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Heavy metal intracellular balance and relationship with metallothionein induction in the liver of carp after contamination by silver, cadmium and mercury following or not pretreatment by zinc

Richard P. Cosson

Service d'Ecotoxicologie, 1356 CNRS, Faculté de Pharmacie, Nantes, France

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Determination of metal levels (copper, zinc, cadmium, silver and mercury) in soluble and insoluble fractions of liver homogenates has been performed after 7 days exposure of carps (*Cyprinus carpio*) to moderate concentrations of cadmium, silver and mercury in water. Metallothionein (MT) levels have been quantified by a polarographic method before and after the contamination and a subsequent decontamination phase (7 days). The influence of pretreatment by zinc (7 days) has also been evaluated. MT level variations have been interpreted as having regard to inter-related flows of metal between subcellular fractions. Special interest has been focused on heat-stable compound (HSC)-bound heavy metal flows within the cytosol, taking in account that MT is the major component of these ligands. Our data showed differences between the ability of metals to bind cytosolic ligands and HSCs, and their respective potency for MT induction in liver. Regardless of pretreatment, mercury gave the highest increase of liver MT, but the MT level decreased during the decontamination step, especially after pretreatment by zinc. Cadmium and silver gave similar increases, but a significant difference with the control appeared only after the decontamination step with cadmium, while 1 week of contamintion was enough for silver. However, silver binding with MT was achieved only by the end of the decontamination step, while cadmium depicted the highest ratio for HSC-bound toxic metals after the contamination. Our experimental conditions gave the following order of potency for MT induction in liver: mercury ≫ silver > cadmium > zinc. Results are discussed comparatively with data obtained with carp gills.

Keywords: fish liver, heavy metals, metallothionein inducibility, subcellular ion balance, zinc pretreatment

Introduction

It is well established and generally accepted that zinc and copper are primary inducers of metallothionein (MT) synthesis (Bremner 1987). Webb (1987) clearly expressed that the primary function of MT is in the homeostasis of these two essential metals. A review of MT participation in toxic metal detoxication led us to hypothesize that it is due to nothing more than fortuitous interactions of foreign cations with their normal homeostasic role (Cosson *et al.* 1991). Binding of metals on MT sites is not only dependant on their respective affinity for cysteinic residues, but is also related to their relative amount in the cell (Hamilton *et al.* 1987).

In this paper, the ion balance between subcellular compartments was investigated with regard to hepatic MT level variations following various experimental protocols, as reported on gills (Cosson 1993). Determination of the metal balance within liver homogenates, between soluble and insoluble fractions, and within cytosolic fractions between heat-stable compounds (HSC) and heat-denaturable compounds (HDC) was performed. Calculating the flows of essential and toxic metal entering or leaving the compartments stated above, we tried to give an interpretation of MT level variations in the liver after pretreatment, contamination and decontamination steps. This interpretation is supported by our knowledge about metal substitution processes at MT metal-binding sites, taking in account results and

Address for correspondence: R. P. Cosson, Service d'Ecotoxicologie, Unité de Recherche Associée 1356 du Centre National de la Recherche Scientifique, Faculté de Pharmacie, 1, rue Gaston Veil, 44035 Nantes Cedex, France. Fax: (+33) 40 41 28 09.

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hypothesis cited by other researchers working on fish or other animals. A comparison is given with results obtained with carp gills (Cosson 1993).

Materials and methods

Fishes (*Cyprinus carpio*) were obtained from a fish hatchery and maintained in unchlorinated water (hardness 92–188 mg l^{-1} as $CaCO_3$) for several weeks prior to the experiments. They were exposed to low concentrations of toxic metals (Cd = 1000 $\mu g\,l^{-1}$; Ag = 10 $\mu g\,l^{-1}$; Hg = 50 $\mu g\,l^{-1}$) in water following or not pretreatment with zinc (2000 $\mu g\,l^{-1}$). Pretreatment and following exposures lasted for 7 days each (Table 1). Static bioassays were performed in 10 l plastic containers. Control, pretreated or exposed fish were fed every other day before renewing the water (contaminated or not). The livers of 10 fishes were dissected and analyzed separately after each treatment. Livers were homogenized in 10 mM Tris–HCl (pH 8.6) buffer and centrifuged for 30 min at 27000 × *g*, at 4 °C. Pellets and aliquots of supernatants were digested with nitric acid at 60 °C for metal analysis. The remaining supernatants were submitted to heat-denaturation (75 °C; 15 min) and centrifuged. Pellets (HDC) and supernatants (HSC) were, respectively, digested (as above) or frozen until metal and MT quantification. Heavy metal determinations (silver, cadmium, copper, mercury and zinc) were performed by atomic absorption spectroscopy to evaluate the ratio of heavy metal available to MT binding. The amount of MT in heat-denatured cytosol has been determined by differential pulse polarography (DPP) (Olafson & Sim 1979, Thompson & Cosson 1984). Results are expressed as concentrations, i.e. nanograms of metal or micrograms of MT per gram of homogenized tissue (Figure 1). To evaluate the variations of metal or MT amounts between successive experimental phases, the ratios were calculated as a percentage of the former metal amount (ng) or the former MT concentration (μg MT g^{-1}) (Tables 2–8). When a decrease was observed, we used the negative sign (−), at the opposite we used (+). Control group values were calculated using control fish sampled at the end of every week of the experiment ($n = 30$). Significant differences between fish groups were determined using analysis of variance (ANOVA) and Fisher's *F*-test. We used the word 'significant' for differences observed with $P < 0.05$, unless otherwise specified ($0.05 < P < 0.10$); other differences were considered as tendencies. To calculate the ratios of metals between the considered fractions (cytosol, pellet; HDC, HSC), amounts of metals are expressed in nanograms (Table 9).

