SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 22, No. 6, pp. 1361–1367, 2003

Printed in the USA
0730-7268/03 $12.00 + .00

MECHANISM OF ACUTE SILVER TOXICITY IN *DAPHNIA MAGNA*

ADALTO BIANCHINI*† and CHRIS M. WOOD‡
†Fundação Universidade Federal do Rio Grande, Departamento de Ciências Fisiológicas, Rua Eng. Alfredo Huch, 475, 96.201-900 Rio Grande, Rio Grande do Sul, Brazil
‡McMaster University, Department of Biology, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

(*Received* 19 *June* 2002; *Accepted* 4 *December* 2002)

Abstract—Daphnids (*Daphnia magna*) were exposed to $AgNO_3$ at 0.303 ± 0.017 μg silver/L (46.9% as Ag^+), in the absence of food, in moderately hard synthetic water under static conditions for up to 48 h. Results from accumulation experiments demonstrated that silver body burden was inversely related to body mass. Daphnids exposed to silver exhibited ionoregulatory disturbance, which was characterized by decreases in whole-body sodium concentration. This ionoregulatory disturbance was explained, at least in part, by a competitive inhibition of the whole-body sodium uptake (six- to sevenfold increase in the Michaelis constant with no change in maximal velocity), which was complete by 1 h of exposure, and resulted in approximately 40% inhibition of sodium influx from the water. A rapidly developing inhibition of whole-body Na^+,K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity, significant by 2 h and complete at 90% blockade by 12 h, also was observed during exposure to $AgNO_3$. Therefore, these findings clearly demonstrate that the key mechanism involved in acute Ag^+ toxicity in *D. magna*, the most sensitive freshwater organism tested to date, resembles that described for freshwater fish— that is, inhibition of active sodium uptake by blockade of Na^+,K^+-ATPase. Furthermore, the results showed that Na^+,K^+-ATPase inhibition was directly related to silver accumulation in the whole body of *D. magna*. However, the nature of the sodium uptake inhibition (competitive vs noncompetitive in fish) and the fact that whole-body chloride concentration was not disturbed in daphnids was different from fish. With regard to the biotic ligand model (BLM) for silver, our results yielded a log *K* value of about 8.9. However, the current version of the BLM uses a rainbow trout log *K* value (7.3) but achieves the correct sensitivity of the model for daphnids by reducing the saturation of toxic sites needed to cause toxicity. An alternative way may be to use the log *K* value derived from the present results.

Keywords—Biotic ligand model Na^+-dependent adenosine triphosphatase Sodium balance K^+-dependent adenosine triphosphatase

INTRODUCTION

Although most silver in surface waters originates from natural leaching, elevated concentrations usually are associated with anthropogenic activities such as mining and photographic processing. Therefore, several different geochemical or biological modeling approaches have been developed in the last few years in an attempt to predict acute silver toxicity in aquatic invertebrate and vertebrate species [1]. For example, acid-volatile sulfide concentrations and silver interstitial water toxic units have been employed to predict the toxicity of silver to estuarine invertebrates in different sediments [2]. Other modeling approaches that emphasize geochemical speciation use the ionic silver (Ag^+) concentration in the water column to directly predict toxicity (e.g., the free ion activity model [3,4]).

A modeling approach to predict silver toxicity to freshwater fish that takes into account the geochemistry of the biological ligand, has been developed over the last few years [5,6]. According to the biotic ligand model (BLM), the gill is considered a negatively charged ligand (the biotic ligand) to which Ag^+ can bind. Toxic effects are considered a function of the degree of saturation of toxic sites on the biotic ligand by Ag^+. The gill modeling approach presents some advantages over simply considering speciation of silver in the water column [1,7]. For example, current versions of the BLM take into account the competition between other cations and Ag^+ for toxic binding sites on the gills as well as the influence of different complexing agents on silver speciation and availability [6,8].

* To whom correspondence may be addressed (adalto@octopus.furg.br).

Parallel to the development of the BLM, evidence elucidating the physiological mechanism of acute Ag^+ toxicity in freshwater fish and crustaceans has been accumulating (for reviews, see Wood et al. [1], Wood [7], and Hogstrand and Wood [9]). The gills are well known to be the main site of active transport of Na^+ and Cl^- from the water into the extracellular fluid of freshwater animals, and branchial Na^+,K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity is well known to be directly related to Na^+ and Cl^- uptake across the gills [10–13]. This uptake is essential to counteract the diffusive ion loss through the gills and excretory organs in freshwater fish and crustaceans. In rainbow trout, the Na^+,K^+-ATPase located at the basolateral membrane of the gill epithelium seems to be the key site for Ag^+ toxicity [14,15]. Recently, the same toxic mechanism has been demonstrated in crayfish [16]. In both cases, the inhibition of Na^+,K^+-ATPase activity is accompanied by an inhibition of Na^+ uptake from the water, and the organisms are thought to die from the pathology associated with the resulting net loss of ions.

Despite the geochemical and toxicological information now available for silver that has contributed to the development of the BLM, one critical area for improvement of the current version of this model is the need for more invertebrate data. The most sensitive freshwater organisms appear to be cladocerans and amphipods with values of acute lethal concentration for 50% of the individuals tested (LC50) as low as 0.3 μg/L of total silver when silver is added as $AgNO_3$ in the absence of food [17,18]. For this reason, the present version of the silver BLM [6] is calibrated with toxicity data for *Daphnia*,

but the actual gill binding constants and most of the water chemistry interaction data come from work on fish. Implicit in this extrapolation is the assumption that the key toxic mechanism in *Daphnia* and other sensitive freshwater invertebrates is the same as in fish, that is, inhibition of active Na^+ uptake by Ag^+ blockade of Na^+,K^+-ATPase. This assumption is based on the facts that essentially all aquatic organisms ionoregulate, requiring Na^+,K^+-ATPase to fuel the process of Na^+ uptake, and that metals such as silver inhibit Na^+,K^+-ATPase, as described above. However, this has never been demonstrated. Therefore, the main goal of our study was to analyze the mechanism of acute toxicity in *Daphnia magna* exposed to $AgNO_3$.

