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Activities of glutamate dehydrogenase and aspartate and alanine aminotransferases in freshwater snails *Helisoma duryi* and *Lymnaea natalensis* exposed to copper

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In this paper we investigate the potential of glutamate dehydrogenase (GDH) and aspartate and alanine aminotransferases (AST and ALT) as biomarkers of water pollution due to copper in the freshwater snails *Helisoma duryi* and *Lymnaea natalensis*. Snails were dosed with copper(II) ion concentrations of 0.01, 0.1 and 1 mg kg^{-1} breeding water for a period of 96 h, after which those surviving were shelled. The copper content in the breeding water, in whole snail tissue and in the snail shells was determined at the end of the period of exposure. For enzyme determinations, whole snail tissue was first homogenized and fractionated by centrifugation at 500 *g* to remove the nuclei. The resulting supernatant was then centrifuged at 10 000 *g* to give a pellet fraction representing the mitochondrial fraction and a supernatant representing the cytosolic fraction. Copper was very toxic to both snail species at concentrations above 0.2 mg l^{-1}, with only 3% of the *Helisoma* and 12% of the *Lymnaea* surviving at concentrations of approximately 1 mg l^{-1}. The copper content in the shells and tissues of snails rose with increasing copper concentration in the breeding water, and was 2.1- to 4.9-fold in snails exposed to copper ion at a dose of 1 mg kg^{-1} water compared with undosed snails. Similarly, the activities of GDH and AST rose by up to 4.7-fold in the homogenate and the mitochondrial and cytosolic fractions with increasing concentrations of copper. These activities, however, fell at copper concentrations of approximately 1 mg l^{-1}, which coincided with massive death of snails. Mitochondrial ALT disappeared at copper ion concentrations of approximately 0.2 mg l^{-1} for *Lymnaea* and 1 mg^{1-1} for *Helisoma*, possibly indicating mitochondrial degeneration. These results show that GDH, AST and ALT have the potential to be biomarkers of sublethal copper pollution in these two snail species, since their activities were significantly altered by low copper concentrations.

Keywords: copper, glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, *Helisoma duryi*, *Lymnaea natalensis*.

Introduction

Copper has been investigated as a control agent for the *Schistosoma* host *Bulinus globosus* (Amin 1972, Ebele *et al*. 1990) and for aquatic weed control (Effler *et al*. 1980). It has been reported to inhibit algal and bacterial growth at a concentration of 1 μg l^{-1} (Albright *et al*. 1972, Steeman-Nielson and Laursen

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1976). While zooplankton appear more tolerant to low level copper pollution (Effler *et al*. 1980), concentrations in the 40–50 μg l^{-1} range have been found to be toxic to microfauna, including *Daphnia magna* (Biesinger and Christensen 1972, Gibson and Grice 1977). The LC_{50} was 0.64 and 5.45 mg l^{-1} copper sulphate for 30-day-old and 6-month-old carp, respectively (Karan *et al*. 1998). Copper sulphate, when applied at a concentration of 20–30 mg l^{-1} as a molluscicide lead to the death of non-target aquatic flora (Shiff 1972). These toxic effects of copper are due to Cu^{2+} and $CuOH^{+}$ species, which are involved in peroxidative reactions and the generation of free radicals (Pagenkopf *et al*. 1974, Andrew *et al*. 1977, Aloj Totaro *et al*. 1986, Roméo and Gnassia-Barelli 1997). Histopathological lesions and changes in enzyme levels have been observed in carp (*Cyprinus carpio* L.) exposed to sublethal concentrations of copper (Karan *et al*. 1998). The metal increased lipid peroxidation in the clam *Ruditapes decussatus* (Roméo and Gnassia-Barelli 1997) and induced mitochondrial degeneration in neurons of the central nervous system in *Torpedo marmorata* (Aloj Totaro *et al*. 1986).

