNO_3 (trace-metal grade; Fisher Scientific, Ottawa, ON, Canada) for determination of ^{110m}Ag radioactivity and total silver concentration to calculate the SA of silver.

Na^+ and Cl^- uptake measurements. For measurement of Na^+ and Cl^- uptake in silver-exposed fish, trout were netted from the exposure water 30 min before each sample time (uptake measurements lasted a total of 30 min) and were placed in a 600-ml Pyrex glass beaker containing 300 ml of continuously aerated water to which the fish had been exposed. Before the uptake measurements, two of these beakers were submerged in the exposure water in the exposure tank to ensure saturation of silver-binding sites on the glass beaker so that the concentration of silver in the water would remain constant during the uptake measurement. For the first uptake measurement, one beaker was removed from the tank, used for the measurement, and at the end

of the measurement rinsed with water and replaced in the exposure tank. For the second uptake measurement, the second beaker was used. For the third measurement, the first beaker was used and so on. Na^+ and Cl^- uptake measurements were also conducted in fish before silver exposure, but for these measurements the fish were netted from the acclimation tank and were placed in a beaker containing acclimation water without added silver. The beaker used for this uptake measurement was not preequilibrated in the silver exposure water. ^{24}Na (6.7 μCi; mean SA 0.004 μCi/μg Na^+; McMaster University Nuclear Reactor, Hamilton, ON, Canada) and ^{36}Cl (2.7 μCi; mean SA 0.0013 μCi/μg Cl^-; ICN Radiochemicals, Irvine, CA) were added to the beaker after the addition of fish. After the start of the measurement (5 min) and again at the completion of the measurement, 2 × 5-ml water samples were taken for measurement of water ^{24}Na and ^{36}Cl radioactivity and of total [Na^+] and [Cl^-]. At the end of the measurement, the fish were sampled as described above and counted for ^{24}Na and ^{36}Cl radioactivity.

Analytical techniques and calculations. During the experiment, fish were exposed to three different isotopes (^{110m}Ag, ^{24}Na, and ^{36}Cl). The isotopes ^{110m}Ag and ^{24}Na are mixed γ- and β-emitters, whereas ^{36}Cl is a pure β-emitter. To determine the ^{110m}Ag radioactivity in the gills, body, and water, the samples were initially counted for the sum of ^{110m}Ag and ^{24}Na by gamma counting. ^{24}Na was then allowed to decay (~2 wk; 15-h half-life), and the samples were counted again by gamma counting to give the ^{110m}Ag radioactivity (256-day half-life). Gamma counting was done according to Hansen et al. (11) using a MINAXI Auto-Gamma 5000 gamma counter (Canberra-Packard, Toronto, ON, Canada) with an energy window of 1,050–2,000 keV. To determine the ^{24}Na radioactivity in the gills, body, and water, the ^{110m}Ag radioactivity, determined after decay of ^{24}Na, was subtracted from the initial sum of ^{24}Na and ^{110m}Ag radioactivity. The ^{24}Na activity was then corrected for decay to a common reference time. The samples were counted a third time by beta counting (LKB-Wallac 1217 Rackbeta Liquid Scintillation Counter, Turku, Finland) to give the ^{110m}Ag plus ^{36}Cl radioactivity (300,000-year half-life). ^{36}Cl radioactivity in the gills, body, and water was obtained by subtracting the ^{110m}Ag radioactivity (after accounting for differences in efficiency of ^{110m}Ag counting by the 2 instruments) from the sum of ^{110m}Ag and ^{36}Cl radioactivity.

The concentrations of silver in the gills, body, and water were calculated by dividing their ^{110m}Ag radioactivities by the SA of silver. The SA of silver was calculated by dividing the ^{110m}Ag radioactivity of the silver stock solution by the total silver concentration, as measured by graphite furnace atomic absorption spectrophotometry (Varian AA-1275 with GTA atomizer; Varian, Mississauga, ON, Canada).

Water chloride was measured using the colorimetric assay of Zall et al. (37), whereas TOC was measured with a Shimadzu TOC-5050A TOC analyzer (Mandel Scientific, Guelph, ON, Canada). TOC is the sum of particulate organic carbon and dissolved organic carbon. Water Na^+ concentration was determined by flame atomic absorption spectrophotometry (Varian AA-1275; Varian).

Uptake rates for Na^+ and Cl^- were determined by monitoring the appearance of radioisotopes in the fish (gill or body) from the water as described above and were calculated as follows:

$$\text{Gill or body ion } (Na^+ \text{ or } Cl^-) \text{ uptake} = CT/(SA \cdot wt \cdot t) \quad (1)$$

where CT is the total counts per minute (cpm) in the gills or body, SA is the measured specific activity of the water, wt is the wet weight of the gills or body (g), and t is the time of exposure (h). The SA of the water was calculated as follows:

$$SA = [(cpm_i/[ion]_i) + (cpm_f/[ion]_f)]/2 \quad (2)$$

where cpm_i represents the cpm per milliliter initially in the water, cpm_f represents the final cpm per milliliter in the water, and $[ion]_i$ and $[ion]_f$ represent the initial and final ion concentrations (nmol/ml) of the water, respectively. Whole body uptake of Na^+ and Cl^- was calculated by adding the gill to the body Na^+ or Cl^- uptake and correcting for whole body weight.