MATERIALS AND METHODS

Daphnid maintenance

Colonies of gravid adult *D. magna* (ARO strain, lot 090600 DM) were obtained from Aquatic Research Organisms (Hampton, NH, USA). The broods originated from the Office of Research and Development of the U.S. Environmental Protection Agency (Cincinnati, OH). According to the Aquatic Research Organisms data sheet, the daphnids had been reared in a freshwater static-renewal system with water saturated in dissolved oxygen, pH 7.5, hardness approximately 150 mg $CaCO_3$/L, and 25°C. The *D. magna* had been fed phytoplankton and a slurry of yeast, cerophyll, and trout chow. Upon arrival at McMaster University, the colonies of *D. magna* were acclimated to synthetic water in an incubator for 3 d. To ensure standardized conditions for water chemistry, synthetic water used for all tests was prepared as a single batch employing 1,000 L of reverse osmosis–purified water in a food-grade polyethylene tank. This water was reconstituted to the following composition: 1.0 mM $CaCO_3$, 0.15 mM $MgSO_4$, and 0.6 mM NaCl. It was bubbled with pure CO_2 for 24 h to ensure that $CaCO_3$ went into solution and then was bubbled with air for 48 h to ensure removal of excess CO_2 and atmospheric equilibration. Water was then left to stand for at least two weeks before use. Final pH was 8.1 to 8.3. Temperature was maintained at 22 to 23°C for the first 24 h of the acclimation period and was then held at 20°C. Photoperiod was fixed at 16:8 h light:dark. During the acclimation period, daphnids were fed a slurry of yeast, cerophyll, and trout chow. Water was not aerated, but was renewed daily.

After acclimation, reproduction rate was measured to ensure it met established criteria for a healthy population (15–20 neonates per adult every 3–4 d). We only proceeded with those colonies where the reproductive rate was satisfactory. To collect neonates, adults were confined in a fine-screen mesh net suspended in a 1-L glass aquarium containing synthetic water at 20°C. During this time, feeding was withheld. Neonates passed through the mesh and into the aquarium of synthetic water, and were maintained under the same conditions described above for up to 25 d.

$AgNO_3$ exposure

In all experiments performed in this study, daphnids were acutely exposed to a single concentration (nominal = 0.5 μg silver/L) of silver as $AgNO_3$, for up to 48 h. This concentration was selected as it is very close to the 48-h LC50 reported for neonatal *D. magna* under the same experimental conditions [18]. Acute silver exposure was performed in borosilicate glass beakers containing 250 ml of synthetic water, preequilibrated to 20°C. This high volume-to-daphnid ratio was used to minimize the accumulation of dissolved organic carbon during the tests, and to maximize the volume-to-wall surface ratio. All glassware used was new and was acid washed in 1% HNO_3 (trace metal grade, Merck, Darmstadt, Germany) and rinsed thoroughly with synthetic water before use.

A stock solution of unlabeled $AgNO_3$ (SigmaUltra, Sigma Co., St. Louis, MO, USA) was added into the test solution 3 h before introduction of *D. magna*. This solution contained a proportion of radioactive ^{110m}Ag (RISØ National Laboratory, Roskilde, Denmark) to facilitate analyses of silver concentrations in the test solution and organisms. The final specific activity of radiolabeled silver in all test solutions was 0.72 μCi/μg total silver. Final silver concentration was obtained by measuring the $AgNO_3$ stock solution (1 mg/L) acidified with 1% HNO_3, by using graphite furnace atomic absorption spectrophotometry (Varian AA-1275 with GTA-9 atomizer, Varian, Palo Alto, CA, USA).

Total and filtered silver concentrations were followed over the experiment. The ^{110m}Ag radioactivity in filtered (Acrodisc 0.45 μM polyethersulfone in-line filters, Pall, Ann Arbor, MI, USA) and nonfiltered water samples (2 ml) was determined by using a gamma counter (MINAXI gamma Auto-gamma 5000 series, Canberra-Packard, Mississauga, ON, Canada), with the precautions on window selection outlined by Hansen et al. [19]. Free Ag^+ ion concentrations also were calculated under the chemical conditions determined in our experiments by using the MINEQL+ geochemical program [20]. Parameters were entered into the program as follows: pH = 8.2; temperature = 20°C; total silver = mean measured over 48 h; Na^+, Cl^-, Ca^{2+}, Mg^{2+}, and HCO_3^- = nominal concentrations. Dissolved organic carbon was not included in the program because measured concentrations of total organic carbon in the synthetic water used in the tests were very low (<0.1 mg C/L [18,21]). Ionic strength corrections were calculated by the MINEQL+ program.

After 3 h of preequilibration, daphnids were placed in beakers containing $AgNO_3$ and maintained without aeration. Measurements were taken to ensure that the water remained sufficiently oxygenated (partial pressure of oxygen = 165 ± 2 mm Hg; n = 10) and that the water pH remained constant (8.25 ± 0.014; n = 10) over 24 h of the experiment. Daphnids were not fed during the tests. Temperature and photoperiod were maintained at 20°C and 16:8 h light:dark, respectively.

After 24 h, daphnids were transferred to a new set of test solutions prepared 3 h before transfer as previously described. For this second 24-h exposure, daphnids were removed from the original beakers and transferred by using plastic pipettes.

