Bull. Environ. Contam. Toxicol. (1997) 59:770-776

Bulletin of Environmental Contamination and Toxicology

Uptake, Tissue Distribution, and Depuration of Cadmium (Cd) in the Frog *Rana ridibunda*

A. K. Vogiatzis, N. S. Loumbourdis

Department of Zoology, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece

Received: 14 May 1997/Accepted: 20 August 1997

Cadmium (Cd) is thought to have no essential biological function in organisms. It occurs naturally and is also released into the environment by human activities such as metal smelting, waste disposal, sewage sludge, use of rock phosphate fertilisers, and mining (Alloway 1990). Cd is taken up by plants from the soil or it is translocated to aquatic environments with rainfall. It accumulates in vertebrates through the food chain and may accumulate over years as elimination is very slow. The elimination of Cd mainly takes place in the kidneys, resulting in severe nephrotoxicity (Savolainen 1995). The half-time of Cd in mammalian kidneys is estimated to be 30 years (Goyer et al. 1995). Once inside the cell, Cd is usually bound to specific metal-binding proteins called metallothioneins (Mts) and to a tripeptide known as glutathione (GSH) (Ochi et al. 1988; Chin and Templeton 1993).

In a previous study (*unpublished data*), we found that exposure of the frog *Rana ridibunda* in water containing 200 ppm of Cd for 30 days, resulted in an accumulation of Cd in the liver and in kidneys. The same study found elevations of the hepatic content of Mts and GSH, that were positively correlated with the Cd concentration of liver.

In the present study, we exposed adult frogs to 200 ppm (mg/l) of Cd (in the form of $CdCl_2$) for 10 days and subsequently transferred them to clean water for a period of 30 days. The aims of this study were: I) to monitor the Cd distribution in various tissues of the frog over the 10 days of exposure and the 30 days of detoxification, II) to study the rate of Cd depuration (if any) in various organs and III) to study the fluctuations in hepatic Mts and GSH content through the detoxification period. We studied the following tissues: liver, kidneys, skin, striated muscle, and the gastrointestinal (GI) tract.

MATERIALS AND METHODS

Adult female *Rana ridibunda* were purchased from a local dealer, who collected them from unpolluted areas of Northern Greece. Fifty frogs were acclimatized in plastic aquariums (35 X 23 X 23.5 cm) in 2-3 cm of dechlorinated tap water, for 6-7 days prior to the experiments. Water was changed every 2 days and aquariums were cleaned thoroughly. Frogs were fed larvae of *Tenebrio molitor*. We decided not to use a circulating water system since the still water in the tanks simulated the semi-stagnant waters of the natural environment of the frog.

Forty frogs were placed in a plastic tank (120 x 65 x 60 cm) in dechlorinated tap water containing 200 ppm of diluted Cd for 10 days. At the end of the 10^{th} day a group of 10 frogs was sacrificed, while the remaining 30 animals were transferred to a plastic tank (of

Correspondence to: N. S. Loumbourdis

the previously mentioned dimensions) and kept in clean water for 30 days, which was the detoxification period. A group of 10 animals was sacrificed every 10 days. A group of 10 frogs served as the control group and was kept in clean water. $CdCl_2$ was prepared as a stock solution in deionized water.

All the glassware and plasticware used in Cd determination, was pre-soaked overnight in 10% v/v HNO_3 (Analytical Grade).

All animals were handled with the same procedure. Animals were sacrificed by a sudden strike to the head. They were weighed to the nearest milligram and body length was measured to the nearest millimeter. Livers and kidneys were weighed to the nearest milligram, and the hepatosomatic index (HSI) of each animal was estimated (HSI = liver weight/body weight X 100). Samples were chilled in liquid nitrogen and then kept in a freezer (-25°C). Tissues were handled with plastic forceps and kept in plastic bijou boxes.

For Cd determination, tissues (liver, both kidneys, striated muscle, skin and the GI tract) were cut in small pieces, dried in an oven at 80°C for about 48 h (to constant weight) and were powdered by a mortar and pestle. About 0.5 g of tissue were used. Tissues were digested in 10 ml HNO_3 (Analytical Grade) over a hot plate, at about 120-150°C, under a reflux cap. Cd was analyzed using an ICP-MS spectrophotometer. The carrier gas was Argon and the internal standard Rhodium. Cd concentration was expressed as ppm (μg/gr dry weight). The Cd concentration in the water used for the experiments during the exposure and detoxification period was estimated by the same method.

