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The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) were exposed to 2 and 10 μg l^{-1} silver (as $AgNO_3$) for up to 75 h in moderately hard freshwater. At 10 μg l^{-1} total Ag, branchial Na^+ and Cl^- influxes were inhibited by over 50% immediately and by almost 100% at 8 h, and showed no signs of recovery over the duration of the experiment. Na^+ and Cl^- effluxes were much less affected. These changes in unidirectional fluxes resulted in a large net loss of both Na^+ and Cl^- across the gills and a significant decrease in plasma [Na^+] and [Cl^-]. The effects of exposure to 2 μg l^{-1} Ag on Na^+ and Cl^- transport were generally similar to those at 10 μg l^{-1}, but were of a lesser magnitude. Unidirectional Na^+ fluxes recovered immediately following removal of silver, after 48 h exposure to 2 μg l^{-1}. Michaelis–Menten kinetic analysis demonstrated that the maximal rate of Na^+ influx (J_{max}) was significantly reduced after 48 h exposure to 2 μg l^{-1} Ag, whereas the affinity of the transport sites for Na^+ ($1/K_m$) was unaffected, indicating that the inhibition of Na^+ influx by silver was of a non-competitive nature. Fish exposed to 10 μg l^{-1} Ag for 48 h also had significantly lower activities of the branchial enzymes Na^+/K^+ ATPase (85% inhibition) and carbonic anhydrase (28% inhibition). The results of this study suggest that a disturbance of branchial ionoregulation, as a result of inhibition of branchial enzymes involved in ion transport, is the principal mechanism of the physiological toxicity of silver nitrate to freshwater fish.

Keywords: Silver nitrate; Physiological toxicity; Osmoregulation; Na^+ and Cl^- transport; Rainbow trout; *Oncorhynchus mykiss*

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1. Introduction

A number of studies have demonstrated that silver nitrate is highly toxic to freshwater fish. Davies et al. (1978) recorded mean 96 h LC_{50} values for rainbow trout (*Oncorhynchus mykiss*) of 0.06 μM and 0.12 μM (6.5 μg l^{-1} and 13.0 μg l^{-1}) in soft and hard water, respectively. Similar values were obtained for rainbow trout by Nebeker et al. (1983) and Hogstrand et al. (1996). The 96 h LC_{50} of silver nitrate for bluegill (*Lepomis macrochirus*), however, was somewhat higher at 0.56 μM (60 μg l^{-1}) (Buccafusco et al., 1981). The toxicity of silver nitrate has been attributed to the presence of Ag^+, the 'free' silver ion; other dissolved forms of silver such as $AgCl_n^-$ and $Ag(S_2O_3)^-$ are much less toxic (LeBlanc et al., 1984; Hogstrand et al., 1997; Wood et al., 1996a, Wood et al., 1996b). Ag^+ is readily complexed by a number of ligands in freshwater such as Cl^-, S^{2-} and dissolved organic carbon (DOC) (Janes and Playle, 1995), and even under laboratory conditions Ag^+ may account for less than 35% of the total silver present in a solution of silver nitrate (Wood et al., 1996a). On this basis, Ag^+ is more toxic than Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+}.

Silver is discharged into the aquatic environment from a number of domestic, agricultural and industrial sources, primarily via sewage treatment plants. The silver in aquatic discharges may occur in a variety of chemical forms, though the concentration of free ionic Ag^+ is probably very low in most circumstances. For example, photoprocessing effluent is almost entirely in the form of silver thiosulphate ($Ag_2S_2O_3$), which, though soluble, is highly stable and produces negligible Ag^+ in solution (LeBlanc et al., 1984). The environmental concentration of Ag^+ is likely to be further reduced by the scavenging of Ag^+ by negatively charged ligands. However, no suitable method has been developed for measuring very low concentrations of Ag^+ (below 0.2–0.5 μg l^{-1}; Schildtkraut et al., 1995). For environmental monitoring a conservative approach has therefore been adopted by regulatory agencies whereby toxicity data obtained from laboratory studies of $AgNO_3$ (i.e. free Ag^+) are applied to total recoverable silver concentrations (e.g. Warrington, 1995).

Recent work suggested that the principal toxic effect of Ag^+ in freshwater fish is a severe ionoregulatory disturbance (Wood et al., 1996a). Branchial Na^+ influx in adult rainbow trout was inhibited by approximately 40% immediately following exposure to 10 μg l^{-1} silver (as $AgNO_3$), and both plasma [Na^+] and [Cl^-] decreased steadily over 6 days. A significant decrease in relative plasma volume and an increase in haematocrit and plasma protein concentration were also observed, leading the investigators to suggest that 'Ag^+ interferes with the mechanism(s) of net Na^+ and Cl^- uptake at the gills, and causes death by secondary fluid volume disturbance, haemoconcentration, and eventual cardiovascular collapse'.

However, the above study did not measure the effects of silver nitrate on Na^+ efflux and net flux or on Cl^- fluxes, nor was the mechanistic origin of the ionoregulatory disturbance ascertained. The aims of the present study, therefore, were to examine more closely the effects of silver nitrate on branchial unidirectional Na^+ and Cl^- fluxes, and to investigate the mechanisms by which any disturbance occurred. Specifically, the effect of Ag^+ on the activity of two branchial enzymes that

may be involved in both Na^+ and Cl^- uptake—carbonic anhydrase (CA) and Na^+/K^+ ATPase—was studied. A further aim of the present study was to compare the mechanisms of Ag^+ toxicity with those of other metals which are known to disrupt branchial osmoregulation in freshwater fish, such as cadmium, copper and zinc. The present experiments exposed fish to two concentrations of silver (as $AgNO_3$), 2 and 10 μg l^{-1}. The higher of these concentrations was chosen as this value is close to the 7 day LC_{50} for rainbow trout measured under similar conditions of aquatic chemistry (9.4 μg l^{-1}; Hogstrand et al., 1996) and has been shown to cause a suite of physiological disturbances, particularly within the circulatory system and to osmoregulation (Wood et al., 1996a), and 2 μg l^{-1} was used as a comparison to determine whether similar physiological effects would be seen at a lower concentration of silver.