Aquatic and terrestrial molluscs have been shown to have a high capacity for metal accumulation (Meincke and Schaller 1974, Martin and Flegal 1975, Coughtrey and Martin 1977, Berger and Dallinger 1989). This has made them suitable bioindicators of metal pollution in the environment. However, when these metals are present in high concentrations in the environment, this propensity to bioconcetrate them results in lethal effects in molluscs (Amin 1972, Tomasik *et al*. 1995). The exposure of molluscs to sublethal concentrations of metals therefore provides an opportunity to investigate biomarkers that indicate metal-induced stress in these organisms. A number of biomarkers of water pollution in snails and other aquatic organisms have been investigated. These include energy molecules, enzymes, RNA/DNA ratios (Giesy *et al*. 1988), heat shock proteins (Sanders 1993, De Pomerai 1996, Clayton *et al*. 2000), metallothioneins (Hamer 1986, Klaassen *et al*. 1999), cellular energy allocation (De Coen and Janssen 1997) and DNA damage (Steinert 1999).

In this study we investigated the enzymes of amino acid metabolism – glutamate dehydrogenase (GDH) and aspartate and alanine aminotransferases (AST and ALT) – as potential biomarkers of pollution due to copper in the freshwater snails *Lymnaea natalensis* and *Helisoma duryi*. *Lymnaea natalensis* is widely distributed in Sub-Saharan Africa, particularly in tropical Africa, but excluding the Eastern Cape and North East Coast, and is found mainly in streams and small dams (Brown 1994). *Helisoma duryi* is found in scattered localities throughout Africa, mainly in ornamental pools and small dams (Brown 1994). In view of their habitats, both gastropod pulmonates are suitable for use in monitoring pollution in rivers and dams. GDH, ALT and AST are key enzymes in amino acid metabolism. Their substrates and products are linked to energy-generating pathways. In bivalve molluscs, it has been shown that environmental stress can result in increased α-amino acid excretion (Bayne 1973), which can be correlated with aminotransferase activity (Hammen 1968). A high degree of ALT, AST and ornithine aminotransferase activity was observed in tissues of the bivalve mollusc *Mytilus edulis*, particularly in the gills and mantle, in response to exposure to organic pollutants (Narvia and Rantamäki 1997). Similarly, AST and ALT were increased in the gill tissue of carp (*Cyprinus carpio* L.) when exposed to copper sulphate (Karan *et al*. 1998). It is therefore of interest to determine the response of

these enzymes in freshwater snails that have a tendency to bioaccumulate metals in common with other terrestrial and aquatic molluscs.

Methods

Chemicals

L-Alanine, L-aspartate, adenosine diphosphate (ADP), reduced nicotinamide adenine dinucleotide (NADH), 2-oxoglutarate, lactic dehydrogenase (LDH) in 50% glycerol, malic dehydrogenase (MDH) in 50% glycerol, bicinchoninic acid solution, copper sulphate pentahydrate 4% solution, bovine serum albumin (BSA), triethanolamine hydrochloride, ethylene diamine tetra-acetic acid (EDTA), ammonium acetate, potassium phosphate (monobasic and dibasic) and sodium phosphate (monobasic and dibasic) were obtained from Sigma-Aldrich, South Africa. Copper chloride ($CuCl_2$) AR was purchased from Saarchem (Pvt) Ltd, South Africa. Solutions were prepared using double distilled water, except those for copper chloride, which were prepared using water from the concrete tanks where the snails were bred. Concentrated nitric and hydrochloric acid, both of analytical grade, were obtained from Merck, Germany.