Gills obtained for Na^+-K^+-ATPase and CA activity determination were homogenized in 500 μl SEID buffer (0.5 g sodium deoxycholate in 100 ml SEI; SEI = 150 mM sucrose, 10 mM EDTA, and 50 mM imidazole, pH 7.3) using a Teflon-glass homogenizer. All preparations were kept on ice during the homogenization process. The homogenates were subsequently assayed for Na^+-K^+-ATPase activity using the microplate method of McCormick (21), and gill CA activity was assayed using the electrometric delta pH method, as described by Henry (12). Because nonperfused gills were used in this study, the CA activity measured reflects both gill and erythrocyte activities. Gill Na^+-K^+-ATPase and CA activities were standardized to protein content, which was measured using the Bradford assay (kit no. B6916; Sigma, Oakville, ON, Canada) with BSA as a standard (Sigma).

Statistical analyses. Data are expressed as means ± SE throughout, except for the water silver, Cl^-, and TOC concentrations, where only the mean of two samples is reported. All comparisons were made using a one-way ANOVA followed by a least-significant difference test (SPSS 10 for Windows). Regression analysis was done using SPSS 10 for Windows. A significance level of $P < 0.05$ was used throughout.

RESULTS

Silver accumulation. Gill silver accumulation increased steadily over the first 18 h of silver exposure, reaching a plateau of ~20 nmol/g wet weight by 24 h of exposure (Fig. 1*A*). Body silver accumulation increased continually throughout the exposure, reaching ~1 nmol/g (1,000 pmol/g) by 24 h of silver exposure (Fig. 1*B*). Whole body silver accumulation increased steadily to a plateau of ~2 nmol/g (2,000 pmol/g) at 24 h of silver exposure (Fig. 1*C*). Note the use of three different scales for silver accumulation in Fig. 1.

Na^+ and Cl^- uptake. Whole body uptake represents the rate of uptake across the apical membrane of the gill cells. Initially, there was a large and rapid inhibition of whole body Na^+ uptake. Uptake was reduced from 1,550 ± 132 to 998 ± 67 $nmol \cdot g^{-1} \cdot h^{-1}$ over the 1st h of exposure, a reduction of ~500 $nmol \cdot g^{-1} \cdot h^{-1}$. Apical uptake remained at this rate for the subsequent 2 h and was followed by another progressive inhibition over the next 21 h to only 137 ± 27 $nmol \cdot g^{-1} \cdot h^{-1}$ (Fig. 2*A*). Body Na^+ uptake represents the rate of basolateral export from the gill to the body compartment and exhibited an almost identical biphasic trend to the inhibition of apical uptake (Fig. 2*A*). This result indicates that it is the movement of Na^+ across the apical membrane that is the rate-limiting step in the movement of Na^+ across the entire epithelium.

The time course of inhibition of whole body Cl^- uptake (apical uptake) was similar to the first phase of the time course of Na^+ uptake inhibition, with uptake rapidly inhibited over the 1st h of silver exposure but reaching a plateau by 2 h of exposure. The degree of whole body Cl^- uptake inhibition was also similar, with uptake reduced by ~500 $nmol \cdot g^{-1} \cdot h^{-1}$ over the 1st h of exposure (from 522 ± 166 to 41 ± 10 $nmol \cdot g^{-1} \cdot h^{-1}$; Fig. 2). The pattern of inhibition of body Cl^- uptake (basolateral export) was very similar to the pattern of whole body Cl^- uptake inhibition (Fig. 2*B*), again, indicating that the apical entry step is rate limiting.

Gill Na^+-K^+-ATPase and CA activity. Gill Na^+-K^+-ATPase activity was approximately stable over the first 6 h of silver exposure, with a tendency to decline thereafter. How-

