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Cadmium Accumulation and Metallothionein Biosynthesis in *Cyprinus carpio* Tissues

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Cadmium is a problem of magnitude and ecological significance due to its toxicity towards organisms. Fishes have the ability to accumulate heavy metal in their tissues by the absorption along the gill surface and gut tract wall to higher levels than the toxic concentration in their environment (Chevreuil *et al.*, 1995). Pollutants rarely distribute evenly throughout the body fish, but are accumulated by particular target organs. Cadmium accumulates essentially in kidney, liver and gill (Norey *et al.*, 1990). When animals are exposed to cadmium they synthesize detoxifying proteins such as metallothioneins (MT) in kidney and liver. The cadmium is accumulated by means of a binding mechanism involving these proteins. So, the metal is sequestered in a relatively inert and thus non-toxic form. Metallothioneins are a class of low molecular weight, cytoplasmic cystein-rich proteins (Cosson, 1992). They are ubiquitous biomolecules present in a large number of organisms including many fish species, *i. e.* goldfish (Carpene *et al.*, 1992) perch (Olsson and Haux, 1986) stoane loach (Norey *et al.*, 1990) and carp (Kito *et al.*, 1986).

Continuing our research line about cadmium uptake by *Cyprinus carpio* (De Conto *et al.*, 1997) this present laboratory study was designed to examine the accumulation of cadmium in the kidney and liver of carp when exposed to a cadmium concentration of 440 $\mu g.L^{-1}$ for 115 days. Thus, cadmium concentration in this experiment is one hundred fold higher than in environment. For example, in the river Seine (France) cadmium concentration is about 5 - 7 $\mu g.L^{-1}$ (Chevreuil *et al.*, 1995). This choice of high cadmium concentration was selected in order to examine the induction of metallothionein biosynthesis in liver and kidney, and to investigate the time course cadmium exchange between this two organs. A relationship between the metallothionein and the cadmium levels in fish tissues was examined.

MATERIALS AND METHODS

The experiments were carried out with 80 one-summer-old carp (*Cyprinus carpio*) weighing 180 ± 35 g ($\overline{x}$ ± SE). They were supplied in December 1996 by a local pisciculture in Dombes and used in experiments from February until May, 1997.

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The experiments have been achieved using previously described exposure system (De Conto *et al.*, 1997). The Cd^{2+} content, 440 ± 17 $\mu g.L^{-1}$ ($\bar{x}$ ± SE; n=60) was monitored three times a week by differential pulse polarography in the course of the experimental period. No fish died during the acclimatization period. No mortalities occurred during cadmium exposure and no weight loss was observed.

In order to conduct analysis of metal intake and metallothionein biosynthesis by carp, ten fish were sequentially removed from aquarium after 0, 50, 86, 106 and 115 days. The kidney and liver were removed from each of them. A part of each tissue sample was used for dry weight determination, after drying at 105 °C for 24 hours. For each organ, the tissues of ten carp were pooled, weighed, and homogenized, in an ice-bath for 5 min, using an Ultra-Turrax homogenizer (Ika Labortechnik) in three volumes of Tris-HCl buffer (25 mM, pH 8.5) with 0.1 mM PMSF to prevent protease action and 1 mM mercaptoethanol to maintain reducing conditions. Each homogenized sample was divided into three aliquots : two ones were prepared for heavy metal determination, and the remaining one was used for metallothionein determination.

