

Season Dependent Response of the Marsh Frog (*Rana ridibunda*) to Cadmium Exposure*

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Cadmium toxicity related to cysteine metabolism and glutathione levels in several tissues of the Marsh frog (*Rana ridibunda*) collected in late spring were investigated after exposure to 80 mg CdCl₂ L⁻¹ for 168 h. The results were compared to those obtained in a previous experiment carried out in autumn. The most striking changes involved the brain which could not maintain a proper glutathione level and the testes in which neither GSH nor sulfane sulfur levels recovered. Substantial damage is expected in the presence of Cd which decreases the antioxidant status of these tissues. It seems that in spring, frogs had lesser tolerance for Cd in comparison with frogs in autumn. This may be caused by the transition to aerobic respiration after hibernation.

Key words: Cadmium, glutathione, sulfurtransferases, sulfane sulfur, *Rana ridibunda* tissues.

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Chronic, low level exposure to heavy metals is an increasing global problem. The symptoms associated with the slow accumulation of heavy metals are multiple and rather nondescript and overt expression of toxic effects may not appear until later in life (QUIG 1998). The sulfhydryl-reactive metals (mercury, cadmium, lead, arsenic) are particularly insidious and can affect a vast array of biochemical and nutritional processes. Recent studies show that metals, including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species (ROS) as superoxide ion, hydrogen peroxide, and hydroxyl radicals (STOHS & BAGCHI 1995; for review see also LINDER & GRILLITSCH 2000). These authors point out that as a consequence, enhanced lipid peroxidation, DNA damage, and altered calcium and sulfhydryl homeostasis occur. Endogenous sulfhydryl compounds serve a critical role in maintaining the function and viability of organisms. Intracellular sulfhydryl levels generally are

higher in cells actively performing some anabolic activity as protein synthesis, lipogenesis, or glycolysis (ELLMAN & GAN 1969) which may be reflected in a higher content of rough endoplasmic reticulum. Sulfhydryl levels therefore represent a measure of the activity potential of cells for maintenance, recovery from damage, or more generally, their ability to react to stressors. Reactive groups of sulfhydryls represent potentially significant sites for reaction with a variety of environmental agents, e.g. metal ions. Reduced glutathione (GSH) is the most abundant of these nonprotein thiols and it has been well demonstrated that, as other sulfhydryls, it also plays an important role in protecting vital nucleophilic sites in the liver from electrophilic attack by numerous classes of reactive chemicals (KLAASSEN *et al.* 1985). Liver GSH concentration is important in modulating Cd-induced hepatotoxicity (DUDLEY & KLAASSEN 1984). Hepatic toxicity of Cd is due to its binding to intracellular sulfhydryl groups and either GSH or metallothionein levels may provide protection against cytotoxicity of Cd (CHAN & CHERIAN

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1992). Histological and histochemical alterations in the liver and kidneys of *R. ridibunda* exposed to aqueous solutions of Cd were recently presented by LOUMBOURDIS (2005). Cd accumulating in vertebrates through the food chain has an extremely long biological half-time (circa 30 years) and its elimination mainly takes place in the kidneys (VOGIATZIS & LOUMBOURDIS 1997, 1998). In amphibians Cd has also a strong inhibitory effect on metamorphosis (FLAMENT *et al.* 2003; JAMES *et al.* 2005).

Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) and 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) are enzymes found in the majority of living organisms. In animal cells MPST is localized in cytosol and mitochondria while rhodanese mainly in mitochondria but in lower vertebrates such as amphibians, reptiles, and fishes it is also present in cytosol (DUDEK *et al.* 1980; NAGAHARA *et al.* 1998). Among tissues, their greatest activity is found in liver and kidney. Rhodanese transfers the sulfur atom from various donors (sulfane sulfur containing compounds) to various thiophilic acceptors. During catalysis, rhodanese cycles between two stable intermediates: a sulfur-free form and a sulfur-substituted enzyme containing a divalent sulfur atom bound by persulfide linkage to the sulfhydryl group of the active-site (HOROWITZ & CRISCIMAGNA 1986). MPST catalyses the transfer of the sulfur atom from 3-mercaptopyruvate (the only one donor) to various acceptors which often produce sulfane sulfur containing compounds (WESTLEY *et al.* 1983). Cysteine in the catalytic site forms persulfide as an intermediate during catalysis (NAGAHARA & NISHINO 1996). γ -Cystathionase (cystathionine γ -lyase, CST, EC 4.4.1.1) is an enzyme found in cytosol of eukaryotic organisms with its greatest activity in liver and kidney (OGASAWARA *et al.* 1994). CST plays a role in the pathway of cysteine (Cys) synthesis from methionine, and more importantly, it is involved in sulfane sulfur generation in cells (for more details see SURA *et al.* 2006). It also functions as a sulfane sulfur carrier – the sulfur is thought to be carried as trisulfide between two cysteine residues (YAMANISHI & TUBOI 1981). All of the above mentioned enzymes contain sulfhydryl groups in their active site and if these groups are not in a sulfur-substituted form, they can be potentially blocked by Cd ions. These may impair the synthesis of cysteine from methionine and the production of sulfane sulfur containing compounds.

Aquatic habitats become polluted with Cd from terrestrial runoff, aerial deposition, and release of effluent directly into water bodies (JAMES *et al.* 2005), so species such as amphibians are at increased risk. Declines and losses of amphibian populations are a global problem. Environmental

toxicants act directly to kill animals, or indirectly by impairing reproduction, retarding growth rates, disrupting normal development, or increasing susceptibility to diseases by immunosuppression or inhibition of immune system development (CAREY & BRYANT 1995; CAREY *et al.* 1999; ALFORD & RICHARDS 1999; LOUMBOURDIS *et al.* 1999; HOULAHAN *et al.* 2000).

The frog tissue distribution of sulfurtransferases and sulfane sulfur-containing compounds have been investigated by WRÓBEL *et al.* (2000a,b). In a previous paper (SURA *et al.* 2006), cadmium administration was found to influence glutathione and sulfane sulfur levels and sulfurtransferases activity in some tissues of *Rana ridibunda* during the autumn season. The aim of the present experiment was to elucidate sulfur metabolism after Cd exposure in frog tissues in spring and to compare these results with those obtained in the previous season.

Material and Methods

Twelve mature frogs *Rana ridibunda* of both sexes were collected at the beginning of June 2004 in the vicinity of Kraków (southern Poland) and were placed for 1 week in plastic aquaria with dechlorinated tap water. The animals were kept in room temperature with a natural day/night rhythm. After acclimatization frogs were divided into 2 groups: control in clean water and the experimental group in water containing 80 mg CdCl₂ L⁻¹ for 168 h. The frogs absorbed Cd from the contaminated water through the highly permeable skin. Water was changed every 24 h to keep a stable concentration of Cd. After that time they were decapitated and the spinal cord was pitched. Liver, kidneys, brain, testes, heart and striated muscle from the thigh were immediately frozen in liquid nitrogen and then kept in a temperature of -20°C. Before assay, tissues were homogenized in four volumes of 0.1M phosphate buffer pH 7.5 and centrifuged. The supernatant was used for the determination of enzyme activities and levels of GSH and sulfane sulfur containing compounds.

The MPST activity was assayed according to the method of VALENTINE and FRANKELFELD (1974). The assays were carried out according to the procedure described by WRÓBEL *et al.* (2004). The enzyme units were defined as nmoles of pyruvate formed during 1 min incubation at 37°C per 1 mg of protein. Rhodanese was assayed according to SÖRBO (1955). The assays were carried out according to the procedure described by WRÓBEL *et al.* (2004). The enzyme units were defined as nmoles of SCN⁻ formed during 1 min incubation at 20°C per 1 mg of protein. The γ -cystathionase activity was determined according to MATSUO and

GREENBERG (1958) with the modification described by CZUBAK *et al.* (2002), using cystathionine as a substrate. The assays were carried out according to the procedure described by WRÓBEL *et al.* (2004). The activity of cystathionase was expressed as nmoles of 2-ketobutyrate formed during 1 min incubation at 37°C per 1 mg of protein. Sulfane sulfur was determined by the method of WOOD (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion. Protein was determined by the method of LOWRY *et al.* (1951) using crystalline bovine serum albumin as a standard.