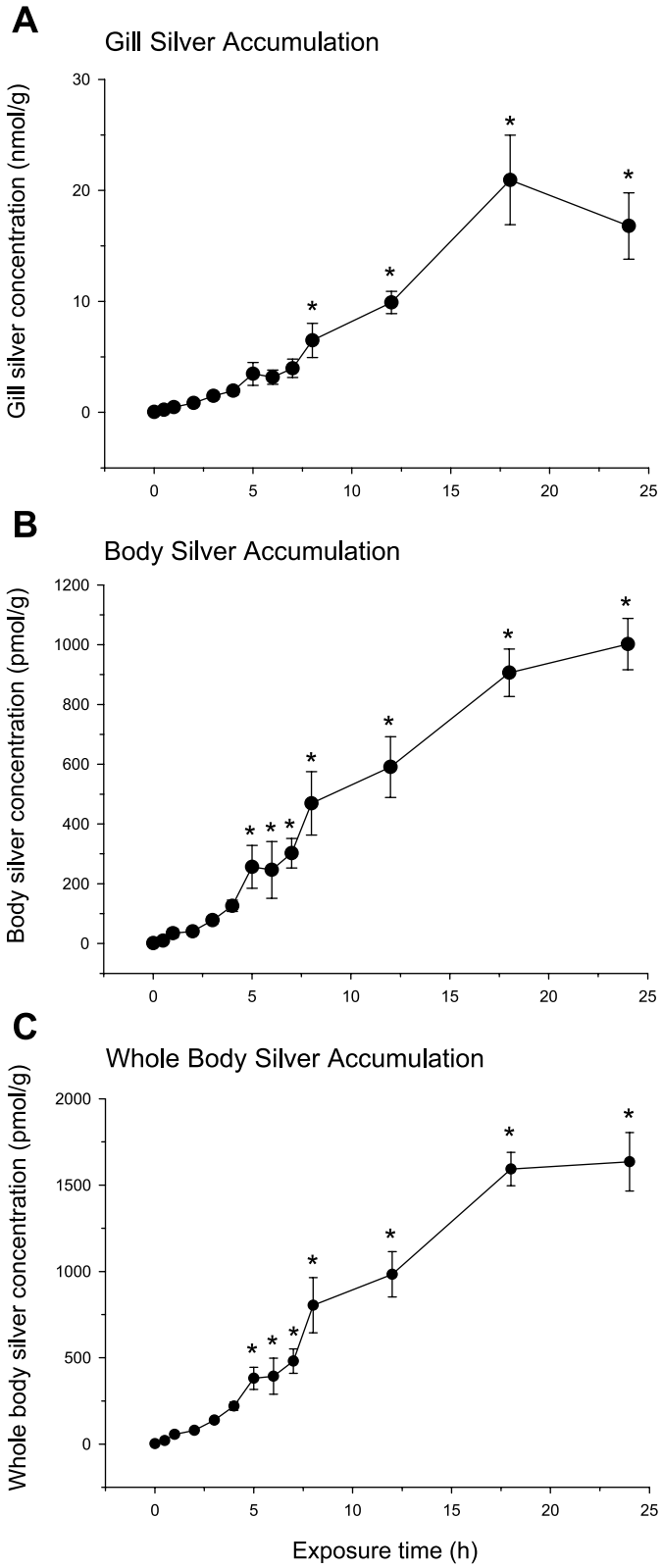


Fig. 1. Time course of silver accumulation in the gills (*A*), body (*B*), and whole body (*C*) of rainbow trout exposed to 4.0×10^{-8} M (4.3 μg/l) total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. The body was that portion of the rainbow trout remaining after the gills were excised, and the whole body is the sum of the gills and body. Values are means ± SE ($n = 8$ at each time point). *Significant differences from the initial value [before silver exposure; ANOVA followed by least significant difference (LSD) test; $P < 0.05$].

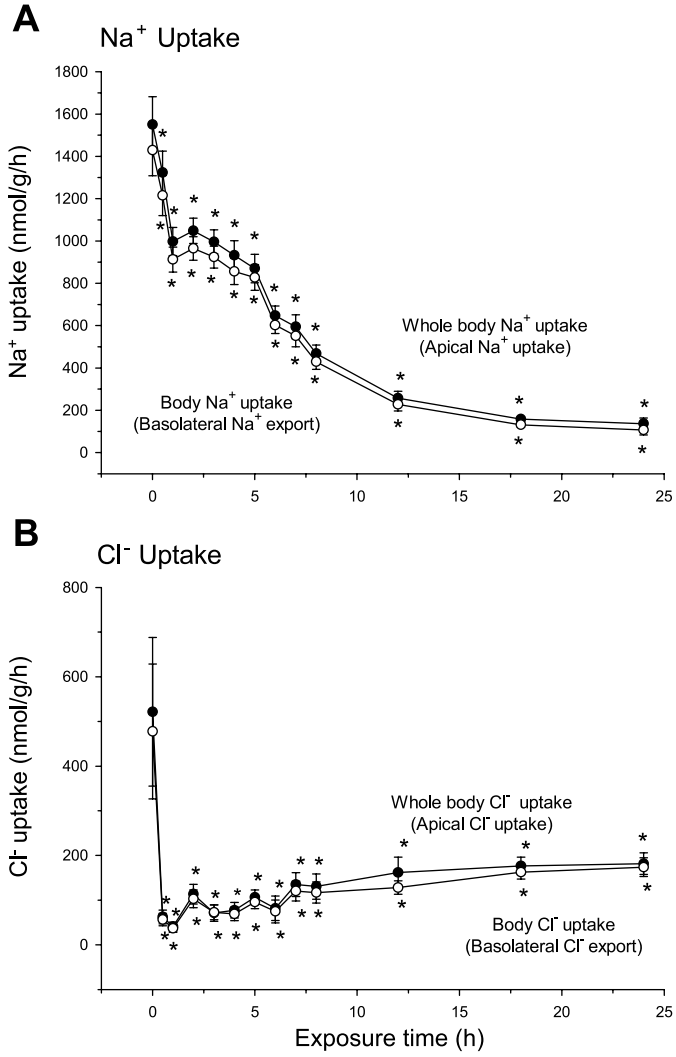


Fig. 2. Time course of inhibition of whole body Na^+ uptake (*A*; ●) and body Na^+ uptake (*A*; ○) and whole body Cl^- uptake (*B*; ●) and body Cl^- uptake (*B*; ○) of rainbow trout exposed to 4.0×10^{-8} M (4.3 μg/l) total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. Values are means ± SE ($n = 8$ at each time point). *Significant differences from the initial value (before silver exposure; ANOVA followed by LSD test; $P < 0.05$).

ever, it was not significantly inhibited until 24 h of silver exposure. At this time, activity was reduced by 41% (from 2.7 ± 0.5 before silver exposure to 1.6 ± 0.2 μmol ADP·mg protein^{-1}·h^{-1} at 24 h of exposure; Fig. 3*A*).

Gill CA activity decreased rapidly over the first 2 h of silver exposure and remained inhibited over the following 22 h of exposure, apart from an apparent increase in activity between 5 and 8 h of exposure (Fig. 3*B*). The possible causes of this transient increase in activity are addressed in the DISCUSSION. Inhibition of CA activity was first significant by 1 h of silver exposure, reaching ~30% (from 1,421 ± 119 before silver exposure to 953 ± 100 μmol CO_2·mg protein^{-1}·min^{-1} at 1 h of silver exposure). Maximal inhibition of the enzyme occurred at 24 h of exposure, with activity inhibited by 42% (to 818 ± 146 μmol CO_2·mg protein^{-1}·min^{-1}; Fig. 3*B*).

