The distribution kinetics of waterborne silver-110m in juvenile rainbow trout

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Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) were subjected to a 2-day radioactive pulse of ^{110m}Ag at 11.9 µg/l (as AgNO$_3$), followed by a 19-day post-tracer exposure to non-radioactive Ag(I) (3.8 µg/l). The distribution of ^{110m}Ag in the gills, liver, intestine, kidney, brain and remaining carcass was investigated over a 19-day post-tracer period. Initially, the intestine contained the highest proportion of the ^{110m}Ag burden (34%), however, by day 8, less than 5% of the total radioactivity remained in this tissue. The majority of the ^{110m}Ag eliminated from the intestine appeared to distribute to the liver. Eventually, the ^{110m}Ag content in the liver accounted for as much as 65% of the total radioactivity in the fish. Apart from the liver and intestine, only the gills and carcass contained any appreciable amount (>5%) of the total body ^{110m}Ag content. Liver and gill samples were fractionated using differential centrifugation techniques to discern the subcellular distribution of ^{110m}Ag in these tissues. In the liver, the ^{110m}Ag levels in the cytosolic fraction increased from 35% to 72% of the total cellular burden between days 8 and 19, respectively. The radioactive pulse in the gills was predominantly found in a membrane compartment termed the nuclear fraction (~60% of the total). Little change was observed over time (day 8 to day 19) to the subcellular distribution of Ag in the gills. Using size-exclusion chromatography, most (~70%) of the ^{110m}Ag content in the liver cytosol eluted at a molecular weight characteristic of metallothionein. The cytosolic distribution of ^{110m}Ag in gills was quite diffuse, occurring primarily in the heavy molecular weight fractions. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Silver nitrate is extremely toxic to freshwater fish, with 96-h LC$_{50}$ values typically between 5 and 65 µg/l Ag(I) (Coleman and Cearley, 1974; Davies et al., 1978; Lemke, 1981; Hogstrand et

al., 1996). Only recently has progress been made towards understanding the physiological mechanisms of silver toxicity. Acute silver toxicity results from an impairment of Na$^+$ uptake processes caused by non-competitive inhibition of branchial Na/K-ATPase activity (Wood et al., 1996a; Morgan et al., 1997). The ensuing ionoregulatory disturbance produces a series of physiological perturbations that can eventually lead to cardiac failure and death (Hogstrand and Wood, 1998). It is believed that the free Ag(I) form of the metal actively binds to sites on the gill, namely the magnesium sites on the Na/K-ATPase (Ferguson et al., 1996), thus causing toxicity (Terhaar et al.,

1977; Galvez and Wood, 1997; Bury and Wood, 1999). Conversely, Ag species such as the AgCl, $AgCl_2^-$ and Ag thiosulfate complexes are much more benign to freshwater fish compared to $AgNO_3$ (Hogstrand et al., 1996). Silver, when presented as these complexes, seems to pass through the branchial epithelium without causing an acute toxic response at the gills, and subsequently accumulates in other parts of the body (Hogstrand et al., 1996; Hogstrand and Wood, 1998). The most notable example is a study in which rainbow trout were exposed for 6 days to extremely high concentrations of silver (30 000 μg/l) as silver thiosulfate. Even though fish accumulated high burdens of Ag in the gills, plasma, kidney and liver, the exposure was not acutely toxic (Wood et al., 1996b).

Bioaccumulated Ag(I) can either directly or indirectly induce metallothionein in fish liver, suggesting that silver can affect biochemical processes (Cosson, 1994; Hogstrand et al., 1996). Moreover, trout exposed to 2.0 μg/l silver (as $AgNO_3$) during a 28-day period exhibited a marked elevation in hepatic Ag levels, accompanied by a modest induction of metallothionein, depressed growth rates, and decreased food-conversion efficiencies (Galvez et al., 1998). Further studies are needed to identify whether chronic toxicity is eventually produced from elevated metal tissue burdens. One of the existing hurdles is the lack of information on the kinetics of uptake, internal distribution, fate and elimination of Ag in teleost fish.

The purpose of this study was to investigate the pharmacokinetics of a two-day radioactive Ag(I) pulse during a 19-day post-tracer period, with continuing exposure to silver at a sublethal level. The continued presence of (non-radioactive) silver during the post-tracer period was felt to best mimic an actual environmental exposure. The Ag(I) isotope ^{110m}Ag was used to monitor Ag distribution and elimination in individual tissues, and silver was added as $AgNO_3$. Differential centrifugation techniques, as well as size-exclusion chromatography were used to elucidate the subcellular distribution of the ^{110m}Ag pulse in gill and liver.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing approximately 10 g were obtained from Wolf Creek Dam National Fish Hatchery (Jamestown, KY, USA), and transported to the University of Kentucky, Lexington. Fish were allocated to three separate 60-l polystyrene tanks supplied with 150 ml/min of dechlorinated Lexington tap water plus aeration. A total of 50 fish were used in this set of experiments. The chemical composition of the water was: (in mmol/l) $[Na^+]$ 0.65; $[Cl^-]$ 0.61; $[Ca^{2+}]$ 0.22; (in mg/l as $CaCO_3$) total alkalinity of 68 and total hardness of 126; and pH of 7.7. Although dissolved organic matter (DOM) was not measured, typical values for groundwater sources approximate 1.3 mg carbon/l. Water temperature was maintained at 15 °C. Fish were allowed to acclimate to laboratory conditions for 2 weeks prior to the start of Ag(I) exposures. Fish were fed commercial trout pellets (Purina Trout Chow) to satiation once daily, except during the 2-day radioactive ^{110m}Ag pulsing period when food was withheld.