Mts were determined according to the method of Kuroshima (1995) with slight modifications. 200-400 mg of frozen liver tissue was thawed and homogenized in 10:1 (v/w) 0.03 M Tris-HCl (pH 8.0) buffer, with a glass homogenizer and teflon pestle. The homogenate, was centrifuged at 10,000 g for 15 min, and the supernatant was heated for 2 min in a boiling water bath. The heated sample was centrifuged at 10,000 g for 10 min to remove precipitated proteins. Volumes of 0.1 ml Cd solution (250 μg/l as $CdCl_2$) and 0.5 ml of the homogenization buffer were mixed with 0.5 ml of sample (heat-denatured supernatant) and incubated at room temperature for 10 min. 0.1 ml of a 6% (w/v) bovine haemoglobin solution was then added and incubated at room temperature for 10 min. The haemoglobin was then denatured in a water bath (100°C) for 2 min, cooled in ice for 3 min, and centrifuged at 10,000 g for 15 min. The haemoglobin addition step was repeated twice, and the concentration of Cd in the supernatant was determined using an ICP-MS spectrophotometer. The carrier gas was Argon and the internal standard Rhodium. The estimated concentration of hepatic Mts was calculated by the equation of Hamilton et al. (1987), which assumes 112.4 nanograms per nanomole of Cd, 7 nanomoles Cd per nanomole of Mts, and 1 nanomole Mts per 6,000 nanograms Mts. Mts concentration was expressed as μg/g of wet tissue.

The amount of hepatic GSH was determined by the method of Richardson and Murphy (1975) with slight modifications. 200-300 mg of frozen liver tissue were thawed and immediately homogenized with the addition of (10:1, v/w) 10% $HClO_4$, with a glass homogenizer and teflon pestle. The homogenate was centrifuged at 5,000 g for 15 min. 40 μl of the supernatant were mixed with 50 μl of 0.01 M DTNB [5,5-dithiobis-(2-nitrobenzoic acid)] and 910 μl of PBS buffer (pH 8.0) and incubated at room temperature in the dark for 15 min. OD was measured at 412 nm. GSH concentration was estimated

according to a standard curve of GSH (0-10 nmole). GSH concentration was expressed as μm/g of wet weight tissue.

Statistical comparisons were based on non-parametric tests (Kruskal-Wallis test, Mann-Whitney U test) since the majority of the studied parameters (Cd concentration in the liver and kidneys, and the concentration of hepatic Mts and GSH) were not normally distributed (Shapiro-Wilk's W test). For the correlation of the means we applied the Pearson r correlation. Differences were deemed statistically significant at $p<0.05$. Statistical analyses were carried out with STATISTICA 4.3 for Windows (Statsoft Inc., 1993).

RESULTS AND DISCUSSION

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

Cd concentration of the tap water used in the tanks was below 0.5 ppb (μg/l). The Cd concentration in the liver increased, compared to control values throughout the experiment (Table 2). The comparison between groups revealed statistically significant differences (Table 2). The rate of Cd bioaccumulation in the liver decreased after the 10th day of exposure, nevertheless the Cd concentration at the end of the 30th day of detoxification was 2.65 times higher than that at the end of the 10th day of exposure. The Cd concentration in the liver was positively correlated with the concentration of Cd in the kidneys ($r=0.997$, $p=0.00015$).

The Cd concentration in the kidneys (Table 2) elevated at the phase of detoxification. The comparison between groups revealed statistically significant differences (Table 2), with the exception being the comparison between the Cd concentration of the 10th and the 20th or 30th day of the detoxification period. The rate of Cd bioaccumulation decreased after the 10th day of exposure. At the end of the 30th day of detoxification the Cd concentration in the kidneys was 2.5 times higher than that at the end of the 10th day exposure.

The Cd concentration in the skin and the GI tract at the end of the 10th day of Cd exposure increased significantly compared to control values (Table 2). At the 20th day of detoxification, the concentration of Cd was significantly decreased in both tissues compared to the concentration of the 10th day of exposure (Table 2). The Cd concentration in skin at the 20th day of detoxification was significantly higher compared to control value, while at the GI tract the Cd concentration recovered to control levels. The Cd concentration in the striated muscle was below the detection limits.

