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Copper kinetics and hepatic metallothionein levels in the frog *Rana ridibunda*, after exposure to $CuCl_2$

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Abstract

Adult female frogs *Rana ridibunda* were exposed to 50 and 100 ppm of Cu (as $CuCl_2$) dissolved in water for 5, 15 and 30 days. We measured the Cu content in the liver, kidneys, ventral skin, and large intestine. Hepatic metallothionein (MT) was also measured and we identified by elution the type of proteins bound to copper. Gross morphological characteristics of the frogs were not affected by Cu accumulation. Cu uptake took place first across the skin, then accumulated first in the large intestine, and then in the liver which was continuously accumulating Cu at all exposure concentrations and times. The highest concentration of the metal was recorded in the kidneys at 30 days and 100 ppm exposure. It appears that the kidneys act as the secondary route of Cu detoxification, probably after a Cu overload of liver. The concentration of hepatic MT increased with the increase of Cu concentration in liver at the 5^{th} and 15^{th} day of exposure but we observed a decrease by the end of the experiment. Cu was observed in the MT-fraction, and in the high-molecular weight protein fraction.

Introduction

The environmental contamination due to both natural factors and human activities has received considerable study. In many ecosystems, human-induced changes have overwhelmed the natural biogeochemical discharges of trace elements (Nriagu 1990). Heavy metals are a major problem because they are toxic and tend to accumulate in living organisms. Cu is a trace element essential to animal and plant life that is required for many biological processes (Underwood 1977). About 30 enzymes are known to use Cu as a co-factor (Harris 1991), including ferroxidase, cytochrome oxidase, and superoxidase dismutase. Cu deficiency leads to severe symptoms. On the other hand, excess Cu is known to be toxic, being a potential source of direct or indirect pollution (Atchison *et al.* 1987). Cu usually enters aquatic ecosystems in organic wastes of industrial and agricultural origin, potentially affecting the inhabitants of these ecosystems.

MT is a low molecular weight cysteine rich, cytoplasmic protein with high affinity for cadmium (Cd), copper (Cu), zinc (Zn) and other heavy metals. The protein usually occurs in two isoforms and has been identified in a wide range of phyla from invertebrates to humans (Posthuma & Van Straalen 1993; Henry *et al.* 1994). MT reduces the toxicity of heavy metals such as Cd and mercury (Hg) ions by binding them, thus playing a role in their detoxification and reducing their harmful effects. Suzuki & Kawamura (1984) found a low molecular weight Cu and or Zn binding protein (MT) in the liver and kidneys of three species of frogs, *Bombina orientalis, Bufo bufo japonicus* and *Hyla arborea japonica*. MT identified in these species consisted of one isoform.

Over the last few years many amphibians populations are declining dramatically and extinction has occurred in a few populations, caused by man-made changes in the environment (Carey & Bryant 1995). Few researchers have worked on the relationship between the frog *Rana ridibunda* and heavy metals. Loumbourdis & Wray (1998) studied the concentration of 14 heavy metals in the liver and whole body of this frog living in a small river of Northern Greece,

known to be polluted by effluents and light industries along its entire length. In recent years we began an effort to establish this frog as a bioindicator of environmental pollution (Vogiatzis & Loumbourdis 1998) since it meets all the criteria listed by Lower & Kendall (1990).

In this study, adult frogs *Rana ridibunda* were exposed to 0 (controls), 50 and 100 mg/l Cu (as $CuCl_2$) for 5, 15 and 30 days. We have two objectives, first we monitored the Cu distribution in liver, kidney, ventral skin and large intestine over time and concentrations of exposure. Second measured the hepatic MT and identified by elution the kind of proteins bound to Cu.