2.2. Silver exposures

At the start of the experiment, water flow to the tanks was stopped, vigorous aeration was instituted, and the fish were statically exposed for 2 days to a radioactive silver pulse of ^{110m}Ag (as $AgNO_3$). During previous static exposures to Ag, we routinely found that Ag would bind non-specifically to the exposure tank and thus reduce the concentration of the metal by up to 50%. As a result, we added ^{110m}Ag at a concentration of 10 μg/l, which represented twice the nominal concentration we tried to achieve during the entire exposure period. However, Ag did not appreciably bind to the exposure tanks, resulting in a measured total [Ag(I)] of 11.9 μg/l Ag(I). Silver concentrations were achieved by adding radiolabeled silver stock (2.2 g/l ^{110m}Ag as $AgNO_3$ at an activity of 303 MBq/ml) (Amersham, Oakville, ON, Canada) to each tank containing 40 l of water. The post-tracer phase of the experiment was commenced by removing ∼85% of the radioactive water from each tank and replacing it with water containing a nominal concentration of 5.0 μg/l non-radioactive Ag(I) (as $AgNO_3$; Sigma, St. Louis, MO, USA). Efficient removal of the radioactive ^{110m}Ag from the water was verified by lack of any measurable gamma-radiation in water samples taken 12 h after starting the post-tracer period (MINAXI Auto-Gamma 5000 series, Canberra-Packard). The measured concentration of Ag dur-

ing the 19-day post-tracer period was 3.8 μg/l Ag(I) and was delivered at a rate of 150 ml/min. Water samples were taken daily from each exposure tank for analysis of total Ag(I). Samples were acidified with trace-metal grade HNO_3 acid (Fisher Scientific) to a concentration of 0.5% (v/v). Total Ag(I) was measured by atomic absorption spectroscopy (Varian SpectrAA-20) with a graphite furnace atomizer (Varian, GTA-96).

2.3. Tissue sampling and analysis

On days 1, 4, 8, 14 and 19 of the post-tracer period, six fish were randomly selected and placed in 20 l of dechlorinated tap water. Each fish was subsequently rinsed in 250 μg/l Ag(I) (as $AgNO_3$) solution for 1 min to displace any superficially bound [110m]Ag. This was followed by another 1-min rinse in a solution containing 150 mg/l sodium thiosulfate and 0.3 g/l MS-222 anaesthetic. This rinsing protocol was found to be the most effective at removing superficially bound Ag from the outer surface of fish as determined during preliminary studies. Sacrificed fish were blotted dry and weighed; blood samples were collected using a sodium-heparinized Hamilton syringe; and plasma aliquots were obtained by centrifuging the blood at $10\,000 \times g$ for 2 min. The liver, intestine (from the pyloric caeca to the anus), entire gill basket, kidney and brain were then dissected out of each fish. Each tissue, in addition to the remnant carcass, was blotted dry, weighed, placed in a 1.5-ml Eppendorf tube, and quick-frozen in liquid nitrogen. Samples were stored at -80 °C until analyzed for [110m]Ag and total Ag burdens. The contents of the intestinal tract were carefully removed prior to cold storage of the tissue. Whole body values for [110m]Ag concentration and content were obtained by summing the component tissues.

Silver-110m was determined by placing Eppendorf tubes, containing the pre-weighed tissues, into scintillation vials for analysis of radioactivity using a gamma counter. Following [110m]Ag analysis, tissues were transferred from the Eppendorf tubes to acid-washed test tubes. Samples were digested in 2.5 ml concentrated HNO_3 for 2 h at 120 °C. Samples were allowed to cool to room temperature and 375 μl of 30% H_2O_2 were added to each test tube. Tubes were then slowly heated to 120 °C and each of the digests was evaporated to dryness. Each sample was resuspended in an appropriate amount of 0.5% HNO_3 and analyzed for total Ag

by graphite furnace AAS. Unfortunately, problems encountered during the tissue digestion of the carcass samples, and some of the day 19 tissues (gills, livers and plasma), prevented us from measuring the total Ag concentrations of these samples.

2.4. Subcellular fractionation

On days 8 and 19 of the post-tracer period, 10 additional fish were killed according to the above methods. Gills and livers were freeze-clamped in liquid nitrogen for subcellular fractionation using a protocol modified from Olson and Hogstrand (1987) and Julshamn et al. (1988). In short, tissues were thawed, and immediately homogenized at 0–2 °C in either a hypertonic (35 mM Tris–HCl, 250 mM sucrose, 200 mM KCl, pH 7.4) or hypotonic (10 mM Tris–HCl, pH 7.6) buffer using a glass–Teflon homogenizer. The two different buffers were used because of concern that some MT-bound [110m]Ag would remain in these fractions during centrifugation in the regular fractionation buffer (Julshamn et al., 1988). It was reasoned that a hypotonic buffer should extract water soluble MT from all intracellular compartments. The hypertonic buffer was used to preserve soluble proteins within intracellular membrane structures, such as nuclei and lysosomes. Subcellular fractions were obtained by a series of centrifugations whereby the pellet of each spin was collected, and the supernatant re-centrifuged at a higher centrifugal force. The original homogenate was first centrifuged at $370 \times g$ for 5 min, and the pellet from this spin was labeled the nuclear (*nuc*) fraction. In addition to nuclear material, this fraction would contain relatively insoluble cellular debris. Likewise, pellets produced from $9200 \times g$ (5 min) and $130\,000 \times g$ (1 h) spins were termed the mitochondrial and lysosomal (*m&l*) and microsomal (*mic*) fractions, respectively. The remaining supernatant was labeled the cytosolic fraction (*cyt*). Individual subcellular fractions were analyzed for [110m]Ag, and the data were expressed as radioactive counts per minute (CPM) per gram tissue. Cytosolic fractions were analyzed frozen and then returned to liquid nitrogen, and stored at -80 °C. Further fractionation of cytosolic fractions was performed by size-exclusion liquid chromatography.