The concentration of hepatic Mts increased with the increase of Cd concentration in the liver (Table 2). The comparison of Mts content between groups revealed statistically significant differences (Table 2), with the exception being the comparison between the Mts concentration of the 10th and the 20th and 30th day of the detoxification period. The concentration of Mts was positively correlated with the Cd concentration in the liver ($r=0.974$, $p=0.005$).

The concentration of hepatic GSH increased with the increase of Cd concentration in the liver up to the 10th day of detoxification (Table 2). The comparison between groups revealed statistically significant differences. At the end of the experiment the GSH

Table 1. Mean values (± SE) of gross morphological characteristics of adult female *Rana ridibunda*, exposed to 200 ppm of Cd for 10 days, and subsequently transferred for 30 days in clean water (n: number of animals).

	Control	10 days in Cd	10 days of detoxification	20 days of detoxification	30 days of detoxification
n	10	10	10	10	10
Body length (mm)	274.66±5.59	266.66±4.84	266.25±5.07	266.24±3.14	266.62±4.41
Body weight(g)	123.19±5.39	119.66±5.79	123.61±8.55	107.2±4.93	99.72±3.41
Liver weight (g)	3.04±0.23	2.86±0.21	2.88±0.37	2.31±0.18	2.17±0.19
Kidney weight(g)	0.36±0.02	0.36±0.01	0.37±0.02	0.33±0.28	0.32±0.01
HSI	2.46±0.15	2.39±0.14	2.37±0.17	2.14±0.12	2.15±0.13

Table 2. Mean values (± SE) of Cd concentration in various tissues and hepatic Mt and GSH concentration, in the adult frog *Rana ridibunda*, exposed to 200 ppm of Cd for 10 days, and subsequently transferred for 30 days in clean water. (n: number of animals; Cd expressed in ppm; Mts expressed in µg/g; GSH expressed in µm/g; n.d.: non-detectable).

	Control	10 days in Cd	10 days of detoxification	20 days of detoxification	30 days of detoxification
n	10	10	10	10	10
Cd in liver	$8.88\pm1.38^{a,b,c,d}$	$133.15\pm15.79^{e,f,g}$	255.46 ± 20.86^{h}	318.66±29.82	352.67±42.51
Cd in kidneys	$21.99\pm3.27^{a,b,c,d}$	$215.57\pm16.78^{e,f,g}$	391.65±51.47	450.5±27.21	532.93±17.65
Cd in GI tract	40.86 ± 4.7^{a}	130.76 ± 4.31^{f}	-	50.6±5.89	-
Cd in shin	$4.93\pm0.82^{a,c}$	62.4 ± 8.01^{f}	-	12.5±0.65	-
Cd in muscle	n.d.	n.d.	-	n.d.	-
Hepatic Mts	$0.7\pm0.14^{a,b,c,d}$	$68.08\pm12.48^{e,f,g}$	369.12±41.13	436.94±48.26	471.71±80.42
Hepatic GSH	$1.11\pm0.08^{a,b,c,d}$	$1.85\pm0.23^{e,g}$	3.19±0.28	2.69±0.16	2.81±0.21

a,b,c,d: Control values differed from values of 10 days in Cd (a), 10 (b), 20 (c) and 30 days (d) of detoxification period respectively.
e,f,g: Values of 10 days in Cd differed from values of 10 (e), 20 (f) and 30 days (g) of detoxification period respectively.
h: Values of 10 days of detoxification differed from values of 30 days (h) of detoxification period respectively.

concentration was almost three times higher compared to control values (Table 2). We found a high trend for a positive correlation between the GSH concentration and the Cd concentration in the liver (r= 0.858, p=0.06), and the Mts concentration (r=0.854, p=0.058).