Sodium 3-mercaptopyruvate, sodium sulfite, N-ethylmaleimide, dithiothreitol, NADH, lactate dehydrogenase (EC 1.1.1.27) from pig heart, sodium thiosulfate, cystathionine, and pyridoxal phosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Potassium cyanide originated from Merck and 2-mercaptoethanol, EDTA-Na₂·2H₂O from Fluka AG.

The statistical significance of differences between experimental group and the control were deter-

mined using the Student's *t*-test. The differences were regarded as significant at $P < 0.05$.

Results and Discussion

In liver, Cd decreases MPST and rhodanese activity, whereas it does not affect the activity of CST and sulfane sulfur levels (Table 1). On the other hand it induces synthesis of GSH, increasing its level two times. The decrease of MPST and rhodanese activity suggests that the synthesis of iron-sulfur proteins, which utilizes sulfane sulfur, is also inhibited. Both enzymes incorporate atoms of sulfur into clusters (Fe-S)_n and so they are indispensable in the synthesis or repair of iron-sulfur proteins (OGASAWARA *et al.* 1995; BONOMI *et al.* 1985; TSE SUM BUI *et al.* 2000).

In kidneys, similarly as in the liver, Cd influences the decrease of MPST and rhodanese activity. The level of glutathione was enhanced by 55% in comparison to the control group. The activity of CST increased five times, which was not observed in other investigated tissues. Simultaneously the

Table 1

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* liver

Liver			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	877±230	640±154**	73
Rhodanese	3255±730	2730±272*	84
CST	0.8±0.3	0.9±0.3	112
GSH	7.3±1.4	16.3±4.2**	223
Sulfane sulfur	183±42	210±40	114

* $P < 0.05$, ** $P < 0.001$

Table 2

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* kidneys

Kidneys			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	977±39	733±39**	75
Rhodanese	4680±260	3990±140**	85
CST	0.3±0.005	1.7±0.25*	567
GSH	1.6±0.18	2.5±0.09**	155
Sulfane sulfur	402±14	192±10**	48

* $P < 0.05$, ** $P < 0.001$

Table 3

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* heart

Heart			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	282±1	644±16**	228
Rhodanese	170±10	330±20**	194
GSH	0.25±0.05	2.7±0.3**	1080
Sulfane sulfur	74±13	128±9**	173

** P<0.001

Table 4

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* muscle

Skeletal muscle			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	142±20	145±6	102
Rhodanese	200±10	110±1**	55
CST	0.9±0.4	0.3±0.1*	34
GSH	1.1±0.07	1.6±0.06**	145
Sulfane sulfur	81±11	96±16	118

* P<0.05, ** P<0.001

Table 5

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* brain

Brain			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	245±11	448±14**	182
Rhodanese	600±10	660±10**	110
GSH	1.11±0.06	0.6±0.02**	50
Sulfane sulfur	322±10	359±18*	111

* P<0.05, ** P<0.001

Table 6

Cd effect on sulfurtransferases activity and the level of GSH and sulfane sulfur in *Rana ridibunda* testes

Testes			
Enzymes (nmol · mg ⁻¹ · min ⁻¹) Glutathione and sulfane sulfur (nmol · mg ⁻¹)	Control	Investigated group (80 mg Cd · L ⁻¹)	% of control
MPST	493±28	214±17**	43
Rhodanese	350±20	130±1**	37
GSH	2.9±0.3	1.7±0.05**	59
Sulfane sulfur	281±35	124±8**	44

** P<0.001

Table 7

Seasonal changes of enzyme activity and the level of glutathione and sulfane sulfur in frog *Rana ridibunda* tissues (comparison of the control groups)

		Sulfane sulfur	GSH	MPST	Rhodanese	CST
		nmol · mg ⁻¹		nmol · mg ⁻¹ · min ⁻¹		
Liver	*fall	308±72	3.7±0.8	903±202	1580±150	1.4±0.3
	spring	183±42	7.3±1.4	877±230	3255±730	0.8±0.3
Kidneys	*fall	185±27	0.29±0.01	799±18	2860±40	1.7±0.5
	spring	402±14	1.6±0.2	977±39	4680±260	0.3±0.01
Brain	*fall	191±11	2.19±0.18	300±13	580±20	ND
	spring	322±10	1.11±0.06	245±11	600±10	ND
Testes	fall	244±9	8.16±0.15	357±21	100±1	ND
	spring	281±35	2.9±0.3	493±28	350±20	ND
Skeletal muscle	*fall	66±6	1.07±0.03	191±16	360±10	ND
	spring	81±11	1.1±0.1	142±20	200±10	ND
Heart	*fall	148±5	2.54±0.02	636±42	280±10	ND
	spring	74±13	0.25±0.05	282±1	170±10	ND