Snail culture, exposure and processing

Two species of freshwater snails, *Helisoma duryi* and *Lymnaea natalensis*, were maintained outdoors in 100 l cement aquaria and fed lettuce once a week as described by Naik *et al*. (1996). Waste in the tanks was removed once a month, and fresh Harare City Council tap water introduced. Adult breeding snails were used in experiments, being at least 12 mm in height for *Lymnaea natalensis* and 14 mm in diameter for *Helisoma duryi*. Experimental snails were removed from the breeding tanks and randomly divided into 2 l rectangular plastic containers in the laboratory. The snails were maintained in their original breeding waters taken from the cement aquaria. They were then dosed for 96 h with 0.01, 0.1 or 1 mg Cu(II) ion as a chloride salt per kg breeding water; undosed snails were used as controls. The final concentrations of copper ions in the breeding waters at the end of the dosage period are shown in table 1. After exposure, the snails were shelled, excluding any dead snails, and the shells were kept in plastic specimen tubes at −20°C prior to metal ion content determination. Whole snail tissue for metal ion content determination was also kept at −20°C. Enzymes were assayed in homogenates and subcellular fractions prepared from pooled whole snails. Five controls were prepared using 10 undosed snails for each preparation. At each copper dosage, three test preparations were each made from 10 snails, the exception being for snails dosed with 1 mg Cu kg^{-1}, where five were used due to the high death rates. Homogenates were prepared by homogenizing the pooled snails in 100 mM potassium phosphate buffer containing 1.15% KCl, pH 7.4, using a Potter-Elvehjem homogenizer. After removing a fraction of the homogenate, the rest of the homogenate was centrifuged at 500 *g* for 10 min at 4°C to pellet nuclei and other cell debri. The resulting supernatant was then centrifuged at 10 000 *g* for 10 min at 4°C. After suspension of the 10 000 *g* pellet in buffer, both the pellet suspension (mitochondrial fraction) and the 10 000 *g* supernatant (cytosolic fraction) were aliquoted and stored at −82°C, as were the homogenate aliquots.

Enzyme assays and protein determination

GDH activity was determined at 25°C by the method of Schmidt (1974), in which glutamate formation is monitored via NADH oxidation at 340 nm. AST and ALT activities were assayed at 25°C in coupled reactions as described by Volman-Mitchell and Parsons (1974). In both assays NADH oxidation was monitored at 340 nm as pyruvate and oxaloacetate, generated respectively by the ALT and AST reactions, were being reduced. Protein was determined using the bicinchoninic acid assay method as described for the Sigma-Aldrich kit.

Determination of copper in breeding waters and in snail tissues and shells

Glassware and plasticware used in sample preparation and storage was cleaned to acceptable analytical standards, involving a 48 h soak in 10% v/v nitric acid followed by a rinse in deionized water. This avoids the use of highly concentrated acid, which is liable to activate the surface, producing ion exchange behaviour (Laxen and Harrison 1981).

Breeding water samples of 150 ml were collected in polyethylene bottles, the pH adjusted to 2, and immediately stored at 4°C. The processing of these samples for analysis involved their 10-fold concentration by heating. Concentrated HCl (one volume) and HNO_3 (one volume) were then added

Table 1. Total copper concentration in *Helisoma duryi* and *Lymnaea natalensis* breeding waters, snail shells and tissues.

	Helisoma duryi	*Lymnaea natalensis*
Initial [Cu] in breeding water (mg l^{-1})	0.13 ± 0.03	0.14 ± 0.01
No copper added to breeding water		
Final [Cu] in breeding water (mg l^{-1})	0.13 ± 0.03	0.14 ± 0.01
[Cu] in snail shells (mg kg^{-1})	3.15 ± 0.18	3.10 ± 0.37
[Cu] in snail tissues (mg kg^{-1})	7.04 ± 0.95	6.10 ± 0.30
% surviving snails	100	100
0.01 mg kg^{-1} copper added to breeding water		
Final [Cu] in breeding water (mg l^{-1})	0.13 ± 0.02	0.15 ± 0.01
[Cu] in snail shells (mg kg^{-1})	3.75 ± 0.03	3.50 ± 0.27
[Cu] in snail tissues (mg kg^{-1})	7.97 ± 0.60	7.41 ± 0.42
% surviving snails	98	98
0.1 mg kg^{-1} copper added to breeding water		
Final [Cu] in breeding water (mg l^{-1})	0.21 ± 0.01*	0.22 ± 0.01*
[Cu] in snail shells (mg kg^{-1})	8.97 ± 0.59*	7.05 ± 0.84*
[Cu] in snail tissues (mg kg^{-1})	13.59 ± 2.59*	15.70 ± 0.39*
% surviving snails	91	81
1.0 mg kg^{-1} copper added to breeding water		
Final [Cu] in breeding water (mg l^{-1})	1.14 ± 0.16*	0.97 ± 0.02*
[Cu] in snail shells (mg kg^{-1})	10.72 ± 0.72*	15.25 ± 0.84*
[Cu] in snail tissues (mg kg^{-1})	15.26 ± 3.40*	21.43 ± 2.15*
% surviving snails	3	12

Snails were exposed for 96 h to 0, 0.01, 0.1 or 1 mg kg^{-1} added copper ion. Results are the mean $\pm$ SD of four determinations. In the mortality studies, $n = 40$ snails of each species for the undosed groups and $n = 32$–49 for the dosed groups at each concentration of the copper ion. *, significantly different ($p < 0.05$) when compared with undosed snails.

to 10 volumes of the breeding water concentrate and heated on a sand bath for 1 h (Chauruka 1997). After this, the remaining solution was made up to 10 ml and stored at 4°C until analysis.