2. Materials and methods

2.1. Experimental animals

Rainbow trout of 200–300 g were obtained from Spring Valley Trout Farm, Petersburg, Ontario. These were maintained indoors in circular glass-fibre tanks (500 l capacity) supplied with flowing, aerated, dechlorinated Hamilton tapwater. The approximate composition of the water was (mM) $[Na^+]$ 0.5, $[Cl^-]$ 0.7, $[Ca^{2+}]$ 1.0, $[Mg^{2+}]$ 0.2, $[K^+]$ 0.05; titratable alkalinity to approximately pH 4.0, 1.0; pH 7.8–8.0. The fish were fed ad libitum on a commercial trout diet until 3 days before the experiments. All experiments were carried out at ambient temperature (11–16°C).

2.2. The effects of silver nitrate on unidirectional Na^+ and Cl^- fluxes

Fish were transferred to individual black Perspex boxes (3 l capacity) supplied with flowing, aerated water and left to acclimate to the chambers for 48 h. The fish were exposed to 2 or 10 μg l^{-1} total silver (as $AgNO_3$) in a flow-through system for 75 h by addition of 32 mg l^{-1} $AgN0_3$ solution (in a light-shielded bottle) to a well-mixed header tank via a peristaltic pump. The unidirectional fluxes of Na^+ and Cl^- were measured using radioisotopes over 3 h under control conditions (i.e. post-acclimation but pre-exposure) and after 0–3, 8–11, 24–27, 48–51 and 72–75 h of silver exposure. For the duration of each measurement, the flow of water to the boxes was turned off and 2 μCi l^{-1} of ^{22}Na and 5 μCi l^{-1} of ^{36}Cl were added and allowed to mix for 15 min. After equilibration and again after 3 h, a 50 ml water sample was taken and analysed for ^{22}Na and ^{36}Cl radioactivity, Na^+ and Cl^- concentration and total silver concentration (see below). At the end of the experiment a blood sample was obtained from each fish by caudal puncture. The blood was centrifuged at 9000*g* for 2 min and the plasma was removed and stored at −20°C for later analysis of Na^+ and Cl^- concentrations. As the fish were not fitted with indwelling catheters, blood samples were not available from these fish before

the silver exposure. Therefore a second group of fish were placed in boxes and exposed to normal freshwater only for a total of 123 h (48 h acclimation plus 75 h 'exposure' period) and plasma samples were obtained as above to act as controls.

A similar experiment was carried out to determine whether the effects of silver on Na^+ fluxes were reversible, i.e. whether any recovery occurred on removal of the silver. Fish were acclimated to the experimental chambers for 48 h and then exposed to 2 $\mu g\ l^{-1}$ silver as above, for 48 h. The fish were then returned to clean (i.e. non-silver-contaminated) water for a further 48 h ('recovery' period). Unidirectional Na^+ fluxes were measured under control conditions, at 8–10 h and 48–50 h of silver exposure, and at 2–4 h, 10–12 h, 24–26 h and 48–50 h of 'recovery'.

Water samples taken at the beginning and end of flux determinations and terminal plasma samples were analysed for ^{22}Na radioactivity by γ-counting (Canberra-Packard, Meridan, CT; Miniaxi γ), for ^{36}Cl radioactivity by β-scintillation counting (LKB Turka, Finland, Rackbeta), for $[Na^+]$ by atomic absorption spectrophotometry (Varian, Palo Alto, CA, USA; AA1275), and for $[Cl^-]$ by the mercuric thiocyanate method (Zall et al., 1956). Water samples were analysed for total [Ag] by graphite furnace atomic absorption spectrophotometry (Varian AA1275 with GTA-95 atomizer). The unidirectional fluxes of Na^+ and Cl^- ($\mu mol\ kg^{-1}\ h^{-1}$) were calculated as follows:

$$\text{Influx, } J_{in}^{ion} = \frac{(cpm_i ion - cpm_f ion)}{MSA} \cdot \frac{V}{W \cdot t}$$

where cpm_i ion and cpm_f ion are respectively the initial and final radioactivity (counts $min^{-1}\ l^{-1}$) of ^{22}Na or ^{36}Cl, MSA is the mean specific activity of Na^+ or Cl^- (see below), V is the volume of the flux chamber (l), W is the weight of fish (kg), and t is time (h). The mean specific activity was calculated from

$$MSA = \left(\frac{cpm_i ion}{[ion]_i} + \frac{cpm_f ion}{[ion]_f} \right) / 2$$

where $[ion]_i$ and $[ion]_f$ are the initial and final concentration of Na^+ or Cl^- (μM). Repeated radioisotope flux determinations may lead to significant internal accumulation of radioactivity. The efflux of this radioactivity during subsequent flux measurements ('backflux') would tend to reduce the calculated J_{in}^{ion} below the true values. Correction of J_{in}^{ion} for radioisotopic backflux was therefore made using the equation of Maetz (1956), when the internal specific activity of the terminal plasma sample was 10% or more of the external specific activity:

$$\text{Corrected } J_{in}^{ion} = \frac{V.\{(cpm_i ion - cpm_f ion) - MSA_{int}.([ion]_i - [ion]_f)\}}{MSA_{ext} - MSA_{int} \cdot Wt}$$

where MSA_{int} and MSA_{ext} are the internal and external mean specific activity, respectively. The MSA_{int} was calculated as

$$MSA_{int} = \frac{\sum cpm_i + \sum cpm_f}{2.V_{int}.ion_p}$$

where Σcpm_i and Σcpm_f are the summated ^{22}Na or ^{36}Cl radioactivity (counts min^{-1}) accumulated by the fish from the water at the start and end of the flux period, respectively, V_{int} is the internal radiospace and ion_p is the plasma concentration of Na^+ or Cl^-. V_{int} was taken as 280.4 ml kg^{-1} and 252.7 ml kg^{-1} for Na^+ and Cl^{-1}, respectively (Wood, 1988), and ion_p was measured in the terminal plasma samples. To estimate Σcpm_i and Σcpm_f, the radioactivity remaining since the previous flux determination was calculated as

$$\text{cpm left} = (\text{cpm fish}^{-1}).\exp(-J_{out}^{ion}.W.MSA_{int}.t/\text{cpm fish}^{-1})$$

where cpm $fish^{-1}$, $J_{out}{}^{ion}$ and MSA_{int} were as calculated at the end of the previous flux period.

Net fluxes and effluxes of Na^+ and Cl^- were calculated from

$$\text{Net flux, } J_{net}^{ion} = ([ion]_i - [ion]_f).\frac{V}{Wt}$$

$$\text{Efflux, } J_{out}^{ion} = \text{net flux} - \text{influx}$$

The mean unidirectional fluxes of Na^+ and Cl^- were calculated and the values for each measurement during exposure to silver and 'recovery' were compared with their respective controls using a two-tailed, paired t-test ($P < 0.05$).

