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The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) 1. The effects of ionic Ag^+

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Abstract

Adult rainbow trout, fitted with arterial catheters, were exposed to $AgNO_3$ for 6 days at a concentration (10 μg Ag l^{-1}, flow-through) close to the 7 day LC50 in moderately hard freshwater. Approximately 35% of total Ag occurred as free ionic Ag^+, and the remainder as silver chlorides. Ag accumulated on the gills and increased about 4-fold above control levels in blood plasma, stabilizing by 48 h. Much greater concentrations of Ag accumulated in the liver, but not the kidney, at 6 days. Metallothionein induction did not occur. Plasma $[Na^+]$ and $[Cl^-]$ declined steadily to 70% of control levels by day 6, accompanied by a progressive metabolic acidosis, a 5-fold increase in blood [glucose], a 40% decrease in relative plasma volume, contraction of the spleen, and marked hemoconcentration. Plasma $[Ca^{2+}]$ and $[K^+]$ were largely unaffected. Respiratory suffocation did not occur: plasma [lactate] remained constant, arterial P_{O_2} increased and P_{CO_2} decreased, the latter compensating the metabolic acidosis so arterial pH fell only moderately. Comparably sampled control fish exhibited negligible disturbance. Unidirectional Na^+ influx from the water, measured in juvenile trout, was inhibited by 42% immediately, and abolished by 48 h of $AgNO_3$ exposure. These symptoms suggested a similar toxic mechanism of action to that of low environmental pH. We speculate that Ag^+ interferes with net Na^+ and Cl^- uptake at the gills, and causes death by secondary fluid volume disturbance, hemoconcentration, and eventual cardiovascular collapse.

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1. Introduction

Silver (presented as silver nitrate) is one of the most toxic metals to freshwater fish, with 96 h LC50s generally in the range of 6.5–65 μg l^{-1} (0.06 to 0.6 μM);(Coleman and Cearley, 1974; Davies et al., 1978; Buccafusco et al., 1981; Lemke, 1981; Nebeker et al., 1983; Leblanc et al., 1984; Diamond et al., 1990). These tests have all been based on the freely soluble salt $AgNO_3$ which dissociates completely (log K value = −0.3; Morel and Hering, 1993) to yield substantial proportions of free ionic Ag^+. Ag is discharged into the aquatic environment from domestic, agricultural, mining, and industrial sources. Within the latter, the largest contributor is photoprocessing effluent in which almost all Ag occurs as silver thiosulfate [$(Ag(S_2O_3)_n^-$] due to the use of sodium thiosulfate as a scavenging agent (Bard et al., 1976; Taylor et al., 1980; Cooley et al., 1988). Almost no free ionic Ag^+ is present in effluent because of the great avidity of thiosulfate (log K values of 8.8, 13.7, and 14.2 for the mono-, di-, and tri-thiosulfate complexes, respectively (Morel and Hering, 1993). Similarly, several anions occurring in natural waters such as Cl^-, $S^=$, and dissolved organic carbon may also complex and/or precipitate Ag^+. Electrode-based measurements in natural waters, both pristine and impacted, have indicated extremely low levels of free Ag^+ (< 0.2 μg l^{-1}) even in circumstances where total Ag was as high as 35 μg l^{-1} (Chudd, 1983; Lytle, 1984).

In fathead minnow (*Pimephales promelas*) complexed forms of silver are much less toxic than free Ag^+ (Terhaar et al., 1972; Leblanc et al., 1984). Recently, we have confirmed this finding in juvenile rainbow trout *Oncorhynchus mykiss* (Hogstrand et al., 1995); 96 h LC50 values were increased by more than 4 orders of magnitude (to $> 100\,000$ μg l^{-1}) when Ag^+ was tested in the presence of excess $Na_2S_2O_3$ or NaCl — i.e. as thiosulfate or chloride complexes. Nevertheless, in most jurisdictions, environmental regulations (e.g. Taylor et al., 1980; EPA, 1980, 1986; Anonymous, 1992) for silver are based on the results of bioassay tests with $AgNO_3$ (i.e. mainly ionic Ag^+) but applied on the basis of total recoverable Ag (i.e. all forms of Ag).

Sensible environmental regulations require information on the toxic mechanism(s) of action (see Sprague, 1990). Environmentally relevant levels of acidity, Cu, Al, Cd, and Zn are all surface-active toxicants at the gills. The first three interfere with Na^+ and Cl^- transport, and that the latter two with Ca^{2+} transport (reviewed by Evans, 1987; McDonald et al., 1989; Wood, 1989, 1992; McDonald and Wood, 1993). The exact mechanisms are metal-specific, but for all the free ionic forms are most toxic. In contrast, almost nothing is known about the toxic mechanism(s) of any of the forms of Ag. At very high concentration, Ag is said to cause a suffocation response (Cooper and Jolly, 1970), but this response is common to virtually all metals at such 'industrial levels' (Mallatt, 1985).