2.5. Chromatography of cytosolic fractions

Cytosolic fractions were thawed individually and fractionated by size-exclusion liquid chroma-

tography using Superdex-75 filtration media (Pharmacia, Uppsala, Sweden) in a 60 cm×1.6 cm gel filtration column (XK 16/70, Pharmacia). The column was calibrated for molecular weight with bovine albumin (mol. wt.$=M_r=66\,000$), cytochrome-*c* Type III (lung tissue; $M_r=12\,400$), rabbit metallothionein (MT; apparent mol. wt.$=10\,000$) and glutathione ($M_r=310$). Gill and liver cytosols (100 μl aliquots) were injected into the Superdex column and eluted at a rate of 1 ml/min with 250 ml of 10 mM Tris–HCl (pH 7.6) at 2 °C. A liver homogenate from Cd-injected rainbow trout was fractionated and the MT fractions (eluted at 9.4 kDa) were partially verified by the presence of Cd, as analyzed by graphite furnace atomic absorption spectroscopy. The elutant was collected in 3-ml fractions, which was then analyzed for radioactivity, and for absorbance at 280 nm. The elution profile was divided into three parts: void volume to 93 ml was termed the heavy molecular weight fractions (HMW), 93–114 ml elution volume (i.e. M_r ranging from 5800 to 22 900 kDa) was referred to as the metallothionein fractions (MT) and >114 ml was termed the low molecular weight fractions (LMW). The MT size region corresponded to the elution of MT in the liver reference, as well as purified rabbit MT.

2.6. Calculations and statistical analysis

Tissue concentrations of non-radioactive Ag (μg/g) and [110m]Ag (cpm/g), and whole body [110m]Ag contents were log-transformed prior to statistical analysis to handle departures from normality. Whole body data were calculated by summing the component tissues. Values were expressed as means ±95% confidence limits following log transformation. All data expressed as percentages of total tissue radioactivities were arcsine transformed prior to statistical analysis. Homogeneity of variances was assessed using the Levene test. Time trends (for tissue concentrations of Ag and [110m]Ag and tissue [110m]Ag contents) were tested for statistical significance using one-way analysis of variance (ANOVA) or using the non-parametric Kruskal–Wallis ANOVA test. The [110m]Ag distribution in the subcellular fractions of gills and liver were statistically compared using multivariate analysis of variance techniques (MANOVA). This test looked for main effects and interactions among three different variables that included time, homogenization buffer and compartments. A combined analysis was performed that accounted for the interdependence among the [110m]Ag values in the compartments. Interaction between the two tissue types (gill vs. liver) was not tested in our analysis. Post-hoc multiple comparisons were performed on these data using the Newman–Keuls test. A *P*-value of 0.05 was considered statistically significant throughout. All statistical analyses were performed using STATISTICA (version 5).

3. Results

The total Ag burdens of tissues sampled during the 19-day post-tracer period are outlined in Table 1. Silver concentrations in the liver were approximately 10-fold higher than the Ag concentrations in any of the other tissues analyzed. The hepatic concentrations of Ag remained constant (~ 9 μg/g) during the entire chronic exposure. In comparison, there was a monotonic increase in the concentration of Ag in the brain, However, even the highest Ag concentration measured in the brain was low compared with that measured in the liver. The Ag concentrations in the other tissues did not significantly change over time, except for fluctuations in the Ag concentrations in the plasma and intestine.

Unlike the relatively few significant differences measured over time in the total Ag concentration of tissues (Table 1), there were marked changes in the concentrations of [110m]Ag (cpm/g basis) in tissues (Table 2). The most pronounced effects on [110m]Ag concentration were seen in the intestine and the liver. By day 19, the [110m]Ag concentration in the intestine was less than 3% of the day 1 level. The decrease in intestinal [110m]Ag concentration over time coincided with a transient increase in the cpm/g value of the liver (day 8). The [110m]Ag concentrations of some of the other tissues tended to fluctuate throughout the experiment including a reduction in the brain (day 4), gill (day 14) and plasma (days 4, 14). In comparison, [110m]Ag did not appear to eliminate readily from the whole bodies of rainbow trout during the 19-day experimental period (Tables 2 and 3).

The internal distribution of [110m]Ag was also monitored in various tissues over the 19-day post-tracer period. Fig. 1 illustrates [110m]Ag content in each tissue as a percentage of whole-body radioactivity levels (Table 3). Initially, the intestine contained the highest proportion of the [110m]Ag

Table 1
Total silver concentrations measured in tissues of juvenile rainbow trout ($n=6$) during days 1, 4, 8, 14 and 19 of the post-tracer period

Tissue	Days				
	1	4	8	14	19
Gill	0.30[a] (0.14–0.64)	0.20[a] (0.10–0.38)	0.31[a] (0.22–0.45)	0.17[b] (0.09–0.35)	N.M.
Intestine	0.57 (0.24–1.32)	0.73 (0.11–4.90)	0.67 (0.19–2.33)	0.35 (0.22–0.55)	2.23 (0.44–11.5)
Liver	8.88 (5.25–15.1)	9.06 (2.69–30.9)	9.28 (6.31–13.5)	8.55 (6.92–10.7)	N.M
Kidney	0.92 (0.47–1.82)	0.49 (0.23–1.02)	0.61 (0.36–1.02)	0.78 (0.50–1.23)	0.88 (0.43–1.82)
Brain	0.08[a] (0.05–0.13)	0.10[a] (0.06–0.15)	0.10[a] (0.07–0.14)	0.21[a,b] (0.09–0.46)	0.25[b] (0.16–0.37)
Plasma	0.02 (0.01–0.04)	0.03 (0.01–0.07)	0.011 (0.01–0.02)	0.04 (0.02–0.09)	N.M.
Carcass	N.M.	N.M.	N.M.	N.M.	N.M.

Total Ag concentrations are expressed as mean values of μg Ag per gram wet weight ± lower and upper 95% confidence limits. Mean values that are significantly different ($P<0.05$) from one another are denoted by different letters. (N.M., not measured—see text for details.)