Table 1. Experimental protocol: carps were sacrified by the end of the first week of the experiment to study zinc pretreatment induced modifications, by the end of the second week to study the influence of the contamination step and by the end of the third week to study the effect of a decontamination step

	Phase I (7 days)	Phase II (7 days)	Phase II (7 days)
T	clear water		
Zn	zinc 2 mg l^{-1}		
TT	clear water	clear water	
ZnT	zinc 2 mg l^{-1}	clear water	
TCd	clear water	cadmium 1 mg l^{-1}	
ZnCd	zinc 2 mg l^{-1}	cadmium 1 mg l^{-1}	
TAg	clear water	silver 0.01 mg l^{-1}	
ZnAg	zinc 2 mg l^{-1}	silver 0.01 mg l^{-1}	
THg	clear water	mercury 0.05 mg l^{-1}	
ZnHg	zinc 2 mg l^{-1}	mercury 0.05 mg l^{-1}	
TTT	clear water	clear water	clear water
ZnTT	zinc 2 mg l^{-1}	clear water	clear water
TCdT	clear water	cadmium 1 mg l^{-1}	clear water
ZnCdT	zinc 2 mg l^{-1}	cadmium 1 mg l^{-1}	clear water
TAgT	clear water	silver 0.01 mg l^{-1}	clear water
ZnAgT	zinc 2 mg l^{-1}	silver 0.01 mg l^{-1}	clear water
THgT	clear water	mercury 0.05 mg l^{-1}	clear water
ZnHgT	zinc 2 mg l^{-1}	mercury 0.05 mg l^{-1}	clear water

Results

Effects of pretreatment by zinc on MT synthesis and metallic ion balance (Table 2)

After 1 week of treatment by zinc (T > < Zn) we did not find any significant change in the hepatic MT level. Total zinc and copper amounts in both studied fractions (cytosol and insoluble) were not significantly different from those encountered in the control, but the amount of copper associated with heat-denatured cytosol had significantly decreased, and the ratio of cytosolic copper bound to HSC had increased slightly (Table 9). Similarly, we did not find significant differences between MT levels from fishes pretreated with zinc (Zn) and fishes for 7 days (ZnT) or 14 days (ZnTT) in clear water, even though MT levels increased, respectively, by 18% (Zn > < ZnT) and 15% (ZnT > < ZnTT). In the cytosol there were increases of copper (significant) and zinc (Zn > < ZnT) amounts. After a further week of decontamination (ZnT > < ZnTT), copper decreased significantly in the insoluble fraction and in the heat-denaturable cytosolic fraction. Increases of zinc were not significant within cytosolic and insoluble fractions, but the decrease of zinc in the heat-denaturable cytosolic fraction was significant.

When control fish were compared with fishes exposed to zinc and subsequently reared in clear water (T > < ZnT), the increase of MT was still not significant. Copper increased in both fractions stu-