Therefore, the objective of the present study was to diagnose the toxic mechan-

ism(s) of action of $AgNO_3$ (i.e. free ionic Ag^+) to adult rainbow trout. The approach employed has been one used successfully in previous studies with acidity (McDonald et al., 1980; McDonald and Wood, 1981; McDonald, 1983), Cu (Wilson and Taylor, 1993; Pilgaard et al., 1994), Al (Wood et al., 1988a,b; Goss and Wood, 1988; Playle et al., 1989), and Zn (Spry and Wood, 1984, 1985). The fish are fitted with indwelling arterial catheters for repetitive blood sampling, and exposed to the toxicant at a level close to the incipient lethal threshold. A concentration of $AgNO_3$ of 10 μg l^{-1} (as Ag) was chosen based on a 7 day LC50 of 9.1 μg l^{-1} determined by Hogstrand et al. (1995) for juvenile rainbow trout in identical water. This approach allowed us to follow the gradual development over time of internal symptoms of toxicity, thereby providing clues as to the mechanism of toxicity.

2. Materials and methods

2.1. Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*; 250–450 g) from Spring Valley Trout Farm, Petersburg, Ontario were held for at least 2 weeks at 10–16°C, on a 1% daily ration of commercial trout pellets, in flowing dechlorinated Hamilton tapwater. Composition was (in mM) $Ca^{2+} = 1.0$, $Mg^{2+} = 0.2$, $Na^+ = 0.5$, $Cl^- = 0.7$, $K^+ = 0.05$, titratable alkalinity to pH 4.0 = 1.9; total hardness was approximately 140 ppm as $CaCO_3$ and pH approximately 8.0. The fish were acclimated to the experimental temperature (15 ± 1°C) for 7 days prior to surgery; food was withheld during this period.

Fish were fitted with indwelling dorsal aortic catheters (PE50 tubing; Soivio et al., 1972), and then transferred to individual darkened plexiglas chambers (volume = 8 l, illustrated by McDonald, 1983) for 48–60 h recovery. The chambers were served with a gravity-driven flow of 400 ml min^{-1} from a vigorously aerated head tank; their effective 50% replacement time was about 15 min. Effluent water ran to waste.

2.2. Experimental protocol

Control and experimental fish were run simultaneously. The experiment was replicated, yielding total n of 8 for the control and 12 for the experimental group (for fish with working catheters). The control and experimental systems were identical. Starting at time 0, $AgNO_3$ was added by peristaltic pump from a light-shielded stock bottle (160 mg l^{-1}; Sigma ultrapure reagent, renewed daily) to a final concentration of 10 μg l^{-1} (as Ag) in the well-mixed head tank on the experimental side. At time 0, experimental chambers were spiked with the stock solution to bring them immediately up to 10 μg l^{-1}.

In both groups, blood samples (0.7 ml) taken prior to exposure ('C' sample), and at 4, 24, 48, 96, and 144 h. Simultaneous water samples were drawn from in front of each fish's mouth for measurement of inspired O_2 tension (P_{iO_2}). Blood was drawn

anaerobically into ice-cold gas-tight glass syringes (Hamilton) for analysis of arterial pH (pH_a), O_2 tension (P_{aO_2}), plasma total CO_2 (C_{aCO_2}), hematocrit (Ht), hemoglobin (Hb), and plasma levels of Na^+, Cl^-, K^+, Ca^{2+}, glucose, lactate, total protein, and total Ag. The sampled volume was replaced by a re-infusion (total volume = 0.7 ml) consisting of Cortland saline (Wolf, 1963) plus resuspended red cells not used in analyses. After the final sample on Day 6, the fish were rapidly killed by an overdose of neutralized MS-222 (1 g l^{-1}), the spleen was quickly excised for weight and Hb measurements, and then samples of soft gill tissue, liver, and kidney were dissected out, blotted, freeze-clamped in liquid N_2, and stored at −70°C for later analyses of total Ag and metallothionein. The *n* for these latter analyses was reduced by the loss of some samples in a freezer failure. Water samples for total Ag levels were taken three times on the first experimental day and at least once per day thereafter.

2.3. Analytical methods

For most blood parameters, analytical methods were identical to those described in detail previously (Wood et al., 1988a; Playle et al., 1989). Spleen hemoglobin content was determined using the homogenization method of Milligan and Wood (1982) and the same cyanmethemoglobin assay as for whole blood. Ag and metallothionein analyses were performed as described by Hogstrand et al. (1995). In brief, Ag levels were determined using graphite furnace atomic absorption spectrophotometry (Varian 1275 fitted with a GTA-95 atomizer), while metallothionein (MT) assays were performed by the method of Hogstrand and Haux (1990). A double antibody radioimmunoassay was employed, using rabbit antiserum raised against MT from perch, *Perca fluviatilis*, as the first antibody, [^{125}I]-labelled rainbow trout MT as tracer, and goat anti-rabbit IgG as the second antibody.