The experiment with larger trout exposed to the same level of silver for 1 h clearly demonstrated that the inhibitory effect

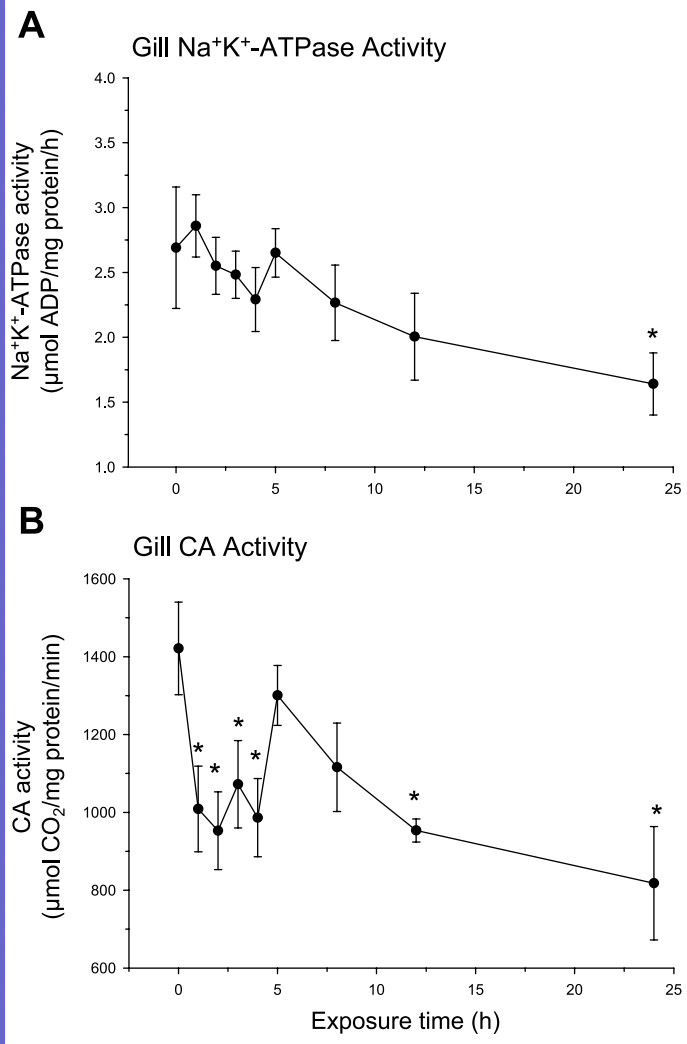


Fig. 3. Time course of inhibition of gill Na^+-K^+-ATPase activity (*A*) and gill carbonic anhydrase (CA) activity (*B*) of rainbow trout exposed to 4.0×10^{-8} M (4.3 μg/l) total silver as $AgNO_3$ for 24 h in synthetic soft water under flow-through conditions. CA activity was measured in nonperfused gills. Values are means ± SE ($n = 8$ fish at each time point in *A*, except the 5-h time point where $n = 7$; $n = 4$ at each time point in *B*, except the 1- and 12-h time points where $n = 3$). *Significant differences from the initial value (before silver exposure; ANOVA followed by LSD test; $P < 0.05$).

of silver was exerted specifically on gill CA activity. In gills perfused free of blood, CA activity was 1,422 ± 75 and 779 ± 114 μmol CO_2·mg $protein^{-1}$·min^{-1} in the absence and presence of silver (1 h exposure), respectively, a significant 45% reduction. Erythrocyte CA activity was 440 ± 71 and 568 ± 39 μmol CO_2·mg $protein^{-1}$·min^{-1} in the absence and presence of silver, respectively (i.e., unchanged). Thus, per unit protein, gill CA activity was about threefold higher than erythrocyte activity.

Na^+ uptake and enzyme activities vs. gill silver accumulation. Na^+ uptake (Fig. 2*A*) appeared to vary inversely with gill silver accumulation (Fig. 1*A*). Indeed, plotting values of whole body Na^+ uptake as a function of corresponding values of gill silver accumulation revealed a significant negative but curvilinear relationship ($r^2 = 0.97$, $P < 0.05$; Fig. 4*A*).

Gill Na^+-K^+-ATPase activity (Fig. 3*A*) also appeared to vary inversely with gill silver accumulation (Fig. 1*A*). Regression analysis revealed a significant negative linear relationship between these two parameters ($r^2 = 0.85$, $P < 0.05$; Fig. 4*B*).

There was no significant relationship between gill silver accumulation and CA activity ($r^2 = 0.3$, $P > 0.05$; Fig. 4*C*).

Water chemistry changes. Although the nominal exposure concentration was 3.3×10^{-8} M (3.6 μg/l), the mean measured total and dissolved water silver concentrations over the 24-h flow-through exposure period were 4.0×10^{-8} M (4.3 μg/l) and 2.8×10^{-8} M (3.0 μg/l), respectively. The water total silver concentration remained relatively constant over the 24-h exposure period, apart from an increase in the concentration of 47% over the first half-hour of exposure (from 3.3×10^{-8} M to 4.9×10^{-8} M; 3.6–5.3 μg/l; Fig. 5*A*). Other than a small initial decrease of 23% over the 1st h of silver exposure (from 2.9×10^{-8} M to 2.2×10^{-8} M; 3.1–2.4 μg/l), the water dissolved silver concentration also remained relatively constant over the exposure period at $\sim 2.8 \times 10^{-8}$ M (3.0 μg/l; Fig. 5*A*).

There was a small increase in the water Cl^- concentration of 13% over the first 2 h of silver exposure (from 122.6 to 138.0 μM). The concentration remained elevated until 8 h of exposure but returned to the initial level by 12 h of silver exposure (Fig. 5*B*).