Snail tissues and shells were washed with deionized water before being stored at −20°C in polyethylene tubes. The processing of these samples for analysis was done by adding 5 ml of concentrated HNO_3 to a 0.5 g sample of tissue or ground shells in Teflon beakers. The mixture was heated to near dryness on a sand bath and then cooled. The gelatinous precipitate was then dissolved in concentrated HCl diluted 1:1 v/v with deionized water, with gentle heating for 5 min. The resulting solution was made up to 20 ml with deionized water and stored in a polyethylene bottle at 4°C.

The metal ion analysis in the breeding water, tissue and shell samples was carried out using a Shimadzu AA6401F flame atomic absorption spectrophotometer equipped with a photodiode detector. Calibration curves were prepared for each metal using nitrate salt standards of 0–10 mg metal ion per kg dionized water. The metal ion content of these standards were themselves checked against certified reference standards, with good agreement in values. The certified reference was Sample No. 269 (lyophilized cod muscle) of Reference material No. 422 produced by the European Community Bureau of Reference.

Statistical analysis

Graphs were plotted using the Graph Pad Prism program, version 3.0, while means were compared by the Student–Newman-Keuls multiple comparison test using the Graph Pad Instat program, version 3.0 (Graph Pad Software Inc., USA).

Results

Mortality rates

The mortality rates for the two snail species at different concentrations of copper are shown in table 1. No deaths were recorded for undosed snails. Copper

was, however, very toxic to both species at concentrations above 0.21 mg l^{-1}. Only smaller snails in the population were amongst the few surviving at approximately 1 mg Cu ion l^{-1}. At this concentration there was mortality of over 85% after 96 h of exposure.

Bioconcentration of copper in snail shells and tissues

The copper content in shells of *Helisoma* and *Lymnaea* (table 1) rose with the increasing concentration of copper in the breeding water. In snails dosed with 1 mg Cu ion kg^{-1} water, this rise was 3.4- and 4.9-fold the metal ion content of undosed *Helisoma* and *Lymnaea*, respectively. Similarly, the copper content in undosed *Helisoma* and *Lymnaea* tissues was 7.04 and 6.1 mg kg^{-1} and rose to 15.3 and 21.4 mg kg^{-1}, respectively, for snails dosed with 1 mg Cu ion kg^{-1} water, representing 2.1- and 3.4-fold increases over undosed snails. The largest increase in tissue and shell content of the metal ion for a 10-fold increase in copper ion dosage to breeding waters occurred between dosages of 0.01 and 0.1 mg Cu ion kg^{-1} water, which resulted in copper ion concentrations of approximately 0.13–0.15 mg l^{-1} and 0.21–0.22 mg l^{-1} in breeding water, respectively. This increase was 71% and 111% for tissue metal ion and 139% and 101% for shell metal ion for *Helisoma* and *Lymnaea*, respectively.

Statistical analysis of the results in table 1 showed that changes in copper ion content in snail breeding waters, tissues and shells were only significant ($p < 0.05$) for snails dosed with 0.1 mg Cu ion kg^{-1} water, or above, when compared with undosed snails.