2.3. The effects of silver nitrate on the kinetics of Na^+ influx

The effects of silver on sodium saturation kinetics were measured using the protocol of Goss and Wood (1990). The external Na^+ concentration was increased sequentially and the rate of Na^+ influx was measured using ^{22}Na at each concentration, such that the kinetic parameters of Na^+ influx could be determined, based on Michaelis–Menten kinetics (see below).

The fish were transferred to boxes similar to those described above but of a smaller volume (1.5 l), and left to acclimate for 48 h. The rate of Na^+ influx was measured over 30 min at nominal external Na^+ concentrations of 50, 150, 300, 600, 1200 and 2400 μM under control conditions and again after exposure to 2 μg l^{-1} silver (as $AgNO_3$) for 48 h. Before each series of Na^+ influx measurements, each box was flushed thoroughly with a Na^+-free synthetic freshwater of an otherwise similar composition to normal Hamilton tapwater. The artificial freshwater was made from deionized water by addition of 1.0 mM $CaCO_3$, 0.2 mM $MgCO_3$ and 0.35 mM $MgCl_2$. The water was gassed with CO_2 for 24 h to dissolve the relatively insoluble $CaCO_3$, and then with air for 12 h to drive off excess CO_2. To achieve the required external Na^+ concentration the necessary volume of 1 M Na_2SO_4 contain-

ing 3 μCi ml^{-1} ^{22}Na was added to the water and was allowed to mix for 15 min. A 25 ml water sample was taken at the start and end of each flux for measurement of ^{22}Na activity and Na^+ concentration. At the end of each flux the water removed owing to sampling (50 ml) was replaced with an equal volume of Na^+-free water, and the appropriate volume of ^{22}Na-labelled 1 M Na_2SO_4 for the next flux measurement was added.

The rate of Na^+ influx, $J_{in}^{Na^+}$ vs. external Na^+ concentration, $[Na^+]_{ext}$, was plotted for each individual fish under control and silver-exposed conditions and curves were fitted to the data by computer (Fig-P, Biosoft) using the Michaelis–Menten equation:

$$J_{in}^{Na^+} = \frac{J_{max} \cdot [Na^+]_{ext}}{K_m + [Na^+]_{ext}}$$

where J_{max} is the apparent maximal rate of Na^+ transport and K_m is the inverse of the affinity of the transport system for Na^+. The mean values of J_{max} and K_m during Ag exposure were compared statistically with those of the controls using a two-tailed, paired t-test ($P < 0.05$).

2.4. The effects of silver nitrate on the activity of branchial enzymes

Fish were placed in boxes, allowed to acclimate for 48 h, and exposed to either 2 μg l^{-1} or 10 μg l^{-1} Ag (as $AgNO_3$) for 48 h as described above. At the end of the exposure the fish were killed with an overdose of MS222 buffered with $NaHCO_3$. The gills were perfused free of erythrocytes using phosphate-free modified Cortland saline (Perry et al., 1984) and were stored at −80°C.

The activity of Na^+/K^+-ATPase in control and silver exposed gills was measured using the method of Holliday (1985). Approximately 50 mg of gill filaments were homogenized in 5 ml of medium (250 mM sucrose, 6 mM EDTA, 20 mM imidazole, pH 7.2) at 0–4°C with 30 strokes of a Teflon-in-glass homogenizer (Thomas Scientific, St. Laurent, Que.; Canada). The protein concentration in these homogenates was measured using the method of Lowry et al. (1951) with bovine serum albumin as a standard. Phosphate liberated from ATP by Na^+/K^+-ATPase was measured in two media. The first medium contained optimal concentrations of all ions (100 mM Na^+, 30 mM K^+, 10 mM Mg^{2+}, 5 mM ATP and 20 mM imidazole; pH 7.2) and therefore the activity of all ATPases present in the crude homogenate was assayed. The second medium lacked K^+ and contained 1 mM ouabain (130 mM Na^+, 10 mM Mg^{2+}, 5 mM ATP, 1 mM ouabain and 20 mM imidazole; pH 7.2) and therefore assayed the activity of all ATPases except Na^+/K^+-ATPase. The reaction was carried out for 30 min at 30°C and was stopped by addition of ice-cold Bonting's colour reagent (178 mM $FeSO_4$, 8.1 mM ammonium molybdate in 580 mM H_2SO_4) and the concentration of inorganic phosphate (P_i) liberated was measured colorimetrically at 700 nm as the reduced phosphomolybdate complex. The activity of Na^+/K^+-ATPase (expressed as μmol P_i mg^{-1} protein h^{-1}) was calculated from the difference in P_i between the two media.

Carbonic anhydrase (CA) activity (expressed in μmol CO_2 hydrated mg^{-1} protein min^{-1}) was measured electrometrically using the delta pH method as de-