2.4. Calculations

Acid-base calculations (P_{aCO_2}, true plasma HCO_3^-) were performed by equations detailed in Playle et al. (1989), using values for CO_2 solubility and pK' in trout plasma at the appropriate temperature from Boutilier et al. (1984). The metabolic acid load in the true plasma (ΔH_m^+) was calculated from measured changes in plasma HCO_3^- and pH_a using the formula of McDonald et al. (1980). In this equation, the whole blood buffer capacity (β) for true plasma was estimated from the measured Hb concentration and the regression relationship between Hb and β determined in rainbow trout blood by Wood et al. (1982). Mean cell hemoglobin concentration (MCHC = g Hb per ml of red blood cells) was calculated as the ratio of simultaneous Hb (g 100 ml^{-1}) to Ht (ml 100 ml^{-1}) measurements. In contrast to Ht and Hb, plasma protein concentration is not affected by repetitive blood sampling in rainbow trout (McDonald et al., 1980; and present study). Therefore in each fish the inverse of the ratio of the final measured plasma protein concentration at 144 h to the initial value taken at time 'C' was calculated as an index of the change in plasma volume (Wood et al., 1988b). The speciation of Ag in the ex-

posure water was calculated using measured water chemistry and the aquatic chemical equilibrium program MINEQL$^+$ (Schecher and McAvoy, 1992).

Data have been expressed as means ± 1 S.E.M. As each fish served as its own control, the statistical significance ($p < 0.05$) of changes within each treatment was assessed with Student's two-tailed paired t-test using the Bonferroni procedure (Nemenyi et al., 1977) to adjust the t-value for multiple comparisons. Comparisons between groups employed Student's unpaired two-tailed t-test. Most changes in the $AgNO_3$ exposed fish were significant with respect to both the pre-exposure value in that group (paired test) and the simultaneous value in the control group (unpaired test). For simplicity in the figures, asterisks (*) have been used to denote only the former, and any discrepancies between the two tests noted in the figure legends. A similar convention has been used in the text.

3. Results

3.1. Water chemistry and survival

The mean measured total Ag concentration during the exposures was 10.9 ± 0.6 μg l^{-1} ($n = 20$), very close to the nominal value of 10 μg l^{-1}. MINEQL$^+$ speciation indicated that 34.5% (3.8 μg l^{-1}) was in the form of Ag^+, 60.2% (6.6 μg l^{-1}) as AgCl(aq), and 5.2% (0.5 μg l^{-1}) as $AgCl_2^-$. Only 1 of 12 fish died (between 96 and 144 h), so these adult fish were somewhat more resistant than the juveniles tested by Hogstrand et al. (1995) which exhibited a 7 day LC50 of 9.1 μg l^{-1}. Data from the individual which died were included in means as they did not bias any of the trends seen. Levels of Ag in the control water were consistently below the detection limit of 0.5 μg l^{-1}. None of the 8 control fish died.

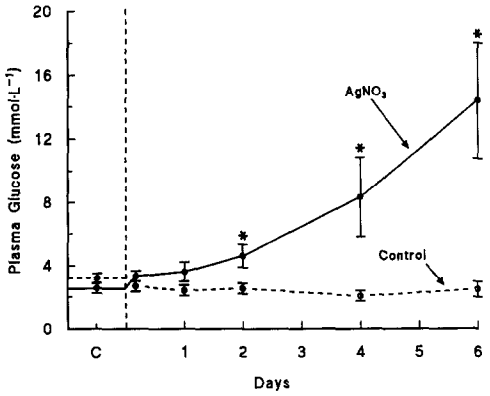

Fig. 1. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on plasma glucose levels in rainbow trout. Means ± 1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times.

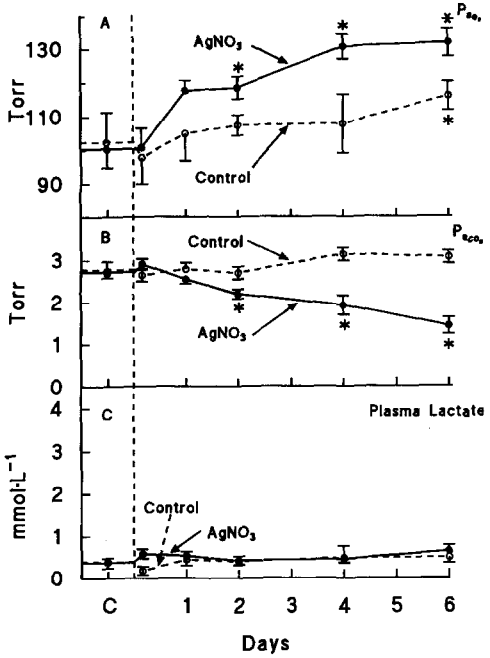

Fig. 2. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on (A) arterial blood O_2 tension (P_{aO_2}), (B) CO_2 tension (P_{aO_2}), and (C) plasma lactate levels in rainbow trout. Means ±1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times.