The most surprising finding of this study was the continuous elevation of the Cd content in the liver and kidneys of the animals, even during the detoxification period. Probably this was the reason for the strong positive correlation between Cd concentration in these two tissues. In contrast, the Cd concentration in the skin and the GI tract decreased by the end of the 20th day of detoxification period almost to control values. The skin and the GI tract in this animal constituted almost 40% of the body weight (our observations). Since animals were kept in clean water during the detoxification period, the only logical explanation for the continuous elevation of the Cd concentration in the liver and kidneys is the mobilization of Cd from skin and GI tract and probably from other tissues to the liver and kidneys. Cd concentration in the liver by the end of the detoxification period was 2.65 times higher compared to the concentration of the heavy metal at the end of the exposure period, and the Cd concentration in the kidneys was 2.5 times higher. The increase of the heavy metal concentration in kidneys, albeit obvious, still was not statistically significant between the animals of the group of the 10 days of detoxification and/or the 20 and 30 days of detoxification (Table 2). This fact was most probably due to the high variance of the mean of the Cd concentration in kidneys in the group of 10 days of detoxification. At the end of experiment kidneys seemed to have greater amounts of Cd than liver. The ability of kidneys to accumulate higher amounts of Cd than liver is common to fishes (Hogstrand and Haux 1991), amphibians (Suzuki and Kawamura 1984) and mammals (Torra et al. 1994; Massanyi et al. 1995). In a previous study (*unpublished data*), we exposed adult *Rana ridibunda* for 30 days to 200 ppm of Cd and once again the Cd concentration in kidneys in the 30th day of exposure was higher than that observed in the liver (Cd concentration in the liver: 239.99±53.32 ppm, Cd concentration in kidneys: 657.31±267.64 ppm). At that study, we also found that after the 10^{th} day of exposure the Cd bioaccumulation rate in kidneys was higher than that in the liver. This might have occurred in the present study if we continued the experiment of detoxification for more than one month. Our results suggest that the Cd depuration from the liver and kidneys in this frog may be a very long process. The Cd concentration in the striated muscle was below the detection limit. This was a very important finding, since frogs' legs are highly regarded as human food in many countries.

The levels of hepatic Mts increased in the frog *Rana ridibunda* throughout the experiment. The comparison of the hepatic Mts content between groups did not reveal statistically significant differences between the groups of 10 and/or 20 and 30 days of detoxification (Table 2). This was most probably due to the high variance of the mean of the hepatic Mts content in the group of 10 days of detoxification. The Mts are a family of small, cysteine-rich proteins that have the capacity for high affinity binding of heavy metal ions, and whose synthesis is regulated by metal ion concentrations (Andrews 1990). These properties suggest that they play pivotal roles in the metabolism of the relatively nontoxic essential metals (zinc and copper), as well as toxic heavy metals such as Cd, a concept supported by a variety of studies of cells in culture as well as in intact animals (Ochi et al. 1988; Benson et al. 1990; Chin and Templeton 1993; Roesijadi 1994). In this study we found a very strong positive correlation between the Cd concentration in the liver and the hepatic Mts content. Binding of Cd ions in the molecule of Mts causes them to be biologically inactive, so that the cell is protected from the cytotoxic effects of the

heavy metal. Our findings are in agreement with the elevation of the hepatic Mts content that we noticed in our previous study (*unpublished data*).

GSH has also been implicated to function in cytoprotection against Cd toxicity. GSH is an important antioxidant and several studies have shown that cadmium induces oxidative stress (Chubatsu 1992; Hatcher et al. 1995). Inside the cell, GSH binds the Cd ions with the -SH group of the cysteine (Hatcher et al. 1995; Liu et al. 1995). Cd induced an increase in GSH levels of red sea bream (Kuroshima 1995) and the same occurred in the liver of rats (Eaton et al. 1980) and guinea pigs (Iscan et al. 1994). In our study the increase of Cd concentration in liver resulted in an increase of the hepatic GSH concentration. At the 20^{th} and 30^{th} day of detoxification period we found a decline of the hepatic GSH level that was not statistically significant compared to the value of the 10^{th} day of detoxification. This decline could indicate that after a certain hepatic Cd concentration, the mechanism of hepatic GSH synthesis may be affected irreversibly. GSH concentration revealed a trend for a positive correlation with the Cd concentration in the liver ($r= 0.858$, $p=0.06$), and the hepatic Mts concentration ($r=0.854$, $p=0.058$). This positive correlation was statistically significant in our previous study (*unpublished data*), since the Cd concentration in the liver was not as elevated as in the present one.

Both GSH and Mts seemed to be of major importance for the frog's defence against Cd toxicity, with Mts having a more conspicuous role since their production seemed not to be affected by the Cd concentration in the liver.