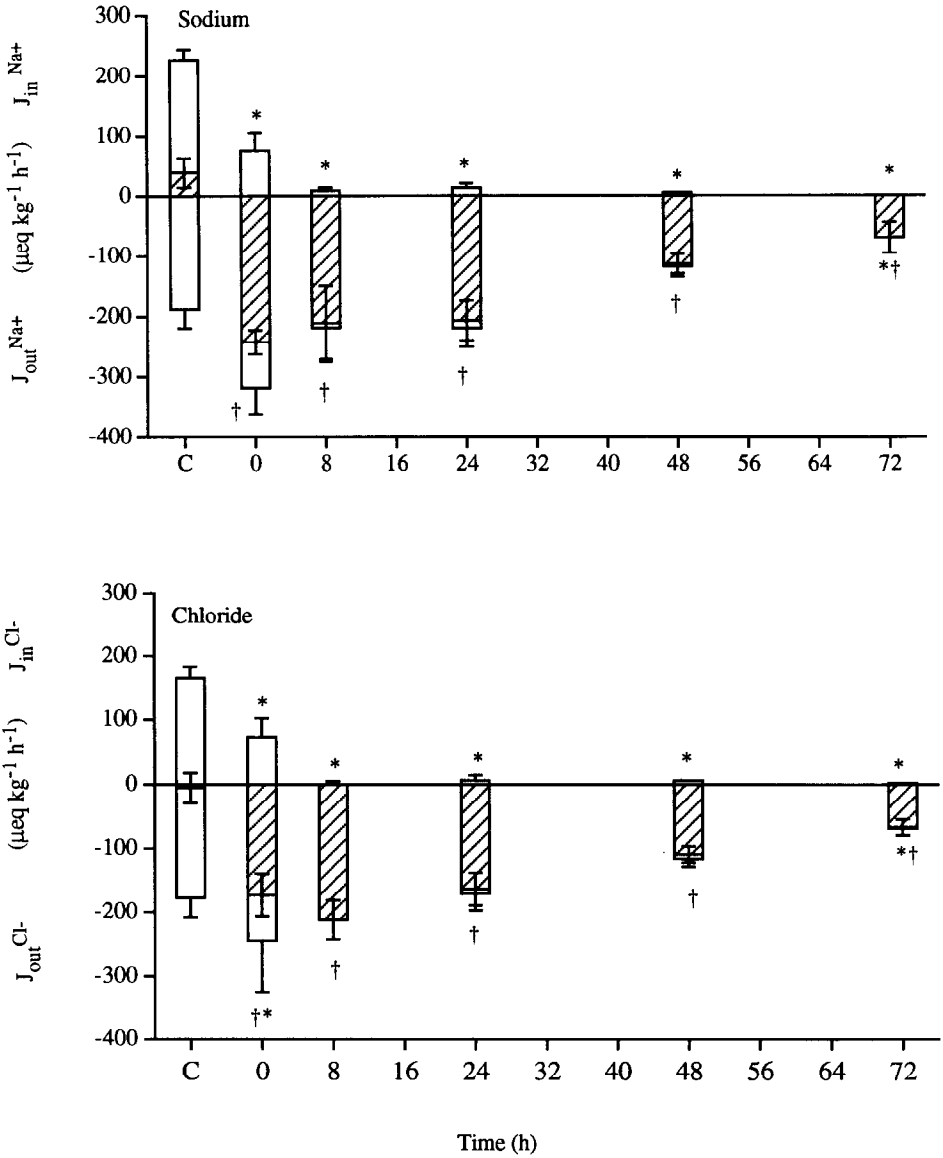

Fig. 1. The effects of exposure to 10 μg l^{-1} silver (as $AgNO_3$) on the unidirectional fluxes of Na^+ and Cl^- in the rainbow trout (*O. mykiss*). Values are means ± SEM ($N = 6$–12). Positive values represent movement into the fish (J_{in}), negative values represent movement out of the fish (J_{out}) and hatched bars represent the net movement of the ion (J_{net}). *Significantly different from the control (C) for J_{in} or J_{out}. †Significantly different from the control for J_{net} ($P < 0.05$).

scribed by Henry (1991). Approximately 0.25 g of frozen gill tissue was homogenized (Omni, Gainesville, VA; Omni 1000) in 4 ml of medium (225 mM mannitol, 75 mM sucrose, 10 mM Trizma base, adjusted to pH 7.4 with 10% phosphoric acid) and sonicated for 30 s at 40 W (Heat Systems, Farmingdale, NY; Micro-son). The homogenate was centrifuged at 4°C for 20 min at 7500*g* and the supernatant was assayed directly. Supernatant (50 μl) was added to 6 ml medium (as above) and rapidly stirred at 4°C. The reaction was started by the addition of 100 μl of CO_2-saturated water and the drop in pH was monitored with a null-point recording pH meter that was calibrated by the addition of 0.1 M HCl. The protein concentration in these supernatants was determined by the Bradford Coomassie Brilliant blue dye method (Bio-Rad, Richmond, CA, USA). The mean enzyme activities after exposure to 2 and 10 μg l^{-1} Ag were statistically compared with the controls using Student's two-tailed *t*-test ($P < 0.05$).

The effects of silver nitrate on the two enzymes were also measured in vitro. Erythrocyte-free gill tissue was obtained as above from fish that had been kept in boxes for 48 h but had not been exposed to silver. Tissue homogenates were prepared and silver nitrate (in the appropriate assay medium) was added directly to the reaction tubes and allowed to equilibrate for 60 s before the initiation of the enzyme assay. The final silver concentrations in vitro were chosen such that the concentration of silver that caused a 50% inhibition of enzyme activity (I_{50}) could be determined.

3. Results

No mortalities occurred in any of the experiments.

3.1. The effects of silver on unidirectional Na^+ and Cl^- fluxes

Exposure to 10 μg l^{-1} Ag resulted in a large and rapid inhibition of both Na^+

Table 1
Plasma concentrations ($[ion]_p$, mM ± SEM; $N = 6$–12) and change in total exchangeable internal pools ($\Delta[ion]_i$, %) of Na^+ and Cl^- in rainbow trout (*Oncorhynchus mykiss*) after 75 h exposure to 2 and 10 μg l^{-1} silver (as $AgNO_3$)

[Ag] (μg l^{-1})	$[Na^+]_p$	$\Delta[Na^+]_i$[a]		$[Cl^-]_p$	$\Delta[Cl^-]_i$[a]	
		Measured	Estimated		Measured	Estimated
Control	151.2 (6.2)			130.2 (2.8)		
2	127.5 (3.2)	15.7	18.1	114.0 (4.5)	12.4	15.7
10	nd	–	26.2	nd[b]	–	29.1

[a]Total exchangeable internal pool=control $[ion]_p \times 42$ mmol kg^{-1} (Na^+) or 33 mmol kg^{-1} (Cl^-); from Wood (1988).
[b]No data; samples lost.