3.2. Physiological responses

Trout exposed to Ag (as $AgNO_3$) exhibited the classic indication of stress, a progressive rise in plasma glucose, significant by Day 2, to almost 5-fold control levels by Day 6 (Fig. 1). Plasma glucose levels remained unchanged in the control group. This stress did not appear to be of respiratory origin. P_{aO_2} gradually increased (Fig. 2A) and P_{aCO_2} reciprocally decreased (Fig. 2B) in exposed fish, significant by Day 2. Plasma lactate remained low and unchanged (Fig. 2C). None of these parameters changed significantly in the control group, except for an increase in P_{aO_2} on Day 6 (Fig. 2A). P_{iO_2} remained uniformly high at 145–155 torr (not shown).

Acid–base measurements revealed a moderate decrease in pH_a (0.2 units; Fig. 3A) significant on Days 4 and 6, and a pronounced fall in plasma HCO_3^- (significant by Day 2, 67% by Day 6; Fig. 3B). The small increase at 4 h was of doubtful importance as it also occurred in the control group. C_{aCO_2} (not shown) directly paralleled the changes in HCO_3^-. The fall in plasma HCO_3^- was associated with the accumulation of a substantial metabolic acid load ($\Delta H_m^+ = 7$ mmol l^{-1} by Day 6) in the blood plasma of Ag exposed trout (Fig. 3C), as well as a decline in P_{aCO_2} (Fig. 2B). The overall acid–base picture was that of 'metabolic acidosis' (ΔH_m^+ accumula-

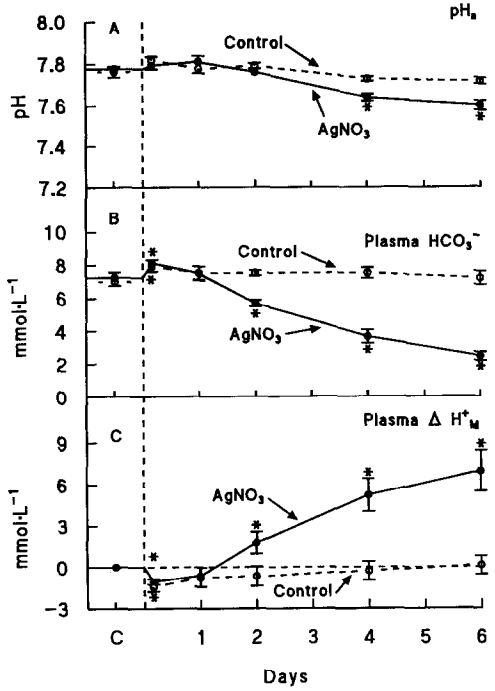

Fig. 3. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on (A) arterial blood pH (pH_a), (B) plasma HCO_3^-, and (C) plasma metabolic acid load (ΔH_m^+) in rainbow trout. Means ±1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times, except at 4 h in (B) and (C).

tion) partially offset by 'respiratory compensation' (decrease in P_{aCO_2}; Fig. 2B). Note that the rise in ΔH_m^+ occurred in the absence of any increase in lactate (Fig. 2C), so lactacidosis was not the cause. Plasma Ca^{2+} was unaffected by Ag (Fig. 4A), and plasma K^+ was only slightly affected with a small rise on the final day (Fig. 4B).

In contrast, plasma Na^+ and Cl^- levels fell rapidly in response to Ag exposure (Fig. 5A,B). The effect was well-developed by 24 h, and levels continued to fall linearly with time throughout the exposure. These losses were equimolar, amounting to about 40 mmol l^{-1}, by Day 6, or about 30% of the pre-exposure plasma concentration. Plasma Na^+ and Cl^- remained constant in the control group.

Remarkably, exposure to Ag (as $AgNO_3$) prevented the significant declines in hematocrit and hemoglobin which occurred with repetitive blood sampling from Day 1 onwards in the controls (Fig. 6A,B). Conversely, plasma protein concentration increased significantly from Day 1 onwards in the exposed fish, but did not change in the controls (Fig. 6C). Table 1 summarizes measurements taken at the end of the experiment which cast some light on these responses. MCHC was not significantly different between the two treatments (Table 1), and did not change over the course of the experiment (not shown), indicating the absence of red blood