The methodology employed ensured the consistency of the silver concentration over the course of the test, as documented in previous studies from our laboratory [18,21].

Silver accumulation studies

In a first experiment, neonates, juveniles, and adults ranging in weight from 0.06 to 6 mg and maintained as previously described were acutely exposed to $AgNO_3$ for 1 (n = 49) or 24 h (n = 45). After silver exposure, daphnids were collected by using plastic pipettes, washed for 15 s in a concentrated (1 mg silver/L) $AgNO_3$ solution to displace loosely bound ^{110m}Ag, blotted dry on filter paper (Whatman No. 1, Whatman, Clifton, NJ, USA), weighed with an electronic microscale (Mettler UMT2, 0.001-mg accuracy, Mettler-Toledo, Columbus, OH, USA), dried (40°C), weighed, and transferred to plastic vials. The ^{110m}Ag radioactivity in daphnids was then measured as

described for water samples. Total silver concentration in *D. magna* was expressed in ng silver/g wet weight. Daphnid water content was 92.2 ± 1.2%.

In a second experiment, juvenile *D. magna* (1.115 ± 0.061 mg) were acutely exposed to $AgNO_3$, and sampled at various times for up to 48 h. At these different times of exposure, daphnids were collected and total silver concentration was assessed in the whole body as described in the first experiment.

Whole-body Na^+,K^+-ATPase activity, Na^+ uptake, and Na^+ and Cl^- concentration measurements

In a third experiment, adult *D. magna* (2.83 ± 0.08 mg; $n = 48$) were used because they provided more biological material than juveniles, thereby ensuring individual enzyme assays and avoiding the use of pooled animals. The adults were exposed up to 48 h to the same nominal concentration of $AgNO_3$ as described above, but using only unlabeled silver. At different times during the exposure, daphnids were collected by using plastic pipettes, washed for 15 s in deionized water, blotted dry on filter paper (Whatman No. 1), transferred to plastic assay tubes, and frozen (−70°C) until Na^+,K^+-ATPase activity was measured. Enzyme activity in the whole body was analyzed in single animals. For the enzyme activity assay, samples were thawed and kept on ice throughout the analysis. They were then homogenized in 0.5 ml of ice-cold buffer solution (150 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 50 mM imidazole, and 11.5 mM sodium deoxycholate), and centrifuged at 5,000 *g* for 30 s at 4°C. Enzyme activity was measured in the supernatant by using the method described by McCormick [22] with modifications according to Wheatly and Henry [23]. Two reaction mixtures were assayed. Reaction mixture A consisted of 20 μl of sample, 50 μl of salt solution A, and 150 μl of working solution A. Reaction mixture B consisted of 20 μl of sample, 50 μl of salt solution B, and 150 μl of working solution B. Salt solution A contained 100 mM NaCl, 10.5 mM $MgCl_2$, 30 mM KCl, and 50 mM imidazole, with pH adjusted to 7.5. In salt solution B, NaCl replaced KCl at the same concentration. Working solution A contained four enzymatic units/ml lactate dehydrogenase, five enzymatic units/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate, 3.5 mM adenosine triphosphate (ATP), 0.22 mM nicotinamide adenine dinucleotide phosphate, and 50 mM imidazole, with pH adjusted to 7.5. To obtain the working solution B, 1 mM ouabain was added to working solution A. Kinetic assays were then run in duplicate at 25°C in a temperature-controlled microplate reader (Molecular Devices, Menlo Park, CA, USA), for 10 min. An adenosine diphosphate standard curve (0–20 nmoles/10 μl) was also run. The Na^+,K^+-ATPase activity was then calculated by considering the difference in adenosine diphosphate production between the two reaction mixtures (A and B). Protein content in the homogenate was measured by using Bradford reagent (Bio-Rad, Richmond, CA, USA). Enzyme activity was then expressed as μmoles adenosine diphosphate/mg protein/h.

Whole-body sodium and chloride concentrations also were analyzed in a separate set of daphnids ($n = 48$) exposed to $AgNO_3$ in a parallel series within this third experiment. After different times of exposure, daphnids were collected as individuals by using plastic pipettes, washed for 15 s in deionized water, dried on filter paper (Whatman No. 1), transferred to plastic assay tubes, and digested in 50 μl of concentrated (95–97%) H_2SO_4 (Merck) overnight at 60°C. After digestion, 1.5 ml of deionized water was added to the digested sample. Sodium concentration was then measured as described for water samples. Chloride concentration was determined by a colorimetric assay [24].

To evaluate Ag^+ effects on kinetic parameters of whole-body sodium influx, some daphnids ($n = 150$) from the same exposure of the third experiment were used. After they had been exposed for 0, 1, or 24 h, they were quickly (15 s) rinsed in deionized water and transferred to a new glass beaker containing 50 ml of solution (1 mM calcium gluconate, sodium gluconate at different concentrations, 20°C, and pH adjusted to 8.2). Chloride replacement by gluconate in this experimental medium was applied to avoid different free Ag^+ concentrations due to silver complexation by chloride. Preliminary tests with control daphnids showed no significant differences in the whole-body Na^+ influx kinetics when using NaCl or sodium gluconate. The $AgNO_3$ (nominal = 0.5 μg silver/L) and ^{22}Na (10 μCi/L, specific activity 303 Ci/g Na^+, Amersham) were then added to this new experimental medium. Water samples for measurement of ^{22}Na radioactivity and total sodium were taken at 0 and 1 h. These samples were used for ^{22}Na radioactivity measurement with the Canberra-Packard MINAXI gamma counter, and total sodium measurement with the Varian AA-1275 atomic absorption unit operated in flame emission mode. After the 1-h flux period, daphnids were collected by using plastic pipettes, washed for 15 s in a concentrated (600 mM) NaCl solution to displace loosely bound ^{22}Na, blotted dry on filter paper, weighed on an electronic microscale (Mettler UMT2, 0.001-mg accuracy), and transferred to plastic vials. The ^{22}Na radioactivity in the whole body was then measured as described for the water samples. The Na^+ uptake rate was calculated based on the incorporation of ^{22}Na in the whole body during the 1-h flux period, the mean measured specific activity of the ^{22}Na in the water, the body weight of the animal, and the elapsed time as above.