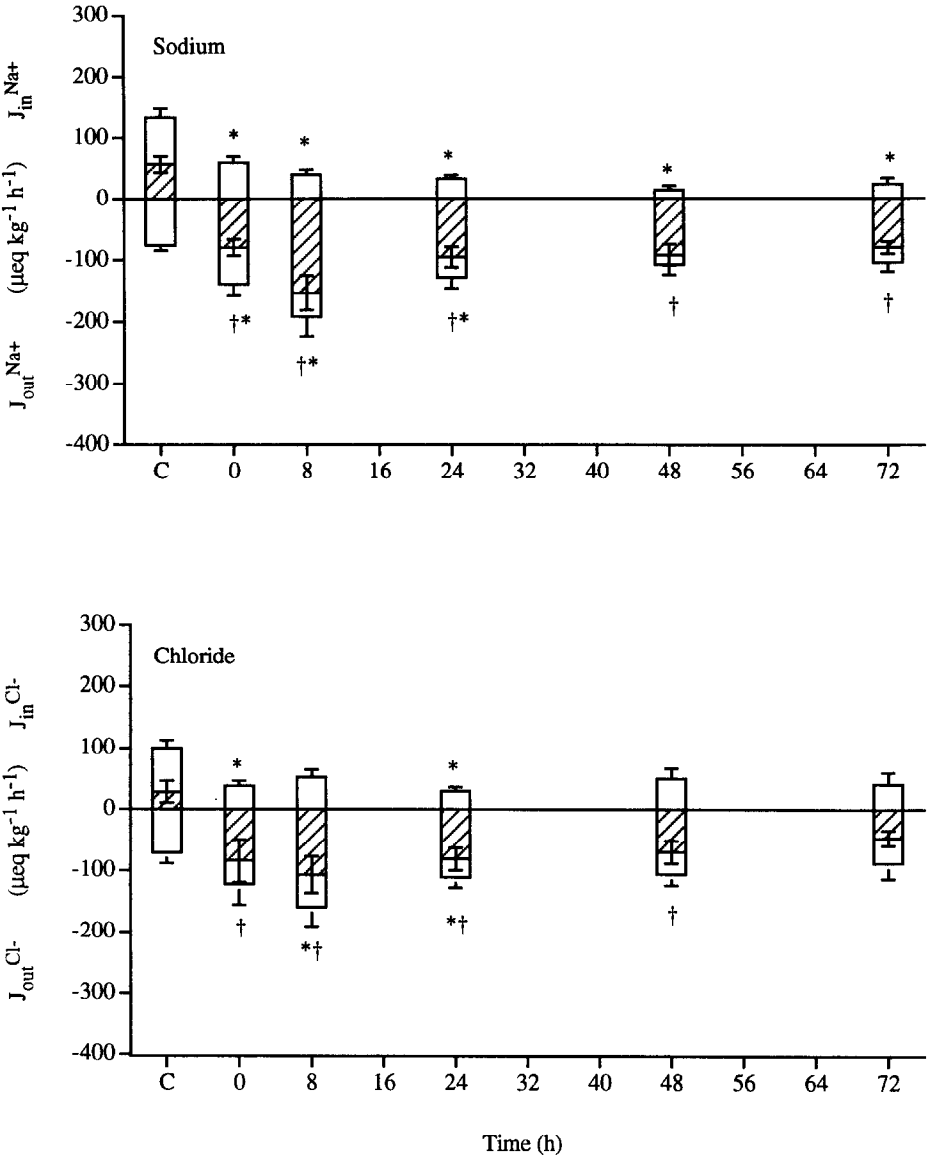

Fig. 2. The effects of exposure to 2 μg l^{-1} silver (as $AgNO_3$) on the unidirectional fluxes of Na^+ and Cl^- in the rainbow trout (*O. mykiss*). Values are means ± SEM (N = 6–12). (See Fig. 1 caption for other details.)

and Cl^- influx (Fig. 1). Influx was reduced by over 50% immediately and by almost 100% at 8 h, and showed no signs of recovery up to 72 h. In comparison, the efflux of Na^+ and Cl^- was not stimulated by exposure to 10 μg l^{-1} Ag. There was an initial increase in efflux (not statistically significant) followed by a gradual decrease

over time, so that at 72 h the Na^+ and Cl^- efflux was significantly lower than control rates. The net result of these changes in unidirectional fluxes was a dramatic loss of ions from the fish (i.e. a significant negative net flux), and although this loss gradually attenuated, ionic balance did not fully recover over the period of the exposure. Unfortunately, the terminal plasma samples from the fish exposed to 10 μg l^{-1} Ag were lost. However, the loss of Na^+ and Cl^- was estimated by taking a time-weighted average value for the net measured Na^+ or Cl^- flux and multiplying this by the total time of the exposure (Table 1). This resulted in an estimated loss of 11.0 mmol kg^{-1} of Na^+ and 9.6 mmol kg^{-1} of Cl^-. The total exchangeable internal pools of Na^+ and Cl^- in rainbow trout kept in water of identical composition are approximately 42 mmol kg^{-1} and 33 mmol kg^{-1}, respectively (Wood, 1988), and therefore the estimated loss of Na^+ and Cl^- was 26% and 29% of the exchangeable pool, respectively.

The effects of exposure to 2 μg l^{-1} Ag (as $AgNO_3$) on Na^+ and Cl^- transport were similar to those observed at 10 μg l^{-1} in that a significant inhibition of influx occurred, but of a lesser magnitude (Fig. 2). However, there was a significant increase in Na^+ and Cl^- efflux over the first 24 h of this exposure. The fish exposed to 2 μg l^{-1} Ag had plasma Na^+ and Cl^- concentrations that were 15.7% and 12.4% lower, respectively, than those of the control fish. As shown in Table 1, these measured losses were in reasonable agreement with the estimated losses (18.1% and 15.7%, respectively) based on branchial net ion fluxes, as above.

The effects of 2 μg l^{-1} Ag on branchial Na^+ fluxes were found to be rapidly

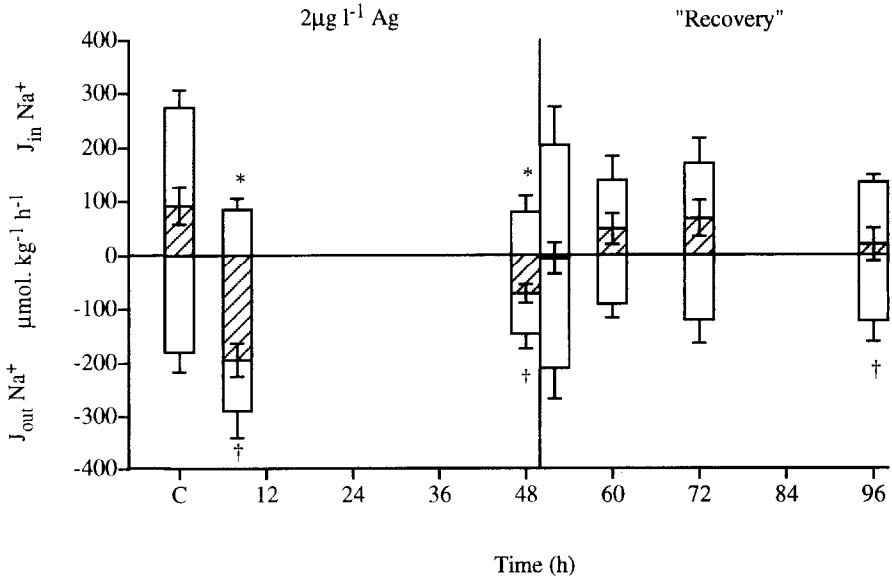

Fig. 3. Unidirectional Na^+ fluxes in rainbow trout (*O. mykiss*) during 48 h exposure to 2 μg l^{-1} silver (as $AgNO_3$), and during recovery thereafter in clean freshwater. Values are means ± SEM ($N=6$). (See Fig. 1 caption for other details.)