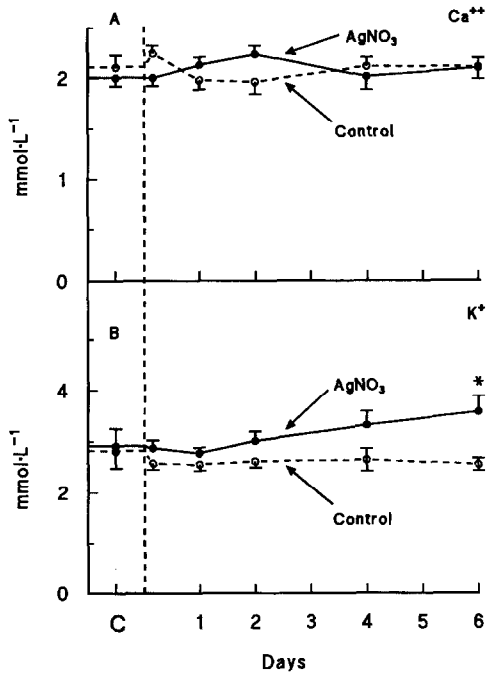

Fig. 4. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on (A) plasma Ca^{2+} and (B) plasma K^+ levels in rainbow trout. Means ± 1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times.

cell swelling or shrinkage. However, fluid was clearly lost from the blood plasma volume, which declined by almost 40% in the Ag exposed trout but remained unchanged in the controls (Table 1). The spleen also contracted, losing 40% of its weight and almost 60% of its Hb content. This combination of hemoconcentration by fluid loss and splenic discharge therefore explained the constancy of Ht and Hb in the face of repetitive removal of blood in the Ag exposed fish (Fig. 6A,B).

3.3. *Internal silver and metallothionein levels*

When fish were exposed to Ag (as $AgNO_3$ – 10.9 μg $l^{-1} \simeq 0.1$ μmol l^{-1}), plasma total Ag concentration increased from pre-exposure levels of about 70 μg kg^{-1} (0.7 μmol l^{-1}) to about 300 μg kg^{-1} (2.8 μmol l^{-1}; Table 2). The increase was significant by Day 1, and by Day 2, plasma Ag had stabilized at this elevated level (see Fig. 7A of Wood et al., 1996). Gill total Ag concentrations were several times higher than plasma levels under control conditions, and were significantly elevated about 3.5-fold when measured after 6 days exposure (Table 2). Total Ag in liver and kidney was about two orders of magnitude higher than in plasma under control conditions. Liver Ag also increased about 3.5-fold in response to Ag (as $AgNO_3$), but kidney levels did not change.

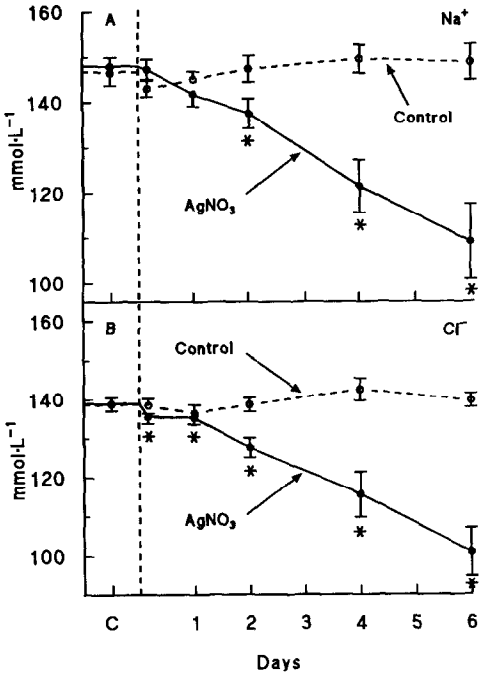

Fig. 5. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on (A) plasma Na^+ and (B) plasma Cl^- levels in rainbow trout. Means ±1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times, except at 4 h and Day 1 in (B).

Under control conditions, metallothionein (MT) concentrations were about 8-fold higher in liver than in gills or kidney (Table 2). However, there was no significant elevation in MT levels in any of these tissues in the $AgNO_3$ exposed fish (Table 2).

4. Discussion

The physiological disturbances seen in trout exposed to 10.9 μg Ag l^{-1} as $AgNO_3$ could have been caused by free ionic Ag^+ (34.5% of the total, according to MINEQL$^+$), or by either of the two dissolved forms of silver chloride (60.2% AgCl(aq), 5.2% $AgCl_2^-$). However LC50 tests have indicated that much higher levels of silver chlorides are extremely non-toxic to rainbow trout (Hogstrand et al., 1995) and fathead minnow (Terhaar et al., 1972; Leblanc et al., 1984). Furthermore, elevations in water Cl^-, which decrease free Ag^+ and reciprocally increase AgCl(aq) and $AgCl_2^-$ in solution, appear to decrease the toxicity of $AgNO_3$ (Galvez and Wood, 1994; Hogstrand et al., 1995). Other metals are most toxic in their free ionic forms, and exert their toxic effects by disrupting essential gill functions (Evans, 1987;