Materials and methods

Test organisms

Adult female *Rana ridibunda* were purchased from a local dealer who collected them from relatively unpolluted areas of northern Greece. Prior to the experiment, 90 frogs were acclimatized for 5–7 days in plastic aquaria (35 × 23 × 23.5 cm) containing 2–3 cm (height) of dechlorinated tap water. Water was changed every 2 days and the aquariums were cleaned thoroughly with detergent first , then with 1% nitric acid and finally with deionized water. Frogs were fed laboratory raised *Tenebrio molitor* ad libitum.

Exposure experiments

Sixty frogs were placed in two plastic aquaria (120 × 65 × 60 cm), 30 frogs in each aquarium, in a room, under seminatural conditions, and exposed to 50 ppm and 100 ppm of dissolved copper, respectively. $CuCl_2$ was prepared as a stock solution in deionized water. Another thirty frogs served as the control group and were kept in clean dechlorinated tap water. Mean water quality parameters during tests were: hardness 288 mg $CaCO_3$/l, pH 7.40, conductivity 650–700 μs/cm, nitrites $<$0.025 mg/l, phosphates $<$0.10 mg/l, ammonium $<$0.05 mg/l, copper below the detection limits. Water was changed every 3 days and the frogs were fed with the larvae *Tenebrio molitor* placed in a tray inside the aquaria. The remaining food was always cleaned out. Ten animals from each group were sacrificed at the end of the 5^{th}, 15^{th} and 30^{th} day of exposure.

Gross morhological characterization

Animals were sacrified by a sudden strike to the head. They were individually weighted to the nearest milligram, and snout to vent length measured to the nearest millimeter. Livers and kidneys were weighted to the nearest milligram. The body length was expressed as mm and the body weight and the tissues weight were expressed as g.

Tissue preparation

Liver, kidneys, a portion of large intestine and a piece from ventral skin were weighed to the nearest milligram. Tissues were handled with plastic forceps, crushed into small pieces, chilled in liquid nitrogen and kept in a freezer (−25 °C) , in plastic boxes. All the glassware and plasticware used in Cu determination were pre-soaked overnight in 10% HNO_3 (analytical grade).

Cu determination

Tissues (liver, kidneys, skin and large intestine) were cut into small pieces, dried in an oven at 80 °C for 48 h (to constant weight) and powdered with a pestle and mortar. About 0.5 g of tissue was used. Tissues were digested in 10 ml HNO_3 (analytical grade) over a hot plate, at about 120–150 °C under a reflux cap. Cu was analysed using a Perkin-Elmer 403 atomic absorption spectophotometer with oxygen-acetylene flame. The spike recovery tests were 95–100%. The Cu concentration was expressed as ppm (μg/g) dry weight.

Preparation of cytosols

Frozen liver tissue of 400–500 mg was thawed and homogenized in 3 vol of ice-cold 10 mM Tris-HCl, 250 mM sucrose, pH 7.4, with a glass homogenizer and teflon pestle. The homogenates were centrifuged at 100,000 *g* (60 min, 4 °C) and the supernatant fractions were used for MT determination and copper distribution.

MT determination

MT were determined according to the thiomolybdate assay of Klein (1990) with some modifications. Supernatant (0.3 ml) was mixed with the same volume of acetonitrile. After 9 min, 1.5 ml of buffer A (10 mM Tris-HCL, 85 mM NaCl, pH 7.4) and 0.3 ml

of CM-Sephadex (66% (v/v) suspension in buffer A) (Sigma Chemie, Germany) were added. The mixture was shaken for 9 min and incubated with 150 μl of bovine serum albumin (BSA) (Sigma Chemie, Germany). After 6 min, 0.06 ml of freshly prepared ammonium tetrathiomolybdate (2 mg/ml buffer A) (Sigma Chemie, Germany) was added and after 10 min the mixture was shaken with 0.3 ml DEAE-Sephacel (66% (v/v) suspension in buffer A) (Sigma Chemie, Germany) for 9 min. After centrifugation (8,000 g, 10 min), 1.8 ml of the supernatant was incubated with 60 μl of $CdCl_2$ (Sigma Chemie, Germany) (110 ppm) for 15 min. 0.6 ml of Chelex 100 (66% (v/v) suspension in buffer A) (Sigma Chemie, Germany) was added and the mixture was shaken for 30 min. After centrifugation (8,000 g, 10 min) 1.5 ml of the supernant was incubated with 1.5 ml of acetonitrile for 9 min. Thereafter, the precipitate was removed by centrifugation (8,000 g, 10 min) and 3 ml of the supernatant was analysed for Cd as descriped previously for the copper determination. The MT concentration was calculated assuming a molar ratio of Cd/MT of 7 and expressed as nmole/g of wet tissue.