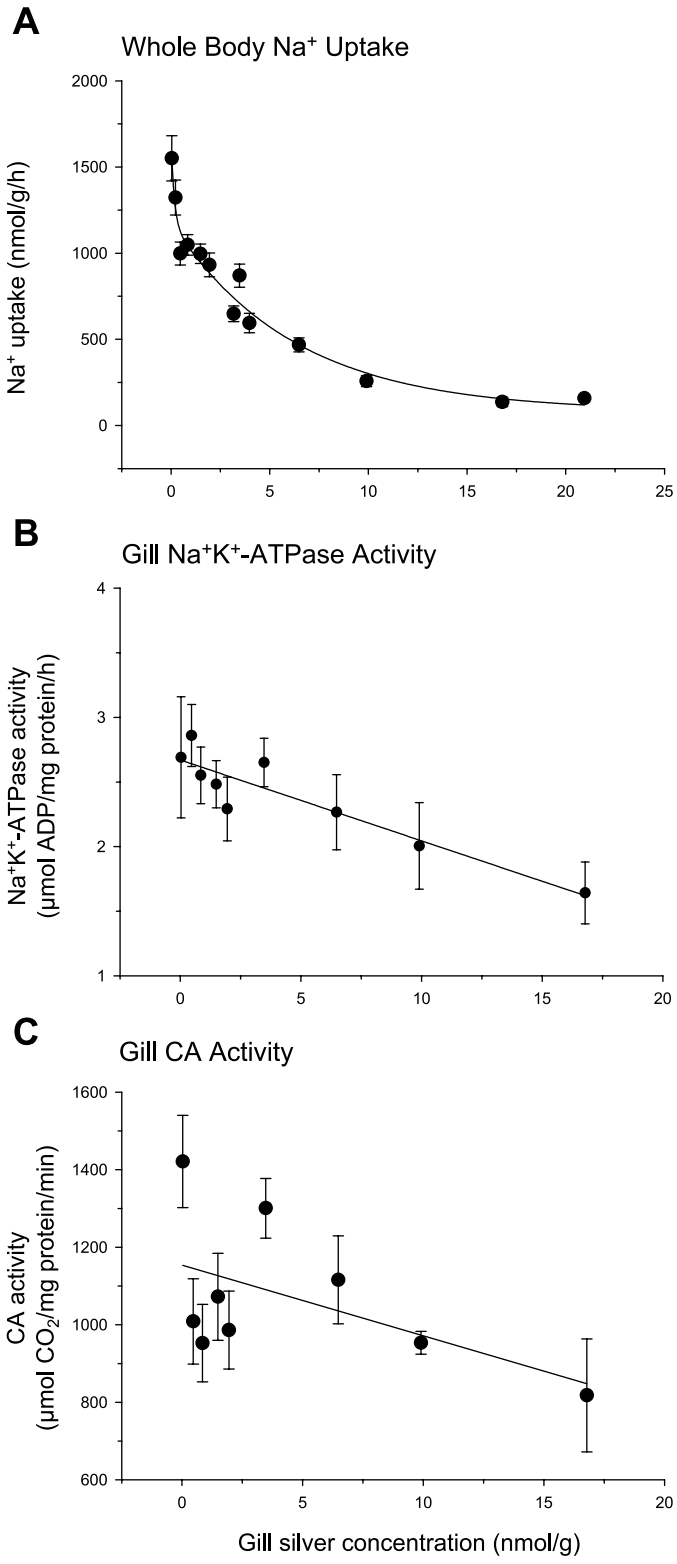
The TOC content of the water increased by 67% over the first 4 h of silver exposure (from 1.2 to 2.0 mg C/l). By 5 h of exposure and for the remaining 19 h of exposure, the TOC content was not different from the initial value (Fig. 5*C*).

DISCUSSION

The mechanism by which silver inhibits Na^+ and Cl^- uptake at the gills of rainbow trout. Na^+ uptake (Fig. 2*A*) declined very clearly before Na^+-K^+-ATPase activity (Fig. 3*A*) during flow-through exposure of rainbow trout to 4×10^{-8} M (4.3 μg/l) total silver. We have demonstrated a similar trend during static exposure of rainbow trout to a slightly lower silver concentration (25). Furthermore, in both studies, the rate of apical Na^+ uptake was clearly the rate-limiting step in transepithelial Na^+ transport (Fig. 2*A*), and the present results demonstrate the same to be true of transepithelial Cl^- transport (Fig. 2*B*).

The present study has shown that the inhibition of CA activity in whole gills by waterborne silver was the result of inhibition of the gill-specific CA, because this also occurred in gills perfused free of blood, whereas there was no inhibition of erythrocytic CA activity. Furthermore, it provides three pieces of evidence that the early decrease in Na^+ uptake is the result of an inhibition of gill CA activity. First, the time courses of inhibition of whole body Na^+ uptake rate (apical uptake) and body Na^+ uptake rate (basolateral export) were identical (Fig. 2*A*), and the time courses of inhibition of whole body Cl^- uptake rate (apical uptake) and body Cl^- uptake rate (basolateral export) were identical (Fig. 2*B*). An identical time course implies that inhibition is exerted on the rate-limiting steps in the movement of these ions across the whole gill epithelium, which are the apical entry, rather than the basolateral export, steps. This implicates CA inhibition in the early decline in Na^+ uptake because the activity of CA provides H^+ and HCO_3^- for the apical uptake of both Na^+ and Cl^-, respectively. Second, the time course and degree of Cl^- uptake inhibition were quantitatively similar to the time course and degree of the first phase of Na^+ uptake inhibition (Fig. 2, *A* and *B*). Inhibition of

CA would be expected to cause a simultaneous and equal inhibition of Na^+ and Cl^- uptake because, as outlined above, the activity of CA provides protons to the H^+-ATPase for apical uptake of Na^+ and HCO_3^- for apical exchange with Cl^- (13, 18). Third, CA activity was inhibited by ~30% by 1 h of silver exposure (Fig. 3*B*), at a time when Na^+ uptake and Cl^- uptake were decreasing (Fig. 2, *A* and *B*), but Na^+-K^+-ATPase activity remained unaffected (Fig. 3*A*).