(34%), although by day 8 less than 5% of the total radioactivity remained in this tissue. The [110m]Ag proportion of the liver increased steadily from 24% (day 1) to ~61% (day 19). Apart from the liver and intestine, only the gills and carcass contained any appreciable amount (>5%) of the whole body [110m]Ag content at any time during the post-tracer period. Worth noting is that the large alterations in the concentration of [110m]Ag in tissues (such as the intestine and liver) (Table 2) and its proportion of the whole body [110m]Ag content (Fig. 1) did not coincide with the relatively few changes in total Ag concentrations of these tissues (Table 1). This observation would suggest that the tissues contained relatively large background levels (pre-exposure) of Ag (Table 1) and that [110m]Ag accumulation (during the pulsing period) was negligible compared with the background Ag concentrations.

The BCF value for the liver is at least 10-fold higher than the estimates for all other tissues, except the intestine (Table 4). Note, however, that the BCF value measured in the intestine decreased over time as [110m]Ag appeared to be redistributed to the liver.

Subcellular fractionation of gills and livers was performed on fish sampled on days 8 and 19 of the post-tracer period. The subcellular fractions of gills and liver in rainbow trout showed distinct patterns of [110m]Ag distribution. The [110m]Ag burden in the gills distributed primarily to the nuclear '*nuc*' fraction (~60%) and marginally to the cytosolic '*cyt*' fraction (~20%) (Fig. 2). The type of homogenization buffer used or the amount of time elapsed from the end of the pulsing period did not affect the distribution pattern of [110m]Ag in the gill. In comparison, [110m]Ag in the liver was primarily distributed to the *cyt*, except in livers sampled on day 8 and homogenized in hypertonic buffer. In these samples, all four subcellular fractions of the liver appeared to have approximately equal amounts of [110m]Ag. However, by day 19, livers homogenized in hypertonic buffer showed significantly greater levels of [110m]Ag in the *cyt* relative to the other three subcellular fractions. There was also a tendency for [110m]Ag burden to increase in the *cyt* of livers between day 8 and day 19, regardless of the homogenization buffer used (Fig. 3). There was an increase in the relative [110m]Ag content of the *cyt*, even though no significant changes in the proportion of [110m]Ag burden in the microsomal fraction '*mic*', mitochondrial and lysosomal fractions '*m&l*' ($P<0.05$) and the *nuc* ($P<0.005$) fraction of the liver existed between day 8 to day 19 (Fig. 3). It appeared that small, yet insignificant reductions in [110m]Ag burden of the *mic*, *m&l* and *nuc* fractions resulted in the observed increase in the *cyt* fraction over time.

There were clear differences in the cytosolic distribution of [110m]Ag between the gills and liver (Figs. 4–6). Fig. 4a,b shows representative gel elution profiles of *cyt* gill samples. The cytosolic distribution of [110m]Ag in gills was diffuse, with binding of the metal to several fractions, including

Table 2
^{110m}Ag concentrations (in cpm g^{-1} wet weight$\times 10^3$) measured in tissues and whole bodies of juvenile rainbow trout ($n=6$) during days 1, 4, 8, 14 and 19 of the post-tracer period

Tissue	Days				
	1	4	8	14	19
Gill	6.62	4.20	6.37	2.98	N.M.
	(5.13–8.45)	(1.51–11.6)	(4.57–8.81)	(2.00–4.45)	
Intestine	122[a]	34.5[b]	15.7[b]	3.22[c]	3.57[c]
	(60.1–249)	(7.67–155)	(7.94–30.9)	(1.98–5.23)	(1.31–9.76)
Liver	68.9[a]	67.5[a,b]	171[b]	106[a,b]	91.9[a]
	(46.1–103)	(44.7–102)	(118–248)	(69.4–162)	(32.1–263)
Kidney	12.6	8.57	8.14	13.8	7.44
	(7.99–19.9)	(2.11–34.7)	(5.61–11.8)	(8.63–22.0)	(2.71–20.4)
Brain	0.68[a,c]	0.25[b]	1.29[a]	0.93[b,c]	0.94[b,c]
	(0.52–0.89)	(0.008–0.72)	(0.92–1.80)	(0.56–1.55)	(0.47–1.89)
Plasma	1.08[a]	0.46[b]	1.30[a]	0.52[b]	N.M.
	(0.69–1.68)	(0.17–1.25)	(0.92–1.83)	(0.23–1.15)	
Carcass	1.18	0.50	0.69	0.66	0.64
	(0.79–1.78)	(0.23–1.11)	(0.55–0.86)	(0.32–1.36)	(0.22–1.93)
Whole body	2.97[a]	1.81[b]	3.06[a]	2.13[a,b]	1.78[a,b]
	(2.09–4.17)	(1.10–2.95)	(2.29–4.07)	(1.45–3.16)	(1.18–3.39)

Values are expressed as means$\pm$lower and upper 95% confidence limits. Mean values that are significantly different ($P<0.05$) from one another are denoted by different letters. (N.M., not measured—see text for details.)

fractions containing MT. The proportions of total ^{110m}Ag in the MT size region of gills were only 17.8% and 8.0% for day 8 and day 19, respectively. In comparison, Ag content in the HMW and LMW regions was 60.7% to 63.0% and 21.5% to 28.3%, respectively (Fig. 6a). In the HMW fractions of gills, the major ^{110m}Ag peak was found at 220 kDa, whereas the main ^{110m}Ag binding region in the LMW fractions was at 1.2 kDa. Fig. 5a,b shows representative elution profiles of liver *cyt* fractions following size-exclusion chromatography. Fractionation of liver cytosols from day 8 produced one major ^{110m}Ag peak eluting at a molecular weight characteristic of MT (Fig. 5a). Approximately 70% of the ^{110m}Ag fractionated within the MT size region, whereas only 11% and 14% of

the radioisotope eluted at the HMW and LMW regions, respectively (Fig. 6b). In the HMW fractions of the liver, ^{110m}Ag was mainly found in the 220 kDa region (same as in the gills), whereas the main ^{110m}Ag binding region in the LMW fractions was at 2.2 kDa. The elution profiles of livers varied slightly with time (Fig. 5b). Although over 65% of the ^{110m}Ag continued to elute at the MT size region, a slight spill over of Ag to HMW fractions (two-fold increase) appeared to occur by day 19 (Fig. 6b).