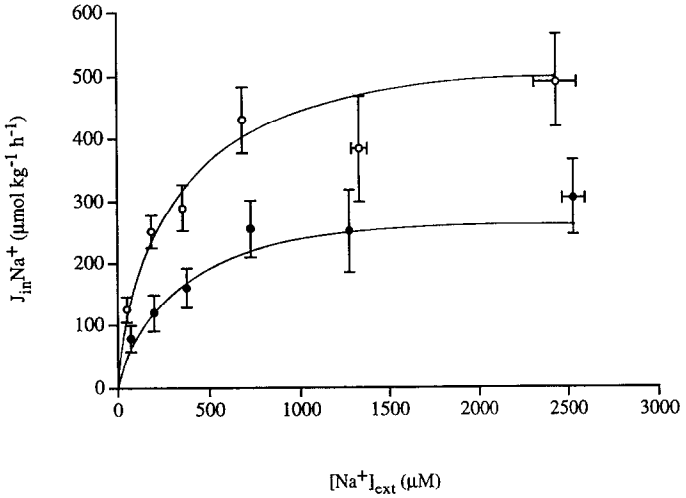

Fig. 4. The effects of exposure to 2 μg l^{-1} silver (as $AgNO_3$) on the kinetics of Na^+ influx ($J_{in}^{Na^+}$) in the rainbow trout (*O. mykiss*). Values are means ± SEM ($N = 10$). ○, Fish under control conditions; ●, silver-exposed fish.

reversible (Fig. 3). The silver exposure ended after 48 h, at which time the Na^+ influx was still significantly less, and the Na^+ net flux still significantly negative, relative to control values. However, within 2 h of removal of the silver exposure, both the Na^+ influx and Na^+ net flux had fully recovered to control values and remained at those levels for the duration of the experimental period (44 h).

3.2. *The effects of silver nitrate on the kinetics of Na^+ influx*

In both control and silver-exposed fish, Na^+ influx ($J_{in}^{Na^+}$) showed typical Michaelis–Menten kinetics in response to increases in external Na^+ concentration ($[Na^+]_{ext}$) (Fig. 4). However, exposure to 2 μg l^{-1} Ag for 48 h resulted in a lower $J_{in}^{Na^+}$ at all values of $[Na^+]_{ext}$ and hence a significant decrease in the maximal rate of $J_{in}^{Na^+}$, J_{max} (Table 2). In contrast, K_m, and therefore the affinity of the site(s) of transport for Na^+ (affinity is $1/K_m$), was not significantly affected by exposure to waterborne silver.

Table 2
Michaelis–Menten kinetic parameters (± SEM, $N = 7$) for Na^+ influx in rainbow trout (*Oncorhynchus mykiss*), under control conditions and after 48 h exposure to 2 μg l^{-1} silver (as $AgNO_3$)

Treatment	K_m (μM)	J_{max} (μmol kg^{-1} h^{-1})
Control	257.1 (89.7)	613.0 (121.0)
2 μg l^{-1} silver	328.0 (126.0)	398.0[a] (126.0)

[a]Significantly different from the control value ($P < 0.05$).

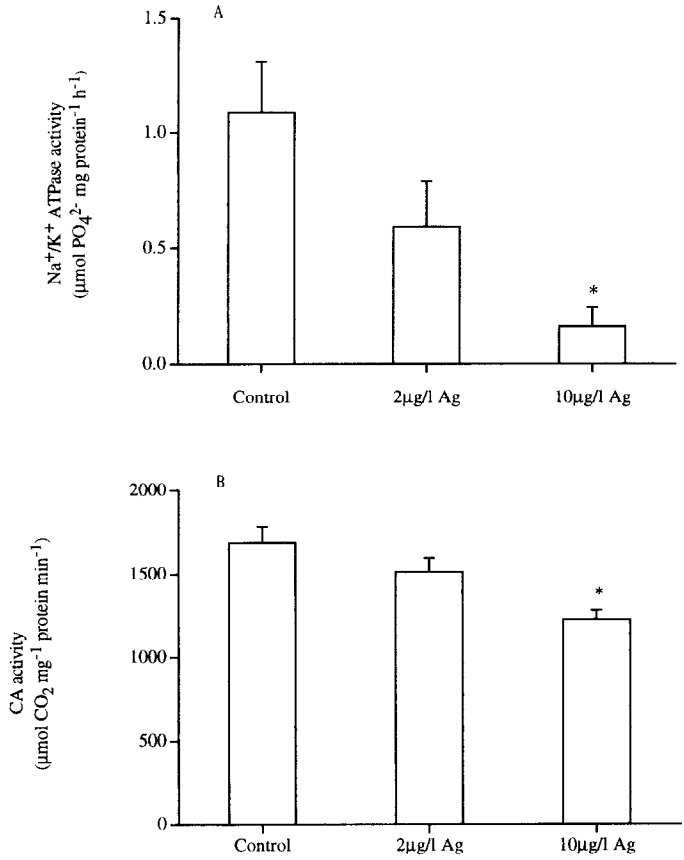

Fig. 5. The effects of 48 h exposure to 2 and 10 μg l^{-1} silver (as $AgNO_3$) in vivo on the branchial activity of (A) Na^+/K^+ ATPase and (B) carbonic anhydrase in the rainbow trout (*O. mykiss*). Values are means ± SEM ($N=6$). *Significantly different from the control ($P<0.05$).

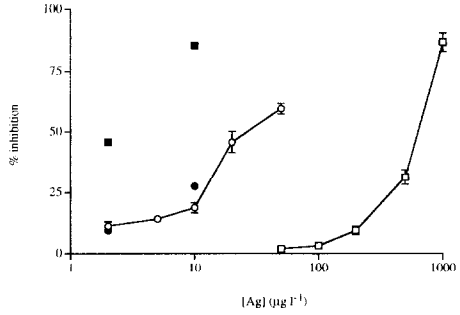

Fig. 6. The effects of silver (as $AgNO_3$) in vitro on the activity of branchial carbonic anhydrase (○) and Na^+/K^+ ATPase (○) in the rainbow trout (*O. mykiss*). Values are means ± SEM ($N=6$). In vivo inhibition values from Fig. 5 (filled symbols) are included for comparison.