Morgan et al. (24) saw a similar degree of inhibition (~30%) of CA activity during exposure of rainbow trout to ~10^{-7} M silver and a simultaneous and equal inhibition of Na^+ and Cl^- uptake but only measured CA activity at 48 h. In our study, CA activity was almost maximally inhibited by 1 h of exposure and remained inhibited at 24 h of exposure, apart from an apparent recovery at 5–8 h (Fig. 3*B*). This suggests that the degree of inhibition of CA activity early in the exposure of Morgan et al. would be similar to the degree of inhibition reported at 48 h and could explain the simultaneous and equal decreases of Na^+ and Cl^- uptake, which were similarly noted in their study. It should be noted, however, that in the study of Morgan et al. and in the present study, we cannot eliminate the possibility of a direct effect of silver on the Na^+ channel, H^+-ATPase, and the Cl^-/HCO_3^- exchanger, and hence, on Na^+ and Cl^- uptake rates.

The mechanism by which silver inhibits CA is not known, but the often striking similarities between copper and silver in terms of physiology and toxicology (15) suggest that silver would inhibit CA by the same or a similar mechanism by which copper inhibits the enzyme. Studies with human CA II have shown that copper inhibits the enzyme by binding to His64, an amino acid located in the active site of the enzyme (33, 34). Binding of copper to His64 blocks its role in the release of a proton from the active site, preventing regeneration of the active form of the enzyme. Properties exhibited by the CA of gills of rainbow trout are similar to those exhibited by human CA II (14), suggesting that copper (and potentially silver) would inhibit gill CA by the same mechanism by which it inhibits human CA II.

It is problematic whether the transient recovery of CA activity observed at the 5- and 8-h sample times (Fig. 3*B*) is real or merely reflects variability in the data, because at these times, the activity levels were intermediate, i.e., not significantly different from either the control level or the inhibited levels. Notably, because of the high CA activity levels in gill tissue, the tissue extract must be subjected to manyfold dilution in the CA assay. Because of this dilution, it is possible that silver may dissociate to some extent from its binding sites on the enzyme. Therefore, if anything, the assay will tend to underestimate, rather than overestimate, the degree of CA inhibition by silver, although we are aware of no reason why this effect might have been greater in the 5- and 8-h samples.

Although the results of this study implicate CA inhibition in the early decline in Na^+ uptake (*phase 1*; Fig. 2*A*), the decline in Na^+ uptake that occurred later (*phase 2*; Fig. 2*A*) is likely because of Na^+-K^+-ATPase inhibition. Because Na^+-K^+-ATPase is located on the basolateral membrane, it would take

Fig. 4. Plot of whole body Na^+ uptake rate (*A*), gill Na^+-K^+-ATPase activity (*B*), and gill CA activity (*C*) as a function of gill silver concentration. Whole body Na^+ uptake rate (nmol·g^{-1}·h^{-1}) and gill Na^+-K^+-ATPase activity (μmol ADP·mg $protein^{-1}$·h^{-1}) were inversely correlated to the gill silver concentration (nmol/g) by the following equations: whole body Na^+ uptake rate = 81.05 + 468.82·$e^{-6.23 \cdot \text{gill silver concentration}}$ + 1,099.92·$e^{-0.16 \cdot \text{gill silver concentration}}$, slope significantly different from 0 ($P < 0.05$), $r^2 = 0.97$; gill Na^+-K^+-ATPase activity = 2.67 − 0.06·gill silver concentration, slope significantly different from 0 ($P < 0.05$), $r^2 = 0.85$. There was no significant relationship between gill silver accumulation and CA activity ($r^2 = 0.3$, $P > 0.05$).