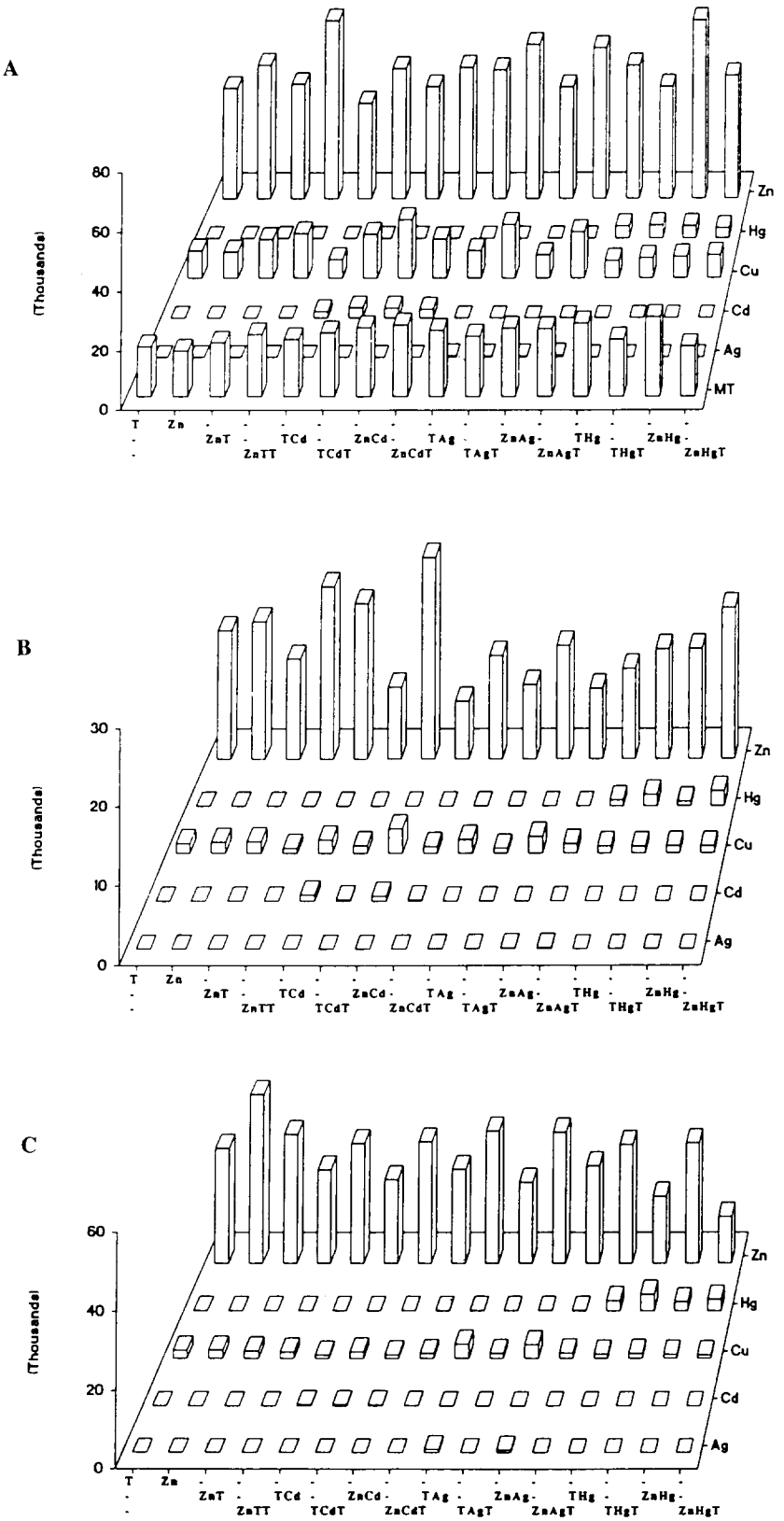

Figure 1. Mean concentrations of metals in the cytosol (**A**) and the pellet (**B**) of liver homogenates; and (**C**) in the heat-denatured cytosolic fractions ($ng.g^{-1}$) and mean concentrations of MT (10 X μg $MT.g^{-1}$) in the heat-stable cytosolic fractions.

Table 2. Variations of metal and MT amounts (%) between different phases: pretreatment by zinc

	Cytosol	Pellet	HDC
T > < Zn	MT − 8		
	Cu − 7	Cu − 6	Cu* − 32
Zn > < ZnT	MT + 18		
	Cu* + 87	Cu + 45[a]	Cu[a] + 38
	Zn + 20	Zn + 5	Zn + 8
ZnT > < ZnTT	MT + 15		
	Cu − 7	Cu* − 67	Cu* − 38
	Zn + 24	Zn + 27	Zn* − 44
T > < ZnT	MT + 8		
	Cu* + 74	Cu + 37	Cu − 7
T > < ZnTT	MT* + 25		
	Cu* + 62	Cu* − 55	Cu* − 43
	Zn* + 50		Zn* − 39

*$P < 0.05$; [a]$P = 0.06$.

Table 3. Variations of metal and MT amounts (%) between different phases: direct exposure to cadmium

	Cytosol	Pellet	HDC
T > < TCd	MT + 15		
	Cd* + 11190	Cd* + 6275	Cd* + 2341
	Cu − 37	Cu + 11	Cu* − 69
	Zn − 22		Zn − 24
TCd > < TCdT	MT + 12		
	Cd* + 91	Cd* − 63	Cd[b] + 41
	Cu* + 215		Cu* + 162
	Zn* + 66	Zn[a] − 36	
T > < TCdT	MT* + 28		
	Cd* + 21475	Cd* + 2282	Cd* + 3347
	Cu* + 99		Cu − 18
	Zn + 30	Zn − 36	Zn[c] − 31

*$P < 0.05$; [a]$P = 0.08$; [b]$P = 0.07$; [c]$P = 0.06$.

died (significantly in cytosol) and its amount associated with HDC decreased.

When control fish were compared with fish exposed to zinc and subsequently reared 2 weeks in clear water (T > < ZnTT), the increase of MT became significant. There was a significant loss of copper in the insoluble fraction and within the cytosolic fraction. The comparison pointed out an increase of copper and zinc in total cytosol and especially in the HSC fraction, the increase of zinc being extremely important.

After 1 week of contamination by zinc, the lack of hepatic MT *de novo* synthesis had to be related to

Table 4. Variations of metal and MT amounts (%) between different phases: exposure to cadmium following pretreatment by zinc

	Cytosol	Pellet	HDC
Zn > < ZnCd	MT* + 51		
	Cd* + 25132	Cd* + 7155	Cd* + 2279
	Cu* + 138	Cu* + 121	Cu* − 44
		Zn* + 75	Zn[c] − 19
ZnCd > < ZnCdT	MT + 4		
		Cd* − 73	Cd* − 39
		Cu* − 66	Cu* + 51
	Zn + 38	Zn* − 67	
T > < ZnCd	MT* + 39		
	Cd* + 17858	Cd* + 6041	Cd* + 2180
	Cu* + 121	Cu* + 108	Cu* − 62
		Zn[a] + 51	Zn − 18
T > < ZnCdT	MT* + 44		
	Cd* + 17795	Cd* + 1577	Cd* + 1301
	Cu* + 70	Cu[d] − 28	Cu* − 43
	Zn[a] + 35	Zn[b] − 49	Zn − 23

*$P < 0.05$; [a]$P = 0.11$; [b]$P = 0.09$; [c]$P = 0.10$; [d]$P = 0.07$.