Data presentation and statistical evaluation

All values, except in the first experiment, were expressed as mean ± one standard error of the mean ($n = 6$). The relationship between size of the *D. magna* and silver accumulation after 1 or 24 h of exposure was assessed by regression analysis. Differences in whole-body Na^+,K^+-ATPase activity and ion concentrations (Na^+ and Cl^-) over the 48-h period of exposure to $AgNO_3$ were assessed by analysis of variance in combination with the least significant difference test. Data were previously tested for normality and homogeneity of variances. Kinetic parameters (Michaelis constant [K_m] and maximal velocity [V_{max}]) for sodium uptake in daphnids under control conditions and exposed to $AgNO_3$ for either 1 or 24 h were determined by means of nonlinear regression analyses (one-site binding). In all statistical analyses, the significance level was 95% ($\alpha = 0.05$).

RESULTS

Mean total and filtered silver concentrations in the first and second experiments were 0.303 ± 0.017 and 0.248 ± 0.014 μg/L, respectively. Free Ag^+ concentration calculated using the MINEQL program was 0.142 μg/L, with the remainder of the silver present as $AgCl_0$.

We found a highly significant negative correlation between body mass of *D. magna* and whole-body silver accumulation in the first and second experiments. Log–log regression lines for silver body burden (ng/g) against body mass (μg) in daphnids exposed to $AgNO_3$ for either 1 or 24 h were parallel with slope values around 0.4 (Fig. 1). Furthermore, sampling at

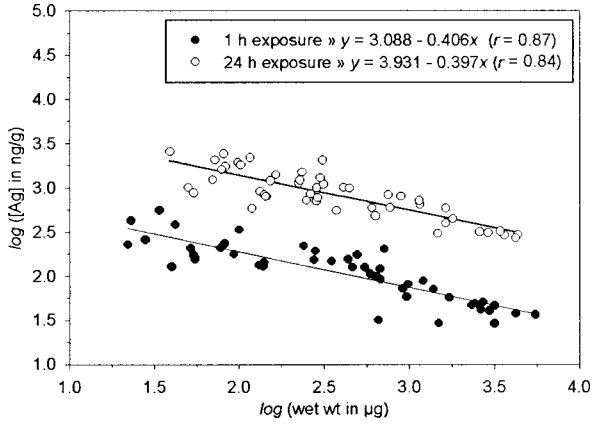

Fig. 1. Whole-body silver accumulation in *Daphnia magna* exposed to 0.3 µg total silver/L, as $AgNO_3$, for 1 or 24 h.

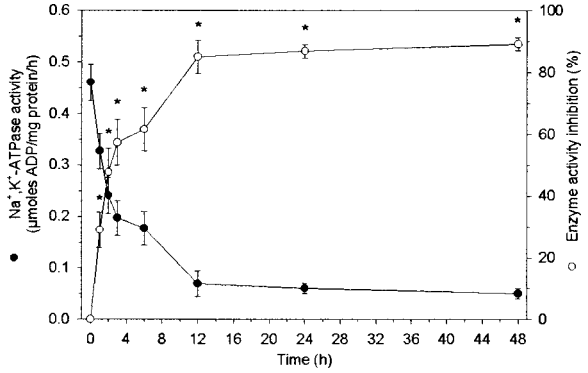

Fig. 3. Whole-body Na^+,K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity in juvenile *Daphnia magna* exposed to 0.3 µg total silver/L, as $AgNO_3$, over 48 h. Data are means ± 1 standard error of mean ($n = 6$). An asterisk (*) indicates means significantly different ($p < 0.05$) from control.

intermediate times demonstrated that silver accumulated very quickly in juvenile daphnids over the first 5 h of exposure, after which the silver accumulation rate decreased markedly (Fig. 2).

Whole-body Na^+,K^+-ATPase activity was significantly inhibited after only 1 h of exposure to $AgNO_3$, and inhibition progressively increased until 12 h (Fig. 3). The maximum enzyme activity was approximately 90% inhibited by this time and remained constantly inhibited up to 48 h of experiment (Fig. 3). Whole-body Na^+,K^+-ATPase inhibition was directly related to whole-body silver accumulation (Fig. 4).

Measurement of unidirectional Na^+ influx rates showed that silver significantly inhibited whole-body sodium uptake after 1 or 24 h of exposure to silver. Kinetic parameters were significantly affected by silver exposure. The K_m values were significantly higher (i.e., lower affinity) in daphnids exposed to $AgNO_3$. The V_{max} values were not significantly affected (Fig. 5). This pattern is characteristic of classical competitive inhibition. Note that this inhibition (as indicated by the six- to sevenfold increase in K_m) was essentially complete by 1 h, indicating a rapid effect. In fact, no significant differences were found in the kinetics of whole-body sodium influx after 1 and 24 h of exposure to silver. In the water Na^+ level (0.6 mM) to which the animals were acclimated, inhibition of Na^+ uptake was about 40%, amounting to a decrement of about 2.4 µEq/g/h.