3.3. The effects of silver nitrate on the activity of branchial enzymes

Although the mean activity of branchial Na^+/K^+ ATPase after 48 h of exposure to 2 μg l^{-1} Ag was approximately 50% lower than that under control conditions, this difference was not statistically significant (Fig. 5(A)). However, exposure to 10 μg l^{-1} Ag in vivo significantly inhibited branchial Na^+/K^+ ATPase activity by approximately 85%. Branchial carbonic anhydrase (CA) activity was inhibited in vivo by Ag but to a lesser degree than was Na^+/K^+ ATPase (Fig. 5(B)). Exposure to 2 μg l^{-1} Ag had no effect on CA activity, but fish exposed to 10 μg l^{-1} had a CA activity that was significantly lower than that of control fish by approximately 30%.

The estimated silver concentration that caused a 50% inhibition of Na^+/K^+ ATPase activity in vitro (I_{50}) was 670 μg l^{-1} (Fig. 6). The in vitro I_{50} value for CA was 31 μg l^{-1} (Fig. 6). When $AgNO_3$ was replaced by $NaNO_3$, no in vitro enzyme inhibition was observed (data not shown).

4. Discussion

Silver speciation analysis using the aquatic chemistry equilibrium programme MINEQL$^+$ (Schecher and McAvoy, 1992) indicated that during exposure to 10 μg l^{-1} Ag (as $AgNO_3$), three main species of silver were present: the free silver ion, Ag^+ (34.5%) and two dissolved silver chloride species, $AgCl_{aq}$ and $AgCl^{2-}$ (60.2% and 5.2%, respectively). However, previous studies have demonstrated that silver chlorides are not toxic to freshwater fish (LeBlanc et al., 1984; Hogstrand et al., 1996), even at concentrations many times greater than those of the present experiments, and therefore the physiological effects observed during exposure to $AgNO_3$ in the present experiments can be attributed primarily to the free silver ion, Ag^+. This concurs both with previous studies of silver toxicity in freshwater (LeBlanc et al., 1984; Wood et al., 1996a, Wood et al., 1996b), and with work on the toxicity of other metals where the free metal ion has been found to be the most toxic form (Borgman, 1983; Luoma, 1983; Brezonik et al., 1991).

Exposure to waterborne $AgNO_3$ resulted in a severe disturbance of branchial Na^+ and Cl^- regulation. Fish exposed to both 2 and 10 μg l^{-1} Ag immediately began to lose ions to the surrounding water as a result of a severe, persistent inhibition of branchial influx rates and a lesser, temporary stimulation of branchial efflux rates. Although this loss of ions decreased over the duration of the experiment, this apparent 'adaptation' may have been due to a depletion of the exchangeable internal pools of Na^+ and Cl^- rather than an indication of any acclimation to silver. The fish exposed to 2 μg l^{-1} Ag lost 16% of their exchangeable Na^+ pool over the 72 h of exposure whereas those exposed to 10 μg l^{-1} lost an estimated 28% of exchangeable Na^+ This estimated loss is somewhat higher than that measured by Wood et al. (1996a), who observed a 19% decrease in plasma Na^+ after 96 h of exposure to 10 μg l^{-1} Ag (as $AgNO_3$). Nevertheless, a loss of plasma Na^+ of over 30% is lethal in rainbow trout (McDonald et al., 1980) and hence a continuation of exposure to 10 μg l^{-1} Ag would have probably resulted in fish mortalities. Indeed,

Wood et al. (1996a) reported that mortalities commenced after 6 days of exposure to 10 $\mu g\ l^{-1}$ Ag, when plasma ion losses reached 30%. It can be concluded, therefore, that disturbance of branchial ion transport is a major factor in the toxicity of Ag^+ to freshwater fish.

Sodium influx in freshwater fish is known to depend on the external (waterborne) Na^+ concentration in a manner well described by Michaelis–Menten kinetics (Avella et al., 1987; Goss and Wood, 1990; Potts, 1994). This approach was therefore used to analyse the nature of the inhibition of Na^+ influx by Ag^+. The values obtained for K_m and J_{max} under control conditions in the present experiments showed good agreement with values in the literature (see Table 3 of Goss and Wood (1990)). The significant decrease in J_{max} and the lack of change in K_m during silver exposure compared with the controls demonstrates that the inhibition of Na^+ influx by Ag^+ is non-competitive. This implies that the binding of Ag^+ to a second site on the Na^+ transport mechanism causes a conformational change in the specific Na^+ uptake site which is consequently rendered non-functional. There is therefore a reduction in the number of available Na^+ transport sites and hence a reduction in V_{max}, whereas the affinity ($1/K_m$) of the remaining functional Na^+ sites is unaffected.

Janes and Playle (1995) demonstrated that silver binds readily to the surface of the gills. This binding was successfully modelled on the assumption that Ag^+ competed with other cations for negatively charged sites on the gill surface as postulated by Pagenkopf (1983). Na^+ and Cl^- are thought to have separate sites and mechanisms of uptake on the apical membrane of the gill epithelium (Evans, 1993; Goss et al., 1995). External Na^+ is taken up in exchange for a proton (H^+) whereas external Cl^- is taken up in exchange for internal bicarbonate ion (HCO_3^-). It is unlikely that the external binding sites of the Cl^-/HCO_3^- exchange mechanism would attract cations. The simultaneous inhibition of both sodium and chloride influx by silver nitrate therefore suggested that the site(s) of inhibition were not on the apical membrane of the gill epithelial cells. However, waterborne silver may cross the gill epithelium and accumulate in the blood (Wood et al., 1996a, Wood et al., 1996b) and as the gills appeared to be the principal target organ of physiological silver toxicity, it was considered likely that the site(s) of inhibition of both Na^+ and Cl^- by Ag^+ occurred within the cells of the branchial epithelium. Ag^+ inhibits the activity of several enzymes from fish in vitro (Jackim et al., 1970; Christensen, 1971; Christensen and Tucker, 1976) and therefore we studied the effects of silver exposure on the activity of two intracellular branchial enzymes that are involved in ion transport, Na^+/K^+ ATPase and carbonic anhydrase (CA).