Table 5. Variations of metal and MT amounts (%) between different phases: direct exposure to silver

	Cytosol	Pellet	HDC
T > < TAg	MT* + 33		
	Ag* + 2475	Ag* + 280	Ag* + 3534
		Cu + 26	Cu[a] + 42
TAg > < TAgT	MT − 9		
			Ag* − 90
	Cu* + 115	Cu* − 59	Cu* − 52
	Zn[b] + 41	Zn − 24	Zn[a] − 34
T > < TAgT	MT* + 21		
	Ag* + 2304	Ag* + 329	Ag* + 252
	Cu* + 125	Cu* − 48	Cu* − 32
	Zn* + 55	Zn − 38	Zn* − 38

*$P < 0.05$; [a]$P = 0.06$; [b]$P = 0.11$.

the stability of zinc levels and its poor binding with heat-stable cytosolic ligands. After 1 week of decontamination (T > < ZnT) the ion balance was modified but the lack of significant MT increase probably resulted from the moderate increases of cytosolic HSC-bound zinc and copper.

Cytosolic HSC-bound zinc and copper ratios in 2 weeks decontaminated fishes were higher than those observed in control (T > < ZnTT). These enhancements could be responsible for the significant MT increase. Compared with the gills, a delay was

Table 6. Variations of metal and MT amounts (%) between different phases: exposure to silver following pretreatment by zinc

	Cytosol	Pellet	HDC
Zn > < ZnAg	MT* + 41		
	Ag* + 1957	Ag* + 476	Ag* + 3496
		Cu* + 35	Cu* + 69
			Zn[a] − 20
ZnAg > < ZnAgT	MT − 1		
	Ag* + 65	Ag + 47	Ag* − 82
	Cu* + 161	Cu − 20	Cu* − 43
	Zn* + 71	Zn − 22	
T > < ZnAg	MT* + 37		
	Ag* + 2086	Ag* + 477	Ag* + 2970
		Cu[b] + 28	
		Zn − 21	Zn − 18
T > < ZnAgT	MT* + 36		
	Ag* + 3502	Ag* + 747	Ag* + 466
	Cu* + 106		Cu* − 35
	Zn* + 55	Zn − 39	Zn[a] − 20

*$P < 0.05$; [a]$P = 0.09$; [b]$P = 0.08$.

Table 8. Variations of metal and MT amounts (%) between different phases: exposure to mercury following pretreatment by zinc

	Cytosol	Pellet	HDC
Zn > < ZnHg	MT* + 75		
	Hg* + 3652	Hg* + 1623	Hg* + 2340
	Cu + 27		Cu − 19
	Zn* + 127	Zn[a] + 47	Zn + 23
ZnHg > < ZnHgT	MT* − 37		
	Hg* − 44	Hg* + 108	Hg − 13
	Cu* − 24	Cu[b] − 36	Cu* − 37
	Zn* − 53	Zn − 10	Zn* − 74
T > < ZnHg	MT* + 60		
	Hg* + 3652	Hg* + 1623	Hg* + 2340
			Cu* − 45
	Zn* + 129	Zn + 26	Zn + 25
T > < ZnHgT	MT + 1		
	Hg* + 2000	Hg* + 3477	Hg* + 2022
		Cu* − 33	Cu* − 66
			Zn* − 68

*$P < 0.05$; [a]$P = 0.10$; [b]$P = 0.08$.

Table 7. Variations of metal and MT amounts (%) between different phases: direct exposure to mercury

	Cytosol	Pellet	HDC
T > < THg	MT* + 47		
	Hg* + 2641	Hg* + 1471	Hg* + 1998
	Cu − 29	Cu* − 31	Cu* − 55
	Zn + 22	Zn − 28	Zn − 13
THg > < THgT	MT* − 22		
	Hg + 34	Hg* + 115	Hg* + 73
	Cu[a] + 31	Cu + 9	Cu + 7
		Zn + 36	Zn* − 39
T > < THgT	MT + 15		
	Hg* + 3578	Hg* + 3272	Hg* + 3520
		Cu − 25	Cu* − 47
			Zn* − 46

*$P < 0.05$; [a]$P = 0.07$.

observed before *de novo* synthesis of MT occurred in the liver, but the relative enhancement of the MT level was twice that observed for the gills (25% > < 12%).

Direct contamination by cadmium (Table 3)

Direct contamination by cadmium resulted in a non-significant increase of the hepatic MT level (T > < TCd). The amount of cadmium entering was extremely high (increase ratio versus control: 11 190%) in the cytosol (Table 3), while a non-significant loss of copper and zinc was observed. Cadmium also increased in the insoluble fraction, while the amount of copper was enhanced, although non-significantly. In the heat-denatured fraction, the cadmium increase was also very important and the copper decrease was significant, while the zinc decrease was not. Within the cytosol, we observed an increase of HSC-associated copper. The ratio of cytosolic cadmium was about 75% of the total entering cadmium, with 86% of it bound to HSC (Table 9).

After 1 week of decontamination (TCd > < TCdT), we still observed a non-significant increase of hepatic MT. Cadmium, copper and zinc cytosolic amounts were increasing significantly. The increase of copper was very important, while it was moderate for cadmium. Cadmium decreased significantly in the insoluble fraction, where we observed a non-significant decrease of zinc (Table 3). The amount of copper associated with HDC exhibited a significant gain, while for cadmium this gain was less significant ($0.05 < P < 0.10$). The ratios of soluble copper, zinc, cadmium and HSC-associated zinc were also enhanced (Table 9).