The $AgNO_3$ exposure induced a continuous decrease in whole-body sodium concentration over the 48-h period of the experiment that first became significant at 15 h. The slope of the loss relationship against time was only about 0.29 µEq/g/h or about 12% of the inhibition of influx, suggesting that a considerable compensating decrease in efflux occurred that slowed the rate of net loss. On the other hand, whole-body chloride concentration was not affected by $AgNO_3$ even after exposure for 48 h (Fig. 6).

DISCUSSION

In rainbow trout, the Na^+,K^+-ATPase located at the basolateral membrane of the gill epithelium seems to be the key site for Ag^+ toxicity [14,15], resulting in a net loss of ions from the animal, with death probably resulting from an associated cardiovascular collapse [9]. Recently, the same toxic mechanism (ionoregulatory failure associated with the inhibition of branchial Na^+,K^+-ATPase) has been demonstrated in crayfish [16]. In spite of this, an elucidation of the mechanism involved in acute Ag^+ toxicity in more sensitive invertebrates, such as *D. magna*, was necessary to validate the use of toxicity data from daphnids in the current version of the BLM [6].

In our study, examination of data obtained in silver accumulation experiments clearly demonstrated that whole-body mass was an important factor affecting silver body burden. Silver accumulation was inversely related to body mass. This

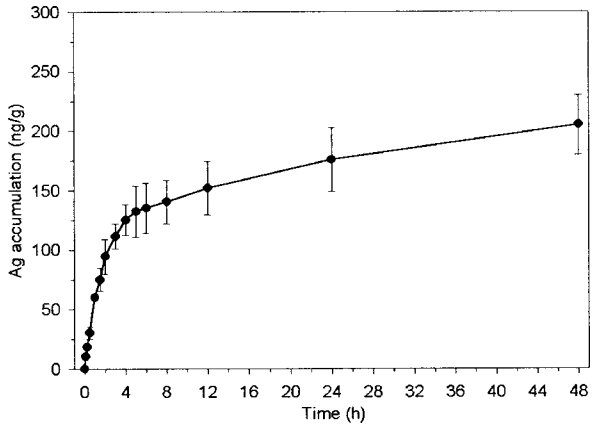

Fig. 2. Whole-body silver accumulation in juvenile *Daphnia magna* exposed to 0.3 µg total silver/L, as $AgNO_3$, over 48 h. Data are means ± 1 standard error of mean ($n = 6$).

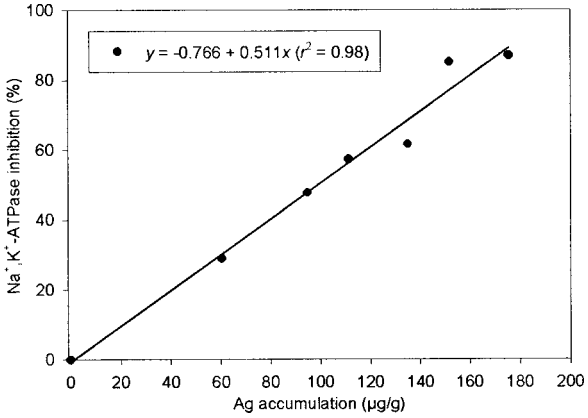

Fig. 4. Whole-body Na^+,K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity in juvenile *Daphnia magna* as a function of whole-body silver accumulation in organisms exposed to 0.3 µg silver/L, as $AgNO_3$, for 48 h (data from Figs. 2 and 3).

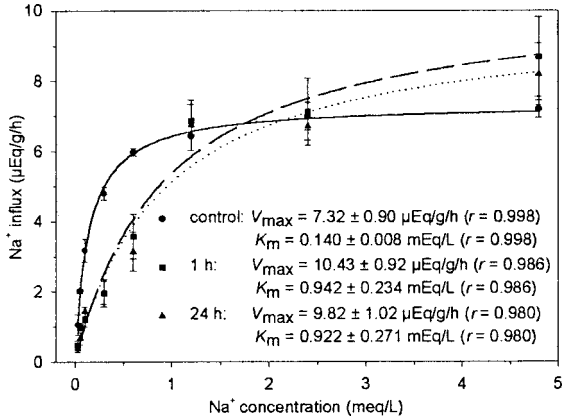

Fig. 5. Whole-body sodium uptake rate as a function of water sodium concentration in juvenile *Daphnia magna* exposed to 0.3 µg total silver/L, as $AgNO_3$, for 1 or 24 h. Data are means ± one standard error of mean ($n = 6$).

kind of relationship can be explained if we consider the gill as the main site for gill binding and the fact that in crustaceans, the mass-specific gill surface area is inversely related to body mass [25]. Note that the slope value (~0.4) obtained from the relationship is very close to that observed for other surface-dependent physiological processes, for example, respiration [26] and whole-body sodium uptake rate [27]. This argument does not preclude the possibility that silver may bind to other surfaces in *D. magna*, or inhibit Na^+,K^+-ATPase activity at extrabranchial sites, or both.