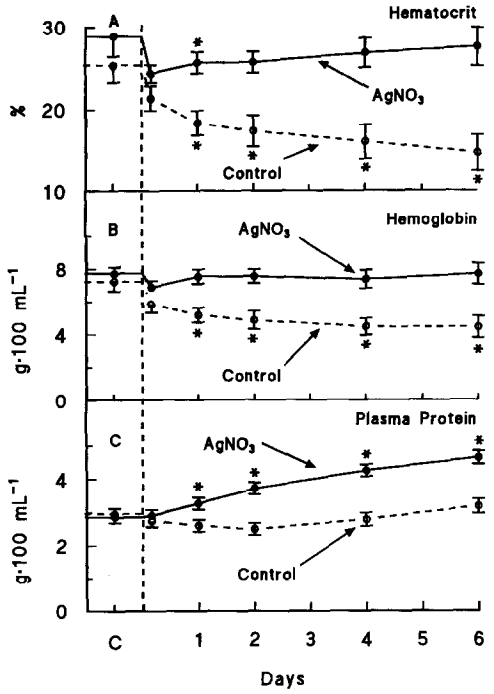

Fig. 6. The influence of 6 days exposure to 10 μg l^{-1} $AgNO_3$ ($n = 10$–12) or control conditions ($n = 8$) on (A) blood hematocrit, (B) blood hemoglobin concentration, and (C) plasma protein concentration in rainbow trout. Means ± 1 S.E.M. Asterisks indicate means significantly different ($p < 0.05$) from the pre-exposure value ('C') in that treatment. Experimental means were also significantly different from simultaneous control means at these times.

McDonald et al., 1989; Wood, 1992). Therefore, physiological disturbances seen in the present study were probably caused by free Ag^+ acting at the gills, and not by silver chlorides.

The gill total Ag levels in the present adult trout (Table 2) were about one third

Table 1
Parameters influencing hematological status in rainbow trout exposed to 10 μg l^{-1} $AgNO_3$ or control conditions for 6 days

	Control	$AgNO_3$
MCHC[a] (g Hb ml RBC^{-1})	0.279 ± 0.009	0.313 ± 0.022
Relative plasma volume (%)	95.2 ± 6.8%	60.8 ± 2.6%[b]
[Spleen weight/body weight] × 100	0.46 ± 0.06	0.28 ± 0.06[b]
Spleen hemoglobin (g $spleen^{-1}$)	0.101 ± 0.022	0.044 ± 0.014[b]

Means ± 1 S.E.M. ($n = 7$–11).
[a] MCHC = mean cell hemoglobin concentration.
[b] $p < 0.05$.

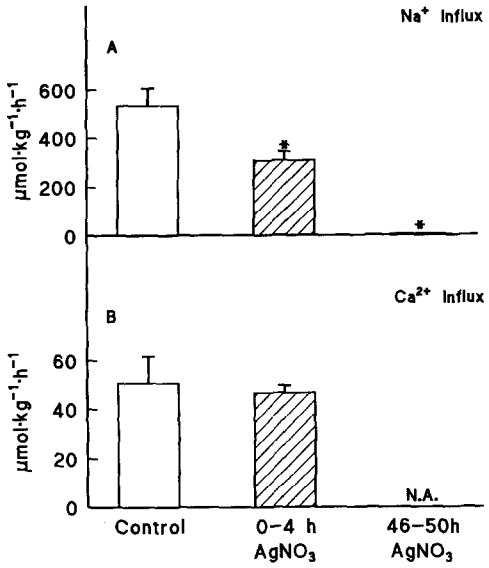

Fig. 7. Unidirectional Na^+ and Ca^{2+} uptake rates from the water in juvenile rainbow trout measured under control conditions, during the first 4 h of exposure to 10 μg l^{-1} $AgNO_3$, and after 46–50 h of exposure to $AgNO_3$. Means ±1 S.E.M (n=8–11). Asterisks indicate means significantly different ($p<0.05$) from the respective control treatment.

of those reported by Janes and Playle (1995) and Hogstrand et al. (1995) in juveniles exposed to a similar $AgNO_3$ concentration in identical water quality, suggesting an allometric difference. The gill surface bears net negative charge, and offers a range of possible binding sites — e.g. junctional, channel, and transport proteins, phospholipids, glycoproteins. Recently, Janes and Playle (1995) have successfully

Table 2
Total Ag and metallothionein levels in tissues of rainbow trout exposed to 10 μg l^{-1} $AgNO_3$ or control conditions for 6 days