When control fish were compared with exposed plus subsequently decontaminated fish (T > < TCdT), the increase of MT appeared to be

Table 9. Ratios of cytosolic metal amount versus total metal content, and ratios of HSC-bound metal amount versus cytosolic metal amount (%)

	Copper		Zinc		Cadmium		Silver		Mercury	
	cytosol	HSC	cytosol	HSC	cytosol	HSC	cytosol	HSC	cytosol	HSC
T	86.57	70.82	69.38	6.12						
Zn	86.43	78.82	72.53	5.12						
ZnT	89.10	84.33	75.13	14.89						
ZnTT	95.84	89.63	74.59	61.64						
TCd	78.61	85.46	64.04	9.08	74.52	85.55				
TCdT	93.40	87.94	82.14	49.98	93.73	89.32				
ZnCd	87.26	95.01	59.39	20.74	82.84	91.51				
ZnCdT	93.87	90.21	85.84	46.24	94.63	94.77				
TAg	84.23	60.51	75.52	19.55			86.86	—		
TAgT	96.57	91.22	85.05	62.34			84.52	85.01		
ZnAg	80.00	57.93	72.38	15.82			78.71	—		
ZnAgT	92.86	90.83	85.23	51.96			80.56	83.92		
THg	86.94	81.49	79.31	33.16					84.89	36.89
THgT	88.85	83.38	72.25	55.51					77.84	18.88
ZnHg	87.93	86.48	80.36	48.55					87.51	46.40
ZnHgT	89.68	88.91	68.44	72.26					65.34	16.73

significant. Amounts of cytosolic cadmium, copper, zinc and insoluble cadmium were increased. Zinc decreased in the pellet and in the HDC cytosolic fraction. Within this latest fraction we observed a significant increase of cadmium and a decrease of copper. As we have noticed in the former comparison (TCd > < TCdT), the ratios of soluble copper and zinc were enhanced, as were their ratios in HSC (Table 9). About 94% of total cadmium was present in the cytosol, with 89% of it bound to HSC.

Cadmium exposure resulted in a non-significant increase of hepatic MT. This increase has to be related, first, to the very important entering flow of cadmium in the cytosol, its association with HSC, second, to the modification of the zinc balance between the cytosol and the pellet, and within the cytosol, between HDC and HSC. After 1 week of decontamination (T > < TCdT), the Zn:HSC ratio was enhanced, the MT increase was significant and copper was still present in the MT containing fraction (HSC).

Contamination by cadmium following prior exposure to zinc (Table 4)

After 1 week of contamination following pretreatment with zinc (Zn > < ZnCd), the level of hepatic MT was raised significantly. Cadmium and copper showed significant increases in the cytosolic amounts. Amounts of insoluble cadmium, copper and zinc were also significantly increased. In the heat-denaturable cytosolic fraction, cadmium and copper exhibited, respectively, a significant increase and a decrease. More than 82% of total entering cadmium was present in the cytosol with about 92% of it associated with HSC (Table 9). The ratios of heat-stable bound copper and zinc were also increased, while the ratio of cytosolic zinc was decreased.

A comparison between contaminated and decontaminated fish (ZnCd > < ZnCdT) showed a slight non-significant increase of the MT level and a non-significant increase of soluble zinc. In the insoluble fraction we observed important significant losses of cadmium, copper and zinc. The heat-denaturable fraction presented a significant loss of cadmium and a gain of copper. Ratios of cytosolic cadmium, copper and zinc increased; however, while cadmium and zinc HSC-bound ratios increased, the copper HSC-bound ratio decreased (Table 9).

Comparisons with control fish (T > < ZnCd and T > < ZnCdT) showed significant increases of MT levels and cadmium amounts in both studied fractions and in the heat-denaturable cytosolic fraction (Table 4). The study of the metal balance showed that entering cadmium was mainly present in the cytosol, bound to HSC, which also bound increasing ratios of soluble zinc. Flows of copper and zinc were

very intense between the soluble and insoluble fractions and/or within the cytosolic fraction between the HDC and HSC fractions.

After zinc pretreatment, cadmium exposure resulted in a significant increase of MT levels, which can be attributed to entering flows of cadmium and copper in the cytosol associated with HSC. The zinc ratio decreased in the cytosol compared with the pellet, but about 50% of cytosolic zinc was bound to HSC. This could be responsible for MT *de novo* synthesis. After 1 week of decontamination (ZnCd > < ZnCdT), more cytosolic zinc was associated with HSC, but MT levels did not change, which suggests the existence of substitution processes at MT metal-binding sites. A large amount of cadmium, as well as zinc and copper, was associated with MT. Compared with direct contamination, pretreatment resulted in an enhancement of hepatic MT levels, with a quicker synthesis due to a quicker elevation of cytosolic HSC-bound zinc.

Direct contamination by silver (Table 5)

One week of exposure to silver (T > < TAg) resulted in a significant increase of MT levels and the amount of cytosolic silver. The amount of silver also increased significantly in the insoluble fraction and the increase of copper in this fraction was not significant. Amounts of silver and copper were enhanced in the cytosolic heat-denaturable fraction. Within the cytosol, we observed an increase of zinc ratio in the heat-stable fraction and a decrease of HSC-bound copper. Silver was essentially represented by soluble forms (87% of total silver), but solely bound to HDC (Table 9).