Cytosol-Cu distribution

0.8 ml of the supernant was applied to a Sephadex G-75 column (1.5 × 60 cm) equilibrated with 0.01 Tris-HCl buffer (pH 8.5). 3-ml fractions were collected and analysed for Cu concentration as described above.

Statistical analyses

The normality of the studied parameters was confirmed with the Kolmogorov-Smirnov test and since all followed normal distribution, parametric analyses were then applied. Values of the studied parameters in each group were compared by analysis of variance (ANOVA) and Scheffe and Dunnett's comparison tests were used to compare the means, and Pearson's for correlation. Differences were deemed statistically significant at $P < 0.05$. Statistical analyses were carried out with SPSS 10.0 for Windows.

Results

Table 1 shows the gross morphological characteristics of the frogs. There were no statistically significant differences between the controls and treated groups.

At the 5th day of exposure, Cu accumulated in the liver, skin and large intestine and the concentration of the metal being statistically significantly higher at 100 ppm exposure compared to control values. The rate of the metal accumulation was greater in the skin compared to the rate of Cu accumulation in the other two tissues (Table 2). At the 15th day of exposure, the concentration of the Cu in liver and large intestine increased at a slower and constant rate. In skin the Cu concentration decreased in both concentrations statistically significantly compared to the values of 5, while in kidneys the metal concentration was increased but the difference was not statistically significant (Table 2). By the the 30th day of exposure, Cu concentration increased in liver, kidneys and large intestine. In the last two tissues the concentration of the metal at 100 ppm was the highest recorded in these tissues, the difference being statistically significant not only with controls but also with values of 5 and 15 days of exposure. The heavy metal concentrations was almost constant in the skin compared to concentrations of day 15 (Table 2).

At the 5th day and 100 ppm Cu exposure, there was a negative Cu correlation between liver and large intestine ($r = -0.803$, $P = 0.05$). Cu in liver and kidneys for both (50 and 100 ppm) concentrations was positively correlated with time (liver 50 ppm $r = 0.594$ $P = 0.01$, 100 ppm $r = 0.535$ $P = 0.01$, kidneys 50 ppm $r = 0.593$ $P = 0.01$, 100 ppm $r = 0.675$ $P = 0.01$). In large intestine only at 100 ppm Cu concentration there was a positive correlation between Cu concentration and time of exposure ($r = 0.665$, $P = 0.01$). In skin at 50 ppm exposure concentration, the Cu concentration was negatively correlated with time ($r = -0.686$, $P = 0.01$) while at 100 ppm the correlation was positive ($r = 0.408$, $P = 0.05$).

At the beginning of the experiment the increase of MT concentration in both 50 and 100 ppm exposure concentrations was significantly different compared to the control values ($P < 0.05$). By day 15 hepatic MT was also increased compared to that of day 5 with statistically significant differences between controls and the group of animals exposed to 50 ppm of exposure concentration ($P < 0.05$). By the end of the experiment MT concentrations in the groups of 50 and 100 ppm of Cu exposure were almost the same as those of the control values but obviously reduced compared to the values of 5 and 15 days of exposure.

Figure 1 shows representative elution patterns of Sephadex G-75 column of the liver extracts from the exposure and the control groups. No peak corresponding to any kind of protein can be detected for controls. Peaks A and B of the groups of 15 and 30 days of

Table 1. Mean values (±SE) of morphological characteristics of adult *Rana ridibunda*, after exposure to various concentrations of Cu for 5, 15 and 30 days.