4. Discussion

This study assessed the uptake of Ag(I) from water and its subsequent internal distribution in

Table 3
Whole body ^{110m}Ag concentrations (cpm/g) and total ^{110m}Ag contents of juvenile rainbow trout measured during days 1, 4, 8, 14 and 19 of the post-tracer period

Days	Fish weight (g)	Fish [^{110m}Ag] (CPM/g)$\times 10^3$	Total ^{110m}Ag content (CPM)$\times 10^3$
1	10.4	2.97 (2.09–4.17)[a]	30.2 (21.7–41.9)
4	9.0	1.81 (1.10–2.95)[b]	14.4 (11.1–18.8)
8	11.7	3.06 (2.29–4.07)[a]	36.1 (22.7–57.5)
14	11.0	2.13 (1.45–3.16)[a,b]	23.4 (14.7–37.3)
19	13.3	1.78 (1.18–3.39)[a,b]	22.3 (12.7–38.9)

Values are expressed as means$\pm$lower and upper 95% confidence limits ($n=6$). Mean values that are significantly different ($P<0.05$) from one another are denoted by different letters.
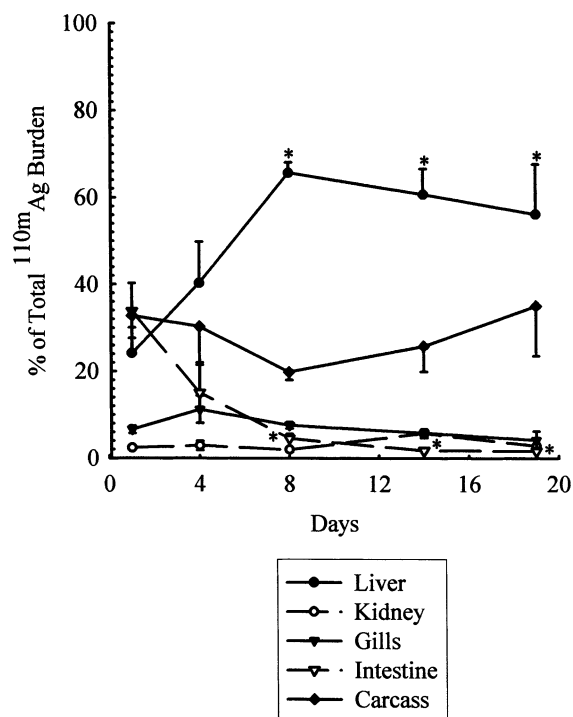
Fig. 1. ^{110m}Ag burden in gills, intestine, liver, kidney, and remaining carcass of rainbow trout as a percentage of the total body radioactivity measured during days 1, 4, 8, 14 and 19 of the post-tracer period. Values are expressed as mean$\pm$S.E. ($n=6$). The plasma and brain data were removed from the figure for clarity, because they contained $<0.2\%$ of the total radioactivity at each of the sample periods. Percentages were arc sine transformed, and ^{110m}Ag burden over time for each tissue was statistically compared with day 1 values using one-way ANOVA. $*P<0.05$.
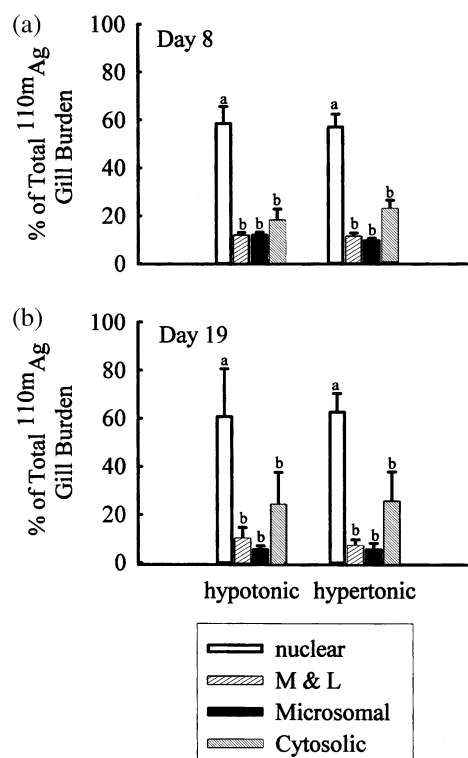

Fig. 2. Subcellular distribution of ^{110m}Ag in gills homogenized either in hypotonic buffer or hypertonic buffer from rainbow trout on (a) day 8 and (b) day 19 of the post-tracer period. Each value is expressed as the percentage mean$\pm$S.E. ($n=5$ per homogenizing medium). The nuclear fraction was obtained after the first centrifugation at $370\times g$ for 5 min; the mitochondrial and lysosomal fraction was obtained after centrifugation at $9200\times g$ for 5 min; the microsomal fraction was obtained after centrifugation at $130\,000\times g$ for 60 min; cytosolic fraction and remaining supernatant. There were no significant effects of depuration time or homogenization medium on distribution of ^{110m}Ag. Different letters were used to denote statistical differences ($P<0.05$) between mean values for subcellular compartments (at any given time or homogenization buffer).