To conduct an analysis of metal intake by fish, two aliquots were used. Indeed, each analysis was carried out in duplicate. The amount of cadmium, copper and zinc was determined using inductively coupled plasma-mass spectrometry. The operating conditions and the analytical process certification were described in De Conto Cinier *et al.* (1997). The third aliquot of the homogenate was centrifuged for 5 min at 5,000 g (Beckman J-21), at 4 °C. The supernatant was separated from the pellet, heated at 80 °C for 5 min in order to precipitate most of the high molecular weight proteins, and subsequently centrifuged for 30 min at 100,000 g (Beckman L5-50B ultracentrifuge), at 4 °C. Aliquots of the heated cytosol were taken for quantification of low molecular weight sulfhydryl metallothioneins by differential pulse polarography (Metrohm VA 663 - Polarecord E506). This technique is based on -SH compound determination according to Brdicka (1965). A modified Brdicka supporting procedure was used as described by Thompson and Cosson (1984). The electrolyte was 1 M NH_4Cl, 1 M NH_3 and 2 mM $[(Co(NH_3)_6]Cl_3$. This solution was prepared weekly and stored at 4 °C when not used. Prior to analysis, the analytical system was purged with oxygen-free N_2 for 4 min and then allowed to equilibrate for 1 min. Calibration of the system was performed using a standard solution of rabbit liver metallothioneins MT- 1 (Sigma). The linear working range was found between 1 and 35 $nmol.L^{-1}$, assuming an average molecular weight of 6500. The detection limit was approximately 0.5 $nmol.L^{-1}$. For each experiment, 200 µL of calibration solution or heated cytosol tissue was added to 20 mL of electrolyte and 200 µL of Triton X-100 (from a working solution of 5.10^{-3} % (v/v)) in the polarographic cell, maintained at a temperature of 0 °C, in order to minimize protein denaturation. Scanning was from - 1.0 V to - 1.7 V (vs Ag/AgCl) at 4 $mV\ s^{-1}$, with a drop time of 1 s. Concentrations of metals and metallothioneins were determined as $mg.kg^{-1}$ dry wt of tissue and $g.kg^{-1}$ dry wt of tissue, respectively. AU data were statistically evaluated for

difference of means by Student's t-test using a probability limit $P \leq 0.05$ as significant.

RESULTS AND DISCUSSION

The data on cadmium bioaccumulation in carp tissues, as a function of exposure time, are presented for kidney and liver in Fig. 1. These curves show clearly that cadmium concentration in both tissues increases significantly ($P \leq 0.05$, Fig. 1.) during the 115 days of exposure. The kidney and liver cadmium concentrations correlated with time of exposure. Indeed, a linear relationship of the form [Cd] = 12.7520 + 1.9483 days (R^2= 0.9462, $P \leq 0.05$) and [Cd] = 8.9711 + 0.9116 days (R^2= 0.9535, $P \leq 0.05$) fitted for kidney and liver data respectively. After 115 days of exposure, cadmium concentration values were 252 $mg.kg^{-1}$ dry wt and 116 $mg.kg^{-1}$ dry wt for kidney and liver respectively. The cadmium distribution, showing decrease in the order kidney > liver, is commonly found in fish (Kraal *et al.*, 1995, Yang and Chen, 1996). The fact that cadmium is not evenly distributed between all the tissues is related to the presence of various ligands, each with different metal-binding characteristics (Bebianno and Langston, 1995). Linear cadmium accumulation with exposure time in carp as found in the present work, has previously been demonstrated in roach liver by Bonwick *et al.* (1991).

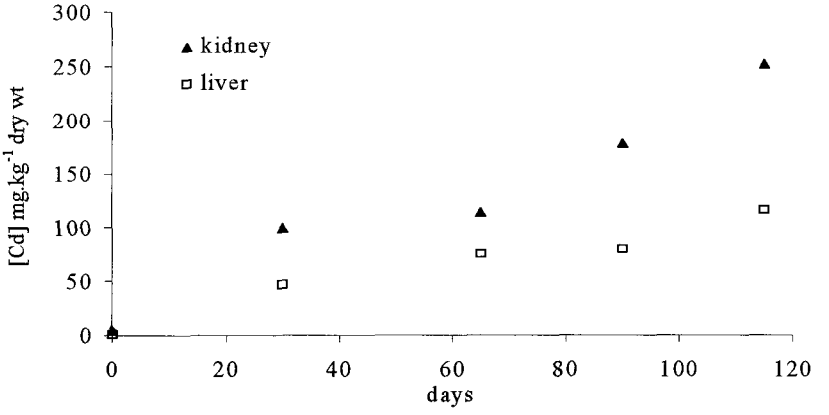

Figure. 1. Cadmium concentrations versus exposure time in kidney and liver of *Cyprinus carpio* exposed to 440 $\mu g.L^{-1}$ Cd. Mean of 10 animals.

Metallothionein concentrations as a function of exposure time for kidney and liver are shown in Fig. 2. These curves show clearly that metallothionein concentrations in kidney and liver also increase significantly ($P \leq 0.05$, Fig. 2.) during the 115 exposure days. There was a linear increase in metallothionein content in both kidney and liver with time. Linear relationships are described for the kidney by the formula [MT] = 1.5762 + 0.0638 days (R^2= 0.9103, $P \leq 50.05$) and for the liver by the formula [MT] = 0.8885 + 0.104 days (R^2= 0.9401, $P \leq 0.05$). After 115 days

of exposure, the metallothionein concentration values were 8.7 $g.kg^{-1}$ dry wt and 13.7 $g.kg^{-1}$ dry wt for kidney and liver respectively. The renal metallothionein content is much lower than the hepatic one during the 115 exposure days. Results are in accordance with that found for turbot (George *et al.*, 1996) and goldfish (Carpené *et al.*, 1992).