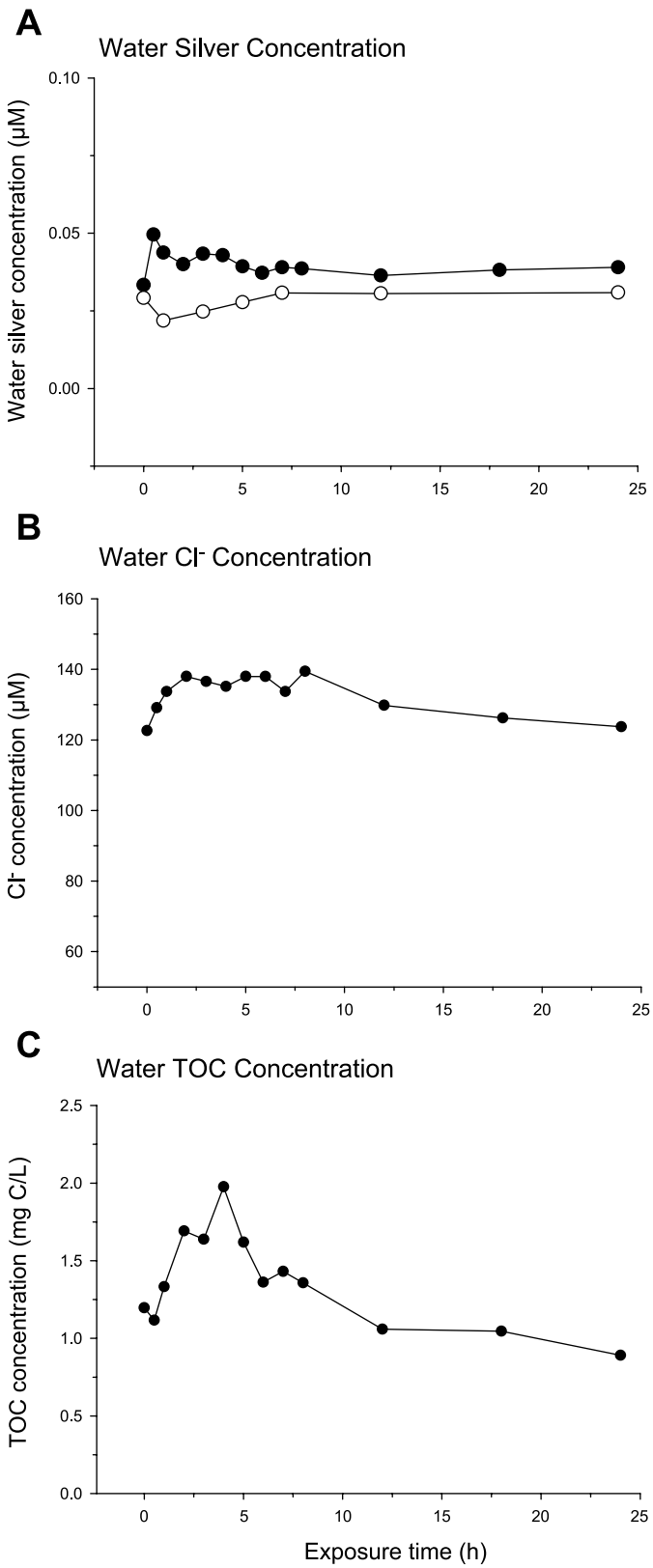


Fig. 5. Measured water total (●) and dissolved (○) silver (*A*), chloride (*B*), and total organic carbon (TOC) concentrations (*C*) over 24 h of flow-through exposure of rainbow trout to $AgNO_3$ in synthetic soft water. The dissolved silver concentration is the concentration of silver in the water after passage through a 0.45-μm filter. Values are means (n = 2 at each time point).

some time for silver to reach and inhibit the enzyme. However, Na^+-K^+-ATPase activity was not significantly inhibited until 24 h of silver exposure yet silver had clearly reached the basolateral membrane after only a few hours, since it was appearing in the body compartment (Fig. 1*B*; also see Fig. 6). This suggests that there may be a kinetic limitation affecting the time course of Na^+-K^+-ATPase inhibition. Nevertheless, Na^+-K^+-ATPase activity was clearly decreasing during *phase 2* of Na^+ uptake inhibition (Figs. 2*A* and 3*A*). Interestingly, although both Na^+ and Cl^- uptake were reduced to a similar absolute extent (~500 nmol·g^{-1}·h^{-1}) over the 1st h of silver exposure, the reductions represented a 36 and 92% inhibition in Na^+ and Cl^- uptake, respectively. The immediate, almost complete inhibition of Cl^- uptake suggests that, in contrast to Na^+ uptake, inhibition of Na^+-K^+-ATPase does not contribute to the decline in Cl^- uptake observed during silver exposure.

Time course of gill silver accumulation during flow-through silver exposure. Our earlier study suggested that the pattern of rapid peak and following decline in gill silver accumulation observed in rainbow trout during static silver exposure (25) could be the result of a decrease in the bioavailability of silver. Because the exposure water was not continually replaced, there was a substantial accumulation of organic matter (300% increase in TOC) and a 70% fall in the dissolved fraction of silver in the water. This likely caused the observed fall in apical silver uptake and, together with constant basolateral export of silver, the rapid peak and following decline in gill silver accumulation. In contrast, during the flow-through exposure of the present study, there were only initial, temporary changes in the chemistry of the exposure water over time. The dissolved silver concentration decreased by <23% for the first few hours of exposure, whereas the Cl^- and TOC concentrations increased, but initial levels were reestablished with continued flow-through exposure (Fig. 5). In consequence, the bioavailability of silver remained relatively constant, and there was no pattern of a peak and decline in gill silver accumulation; rather, gill silver levels increased steadily to a plateau with time (Fig. 1*A*). This pattern of accumulation is similar to that which has been reported for other metals, such as copper, cadmium, and zinc (1, 8, 9, 16, 19, 29).

By employing lines of best fit to the whole body (apical silver uptake; Fig. 1*C*) and body (basolateral silver export; Fig. 1*B*) silver accumulation data, we were able to calculate the actual rates of apical silver uptake and basolateral silver export from the gills (Fig. 6). This was done by first using the equation of the line of best fit to determine silver accumulation at each time point and then dividing the change in silver accumulation over each time interval by the length of the time interval. The calculated rates were expressed as rates per whole body weight and were plotted at the midpoint of the time interval.

This analysis demonstrated that there was no early decline in apical silver uptake rate but rather a steady increase that did not end until 10 h of silver exposure (Fig. 6). The result was surprising because silver is thought to enter gill cells by the same mechanism as Na^+ (4), and Na^+ uptake was declining over this period (Fig. 2*A*) due first to the inhibition of CA (Fig. 3*A*) and later perhaps to the inhibition of Na^+-K^+-ATPase (Fig. 3*B*). AgCl entry (36) was unlikely to be important in this pattern, since speciation calculations using MINEQL+ (31) showed that AgCl was <3% of the total silver concentration. Rather, the result suggests that Ag^+ entry via the apical Na^+