	5 days	15 days	30 days
Body length(mm)			
Control (N=10)	255.0 ± 3.7	265.0 ± 4.7	249.0 ± 3.4
50 ppm (N=10)	269.0 ± 3.4	256.0 ± 2.8	258.0 ± 1.8
100 ppm (N=10)	262.0 ± 5.3	253.0 ± 2.2	248.0 ± 2.8
Body weight(g)			
Control (N=10)	101.5 ± 4.1	109.3 ± 6.1	100.4 ± 3.2
50 ppm (N=10)	112.5 ± 5.1	106.6 ± 3.5	105.6 ± 2.9
100 ppm (N=10)	108.6 ± 6.2	105.5 ± 4.9	91.7 ± 3.2
Liver weight(g)			
Control (N=10)	1.95 ± 0.09	1.85 ± 0.18	1.70 ± 0.06
50 ppm (N=10)	1.52 ± 0.03	1.92 ± 0.03	1.57 ± 0.06
100 ppm (N=10)	1.76 ± 0.12	1.68 ± 0.09	1.57 ± 0.12
Kidney weight(g)			
Control (N=10)	0.31 ± 0.05	0.28 ± 0.05	0.28 ± 0.04
50 ppm (N=10)	0.25 ± 0.05	0.31 ± 0.05	0.29 ± 0.04
100 ppm (N=10)	0.27 ± 0.06	0.30 ± 0.03	0.26 ± 0.03

Table 2. Mean values (±SE) of Cu concentration in various organs and hepatic Mts (nmol g) in adult frog *Rana ridibunda* after exposure to various concentrations of Cu for 5, 15 and 30 days.

	5 days	15 days	30 days
Cu in liver (ppm)			
control (N=10)	160.25 ± 17.62	169.41 ± 19.08	190.70 ± 16.17
50 ppm(N=10)	230.20 ± 22.40	274.11 ± 22.43	464.34 ± 33.48^{a,*}
100 ppm(N=10)	315.97 ± 16.89*	382.00 ± 28.95*	473.98 ± 3.26*
Cu in kidney (ppm)			
control (N=10)	4.31 ± 0.31	5.41 ± 0.43	6.47 ± 0.69
50 ppm(N=10)	7.07 ± 0.37	15.05 ± 1.6	18.42 ± 3.06
100 ppm(N=10)	8.30 ± 0.44	25.40 ± 2.05	163.09 ± 15.88^{a,b,*,**}
Cu in skin (ppm)			
control (N=10)	5.91 ± 0.79	10.97 ± 0.46	5.56 ± 0.39
50 ppm(N=10)	109.60 ± 13.76*	61.60 ± 5.63^{a,*}	39.89 ± 0.33^{a,*}
100 ppm(N=10)	102.66 ± 10.03*	49.00 ± 5.56^{a,*}	67.96 ± 7.43*
Cu in intestine(ppm)			
control (N=10)	19.06 ± 2.43	26.00 ± 5.03	18.08 ± 3.00
50 ppm(N=10)	67.57 ± 6.80	59.25 ± 5.34	56.99 ± 3.73
100 ppm(N=10)	83.80 ± 9.30*	141.24 ± 15.70*	350.54 ± 40.47^{a,b,*,**}
Mts(nm/g)			
control (N=10)	2.85 ± 0.07	2.89 ± 0.26	3.12 ± 0.05
50 ppm(N=10)	7.12 ± 0.60*	8.61 ± 1.00*	3.58 ± 0.23
100 ppm(N=10)	5.72 ± 0.27*	8.57 ± 0.99*	4.17 ± 0.25

^{a,b}For each tissue, different letters indicate significant differences among exposure times within a dose. no letter = not different, a = difference from 5 days, b = difference from 15 days.
*within an exposure time, indicates significant differences compared to control.
**within an exposure time, indicates significant differences between Cu doses.