Na^+/K^+ ATPase is located on the basolateral membranes of the ion-transporting cells of the branchial epithelium and actively transports Na^+ from these cells into the blood (Wood, 1992; Evans, 1993). Although this enzyme is not thought to be directly involved in Cl^- transport in freshwater fish, it has been shown that application of the specific Na^+/K^+ ATPase inhibitor, ouabain, reduces the influx of both Na^+ and Cl^- (Richards and Fromm, 1970; Battram et al., 1989). Carbonic anhydrase occurs in the apical region of the gill epithelial cells, both internally and externally (Rahim et al., 1988) and catalyses the hydration of CO_2 to produce

H^+, which may be exchanged for external Na^+, and HCO_3^-, which may be exchanged for external Cl^-. Both Na^+/K^+ ATPase (Nechay and Saunders, 1984) and CA (Christensen and Tucker, 1976) have been shown to be inhibited by silver nitrate in vitro in other animals. Hence, a poisoning of one or both of these enzymes by Ag^+ in vivo could result in an inhibition of Na^+ and Cl^- transport across the gills of rainbow trout.

Indeed, in vivo exposure to 10 μg l^{-1} silver for 48 h inhibited branchial Na^+/K^+ ATPase activity by 85% and inhibited CA activity by 28%. The greater inhibition of Na^+/K^+ ATPase activity suggested that a non-competitive inhibition of this enzyme was the primary cause of the ionregulatory disturbance observed during exposure to 10 μg l^{-1} Ag, though as CA activity was also significantly inhibited, the relative contribution of the two enzymes cannot be stated for certain. However, the Ag concentration that caused a 50% inhibition of Na^+/K^+ ATPase in vitro (I_{50}) was 670 μg l^{-1} (5.9 μM), much greater than the waterborne concentrations used in the present study. This implies that considerable accumulation of Ag within the gill tissues must have occurred during exposure to 10 μg l^{-1} Ag to cause the 85% inhibition of Na^+/K^+ ATPase activity that was observed. This suggestion is supported by both Wood et al. (1996a) and Hogstrand et al. (1996), who recorded gill Ag concentrations of 275 μg kg^{-1} in 250–450 g rainbow trout and 4.5 mg kg^{-1} in 1–4 g rainbow trout, respectively, after 6 days of exposure to 10 μg l^{-1} Ag (as $AgNO_3$).

In contrast to Na^+/K^+ ATPase, the inhibition of CA by Ag in vitro was very similar to that in vivo, which does not appear to support the above conclusion that Ag accumulated in the gill tissue during exposure to waterborne silver nitrate. This apparent anomaly may be explained, however, by the exact technique used to assay branchial CA. The gill tissue of rainbow trout has a very high activity of CA and hence the slopes of the assay curves used in the delta pH method (Henry, 1991) were very steep, which tended to magnify small differences in the raw data. To overcome this problem, it was necessary to dilute the gill homogenates several-fold. This had no effect on the silver concentration present in vitro, as the $AgNO_3$ was added post-dilution. However, in the in vivo assays the only silver present was that from the waterborne exposures, and so dilution of the homogenates could have reduced the actual silver concentration. The inhibition of CA recorded in vivo may therefore have been an underestimate of the actual inhibition, particularly if Ag is only a weak inhibitor of CA. The rapid recovery of Na^+ fluxes when the silver exposure was removed indeed suggests that the binding of silver to the Na^+/K^+ ATPase and/or CA was weak and that the inhibition was reversible.

In comparison with other waterborne toxicants, the pattern of osmoregulatory disturbance in freshwater fish owing to Ag^+, i.e. a reduction of branchial Na^+ and Cl^- influx, shows most similarity to that seen in fish exposed to environmental acidification (H^+) and to waterborne copper. In contrast, exposure to aluminium, cadmium or zinc causes only moderate disturbance to Na^+ and Cl^- regulation at levels of water hardness and pH comparable with those of the present study (Wood, 1992; Heath, 1995). Environmental acidification causes a simultaneous inhibition of both Na^+ and Cl^- influx and a stimulation of Na^+ and Cl^- effluxes, which result in

a decrease in plasma Na^+ and Cl^- (Wood, 1989). Plasma K^+ and Ca^{2+} concentrations, however, are unaffected by H^+ (McDonald et al., 1980; Audet et al., 1988). This is precisely the pattern of plasma ion concentration changes that was observed by Wood et al. (1996a) when rainbow trout were exposed to 10 $\mu g\ l^{-1}$ Ag, and indeed Wood et al. (1996a) suggested that Ag^+ acts as an apical membrane blocker of ion transport sites, a mechanism that is generally accepted for H^+ toxicity (Wood, 1989). However, the present study suggests that Ag^+ toxicity acts internally within the gills, primarily by inhibition of branchial Na^+/K^+ ATPase and carbonic anhydrase. Low pH alone does not appear to appreciably inhibit Na^+/K^+ ATPase (Reite and Staurnes, 1987; Audet and Wood, 1993). The only studies to observe lower Na^+/K^+ ATPase activities in acid-exposed fish were in smolting Atlantic salmon, *Salmo salar* (Saunders et al., 1983; Johnston et al., 1984; Staurnes et al., 1993), which have high ATPase activities in preparation for migration to seawater and are not comparable with the fish used in the present study. We therefore conclude that the mechanisms of Ag^+ toxicity are not the same as those of H^+.

Copper also inhibits branchial Na^+ influx (Laurén and McDonald, 1985; Reid and McDonald, 1988) and like Ag^+, but in contrast to H^+, has been clearly shown to inhibit branchial Na^+/K^+ ATPase (Lorz and McPherson, 1976; Laurén and McDonald, 1987). However, copper is also an inhibitor of branchial Ca^{2+} ATPase (Shephard and Simkiss, 1978) and Ca^{2+} fluxes (Sayer et al., 1991), and causes a decrease in plasma Ca^{2+} concentration (Pilgaard et al., 1994), which was not seen during Ag^+ exposure (Wood et al., 1996a). Therefore, although the physiological mechanisms of Ag^+ toxicity share some features with both H^+ and copper, there are also some important differences such that the full pattern of physiological toxicity seems to be characteristic of Ag^+ alone.

The present definition of the physiological mechanisms of silver nitrate toxicity in freshwater rainbow trout provides a well-defined biological response that may be used to detect the presence of bioavailable Ag^+ under various conditions of water quality. Traditionally, $[Ag^+]$ has been measured by physico-chemical methods such as silver electrodes and anodic stripping voltammetry, or has been calculated using computer programs such as MINEQL$^+$. These methods provide only indirect approximations of the biological impact of the measured Ag^+. Measurement of physiological (branchial Na^+ influx) or biochemical (branchial Na^+/K^+ ATPase activity) parameters, however, gives a direct and immediate indication of a biological impact, which is the important factor from a regulatory standpoint.

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