Changes in activities of enzymes

The changes in activities of GDH, AST and ALT in snail tissue homogenates, and in 10 000 *g* pellet suspensions and supernatants are summarized in figure 1. The ratios of enzyme activities in snails dosed with 0.1 mg Cu ion kg^{-1} water to enzyme activities in undosed snails are shown in table 2. Generally, the activities of the three enzymes in the three fractions increased with copper concentration in breeding waters up to 0.22 mg Cu ion l^{-1}, but decreased when the concentration reached approximately 1 mg l^{-1}. With regard to GDH, this pattern was most clearly shown in the 10 000 *g* pellets of both species of snail. At the dosage of 0.1 mg Cu ion kg^{-1} water (0.21–22 mg Cu ion l^{-1} final), the increase in GDH activity was 1.8- and 1.6-fold over values for undosed *Helisoma* and *Lymnaea*, respectively. Also at this dosage, AST showed very significant ($p < 0.01$) increases of at least 1.5-fold over values for undosed snails in all three fractions in both snail species. ALT activity decreased early in snails dosed with 0.1 mg Cu ion kg^{-1} water after having shown an initial increase at the dosage of 0.01 mg Cu ion kg^{-1} water in some fractions. The most remarkable response of ALT activity was in the 10 000 *g* pellet fractions, where it disappeared at dosages of 1 mg Cu ion kg^{-1} water in *Helisoma* and at 0.1 mg Cu ion kg^{-1} water in *Lymnaea*.

It was also noted that considerable amounts (up to 30–35%) of GDH and AST activity were recovered in the 500 *g* pellets (nuclear fractions) of the homogenate in dosed and undosed snails. This was also the case with undosed snail ALT activity. The homogenates had higher than expected specific activities of these enzymes.