After 1 week of decontamination (TAg > < TAgT) the level of MT was lower, and copper and zinc amounts increased in total cytosol while they decreased in the insoluble fraction and in the heat-denaturable cytosolic fraction. The amount of silver also decreased in this latter fraction. Ratios of copper, zinc and silver bound to HSC were enhanced (Table 9).

A comparison with control (T > < TAgT) revealed that by the end of the decontamination step, the level of MT was still higher than before silver contamination. Amounts of cytosolic silver, cadmium and zinc were higher too, the amount of silver increased in the heat-denaturable fraction, while there were significant losses of both essential metals. The amount of silver also increased significantly in the insoluble fraction, while the amounts of copper and zinc decreased. Ratios of copper and zinc were higher in total and in heat-stable cytosol (Table 9).

Hepatic MT increase has to be related to the increase of zinc bound to cytosolic HSC. As we did not observe a significant increase of global cytosolic zinc, we can assume that the important entering flux of silver in the cytosol removed zinc from HDC towards HSC. However, this modification of cytosolic zinc status was not quantitatively important enough to result in a significant decrease of HDC bound zinc. A relevant observation was the lack of HSC bound silver within the cytosol. One week later after the decontamination step, 85% of cytosolic silver was associated with HSC. The level of MT did not show a significant change while fluxes from the insoluble fraction towards cytosol and within the cytosol from HDC towards HSC were observed for copper (significant) and zinc. Two hypothesis were proposed to explain the stability of MT levels while silver was entering the HSC fraction. First, silver was bound to a heat-stable cytosolic compound which did not give any polarographic response interfering with the MT response. Second, silver was bound to MT newly synthetised during the silver contamination step, leading to Cu,Ag,Zn–MT. These hypotheses will be discussed in the next section.

Contamination by silver following prior exposure to zinc (Table 6)

After 1 week of contamination following pretreatment with zinc (Zn > < ZnAg), the level of hepatic MT was raised significantly. The amount of silver increased in both studied compartments, as it increased in the heat-denaturable cytosolic one. The amount of copper increased only in the heat-denaturable cytosolic and insoluble compartments. There was also a loss of zinc in the heat-denaturable cytosolic fraction. About 79% of silver was present in the cytosol, but none of it was associated with HSC which bound more zinc and less copper than after pretreatment alone (Table 9).

After 1 week of decontamination (ZnAg > < ZnAgT) the level of MT remained stable. Amounts of cytosolic silver, copper and zinc increased significantly, but amounts of silver and copper in the heat-denaturable fraction decreased. Changes in the insoluble fraction were not significant. About 81% of silver was present in the cytosol with 84% of it associated with HSC (Table 9). The ratios of copper and zinc bound to HSC reached 91 and 52%, respectively, of cytosolic essential metal amounts.

Comparison with control fish (T > < ZnAg and T > < ZnAgT) showed an increase of hepatic MT

levels and an enhancement of the amounts of silver. Copper and zinc increased in the cytosol (T > < ZnAgT), but decreased in the heat-denaturable fraction.

In the liver, we did not observe noticable differences between results obtained with or without pretreatment. Our interpretation is the same for both experiments. An important entering flux of silver in the cytosol removed zinc from HDC towards HSC, consequently enhancing the MT level (Zn > < ZnAg). By the end of the decontamination step, about 84% of silver, which was not formerly bound to HSC, was then associated to HSC. Both former hypotheses are proposed: synthesis of a specific silver-binding HSC or of Cu,Ag,Zn–MT.

Direct contamination by mercury (Table 7)

Fish exposure to mercury showed in a significant increase of hepatic MT levels (T > < THg). Amounts of mercury increased in cytosol, insoluble and heat-denaturable cytosolic fractions. Copper was lost by cytosol, especially in the heat-denaturable fraction, and also in the insoluble fraction. Zinc fluctuations were not significant but the observed tendency was expressed by a shift from the insoluble fraction towards HSC. About 85% of total mercury was present in the cytosol, with only 37% of it bound to HSC. The ratios of copper and zinc bound to these ligands were increased (Table 9).

MT levels decreased after 1 week of decontamination (THg > < THgT) but the amounts of mercury in the studied fractions were still increasing, even if the phenomenon was less important than during the contamination. Copper was enhanced in the total cytosol, while a zinc decrease was limited to heat-denatured cytosol. The amount of zinc was enhanced in the insoluble fraction. Mercury cytosolic ratio was lowered, with less mercury bound to HSC which, however, bound more zinc (Table 9).

Comparison with control fish (T > < THgT) showed a non-significant increase of MT levels, a general significant increase of mercury amounts, a non-significant loss of copper from the pellet, a shift of copper from HDC towards HSC and a significant decrease of zinc in the cytosolic heat-denaturable fraction. Only 78% of total mercury was present in the cytosol with only about 19% of it bound to HSC. These ligands bound 56 and 83%, respectively, of cytosolic zinc and copper (Tables 3 and 4).

In the liver the significant increase of MT resulted from entering fluxes of zinc and mercury in the cytosol and their binding with HSC. The copper ratio associated with HSC also increased, leading to the formation of Cu,Hg,Zn–MT. After the decontamination step, the decrease of MT was related to the loss of mercury bound to HSC. As zinc bound HSC with a high ratio, we can assume, that by the time the whole content of MT in the organ decreased, we had a substitution of mercury by zinc on the remaining MT molecules, leading to the formation of Cu,Zn,Hg–MT.