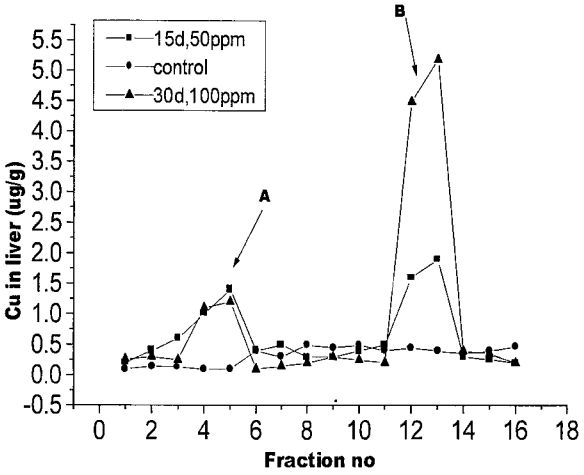

Fig. 1. Distribution of Cu in the liver cytosol fraction of the adult frog *Rana ridibunda* after exposure to 50 and 100 ppm of Cu for 5,15 and 30 days.

exposure to 50 and 100 ppm are estimated to be about 100 kD and 8–9 kD, respectively, from their positions on the Sephadex column. Therefore, the substances were considered to be high and low molecular weight proteins (probably MT).

Discussion

In this study, we did not find any association between the Cu concentration and the gross morphological characteristics of the frogs. Similarly, Handy (1993) found no changes in body weight of rainbow trout *Oncorhyncus mykiss* fed for 28 days on a commercial diet containing 10 g Cu kg^{-1} dry mass.

Amphibians typically do not drink water and obtain water through the ventral skin, but accidental drinking of water could not be excluded. The frogs in our experiment were constantly kept in 2–3 cm of water, thus always remaining exposed to copper. We found a remarkable increase in Cu concentration in the ventral skin at the beginning of the experiment with almost a 22-fold increase compared to the control values. Thus, it seems likely that copper uptake takes place first across the skin. The uptake and release of copper across the skin is similar to that observed for cadmium (Vogiatzis & Loumbourdis 1997). At the 15^{th} and 30^{th} day of exposure for both concentrations, 50 and 100 ppm, the levels of the metal were lower compared to those of the 5^{th} day of exposure. This decrease may indicate that copper kinetics follows the two-compartment model, which implies that easily mobilised metals are absorbed to external surfaces, complexed to low affinity ligands (Scholz 1980). The release from these sites may take place in a short time (days or weeks). This is also implied by the negative correlation between time and Cu concentration, observed in 50 ppm exposure.

The rapid decline in the integumentary copper concentration together with the concomitant increase in the intestinal copper, indicates that, for some unexplainable reason, copper permeability is inhibited across the skin but increases across the intestine. Cu entered the water-permeable skin and then dispersed to the tissues deeper in the body via the blood circulation. Preliminary study showed that Cu accumulated only in this part of the gastrointestinal tract, as the concentration of the metal in oesophagus, stomach and small intestine was below the detection limit of the procedure.

The intestinal copper storage seems to follow the pattern of temporary and long-term storage. At 50 ppm and over the first 5 days of exposure, intestinal Cu increased 3-fold compared to the control values and remained constant throughout the experiment. The fact that there were no significant changes implies that Cu from the intestine moved continuously to other more stable binding sites. The remarkable accumulation in the liver at that time reinforces this observation. This is the temporary storage. The situation at 100 ppm exposure concentration was quite different; the continuous increase of intestinal Cu is indicative of continuous storage. The keyfactor in differential storage seems to be the concentration of dissolved copper. Atlantic salmon *Salmo salar* (Berntssen *et al.* 1999) exposed to low concentrations of Cu did not show any accumulation in the intestine, an indication of copper homeostasis; on the other hand, exposure to higher concentrations led to high accumulation in the tissue. The presence of Cu below the small intestine, indicates that this region either engaged active Cu and propelled it to the liver, or that the metal entered the intestine from the liver through the bile. The negative correlation between Cu in liver and Cu in large intestine at the concentration of 100 ppm at the beginning of the experiment reinforce the first speculation.