Table 4
Bioconcentration factors (BCF) measured for tissues and whole bodies of juvenile rainbow trout ($n=6$) during days 1, 4, 8, 14 and 19 of the post-tracer period

Tissue	Days				
	1	4	8	14	19
Gill	5.5	3.5	5.3	2.5	N.M.
Intestine	102.0	28.8	13.1	2.7	3.0
Liver	57.4	56.3	142.6	88.3	76.5
Kidney	10.5	7.1	6.8	11.5	6.2
Brain	0.6	0.2	1.1	0.8	0.8
Plasma	0.9	0.4	1.1	0.4	N.M.
Carcass	1.0	0.4	0.6	0.5	0.5
Whole body	2.5	1.5	2.5	1.8	1.5

BCF values are expressed as the ratio of CPM/g tissue (Table 2) to the measured radioactivity of the water in the 2-day ^{110m}Ag pulse (1200 CPM/ml). (N.M., not measured.)

freshwater rainbow trout. Metal uptake in teleost fish occurs primarily at the gill surface and in the gastrointestinal tract. Although waterborne metals are predominantly taken up via the gills in freshwater fish, the inadvertent consumption of water during feeding may lead to increased gastrointestinal metal uptake. Drinking rates in starved seawater fish are approximately 3.0 ml/kg per h, but only 0.25 ml/kg per h in starved freshwater fish (Fuentes and Eddy, 1997). The present study attempted to look at Ag(I) accumulation across the gills alone. Therefore, feeding was suspended during the 2-day ^{110m}Ag pulsing period to reduce
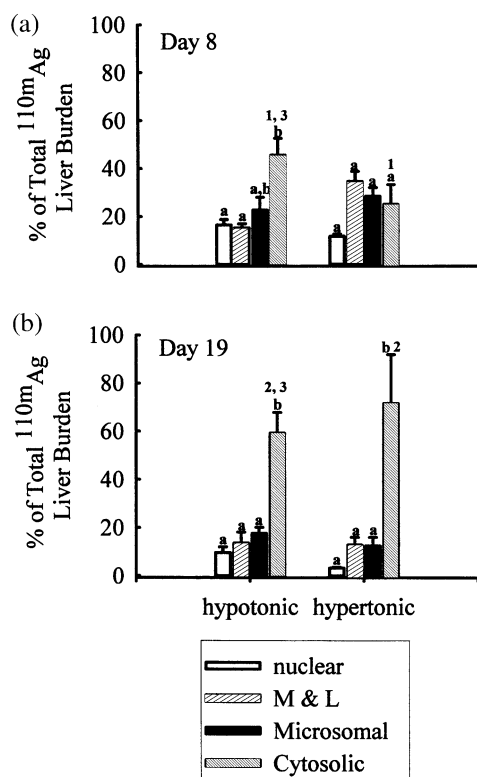
Fig. 3. Subcellular distribution of ^{110m}Ag in livers homogenized either in hypotonic buffer or hypertonic buffer from rainbow trout on (a) day 8 and (b) day 19 of the post-tracer period. Each value is expressed as the percentage mean $\pm$ S.E. ($n=5$ per homogenizing medium). See legend of Fig. 2 for further details. Different letters were used to denote statistical differences ($P<0.05$) between mean values for subcellular compartments (at any given time or homogenization buffer). Different numbers were used to denote statistical differences between the mean values of each subcellular compartment (test for effects of time and homogenization buffer).

gastrointestinal uptake of the metal. As will be discussed in more detail below, Ag(I) uptake from the water probably occurred almost exclusively across the gill epithelium.

Radioisotopic ^{110m}Ag was used to distinguish between newly accumulated Ag(I) and background Ag levels in the fish. ^{110m}Ag was completely removed from the water shortly after the start of the post-tracer period during Day 0. Therefore, any radioactivity in the fish would have accumulated solely during ^{110m}Ag pulsing (days -2 to 0). Based on day 1 levels for whole body CPM (Table 3), the accumulation rate (AR) of Ag(I) into rainbow trout from water was calculated to be 15.8 ng/(g·day). To further assess the bioavailability of waterborne Ag(I), a concentration

specific accumulation rate (CSAR) was determined (see Hogstrand and Wood, 1998). The CSAR value, calculated by normalizing the Accumulation Rate (AR) to the exposure concentration in parts per billion, was 1.3 ng/(g·day·ppb). This is in contrast with the AR of 72.3 ng/(g·day) and CSAR of 9.0 ng/(g·day·ppb) reported for rainbow trout subjected to 4-h fluxes of 8 μg/l Ag(I) (as ^{110m}AgNO$_3$) in synthetic soft water (Bury et al., 1999a). The discrepancy in AR and CSAR values between studies is likely due to variations in water chemistry. Water from the present study contained three-fold higher DOM and two-fold higher sodium than Bury et al. (1999a,b). The combined effect of increased levels of a complexing ligand and a competing ion in water is expected to decrease the bioavailability of Ag(I) to whole fish. Differences in aqueous chloride are likely inconsequential to bioavailability (Bury et al., 1999a,b), and only Ag(I) is of acute toxicological concern (Galvez and Wood, 1997; Bury et al., 1999a; Wood et al., 1999).

Any apparent loss of whole-body ^{110m}Ag on a per gram basis was likely due to dilution resulting from fish growth (Table 3). It is not expected that Ag is easily eliminated from rainbow trout. The only other published study on Ag depuration in salmonids reported a 23% reduction in radioactivity after 28 days in clean water (Garnier et al., 1990), but attributed much of the apparent elimination of Ag to growth dilution by the fish. The carcass (as defined herein) is a relatively important sink for silver in fish, accounting for between 30 and 35% of the total body radioisotope burden (Fig. 1). Nonetheless, because of the relatively large mass of the carcass, the concentration of silver in the carcass was low when expressed on a per gram basis (Table 2), and therefore probably was not toxicologically relevant.