Examination of silver accumulation data showed that the slopes of the regression lines estimated from the relationship between body mass and silver body burden in daphnids exposed for 1 or 24 h were similar (~0.4). This result indicates that body mass effects on silver accumulation do not depend on the time of exposure. Results obtained in the second experiment indicate that silver accumulation occurred very quickly in juvenile daphnids. This may be explained by considering the high turnover of sodium in *D. magna* compared to other crustacean species [27], and the fact that Ag^+ may be acting as an analogue for Na^+ in a competitive fashion. This statement is based on the observation that the inhibition of whole-body sodium uptake induced by silver in daphnids is competitive, indicating that silver and sodium are sharing the

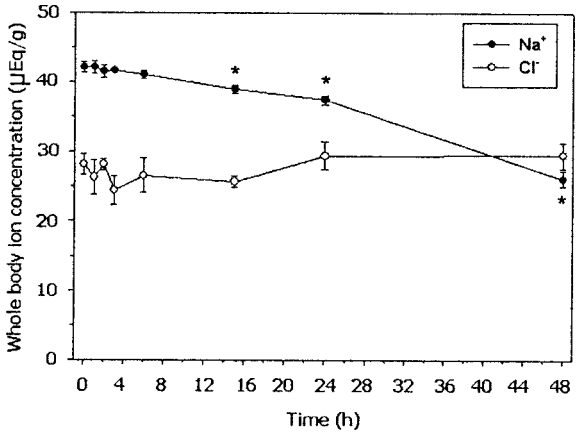

Fig. 6. Whole-body sodium and chloride concentrations in juvenile *Daphnia magna* exposed to 0.3 µg total silver/L, as $AgNO_3$, over 48 h. Data are means ± one standard error of mean ($n = 6$). An asterisk (*) indicates means significantly different ($p < 0.05$) from control.

same mechanism(s) of sodium transport across the gill epithelium. In the rainbow trout, Bury and Wood [28] demonstrated that silver, probably as Ag^+, enters the branchial epithelial cells via the Na^+ channel coupled to the proton ATPase in the apical membrane of the gills. Recently, we have demonstrated the presence of this same mechanism of transport in *D. magna* [29]. Therefore, it seems very likely that Ag^+ is sharing the Na^+ channel coupled to the proton ATPase to enter the branchial epithelial cells in *D. magna*, as it does in fish.

As previously mentioned, gills are the main site of active transport of Na^+ and Cl^- from the water into the extracellular fluid of freshwater crustaceans, and branchial Na^+,K^+-ATPase activity is directly related to the Na^+ and Cl^- uptake across the gills [10,13]. This uptake is essential to counteract the ion loss through the gills and excretory organs in freshwater crustaceans. We observed a progressive and profound inhibition of Na^+,K^+-ATPase activity after silver exposure. In this case, an inhibition of the enzyme powering the active transport of sodium should lead to decreases in hemolymph and consequently in whole-body sodium concentrations. Thus, this inhibitory effect of Ag^+ on Na^+,K^+-ATPase activity can also explain, at least in part, the reduced sodium uptake rate and whole-body sodium concentration recorded in Ag^+-exposed daphnids. At this point, it is interesting to note that the inhibition of the whole-body sodium influx was almost complete by 1 h of exposure to silver, indicating a rapid effect. However, at this time inhibition of Na^+,K^+-ATPase activity was only about 30%. Thereafter, a further progressive inhibition of Na^+,K^+-ATPase activity occurred, reaching 90% by 12 to 48 h, but virtually no additional effect occurred on whole-body sodium influx. The explanation for this phenomenon is presently unclear and worthy of a future study.

Although in broad view, this mechanism of Ag^+ toxicity in *D. magna* appears similar to that in fish (i.e., ionoregulatory failure associated with inhibition of branchial Na^+,K^+-ATPase activity [1,7,9]), in fine detail there are two substantial differences. First, in *D. magna* the inhibition of Na^+ uptake by Ag^+ is clearly competitive, whereas in the rainbow trout it is clearly noncompetitive [14], as is the inhibition of Ag^+ uptake by Na^+. Other kinetics experiments on *D. magna* that used different concentrations of silver (lower and higher than that employed here) in the scope of another study in our laboratory again showed significant changes in the K_m without significant changes in V_{max}, thus supporting the present results. Possibly, this reflects a different balance in the degree of apical channel competition versus Na^+,K^+-ATPase inhibition in daphnids relative to trout. Second, in trout, Cl^- uptake is similarly inhibited by Ag^+ exposure [14,30,31], such that plasma Na^+ and Cl^- concentrations fall almost in parallel [15,30–33]. Very clearly, whole-body Cl^- concentration did not fall in Ag^+-exposed daphnids of the present study (Fig. 6), nor did hemolymph Cl^- concentration in the Ag^+-exposed crayfish of Grosell et al. [16]. Unfortunately, Cl^- uptake was not measured in either study. Reductions in whole-body Na^+ concentration would be expected to be accompanied by similar reductions in Cl^- concentration to maintain the charge balance. The lack of an effect of Ag^+ on Cl^- whole-body concentration suggests that other ionoregulatory shifts could be occurring in freshwater crustaceans exposed to silver. This lack of an effect of Ag^+ on Cl^- whole-body concentration in crustaceans is reminiscent of a similar lack of effect in eels [31], but there the difference is explained by the fact that eels take up Cl^- through the diet rather than through the gills [34]. This is unlikely to be the

explanation in crustaceans; both daphnids [35] and crayfish [36] exhibit a vigorous active uptake of Cl^- at the gills, although at least in the latter, this appears to be partially independent of Na^+ uptake, and vice versa [37,38]. However, in fish, the coupling also is loose, and indeed the mechanism by which Na^+,K^+-ATPase activity is linked to Cl^- uptake remains poorly understood (reviewed by Wood [7]). Interestingly, the chloride transport pathway has been demonstrated to be less sensitive to $AgNO_3$ exposure than the sodium transport pathway in the intestine of marine teleost fish [39]. Clearly, much remains to be learned about the fine details of these transport mechanisms in both fish and crustaceans, and Ag^+ may prove to be a very useful tool in this regard.