The liver accumulated the highest levels of Cu, probably transferred from the skin and later from the intestine. It is the main organ in metal homeostasis, being the site of metalloenzyme production and metal storage, as well as excretion via production of bile. For both exposure concentrations, 50 and 100 ppm, liver accumulated Cu from the beginning of the exper-

iment until the 30th day of exposure with differences statistically significant compared to control values.

Under normal circumstances, much of copper in the liver occurs in the cytosol but as copper accumulates an increasing proportion occurs in particular fractions (Bremner 1998). First is the nucleus; X-ray microanalysis of the livers of copper-loaded rats confirmed the presence of copper in the nuclei (Haywood 1985). The lysosomal accumulation has generally been assumed to be also part of a detoxification process. Lysosomal copper accumulation has been reported in neonates of the toads (Goldfisher *et al.* 1970), Bedlington terriers (Johnson *et al.* 1981), patients with Wilson disease (Goldfisher 1966) and copper-loaded rats (Haywood *et al.* 1985). The lysosomal accumulation is a prelude to biliary excretion of the metal (although there are also non lysosomal pathways for biliary copper ecretion).

The other mechanism that hepatocytes have developed for adaptation to the presence of Cu at toxic levels is that in which Cu is bound to cytoplasmic metallothionein or other low or high molecular weight copper binding proteins such as ceruloplasmin (Cousins 1985). In our results, compared to the control values, hepatic MT increased significantly in *Rana ridibunda* with the increase of Cu concentration compared to the control values at both 50 and 100 ppm until the 15th day of exposure. By the end of the experiment we noticed an increase in MT concentration concerning the group of animals exposed to 100 ppm of Cu, we noticed a slight increase in MT concentration, but this was not statistically significant compared to the control values. The increase in hepatic MT concentration was much less compared to almost 600 times increase observed in the same frog exposed to Cd for the same days (Vogiatzis & Loumbourdis 1998).

In Atlantic salmon, after exposure for 28 days to 700 mg Cu kg^{-1} diet, hepatic MT did not appear to have a strong detoxifying role in Cu-homeostasis (Berntssen *et al.* 1999). Pedersen *et al.* (1998), showed that copper injected into crustaceans increased the mean MT level to approximately twice that found in control animals but with no significant difference. Thus, it seems likely that copper is a very poor inducer of MT.

By separation with a Sephadex G-75 column, soluble Cu was found to exist not only in the MT-fraction but also in the fraction of high-molecular weight proteins. It seemed that Cu is binding not only with MT but also with other proteins. Sugawara *et al.* (1995) found that most Cu existed in the high-mol-wt proteins ($74.3 \pm 2.0\%$) in the group of animals fed with 600 ppm of copper-chloride. A protein peak of large molecular mass also is found in the initial fractions of liver homogenates of carp peritoneally treated with copper (Toth *et al.* 1996). The distribution of Cu in the fractions obtained on the column of the liver homogenates were in agreement with the results of the concentrations of hepatic MT. The low concentrations concerning the exposure groups were due to the binding of copper to the other proteins beside MT, which may serve as a temporary reservoir for the metal.

Apart from the large intestine and liver, kidney was the tissue with a greatest Cu accumulation at the end of the experiment and 100 ppm concentration. It seems likely that, under conditions of high accumulation for a long period, a second detoxification route appears; that through kidneys. For both 50 and 100 ppm Cu kidneys content were positively correlated with time of exposure. After a continuous and in high concentrations entering of copper, the detoxification process begins, partly through the kidneys, partly through the digestive system and partly through the bile. The kidneys may be only a secondary route for copper detoxification in *Rana ridibunda*, a condition also observed in other vertebrates (Grosell *et al.* 1998).

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