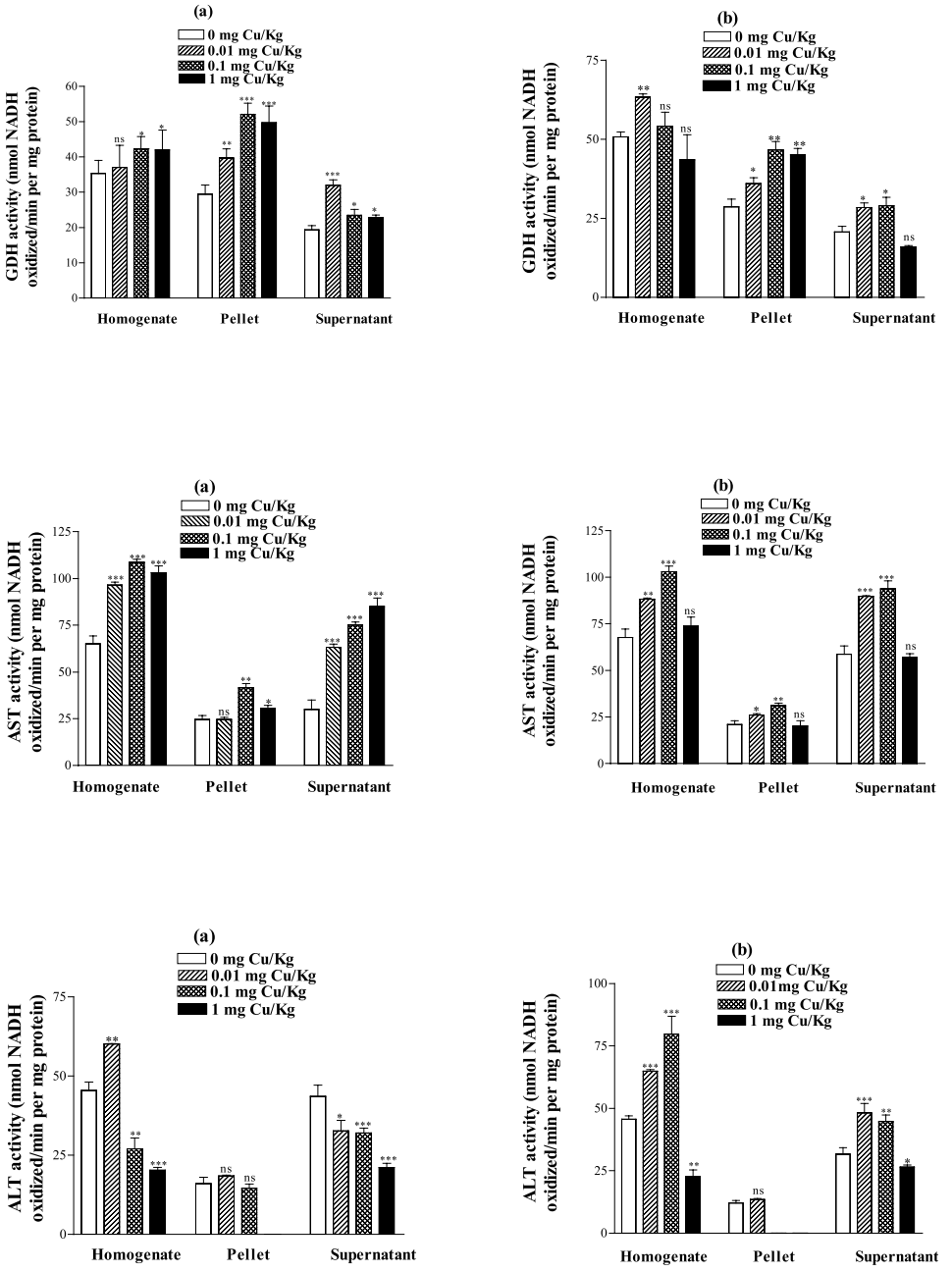

Figure 1. Bar graphs showing the activities of GDH, AST and ALT in tissue fractions of (a) *Helisoma duryi* and (b) *Lymnaea natalensis* snails exposed to breeding water that was either undosed or dosed with copper at the concentrations indicated in the key. The initial concentration of copper in undosed breeding water was 0.13–0.14 mg l^{-1}, to which copper was added at the concentrations indicated. The fractions tested were the homogenate, the 10 000 *g* pellet and the 10 000 *g* supernatant. The values given are the mean ± SEM of five preparations for undosed snails (controls) and three preparations for dosed snails, each preparation being made from at least 10 shelled snails, except for 1 mg kg^{-1} where five snails were used due to the high mortality rate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, not significant, when activities in dosed snails were compared with those in undosed snails.

Table 2. Ratios of enzyme activities in snails dosed with copper at a concentration of 0.1 mg kg^{-1} water to those in undosed snails.

Enzyme	Homogenate		10 000 *g* pellet		10 000 *g* supernatant	
	Helisoma duryi	*Lymnaea natalensis*	*Helisoma duryi*	*Lymnaea natalensis*	*Helisoma duryi*	*Lymnaea natalensis*
GDH	1.2	1.1	1.8	1.6	1.2	1.4
AST	1.7	1.5	1.7	1.5	2.5	1.6
ALT	0.6	1.8	0.9	0	0.5	1.4

Discussion

The bioconcentration of copper in *Helisoma duryi* and *Lymnaea natalensis* was consistent with results obtained for other snail species (Deschiens *et al*. 1957, Coughtrey and Martin 1977, Berger and Dallinger 1989). Although copper is an essential trace element, the tendency for snails to bioconcentrate this metal beyond normal requirements is probably the reason for its potency as a molluscicide (Amin 1972, Ebele *et al*. 1990). The accumulation of copper and other heavy metals is mediated by metal-inducible metallothioneins, which are cysteine-rich metal binding proteins with a high affinity for divalent metals (Hamer 1986). The metallothioneins are also thought to be involved in the detoxification of heavy metals. Their inducibility by metal ions has made them potential biomarkers of heavy metal pollution in fish (Chan, 1995). Although there is no direct evidence, it is likely that the bioconcentration of copper in *Helisoma* and *Lymnaea* tissues was mediated by metallothioneins, resulting in lethal toxic effects at higher (>0.21 mg l^{-1}) concentrations of the metal in breeding waters. The copper concentrations used in the current experiments were well below the 20–30 mg l^{-1} level used to control molluscs, but were in the range of copper concentrations that can cause death in zooplankton and fish (Biesinger and Christensen 1972, Gibson and Grice 1977, Shiff 1972, Karan *et al*. 1998).