Finally, it is important to note that inhibition of Na^+,K^+-ATPase activity is well correlated with silver accumulation in the whole body of juvenile daphnids, the most sensitive freshwater organism [17]. This supports the idea that silver binds to a specific biotic ligand, that is, the Na^+,K^+-ATPase, and that this binding is directly associated with toxicity in *D. magna*, just as in less sensitive crustaceans [16] and fish [1]. In this regard, it is noteworthy that in rainbow trout, the concentration of free Ag^+ ion in the water associated with 85% inhibition of Na^+,K^+-ATPase is about equal to the 96-h LC50 value (2.7–5.5 μg/L), and thereby provides the log *K* value for the BLM—7.6 according to McGeer et al. [8] or 7.3 according to Paquin et al. [6]. Similarly in *D. magna*, the concentration of free Ag^+ ion in the water associated with 90% inhibition of Na^+,K^+-ATPase in our study (0.142 μg/L = 1.3 nM) is about equal to the 48-h LC50 value [18]. By analogy this would yield a log *K* value of approximately 8.9 for a daphnid BLM. However, the current version of the silver BLM for daphnids uses a rainbow trout log *K* value (7.3) but achieves the correct sensitivity of the model by reducing the saturation of toxic sites needed to cause toxicity [6].

The original log *K* values (7.3–7.6) were worked out in fish gills on the basis of correlation with acute toxicity (i.e., 96-h LC50 values) before we understood the exact physiological basis. Once we learned the physiological basis in the fish gill (Na^+,K^+-ATPase inhibition with resulting ionoregulatory failure), it became apparent that sufficient inhibition (50–85%) to cause death occurred in this log *K* range. Thus, we can now associate a log *K* value with a physiological endpoint [1,8]. The original developers of the BLM for silver in *Daphnia* did not have the advantage of this knowledge, so they had to make a fish-based model work in *Daphnia* without knowledge of mechanism. To account for the toxicological data (many-fold greater sensitivity in *Daphnia*) they had the choice to either greatly reduce the saturation of binding sites needed to cause 50% mortality or to change the log *K* value. Because both constants are operationally defined, either modification would work equally well. They chose to reduce the saturation of binding sites needed to cause 50% mortality and developed a model that worked extremely well [6]. However, with the new physiological knowledge presented in this paper (i.e., that the toxic mechanism is the same as in fish, and associated with the same degree of Na^+,K^+-ATPase inhibition), it is apparent that it may have been preferable to raise the log *K* value to the point where comparable Na^+,K^+-ATPase inhibition occurred (i.e., around 8.9).

This is not meant as a criticism of the BLM, but simply an improvement, which allows it to be founded on a common mechanistic basis with that for fish. Relating the BLM to its mechanistic basis may become important when it is applied to new circumstances (e.g., multimetal mixtures or different water qualities). This approach offers a way to also adapt the model to other species with higher or lower tolerance. It demonstrates the beauty of the BLM—that it can evolve as new information becomes available. In this regard, it is interesting that De Schamphelaere and Janssen [40] recently developed a modification of the BLM for copper that works better in *Daphnia*, and they did this principally by raising the log *K* value. Because copper and silver are thought to work through a similar physiological mechanism, this offers additional support for our conclusion.

Acknowledgement—We acknowledge the financial support of Kodak Canada and the National Sciences and Engineering Research Council Cooperative Research and Development Program. We also wish to thank Joseph W. Gorsuch (Eastman Kodak Company USA) and David Rollo (McMaster University). A. Bianchini is a fellow from the Brazilian CNPq (Process 300536/90-9) and C.M. Wood is supported by the Canada Research Chair Program.

REFERENCES

1. Wood CM, Playle RC, Hogstrand C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environ Toxicol Chem* 18:71–83.
2. Berry WJ, Cantwell MG, Edwards PA, Serbst JR, Hansen DJ. 1999. Predicting toxicity of sediments spiked with silver. *Environ Toxicol Chem* 18:49–55.
3. Campbell PGC. 1995. Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. In Tessier A, Turner DR, eds, *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley, Chichester, West Sussex, UK, pp 45–102.
4. Simkiss K, Taylor MG. 1995. Transport of metals across membranes. In Tessier A, Turner DR, eds, *Metal Speciation and Bioavailability in Aquatic Systems*. John Wiley, Chichester, West Sussex, UK, pp 1–44.
5. Janes N, Playle RC. 1995. Modelling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 14: 1847–1858.
6. Paquin PR, Di Toro DM, Santore RS, Trevedi D, Wu KB. 1999. A biotic ligand model of the acute toxicity of metals III: Application to fish and *Daphnia* exposure to silver. Integrated approach to assessing the bioavailability and toxicity of metals in surface waters and sediments, section 3. EPA 822-E-99-001. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Protection Agency, Washington, DC.
7. Wood CM. 2001. Toxic responses of the gill. In Benson WH, Schlenck DW, eds, *Target Organ Toxicity in Marine and Freshwater Teleosts*, Vol 1—Organs. Taylor and Francis, London, UK, pp 1–89.
8. McGeer JC, Playle RC, Wood CM, Galvez F. 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to rainbow trout in fresh waters. *Environ Sci Technol* 34:4199–4207.
9. Hogstrand C, Wood CM. 1998. Towards a better understanding of the bioavailability, physiology, and toxicology of silver in fish: Implications for water quality criteria. *Environ Toxicol Chem* 17: 547–561.
10. Péqueux A. 1995. Osmotic regulation in crustaceans. *J Crustacean Biol* 15:1–60.
11. Flik G, Kaneko T, Greco AM, Li J, Fenwick JC. 1997. Sodium dependent transport. *Fish Physiol Biochem* 17:385–396.
12. Perry SF. 1997. The chloride cell: Structure and function in the gills of freshwater fishes. *Annu Rev Physiol* 59:325–347.
13. Castilho PC, Martins IA, Bianchini A. 2001. Gill Na^+,K^+-ATPase and osmoregulation in the estuarine crab, *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). *J Exp Mar Biol Ecol* 256:215–227.
14. Morgan IJ, Henry RP, Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. *Aquat Toxicol* 38:145–163.
15. McGeer JC, Wood CM. 1998. Protective effects of water Cl^- on