We can summarize our findings with this frog: a) Cd is mainly accumulated in the liver and kidneys, b) during the detoxification period, Cd was most probably mobilized from the peripheral tissues to the liver and kidneys, c) the striated muscle of this frog contained Cd below the detection limits, a fact very important since the legs of this frog are widely eaten, and, d) exposure to Cd resulted in a significant increase of hepatic Mts and GSH content, even during the detoxification period.

Acknowledgements. We are grateful to Dr Adrian Hailey for critically reading an early draft of this manuscript and correcting it linguistically and phraseologically.

REFERENCES

Alloway BJ (1990) Cadmium. In: Alloway BJ (ed) Heavy metals in soils, Blackie, Glasgow and London, John Wiley & Sons, New York, p 100- 124

Andrews GK (1990) Regulation of metallothionein gene expression. Prog Food Nutr Sci 14:193-258

Benson WH, Baer KN, Watson CF (1990) Biological markers of environmental contamination. In: McCarthy JF, Shugart LR (eds) Biomarkers of environmental contamination. Lewis Publishers, Florida, p 255-266

Chin TA, Templeton DM (1993) Protective elevations of glutathione and metallothionein in cadmium-exposed mesangial cells. Toxicology 77: 145-56

Chubatsu LS, Gennari M, Meneghini R (1992) Glutathione is the antioxidant responsible for resistance to oxidative stress in V79 Chinese hamster fibroblasts rendered resistant to cadmium. Chem Biol Interact 82:99-110

Eaton DL, Stacy NH, Whong KL, Klaassen CD (1980) Dose-response effect of various metal ions on rat liver metallothionein, glutathione, heme oxygenase and cytochrome P-450. Toxicol Appl Pharmacol 55:393-402

Goyer RA, Cherian MG, Jones MM, Reigart JR (1995) Role of chelating agents for prevention, intervention and treatment of exposures to toxic metals. Environ Health Perspect 103: 1048-1051

Hamilton SJ, Mehrle PM, Jones JR. (1987) Evaluation of metallothionein measurement as a biological indicator of stress from cadmium in brook trout. Trans Am Fish Soc 116:551-560

Hatcher EL, Chen Y, Kang-YJ (1995) Cadmium resistance in A549 cells correlates with elevated glutathione content but not antioxidant enzymatic activities. Free Radic Biol Med 19:805-812

Hogstrand C, Haux C (1991) Mini-review. Binding and detoxification of heavy metal in lower vertebrates with reference to metallothionein. Comp Biochem Physiol 100C: 137-141

Iscan M, Cobna T, Eke BC (1994) Deferential combined effect of Cadmium and Nickel on hepatic and renal glutathione S-transferases of the guinea pig. Environ Health Perspect 102:69-72

Kuroshima R (1995) Hepatic metallothionein and glutathione levels in Red Sea bream Comp Biochem Physiol 110C:95-100

Liu JH, Miyakawa H, Takano T, Marumo F, Sato C (1995) Effects of cadmium on glutathione metabolism in Hep G2 cells. Res Commun Mol Pathol Pharmacol 90:143-152

Massanyi P, Toman R, Uhrin V, Renon P (1995) Distribution of cadmium in selected organs of rabbits after an acute and chronic administration. Ital J Food Sci 3:311-316

Ochi T,Otsuka F, Takahashi K, Ohsawa M (1988) Glutathione and metallothionein as cellular defense against cadmium toxicity in cultured Chinese hamster cells. Chem Biol Interact 65:1-14

Richardson RJ, Murphy SD (1975) Effect of glutathione on tissue deposition of methylmercury in rats. Toxicol Appl Pharmacol 31:505-519

Roesijadi G (1994) Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environ Health Perspect 102:91-100

Savolainen H (1995) Cadmium-associated renal disease. Ren Fail 17:483-487

Suzuki KT, Kawamura R (1984) Metallothionein present or induced in the three species of frogs *Bombina orientalis, Bufo bufo japonicus* and *Hyla arborea japonica.* Comp Biochem Physiol 79C:255-260

Statistica for Windows Release 4.3 (1993) Statsoft Inc.

Torra M, To-Figueras J, Brunet M, Rodamilans M, Corbella J (1994) Total and metallothionein-bound cadmium in the liver and the kidney of a population in Barcelona (Spain). Bull Environ Contam Toxicol 53:509-515