The bioconcentration of copper in the test snails resulted in significant changes in the activities of GDH, AST and AST. Generally the enzymes were elevated by copper concentrations of up to 0.21 mg l^{-1}, but tended to fall at copper concentrations of between 0.21 and 1 mg l^{-1}. This fall in enzyme activities coincided with the high death rates of snails dosed with 1 mg Cu ion kg^{-1} water, showing that high levels of copper were extremely toxic to the snails. These enzymes can therefore be useful as biomarkers of water pollution due to copper only in the sublethal range of copper concentrations where they are induced. It may also be noted that the dosage of 0.01 mg Cu ion kg^{-1} water (approximately a 7% increase with respect to breeding water) resulted in quite significant responses in the enzymes. This could be due to the bioconcentration of copper by the snails, since the corresponding increase in copper in snail tissues at this dosage was 13–21% with respect to undosed snails. The higher level of copper accumulation in shells compared with tissues could indicate some adsorption of the metal rather than its total incorporation into the shell structure as a result of growth. Recent data (unpublished) in our laboratories seem to indicate metal–metal exchanges between breeding water, snail tissue and snail shells, raising the possibility that some of the copper accumulating in shells could be incorporated into the shell structure through the displacement of other metals.

Elevations of serum AST and ALT have long been used as indicators of damage to hepatic and cardiac tissues (Wroblewski and LaDue 1956). These aminotransferases have both a mitochondrial and cytosolic localization (Fleisher *et al*. 1960, Boyd 1961, Volman-Mitchell and Parsons 1974, DeRosa and Swick 1975), whereas GDH has a mitochondrial localization (Elduque *et al*. 1982). Various studies have also shown that ALT and AST are increased in the sera and tissues of organisms exposed to metal and organic pollutants. Examples of these include fish exposed to organic pollutants (Lane and Scura 1970, Lockart *et al*. 1975, Reddy and Yellamma 1991) or metals (Karan *et al*. 1998), and molluscs exposed to organic pollutants (Heitz *et al*. 1974, Narvia and Rantamäki 1997). It has been proposed that elevations of these enzymes in tissues is related to increased energy demand as organisms attempt to overcome the toxic effects of pollutants (Reddy and Yellamma 1991). In general, alterations in enzyme activities in organisms exposed to pollutants reflect homeostatic adjustments in metabolic pathways as part of the general adaptation syndrome (Giesy *et al*. 1988). Certainly GDH, AST and ALT are very much involved in energy metabolism, since the reactions they catalyse have substrates and products that are important intermediates in energy-generating pathways. It is also possible that increases in activities of these enzymes were partly due to a need to deaminate amino acids resulting from possible increased tissue breakdown as a result of the toxic effects of pollutants. Calculations of the percentage distribution of the enzymes in the 10 000 *g* pellet suspensions and 10 000 *g* supernatants of undosed and dosed snails also show that there was little or no redistribution of enzymes between the mitochondrial and cytosolic cell compartments as a result of copper dosage. GDH, AST and ALT are not copper-requiring enzymes, hence the most likely cause of the increases in enzyme activities was increased expression of genes encoding these enzymes. Our results (figure 1) show higher than expected specific activities of the enzymes in the homogenates compared with the mitochondrial and cytosolic fractions, especially with respect to GDH, which has an entirely mitochondrial localization in other species (Frieden 1976, Elduque *et al*. 1982). Since the homogenate and, in particular, the 500 *g* pellet (nuclear fraction) were extremely viscous, entrapment by the latter of mitochondria containing all three enzymes cannot be ruled out. This may be so in view of the high levels of enzymes retained in the nuclear fraction despite thorough homogenization. ALT activity in the 10 000 *g* pellet suspension, representing the mitochondrial fraction, decreased with increasing copper dosage and disappeared at 1 mg Cu ion kg^{-1} water. The decrease in mitochondrial ALT could indicate mitochondrial degeneration in the snails as a result of high tissue levels of copper. Copper has been observed to induce degeneration of neuron mitochondria in *Torpedo marmorata* (Aloj Totaro *et al*. 1986). Mitochondrial ALT is also known to be very unstable (Swick *et al*. 1965, Mukorah *et al*. 1998). It would appear that the mollusc enzyme is similarly unstable and that its instability is increased in the presence of high copper levels.

Our results indicate that GDH, AST and ALT in *Lymnaea natalensis* and *Helisoma duryi* all have the potential of being biomarkers of low level copper pollution in water, since their activities are altered significantly by low concentrations of the metal. The fact that copper can induce these enzymes as well as other proteins such as heat shock proteins (Clayton *et al*. 2000) might mean that many other genes have metal-responsive elements, as is the case with metallothionein genes (Hamer 1986).

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