*On the base of values received for frogs *R. ridibunda* in autumn (SURA *et al.* 2006)

ND – not detected

sulfane sulfur level dropped two times in comparison to the control value (Table 2). It seems that cysteine from the plasma glutathione is directed in the kidneys to glutathione synthesis. Cd causes the intensification of glutathione synthesis, presumably also through the rise of CST activity involved in the production of cysteine from methionine, and the intensification of sulfane sulfur conversion through increasing consumption (its level decreases). This is accompanied by high rhodanese activity (Table 7).

In heart a higher activity of MPST and rhodanese and increased levels of glutathione and sulfane sulfur (Table 3) were detected. These results reveal an increase of cysteine availability probably due to the increase of the level of plasma GSH synthesized in liver. Conversions to sulfane sulfur and glutathione are subject to intensification.

Table 4 shows a slight increase of the level of GSH in skeletal muscle, namely by 45% in comparison to the control value, however activity of rhodanese and CST decreased. Because the activity of MPST is stable, the level of sulfane sulfur probably does not decrease. In this tissue the desulfurative conversions of cysteine do not play a significant role and this is manifested by lower activity of MPST, rhodanese and CST as well as a low level of sulfane sulfur in comparison to other tissues (Table 7).

Table 5 shows changes caused by Cd in frog brain. A dramatic decrease of the GSH level is ob-

served accompanied with an increase of activity of MPST and rhodanese and increase of sulfane sulfur. This suggests that cerebral glutathione is used in detoxification processes, however its synthesis is inhibited, for unknown reasons, and cysteine delivered by the plasma glutathione is directed to the desulfurative conversions pathway which is manifested by the increase of activity of MPST and rhodanese and increase of sulfane sulfur level.

In testes the activity of the investigated enzymes as well as levels of GSH and sulfane sulfur dropped significantly after exposition to Cd (Table 6). Gonads cannot maintain the GSH and sulfane sulfur levels, probably because of a lack of cysteine. Acute reproductive effects of Cd in mammals are well known and include testicular necrosis, ovarian hemorrhaging, and delayed embryo implantation. In amphibians Cd disrupts oogenesis (LIENESCH *et al.* 2000). The seminiferous tubules of frogs *Hoplobatrachus tigerinus* injected intraperitoneally with one dose of 0.45 mg CdCl₂ also showed mild shrinkage, the tubular epithelium displaying distortion of the cellular arrangement and exfoliation 48 h after injection (MATHUR & RAMASWAMI 1976). Spermatids and spermatozoa were very few in number, but after 96 h the tubule showed signs of regeneration. However, a lack of a direct effect of Cd on sex determination has been shown – differentiation was found in larvae of urodele amphibian *Pleurodeles waltl* (FLAMENT *et al.* 2003).

Summing up, the most striking changes observed within 7 days after administration of 80 mg CdCl₂ L⁻¹ concern the brain which cannot maintain a proper glutathione level and thus is prone to reactive oxygen species (ROS) generated by Cd (see also WANG *et al.* 2004; SURA *et al.* 2006), and the testes in which neither GSH nor sulfane sulfur levels are recovered leading to substantial damage in the presence of Cd or ROS. In brain a significantly higher level of sulfane sulfur was observed compared to experiments carried out in the autumn (Table 7). Frogs during the spring season had inferior tolerance for Cd in comparison with frogs in the fall (SURA *et al.* 2006) which may be due to the transition to aerobic respiration after hibernation. Some of them were even moribund just before decapitation and this never happened in the fall even after longer exposure to CdCl₂. Frogs subjected to Cd may show various behavioral disorders such as loss of balance, respiratory difficulty, slowness in motion, capsizing in water, sinking to the bottom and increased mucus secretion (SELVI *et al.* 2003; present study).

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