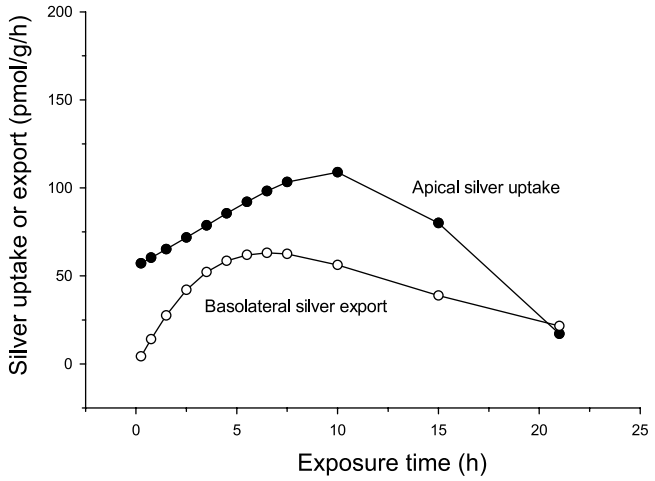


Fig. 6. Apical silver uptake (●) and basolateral silver export (○) across the gills of rainbow trout over 24 h of flow-through exposure to 4.0×10^{-8} M (4.3 μg/l) total silver as $AgNO_3$ in synthetic soft water. Silver uptake was determined by first calculating silver accumulation at each time point using the equation of the line of best fit for the whole body and body silver accumulation data (adjusted to whole body wt) and then dividing the change in silver accumulation over each time interval by the length of the time interval.

channel may not depend on the same driving force for entry as Na^+. Alternatively or additionally, if multiple pathways exist for apical silver uptake at the gill cells (in addition to the Na^+ channel), then silver uptake may not decrease unless more than one pathway is disrupted. Bury and Wood (4) demonstrated that approximately one-third of silver uptake continued in the presence of phenamil and bafilomycin A_1 (Na^+ channel and V-type ATPase blocker, respectively) or high levels of competing Na^+ in the water, suggesting that, in addition to the proton-coupled Na^+ channel, other apical uptake pathways for silver exist. Recent evidence that silver may replace copper in some transport processes in bacteria (32) suggests that the other apical uptake pathway for silver may involve a copper transporter. The Ctr-type copper transporter, although as of yet unidentified in teleost fish gills, is a potential mechanism for apical copper (and silver) uptake in rainbow trout because this transporter has been documented in species from yeast to humans (5). Recently, Grosell and Wood (10) identified two pathways of apical copper uptake in freshwater trout gills, an Na^+-sensitive, phenamil-sensitive, bafilomycin-sensitive pathway (likely the Na^+ channel) and an Na^+-insensitive pathway, possibly Ctr-type transporter. By analogy, these pathways could both contribute to apical silver uptake.

Toxicological implications. The difference in the time course of Na^+ uptake inhibition between static (transient inhibition; see Ref. 25) and flow-through exposures (rapid, progressive, and permanent inhibition; Fig. 2*A*) has important toxicological implications. Because static exposures show recovery of gill function with continued silver exposure, these exposures may underestimate the toxic effects of silver. In fact, Nebeker et al. (26) and Erickson et al. (7) reported that flow-through LC_{50} values were lower than static median lethal concentration (LC_{50}) values for both rainbow trout and fathead minnow (i.e., silver was more toxic under flow-through conditions). Those workers attributed the differences to the accumulation of organic matter in the static exposure system, a conclusion supported by the present study. Clearly, exposure conditions are critically important when assessing the risk of silver to freshwater fish in natural waters, and results of flow-through tests probably are more representative of natural freshwater lotic habitats.

In both this and our earlier static study (25), strong negative relationships existed between gill silver accumulation and the inhibition of whole body Na^+ uptake (Fig. 4*A*). A negative relationship also existed between gill silver accumulation and Na^+-K^+-ATPase activity (Fig. 4*B*). These observations suggest that there may also be a relationship between gill silver accumulation and mortality, because inhibition of Na^+ uptake and Na^+-K^+-ATPase activity are the causes of eventual mortality in rainbow trout (24, 35, 36). These findings, together with the observation that an equilibrium level of silver accumulation at the gills is achieved over time during flow-through exposure (Fig. 1*A*), lend support to the new Biotic Ligand Model (BLM) approach for predicting metal toxicity (22, 27, 30). The BLM attempts to predict mortality in different water qualities by predicting gill metal accumulation (27). Because silver accumulation on the gills appeared to constantly change over time (4, 15, 23, 25, 36) and silver accumulation on the gills has not yet been satisfactorily correlated with mortality, the present silver versions of the BLM (27) have not yet been fully accepted by regulatory agencies. Although the results of the present study suggest a relationship between gill silver accumulation and mortality and provide a mechanism, definitive evidence would come from experiments establishing a direct relationship between short-term gill silver accumulation and eventual mortality. For example, MacRae et al. (20) were able to demonstrate a relationship between gill copper accumulation at 24 h and mortality of juvenile rainbow trout after 120 h of copper exposure, a finding that was instrumental in the acceptance of the copper version of the BLM (30). Demonstration of such a relationship for silver, together with the results presented here, would help promote incorporation of the silver BLM into regulatory practice.

Perspectives

By the simple technique of assaying gill and body radiotracer uptake separately, we have been able to separate apical from basolateral events in gill transport. Returning now to the two possible interpretations raised in the Introduction, our results strongly support the conclusion that, when the bioavailability of silver is held relatively constant by preventing organic matter buildup, the pattern of peak and decline in gill silver accumulation (25) is prevented, and gill silver burden equilibrates at a plateau. Furthermore, under these conditions, silver exposure does cause a rapid, equimolar inhibition of both Na^+ and Cl^- uptake by an apical event associated with the rapid inhibition of CA, long before the inhibition of basolateral Na^+-K^+-ATPase occurs. However, the apical entry of silver itself is not inhibited. Previous research that attributed silver toxicity and blockade of Na^+ uptake entirely to the inhibition of basolateral Na^+-K^+-ATPase (24) overlooked this important early step. These results call for a renewed focus on apical processes as key regulatory points for ion transport, particularly the role of CA, and on the possible mechanisms by which silver and other metals are taken up across the apical membranes of the gill cells.

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GRANTS

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