		Control	$AgNO_3$
Total Ag			
(μg kg wet tissue^{-1})	Plasma	72 ± 7 (7)	300 ± 30[b] (8)
	Liver	10 520 ± 890 (6)	34 140 ± 6 360[b] (6)
	Gills	350 ± 50 (7)	1 250 ± 290[b] (6)
	Kidney	6 850 ± 1 740 (6)	6 190 ± 530 (6)
Metallothionein			
(mg kg wet tissue^{-1})	Liver	124.08 ± 29.48 (6)	131.02 ± 44.10 (7)
	Gills	16.51 ± 5.84 (6)	15.66 ± 2.63 (8)
	Kidney	13.32 ± 2.28 (4)	10.37 ± 1.06 (3)

Means ±1 S.E.M. (n).
[a] $p<0.05$.

modelled gill binding on the assumption that Ag^+ competes with other cations for anionic sites on trout gills, and these in turn compete for Ag^+ with anionic ligands in solution. The characteristics of these gill Ag^+-binding sites were different from the sites which bound Cd^{2+}, and their conditional equilibrium binding constants suggested possible co-valent bonding to sulfhydryl groups. The ability of Ag^+ to inhibit a variety of enzymes in vitro through binding to sulfhydryl groups is well-known (Cooper and Jolly, 1970; Taylor et al., 1980).

In addition to binding to the gills, Ag clearly entered the fish (Table 2) and accumulated in the blood plasma, reaching equilibrium by Day 2 (see Fig. 7A of Wood et al., 1996). Kidney total Ag levels did not change, but there was a significant accumulation of Ag in the liver by Day 6, indicating that the liver serves as an important clearance site. However there was no induction of metallothionein (Table 2). In juvenile trout exposed to a comparable level of $AgNO_3$ for 7 days, there was accumulation in liver and a small induction of metallothionein (Hogstrand et al., 1995). More substantial accumulation in internal organs has been reported in much longer exposures (2–4 months; Coleman and Cearley, 1974; Terhaar et al., 1977). Interestingly, in the present control trout (adults), plasma total [Ag] was appreciable and liver [Ag] was several-fold higher than in the juvenile trout studied by Hogstrand et al. (1995), suggesting that as the fish grows, it may continually sequester Ag even from background concentrations in the environment.

The complex suite of internal physiological disturbances observed in the present study were probably not caused by Ag acting internally, because much higher Ag levels accumulated in plasma and tissues of trout in the subsequent study, without any such internal disturbances (Wood et al., 1996). This suite included pronounced decreases of plasma Na^+ and Cl^-, metabolic acidosis, disruption of fluid volume regulation, hemoconcentration, and splenic contraction. Arterial P_{O_2} and P_{CO_2} were not detrimentally affected, and there was little or no effect on plasma Ca^{2+} and K^+. These symptoms of Ag exposure (as $AgNO_3$) were very different from those produced by comparable levels (relative to the LC50) of Zn (e.g. Spry and Wood, 1984, 1985; Hogstrand et al., 1994) and Cd (e.g. Giles, 1984; Verbost et al., 1989), both of which appear to act mainly as Ca^{2+} uptake antagonists. The actions of Ag bore partial resemblance to Cu and Al in causing a decline in plasma Na^+ and Cl^-, fluid volume disturbance, and hemoconcentration. However with Cu (Lauren and McDonald, 1987; Pilgaard et al., 1994), these effects are attenuated and reversed with continued exposure, metabolic alkalosis occurs, and losses of plasma Ca^{2+} and K^+ are also seen. None of these responses occurred with Ag (Fig. 3–Fig. 5). The principal difference from Al (Wood et al., 1988a,b; Goss and Wood, 1988; Playle et al., 1989) was the complete absence of any evidence of respiratory toxicity — e.g. hypoxemia, respiratory acidosis, or lactacidosis (Fig. 2). In contrast to early anecdotal reports of 'suffocation' at very high Ag levels (Cooper and Jolly, 1970), Ag is not a respiratory toxicant at more realistic concentrations.

The responses to Ag (as $AgNO_3$) appear closest to those to low pH. Indeed, the symptoms are almost identical to those documented earlier for rainbow trout of the same stock exposed to pH 4.0–4.5 in water of identical quality (McDonald et al., 1980; McDonald and Wood, 1981; Milligan and Wood, 1982; McDonald, 1983;

Wood, 1989). The primary toxic effect of acid exposure is a disruption of net Na^+ and Cl^- uptake at the gills, which in turn leads to all the other internal symptoms (Wood, 1989, 1992; McDonald and Wood, 1993). Death ensues when plasma Na^+ and Cl^- levels fall by about 40 mmol l^{-1}, equivalent to the decline seen by Day 6 in the present study (Fig. 5). The most important element in this disruption is an inhibition of the active Na^+ transport mechanism.