physiological responses to waterborne silver in rainbow trout. *Can J Fish Aquat Sci* 55:2447–2454.
16. Grosell M, Brauner C, Kelly SP, McGeer JC, Bianchini A, Wood CM. 2002. Physiological responses to acute silver exposure in the freshwater crayfish (*Cambarus diogenes diogenes*)—A model invertebrate? *Environ Toxicol Chem* 21:369–374.
17. Ratte HT. 1999. Bioaccumulation and toxicity of Ag compounds: A review. *Environ Toxicol Chem* 18:89–108.
18. Bianchini A, Bowles K, Brauner CJ, Gorsuch JW, Kramer JR, Wood CM. 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver(I) to *Daphnia magna*. Part II: Toxicity results. *Environ Toxicol Chem* 21:1294–1300.
19. Hansen HJM, Jacobsen U, Jorgensen JC, Grosell M, Hogstrand C, Wood CM. 2002. Precautions in the use of ^{110m}Ag, containing transmutation produced ^{109}Cd, as a tracer of Ag metabolism in ecotoxicology. *Environ Toxicol Chem* 21:1004–1008.
20. Schecher WD, McAvoy DC. 1998. *MINEQL+. A Chemical Equilibrium Modeling System.* Environmental Research Software, Hallowell, ME, USA.
21. Bowles KC, Bianchini A, Brauner CJ, Kramer JR, Wood CM. 2002. Evaluation of the effect of reactive sulfide on the acute toxicity of silver(I) to *Daphnia magna*. Part I: Description of the chemical system. *Environ Toxicol Chem* 21:1286–1293.
22. McCormick SD. 1993. Methods for non-lethal gill biopsy and measurements of Na^+,K^+-ATPase activity. *Can J Fish Aquat Sci* 50:656–658.
23. Wheatly MG, Henry RP. 1987. Branchial and antennal gland Na^+/K^+-dependent ATPase and carbonic anhydrase activity during salinity acclimation of the euryhaline crayfish *Pacifastacus leniusculus*. *J Exp Biol* 133:73–86.
24. Zall DM, Fisher D, Garner MD. 1956. Photometric determination of chlorides in water. *Anal Chem* 28:1665–1678.
25. Santos EA, Baldisserotto B, Bianchini A, Colares EP, Nery LEM, Manzoni GC. 1987. Respiratory mechanisms and metabolic adaptations of an intertidal crab, *Chasmagnathus granulata* (Dana, 1851). *Comp Biochem Physiol A* 88:21–25.
26. Barnes RSK, Calow P, Olive PJW. 1993. *The Invertebrates: A New Synthesis.* Blackwell Science, Oxford, UK.
27. Bianchini A, Grosell M, Gregory SM, Wood CM. 2002. Acute silver toxicity in aquatic animals is a function of sodium uptake rate. *Environ Sci Technol* 36:1763–1766.
28. Bury NR, Wood CM. 1999. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. *Am J Physiol* 277:1385–1391.
29. Bianchini A, Wood CM. 2001. Sodium uptake in different life stages of crustaceans. *Proceedings*, Society for Experimental Biology Annual Meeting, Canterbury, Kent, UK, April 2–6, p 37.
30. Webb NA, Wood CM. 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout. *Environ Toxicol Chem* 17:579–588.
31. Grosell M, Hogstrand C, Wood CM, Hansen HJM. 2000. A nose-to-nose comparison of the physiological effects of exposure to ionic silver versus silver chloride in the European eel (*Anguilla anguilla*) and the rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 48:327–342.
32. Wood CM, Hogstrand C, Galvez F, Munger RS. 1996. The physiology of waterborne silver exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). I. The effects of ionic Ag^+. *Aquat Toxicol* 35:93–109.
33. Rose-Janes NG, Playle RC. 2000. Protection by two complexing agents, thiosulphate and dissolved organic matter, against the physiological effects of silver nitrate to rainbow trout (*Oncorhynchus mykiss*) in ion-poor water. *Aquat Toxicol* 51:1–18.
34. Rankin JC. 2000. Can reduced plasma chloride concentration be used as a measure of environmental stress in freshwater teleost fishes? *Abstract*, The Society for Experimental Biology Annual Meeting, Exeter, UK, March 27–31, p 27.
35. Holm-Jensen IB. 1948. Osmotic regulation in *Daphnia magna* under physiological conditions and in the presence of heavy metals. *K Dan Vidensk Selsk Biol Medd* 20:1–64.
36. Shaw J. 1960. The absorption of chloride ions by the crayfish *Astacus pallipes* Lereboullet. *J Exp Biol* 37:548–556.
37. Shaw J. 1960. The absorption of sodium ions by the crayfish *Astacus pallipes* Lereboullet. II. The effect of the external anion. *J Exp Biol* 37:534–547.
38. Shaw J. 1960. The absorption of sodium ions by the crayfish *Astacus pallipes* Lereboullet. III. The effect of other cations in the external solution. *J Exp Biol* 37:548–556.
39. Grosell M, DeBoeck G, Johannsson O, Wood CM. 1999. The effects of silver on intestinal ion and acid–base regulation in the marine teleost fish, *Parophrys vetulus*. *Comp Biochem Physiol* 124:259–270.
40. De Schamphelaere KA, Janssen CR. 2002. A biotic ligand model predicting acute copper toxicity to *Daphnia magna*: The effects of calcium, magnesium, sodium, potassium and pH. *Environ Sci Technol* 36:48–54.