Fig. 3.a. and Fig. 3.b. show the relationship between cadmium and metallothionein levels in kidney and liver respectively. Metallothionein concentrations increase linearly versus cadmium concentrations and may be described by the following regression equations : [MT] = 1.1596 + 0.0327 [Cd] (R^2 = 0.9618, $P \leq 0.05$) for kidney and [MT] = 0.3128 + 0.1070 [Cd] (R^2 = 0.8682, $P \leq 0.05$) for liver. Accumulated cadmium in perch or roach liver was found to correlate with an increase of metallothioneins (Bonwick *et al.*, 1991, Olsson and Haux, 1986).

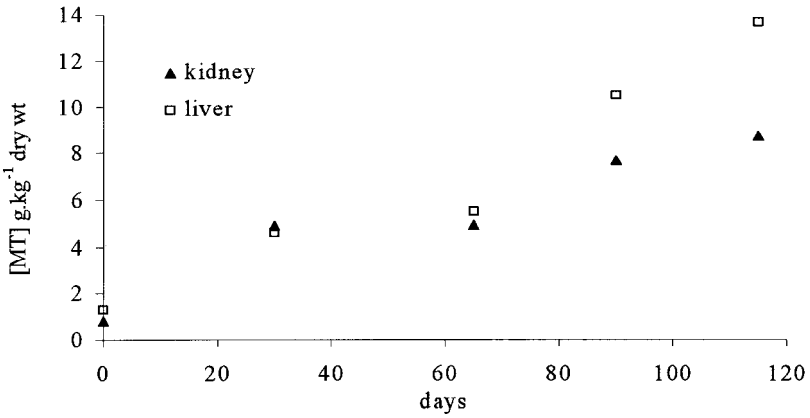

Figure. 2. Metallothionein concentrations versus exposure time in kidney and liver of *Cyprinus carpio* exposed to 440 $\mu g.L^{-1}$ Cd. Mean of 10 animals.

Such results support the opinion that induction of metallothionein biosynthesis in kidney and liver occurs during the cadmium exposure. Indeed, carp have developed cadmium-sequestering detoxifying systems which make this metal harmless to the organism. Then, carp are able to accumulate high cadmium concentrations without any apparent detrimental effect. Previous laboratory investigations on fish have indicated that metallothionein synthesis can be induced by cadmium during acute exposure (Kito *et al.*, 1986). Comparing cadmium accumulation data reported in the literature for fish (Kito *et al.*, 1986, George *et al.*, 1996) with the results of the present study, we can postulate a scheme of cadmium metabolism in carp. The results indicate that the increase rate of cadmium bioconcentration was 2 $mg.kg^{-1}$ dry $wt.day^{-1}$ for kidney and 0.9 $mg.kg^{-1}$ dry $wt.day^{-1}$ for liver.