High amounts of ^{110m}Ag were accumulated in the intestinal tract up to 4 days following the radioactive exposure (Fig. 1, Table 2). Based on the normal drinking rate of starved, freshwater fish (Fuentes and Eddy, 1997), only ~0.12 ml of water would have entered the gastrointestinal tract during the 48-h radioactive pulsing period. Consequently, only 140 CPM or approximately 1% of the observed intestinal ^{110m}Ag burden at day 1 could be explained by inadvertent drinking of water. Therefore, Ag accumulated within the intestines was probably taken up at the gills and transported via the bloodstream. The high accu-
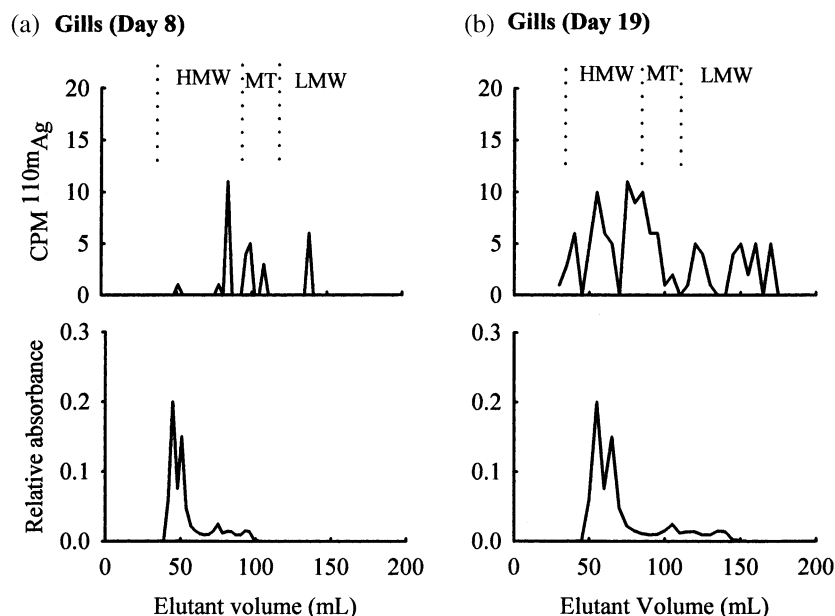
Fig. 4. Representative elution profiles from gel filtration chromatography (Superdex-75) from the cytosolic fraction of rainbow trout gills on (a) day 8 and (b) day 19 of the post-tracer period. Top panels are the radioactive counts (CPM) of each fraction. Bottom panels are relative absorbances at 280 nm for each fraction.

mulation potential for Ag in the intestine could be due to the tissue's abundance of basement membranes (Baudin et al., 1994). Basement membranes have a high content of cysteinyl residues and Ag binds preferentially to such sulfhydryl groups. This is consistent with studies by Martoja et al. (1988) and Baudin et al. (1994), which also observed high concentrations of Ag in the digestive tract of other aquatic organisms.

Most of the intestinal radioactivity observed during the early portions of the study was quickly redistributed to the liver. By day 8, the liver contained approximately 65% of the [110m]Ag. Similarly, Garnier et al. (1990) found that the liver contained from 62% to 70% of the whole body content in brown trout after waterborne [110m]Ag exposure. Clearly, the liver appears to be the primary site for silver accumulation in most aquatic (Hibiya and Oguri, 1961; Pentreath, 1977; Hogstrand et al., 1996; Wood et al., 1996b) and terrestrial vertebrates (reviewed by Eisler, 1996). According to Pentreath (1977), the accumulation potential of the liver for Ag will subsequently determine the biological turnover rate of Ag from
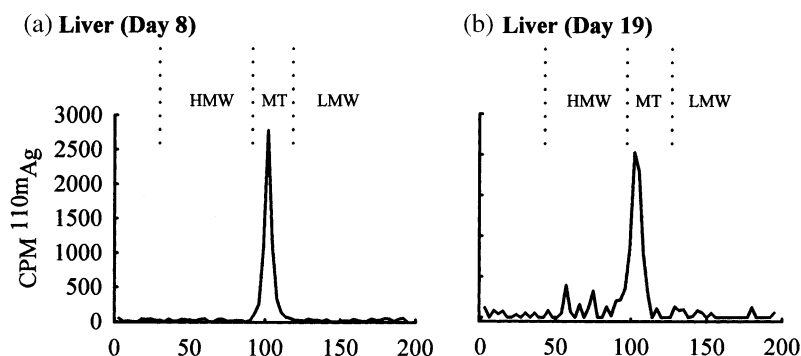

Fig. 5. Representative elution profiles from gel filtration chromatography (Superdex-75) from the cytosolic fraction of rainbow trout livers on (a) day 8 and (b) day 19 of the post-tracer period. Data expressed as radioactive counts (CPM) per fraction.
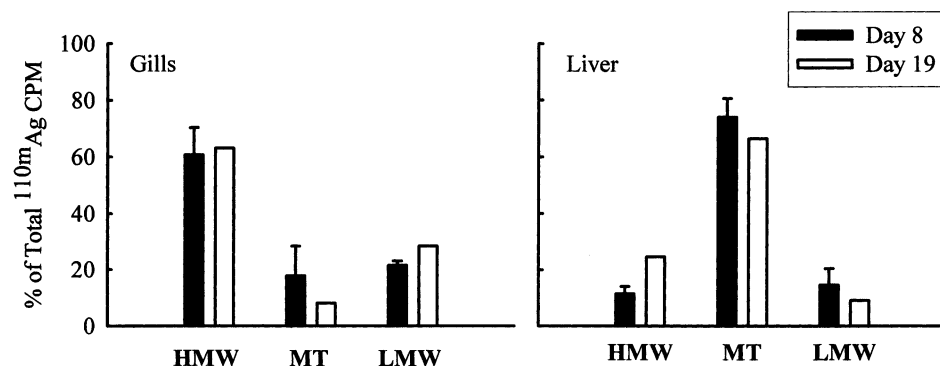
Fig. 6. Subcellular distribution of ^{110m}Ag burden in the cytosolic fractions of (a) liver and (b) gill homogenates of juvenile rainbow trout on days 8 and 19 of post-tracer exposure. Heavy molecular weight (HMW), metallothionein-sized (MT) and low molecular weight (LMW) fractions isolated using gel filtration chromatography (Figs. 4 and 5 S.E. are shown for day 8 data ($n=10$)). However, all livers ($n=10$) and gills ($n=10$) from day 19 were pooled prior to gel filtration chromatography due to concern that growth dilution would have reduced specific activity of the tissues below the detection limit. Consequently, no S.E. could be calculated.

the whole organism. In seawater, fish such as *Pleuronectes platessa,* which have low hepatic accumulation potentials for Ag, will tend to have relatively fast whole-body Ag elimination rates (e.g. $t_{1/2}=12$ days). In comparison, *Raja clavata* accumulated high concentrations of Ag in the liver and had a correspondingly long half-time for Ag elimination ($t_{1/2}=315$ days) (Pentreath, 1977). Our study is consistent with the hypothesis by Pentreath (1977), however, more experimental evidence is still required to support this statement.