A preliminary test strongly supported a mechanism of action similar to that of low environmental pH. We exposed juvenile rainbow trout (2–4 g) to 10 μg l^{-1} $AgNO_3$ and measured unidirectional Na^+ and Ca^{2+} uptake from the water using ^{22}Na and ^{45}Ca by the methods of Lauren and McDonald (1987) and Hogstrand et al. (1994) respectively. Unidirectional Na^+ influx, determined over the first 4 h of $AgNO_3$ exposure, was reduced by 42%, and it was abolished (99% inhibition) at 46–50 h of exposure (Fig. 7). In contrast, Ca^{2+} uptake, measured over the first 4 h as a negative control, was unaffected. Model calculations using exchangeable body Na^+ pools and diffusive loss rates (cf. Wood, 1989; Goss and Wood, 1990) indicate that this inhibition of active uptake alone would be sufficient to explain the measured decreases in plasma Na^+ concentration (Fig. 5A). However elevated diffusive losses (not assessed in our test) may also contribute, because environmental acidity is also known to increase the diffusive efflux of Na^+ and Cl^- across the gills, apparently by increasing paracellular permeability (Wood, 1989, 1992).

By analogy to acid exposure (see Wood, 1989), we propose the following sequence of events leading to eventual death of fish from exposure to waterborne Ag^+. The net loss of Na^+ and Cl^- at the gills causes a steady decline in plasma Na^+ and Cl^- (Fig. 5). A stress response ensues — rapid activation of the sympathetic nervous system — chromaffin axis (catecholamine mobilization) and a slower activation of the pituitary interrenal axis (cortisol mobilization). Splenic discharge (Table 1) results from the former, and a progressive mobilization of glucose (Fig. 1) from the latter or both. As plasma Na^+ and Cl^- fall, the resulting osmotic imbalance is compensated by a shift of fluid out of the extracellular space into the intracellular compartment. The consequence is a decline in plasma volume (Table 1) and hemoconcentration (Fig. 6), exacerbated by the red blood cells added by the spleen (Table 1). Arterial blood pressure and heart rate increase due to sympathetic activation, the rise in pressure is intensified by the increase in blood viscosity resulting from hemoconcentration, and the fish eventually dies from circulatory failure. While these cardiovascular responses were not monitored in the present study, they have been recorded in both acid (Milligan and Wood, 1982) and Cu exposed trout (Wilson and Taylor, 1993). One possible explanation for the better than expected survival of the present trout (relative to toxicity tests with juveniles; Hogstrand et al., 1995) may be that repetitive blood sampling attenuated the hemoconcentration response (Fig. 6).

Blood metabolic acidosis (Fig. 3C) in the absence of lactacidosis (Fig. 2C) is also seen in rainbow trout exposed to low environmental pH in water of identical quality (McDonald et al., 1980; McDonald and Wood, 1981). The response reflects 'acid loading' from the water caused either by a preferential inhibition of Na^+ uptake (which is coupled to acid excretion) relative to Cl^- uptake (which is coupled to base

excretion) and/or a greater diffusive loss of Na^+ relative to Cl^- (McDonald, 1983; Wood, 1989). To ascertain whether the same explanation applies to Ag exposure (as $AgNO_3$) will require measurements of unidirectional Na^+ and Cl^- fluxes and net acidic equivalent fluxes with the water.

Metabolic acidosis probably caused the accompanying increase in P_{aO_2} (Fig. 2A) and decrease in P_{aCO_2} (Fig. 2B). Decreases in arterial blood pH can play an important secondary role in stimulating ventilation in fish (e.g. Heisler, 1989; Kinkead and Perry, 1991; Wood and Munger, 1994). We noticed that by Day 3 of $AgNO_3$ exposure, the fish were clearly breathing more deeply. This hyperventilation, perhaps in combination with catecholaminergic gill vasodilation, likely explained the improvement of arterial oxygenation. The decrease in P_{aCO_2} associated with hyperventilation ('respiratory compensation') helped moderate the fall in pHa caused by metabolic acidosis.

In summary, Ag (as $AgNO_3$) at levels approximating the 7 day LC50 causes internal physiological disturbances almost identical to those caused by low environmental pH. The actions are likely caused by ionic Ag^+ binding to the gill surface and interfering with the mechanism(s) of active Na^+ (and Cl^-?) uptake, resulting in a profound decrease of plasma Na^+ and Cl^- concentrations, internal fluid-volume disturbance, hemoconcentration, and cardiovascular collapse. These findings suggest that silver may be most toxic in soft ion-poor water (low in anionic complexing agents) of low pH, such as streams impacted by acid drainage from silver mining. However, do such findings on toxic mechanism at an LC50 concentration (10 μg l^{-1}) have any relevance to natural waters where Ag levels may be only a few percent of this figure (Chudd, 1983; Lytle, 1984)? The answer is certainly yes, for recently we have shown that the principal sublethal effect in trout during chronic exposure to only 0.5 μg l^{-1} Ag (as $AgNO_3$) is a qualitatively similar, though much smaller, depression of plasma Na^+ and Cl^- (F. Galvez et al., unpublished results).

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