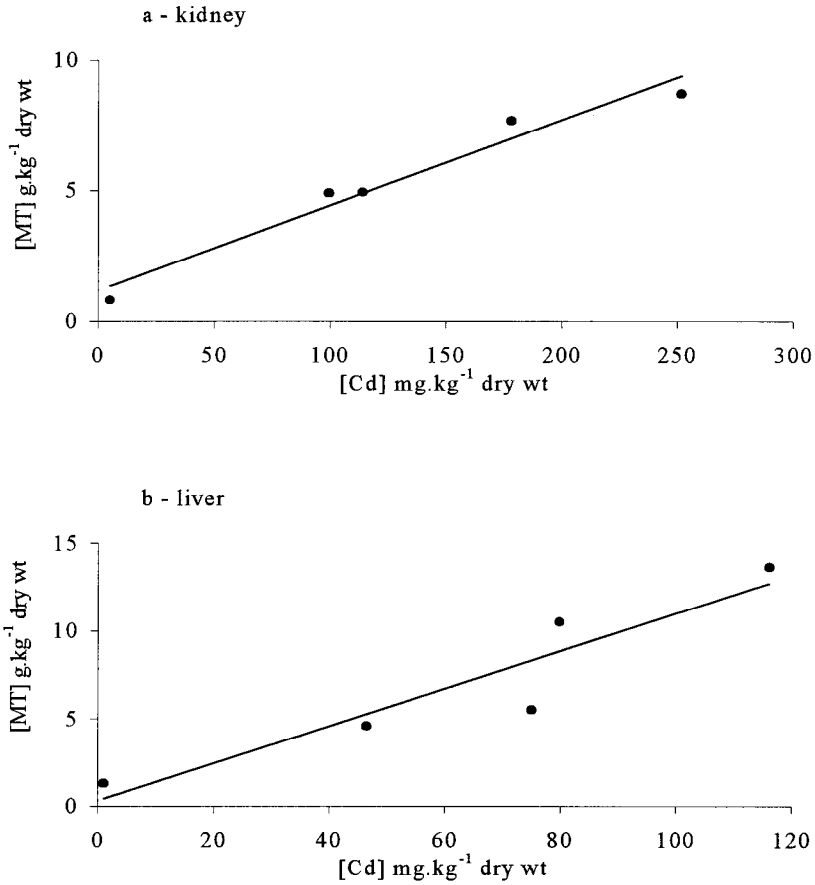

Figure. 3. a. b. Metallothionein concentrations ($g.kg^{-1}$ dry wt) versus cadmium concentrations ($mg.kg^{-1}$ dry wt) for liver and kidney respectively.

The kinetic of kidney metallothionein synthesis, 0.064 $mg.kg^{-1}$dry $wt.day^{-1}$, was slower than the kinetic in liver, 0.104 $mg.kg^{-1}$dry wt. day^{-1}. We can assume that a specific role of metal metabolism has been developed for each tissue. During cadmium exposure, induction of metallothionein synthesis occurs first in liver. Indeed, it is well known that liver is the primary organ for detoxification. As the liver reaches saturation, Cd-MT complexes are transported to kidney, which is the main cadmium storage site. However, if metal intoxication is acute, kidney is also considered to take up cadmium directly from the blood. And then, metallothionein biosynthesis occurs in fish kidney too. An important result is that all metallothioneins are closely similar. Consequently, there is no appreciable difference in their metal-binding properties since kidney and liver contain the same set of metallothioneins. It is noteworthy that the ratio [MT]/[Cd] is around 3 fold higher in liver than in kidney. The generally higher cadmium content in the kidney is not the result of a difference in metallothionein primary structure but must be

attributed to a difference in chemical environment in both organs. This lead us to consider the possibility of a metal-binding by high molecular weight proteins. Indeed, Kito *et al.* (1986) show that in kidney and liver, the cadmium content in metallothioneins increased first, but reached a limited value, and that cadmium binding to high molecular weight proteins increased. In kidney, the cadmium content in high molecular weight proteins increased more quickly than in liver with time of exposure to cadmium. This result explains the different rates observed for metallothionein synthesis and cadmium accumulation between kidney and liver.

It is shown that cadmium copper and zinc may be linked to the same metallothioneins. Thus, internal competitions between these metals for metallothionein binding sites probably modify the concentration of copper and zinc during the exposure. When fish are exposed to cadmium this ion in the cytosol can displace copper and/or zinc ions from native metallothioneins and then copper and zinc concentrations decrease. At the opposite, metallothionein biosynthesis during cadmium exposure results in a concomitant increase in zinc and/or copper content (Olsson and Haux, 1986). It is noteworthy that after cadmium exposure, there is no change in the copper and zinc carp tissues levels (De Conto *et al.,* 1997).

When amount of accumulated metal exceeds the animal ability to synthesize the detoxifying metallothioneins, metal binding to high molecular weight proteins occurs (Kito *et al.,* 1986). Storage of cadmium in other parts of the body assumes an increasing importance, notably in muscle (De Conto *et al.,* 1997) and causes cadmium toxicity. This cadmium toxicity in carp manifests itself and thus leads to the development of structural abnormalities. Fish may suffer from anemia, anorexia and respiratory distress. The anorexia in cadmium-exposed fish contributes to the depression in antibody production. Biosynthesis of metallothionein leads to detoxification of the metal but can also interfere with other mechanisms in which metallothioneins may be involved. This phenomenon deserves further attention. From the present results, it can be concluded that metallothioneins appear to be an important factor in carp cadmium accumulation. However, further investigations are needed to clarify the biochemical mechanisms for cadmium accumulation in kidney and liver, and cadmium exchange between this two organs. It is noteworthy that the mechanism depends on routes of exposure as well as fish species (Thomas *et al.,* 1983).

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