Contamination by mercury following prior exposure to zinc (Table 8)

Pretreatment by zinc resulted in an important increase of MT levels after subsequent contamination by mercury (Zn > < ZnHg). The amounts of mercury in both studied fractions were enhanced significantly. Mercury increase in the heat-denaturable fraction was very high. There was an increase of zinc in the cytosol (significant) and in the insoluble fraction. About 88% of mercury was present in the cytosol, with 46% of it bound to HSC. The ratio of heat-stable associated zinc was enhanced (Table 9).

After 1 week of decontamination (ZnHg > < ZnHgT) we observed a decrease of MT levels, and cytosolic amounts of mercury, copper and zinc. These decreases were significant in the heat-denaturable cytosolic fraction, except for mercury. Mercury exhibited a significant increase in the insoluble fraction, for zinc the decrease was not significant. The ratio of total cytosolic mercury was lowered, as was the ratio of mercury bound to HSC, but for zinc the corresponding ratio was enhanced (Table 9).

Compared with control, MT levels at the end of the experiment (T > < ZnHgT) were unchanged. There was a general significant increase of mercury in all the studied fractions, and a significant decrease of copper in the pellet and within the cytosol in the heat-denaturable fraction. The amount of zinc associated with the heat-denaturable cytosol was also significantly decreased. About 65% of total mercury remained in the cytosol, with only 17% of it bound to HSC, which also bound about 72% of the cytosolic zinc.

We have the same pattern than without pretreatment: an increase of MT levels due to entering fluxes of mercury and zinc in the cytosol, associated with HSC. Pretreatment with zinc prior to mercury exposure resulted in an higher elevation of hepatic MT levels (75% > < 47%) related to a more important flux of entering zinc in the cytosol and a higher association with HSC. Pretreatment influenced the decontamination step: we noted a decrease of cytosolic mercury and a higher MT relative decrease.

Discussion

Exposure to zinc

Kito *et al.* (1982a,b), Bradley *et al.* (1985) and Klaverkamp & Duncan (1987) have shown that exposure to zinc increases hepatic MT levels in fish. Our experimental conditions led to an increase only by the end of the third week of the experiment (T > < ZnTT), together with entering amounts of copper and zinc within the heat-stable fraction of the cytosol. This increase was twice that observed for gills, in agreement with hepatic MT level, which is generally higher than the gill MT level, as the liver is considered as a major MT synthesis site.

Taking in account our experimental parameters, the results obtained are consistent with the literature about fish MT hepatic induction by zinc. The observed delay itself is in agreement with Hogstrand & Haux's (1990) statement that the MT induction time-course depends on species and doses.

Exposure to cadmium

Displacement of zinc from native MT has already been cited by others (Noël-Lambot *et al.* 1980, Kito *et al.* 1982a,b; Klaverkamp & Duncan 1987) investigating cadmium-exposed fish. In liver, the abundance of copper associated with native MT (already mentioned by several authors studying fishes: Harrison & Lam 1986) is important enough to prevent its disappearance from the cytosolic HSC fraction. Interactions between cadmium and copper have already been questioned (Klaverkamp & Duncan 1987). These authors have shown that cadmium exposure resulted in a decrease of hepatic MT levels. In our experiments, hepatic MT levels were not decreased but the increases were not significant by the end of the 7 days of exposure. However, 1 week later (TCdT) or after pretreatment by zinc (ZnCd), we observed an increase of MT, related to an increase of the Zn:HSC ratio. The ratios of HSC-bound cadmium in *C. carpio* livers (86–95%) were in agreement with those observed in equine liver (80%), where the MT-bound cadmium concentration is related to that of MT-bound zinc (Elinder *et al.* 1981, cited by Webb 1987). In carp liver, pretreatment by zinc improved cadmium binding with MT and *de novo* synthesis of MT, while pretreatment did not act on MT level recovery, which remained significantly higher than control (T > < ZnCdT). If one considers MT synthesis as a detoxication process we can assume that pretreatment with zinc improves cadmium tolerance at the level of the liver by the HSC binding of a higher ratio of cadmium.

Differences noticed between gills (Cosson 1993) and liver regarding MT induction by cadmium could be attributed to the relative abundance of zinc and copper in the considered organs. Levels of copper and zinc are higher in liver cytosol than in gill cytosol, and the ratio of copper associated with HSC is higher in the liver than in the gill, while hepatic cytosolic zinc is very poorly associated with these ligands. When fish are directly exposed to cadmium, the lack of *de novo* synthesis of hepatic MT could be related to the inefficiency of cadmium ions to displace copper ions from native MT and the small amount of zinc ions released in the cytosol by cadmium substitution on pristine MT. At the opposite, more zinc is available for cadmium substitution on gill MT and its release could be considered as responsible for higher *de novo* MT synthesis.

Exposure to silver

Evidence for Ag,Zn–MT has been demonstrated by Takashima *et al.* (1987) in the liver of rats, 24 h after injection of silver sulfadiazine (100 mg kg^{-1} body weight). This MT, resulting from a non-natural contamination route, gave a ratio of silver to zinc of more than 4.5.