Bioconcentration factors (BCF) have also been used to assess the relative bioavailabity of waterborne Ag(I) exposures in aquatic organisms. In this study, the BCF (as calculated from the whole-body radioactivity at day 1) was only 2.5 ± 0.3. This value approximates the whole-body BCF value of 2.7 for rainbow trout following a 57-day exposure to waterborne $AgNO_3$ and 1.8 for fathead minnows exposed to Ag thiosulfate (Ewell et al., 1993). The highest BCF of any tissue in the trout was observed in the liver, with a mean of 84 ± 15 over the entire post-tracer period, and a maximum on day 8 of 143 (Table 4). This is consistent with the BCF value of 282 for liver of freshwater brown trout after 57 days of exposure (Garnier et al., 1990). Intestine, gills and kidney also exhibited BCF values substantially above the whole-body mean.

Subcellular fractionation of liver and gills of Ag(I)-exposed fish suggests that clear differences exist in the way that silver is handled between these tissues. The gill Ag load was primarily

distributed to the *nuc* ($\sim60\%$) and *cyt* ($\sim20\%$) fractions (Fig. 2). The fact that the ^{110m}Ag in the *nuc* fraction was not extractable with the hypotonic buffer suggests that the ^{110m}Ag is bound to membranes. In bivalves, Ag in the gills was primarily associated with insoluble fractions, including basement membranes (Berthet et al., 1992; Truchet et al., 1990). Nonetheless, this does not preclude the possibility that Ag in the *nuc* fraction was bound to mucus and/or membrane fragments. The distribution pattern of Ag in the gills was not significantly different between days 8 and 19, suggesting that Ag was bound in a stable form, likely to sulfides. Certainly, work on bivalves has shown that Ag primarily exists as a sulfide in the gills of these organisms. Moreover, this insoluble fraction was found to depurate extremely slowly from the gills, in comparison to water-soluble protein-bound Ag (Berthet et al., 1992). Of the 20% of ^{110m}Ag distributed to the *cyt* fraction of the gills, less than 20% was found in the MT fraction (Fig. 6a). The fact that Ag is not readily sequestered in the gills may, in part, be due to the low background levels of MT in the tissue as a whole. In general, the concentration of MT in the gills is at least three-fold lower than hepatic levels following waterborne Ag(I) exposure (Hogstrand et al., 1996). Most of the cytosolic ^{110m}Ag burden in the gills was found associated with HMW proteins. Many studies have related toxic effects of metals with the amount of metal eluted with the non-MT metal ligands (reviewed by Hogstrand and Haux, 1990).

The subcellular distribution of Ag in the liver was quite different from the distribution observed in the gills. The proportion of ^{110m}Ag in the cytosolic fraction was increased from ∼40% to ∼65% between days 8 and 19 due to the redistribution of ^{110m}Ag from the other three fractions. The distribution of Ag in the livers of rainbow trout was similar to that of squid with approximately 60% of the Ag compartmentalized within the cytosol (Tanaka et al., 1983). This is in stark contrast to the distribution of Ag seen in some mammalian studies that suggest microsomal compartmentalization of hepatic Ag. Incorporation of Ag in the microsomal fraction is associated with decreased activity of copper enzymes such as ceruloplasmin (Hirasawa et al., 1994; Sugawara and Sugawara, 1984). In the present study, greater than 70% of the Ag in the liver cytosol eluted in the MT fractions. Consequently, cytosolic MT would bind approximately 24% and 46%, of the total ^{110m}Ag burden on days 8 and 19, respectively. This may explain why Ag accumulated in the liver from waterborne sources appears to be non-toxic in fish (Hogstrand et al., 1996; Wood et al., 1996b). Silver that eluted with the HMW and LMW fractions was primarily found in the 220 and 2.2 kDa fractions. Ag was associated with the same 220 kDa HMW fraction in both the liver and gills, but appeared to elute at different molecular weights (gills: 1.2 kDa; liver: 2.2 kDa) in the LMW region. Thus, in the present study, we show evidence of a lower capacity of the gills than the liver to detoxify ^{110m}Ag. These differences may partly explain why the gill is the primary site for acute Ag(I) toxicity in fish during exposure via the water.

Recent work suggests that Ag is an inducer of hepatic metallothionein in fish (Cosson, 1994; Hogstrand et al., 1996). Silver has been shown to accumulate in the liver to concentrations of 900 μg/g wet weight following exposure to Ag thiosulfate (with Ag levels approx. 2000-fold above controls), without any discernable toxic effects (Hogstrand et al., 1996; Wood et al., 1996b). This ability to induce MT up to 400% above control concentrations may explain why bioaccumulated Ag in liver is not acutely toxic. Once Ag(I) accumulates in the tissue it is believed to directly interact with the transcriptional machinery of the rainbow trout MT-A gene to induce MT production (Mayer et al., 1996). Exposure of juvenile rainbow trout for 7 days to waterborne Ag(I) at 9.3 μg/l

(as $AgNO_3$) increased the hepatic MT level by 80%, or approximately 40 μg/g (Hogstrand et al., 1996). The present study is the first to demonstrate that almost all of the cytosolic ^{110m}Ag and 25–50% of the total ^{110m}Ag in the liver is bound to MT in vivo. This relatively high capacity of the liver to sequester Ag in MT likely offers protection against silver-induced hepatotoxicity. However, our results suggest that up to 75% of the hepatic ^{110m}Ag was present in other fractions of the liver. The possibility exists that Ag is toxic in subcellular compartments other than the cytosol. In the gill, for example, Ag acts by blocking the Na/K-ATPase found in the basolateral membrane, and would be expected to separate to the *nuc* and *mic* fractions.

Acknowledgments

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