Silver is known to displace zinc from MT binding sites; however, after our exposures, the binding of silver with HSC was ineffective. This result has to be related to the persistent low ratio of hepatic HSC-bound zinc, observed even after zinc pretreatment. The amounts and ratio of cytosolic copper and zinc were stable, but these ions shifted in an opposite way between HSC and HDC. This ion movement was sufficient to trigger the induction of MT in liver, whose inducibility is known to be important. During the decontamination step a re-arrangement of ions took place within the cytosol, and especially within the HSC fraction, but the MT hepatic level remained stable whereas copper and zinc HSC-bound ratios increased hugely. Considering that MT synthesized during the decontamination step is supposed to be already ion-saturated, the binding of entering ions within the HSC fraction could be questioned. A hypothesis is the association of silver with a heat-stable ligand, whose polarographic response does not interfer with MT and, therefore, we should have to admit that this ligand had been synthesized during the decontamination step, because it was lacking by the end of silver contamination. Is this ligand binding all together zinc, copper and silver? We cannot answer this question for the moment. *In vitro* experiments have demonstrated that MT is able to

bind important amounts of copper and silver (12 to 20 equivalents). We could also hypothesize that MT synthesized during the silver contamination step (Cu,Zn–MT) had its spatial structure modified during the decontamination step, leading to the binding of excessive ions such as copper and silver, resulting in Cu,Ag,Zn–MT. What happened during this decontamination step is unclear and needs more research. Moreover, we need to study the cytosolic fraction containing HSC and to determine the existence or lack of a metal-binding compound, different from MT. Answering these questions is important because Garnier *et al.* (1990) have shown that 70% of total silver is stored in the liver of experimentally contaminated fish (*Salmo trutta* L.).

With our experimental conditions, zinc pretreatment did not influence the subsequent silver contamination and liver intracellular ion balance.

Our experiments have pointed out important differences between silver incorporation in liver or gill, even if, after the decontamination step, most cytosolic silver is bound to HSC within both organs. In gills, the amount of HSC-bound zinc is important enough to allow the partial substitution of zinc by silver on pristine MT, resulting in the formation of, presumably, Cu,Ag,Zn–MT. During the decontamination step, more silver binds MT and more zinc is released in the cytosol stimulating *de novo* synthesis of MT. Newly synthesized MT was an Ag, Zn–MT (as in Takashima *et al.*'s experiment), because copper ions have shifted towards HDC.

Exposure to mercury

After exposure of carps to mercury, MT levels have been raised and entering mercury was associated with zinc. Such an observation is consistent with Klaverkamp & Duncan (1987).

Mercury is known to displace copper *in vitro* and zinc *in vivo*; however, here, mercury binding with MT by substitution was poor. MT synthesis induction needs zinc entering into the cytosolic fraction, or its shift from HDC towards HSC.

Zinc pretreatment efficiency found expression in a high increase of MT levels, due to a significant entering flow of zinc within the cytosol, in a more effective binding of mercury with MT and in a decrease of cytosolic mercury which shifted towards the insoluble fraction during the decontamination step.

Comparison between the studied toxic metals

Cadmium, mercury and silver are known to be very toxic pollutants, whose deleterious effects are minimized when they are bound with MT. Our results emphasize the differences between their respective ability to achieve such a detoxicification process. Several parameters have to be considered comparing their order of potency for metal-induced MT. First, is their bioavailibility in the experimental medium and, second, as binding sites are supposed to be the target of these metals, their relative abundance, expressed as the amount of ions per liter instead of concentrations. Following such a basis we have a Zn:Cd:Hg:Ag ratio of 329:96:2.7:1. Another parameter to be considered to estimate their potency is the relative increase of MT levels after exposure but, as this increase has been sometimes observed with a delay, this delay is also an important evaluation parameter. Taking in account this latest remark we propose the following order mercury > silver > cadmium > zinc, but whether mercury's supremacy versus silver is due to its own induction potency or to its higher abundance in the medium (2.7 times) is not clear. However, important data is the fact that silver is not bound to HSC after the contamination step but only after the decontamination, while the HSC-bound mercury ratio reaches about 40% of cytosolic mercury and decreases (about 17%) after the decontamination.

In terms of toxicity, toxic metal binding by hepatic MT is very efficient with cadmium (85–94% of cytosolic cadmium associated with HSC), less efficient with mercury (only 36–46%) and poorly efficient with silver (1 week delay needed).

Comparison between organs: liver > < gill

Our results emphasize the higher inducibility of the liver concerning MT synthesis. The increases of MT levels were more important within the liver than within the gill after an identical exposure to metals. When we used silver as a potential inducer, we noticed a delay before MT level increases became significant within both organs. This delay was longer for gills. Within both organs, mercury has been encountered as the highest inducer. Concerning the ability of the studied organ to bind entering toxic ions, we noticed important differences. The ratios of cytosolic metals were very high within the liver, while insoluble forms were predominant within the gill. HSC-binding of cadmium was less efficient within the gill than within the liver. At the opposite, the ratios of HSC-bound silver and mercury were higher within the gill. Our interpretation of toxic metal balance within the studied organs relies on their anatomical situation and physiological function. At the level of the gill, we can assume that the

bio-transformation of toxic metals is partially performed, while at the level of the liver, it is achieved. However, the higher metabolic rate of the liver results in higher MT synthesis and also in an increased competition between metal soluble ligands (HDC and HSC) and higher toxic consequences, since toxic metals are not definitely detoxified.

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