

Cadmium-Binding Protein (Metallothionein) in Carp

by Hideaki Kito,*† Youki Ose,* and Takahiko Sato*

When carp (*Cyprinus carpio*) were exposed to 5 and 30 ppm Cd in the water, the contents of Cd-binding protein, which has low molecular weight, increased in the hepatopancreas, kidney, gills and gastrointestinal tract with the duration of exposure. This Cd-binding protein was purified from hepatopancreas, kidney, gills, and spleen of carp administered 2 mg/kg Cd (as CdCl₂), intraperitoneally for 6 days. Two Cd-binding proteins were separated by DEAE-Sephadex A-25 column chromatography. These proteins had Cd-mercaptide bond, high cysteine contents (ca. 29–34%), but no aromatic amino acids or histidine. From these characteristics the Cd-binding proteins were identified as metallothionein.

By using antiserum obtained from a rabbit to which carp hepatopancreas MT-II had been administered, immunological characteristics between hepatopancreas MT-I, II and kidney MT-II were studied, and a slight difference in antigenic determinant was observed among them.

By immunological staining techniques with horseradish peroxidase, the localization of metallothionein was investigated. In the nontreated group, metallothionein was present in the acinar cells of hepatopancreas and renal convoluted tubules. In the Cd-treated group (2 mg/kg IP daily for 3 days), metallothionein was present in the nuclei, sinusoids, and extracellular space of hepatopancreas, in addition to the acinar cells. Carp were bred in 1 ppm Cd, 5 ppm Zn solution, and tap water for 14 days, following transfer to 15 ppm Cd solution, respectively. The survival ratio was the highest in the Zn group followed by Cd-treated and control groups. The metallothionein contents increased in hepatopancreas and kidney in the order: Zn>Cd>control group.

Introduction

It is well known that cadmium-induced renal dysfunction may be the causal substance of Itai-Itai disease. During the 1960s fish kills occurred several times following an increase in water pollution of rivers, lakes and seas in Japan. Some of those were caused by Cd. In Japan the environmental quality standard on water pollution of Cd has been established as <0.01 mg/mL. As an indicator of heavy metal toxicity of fish, LC₅₀ has been available, and reported by many researchers. For example, LC₅₀ values were 0.34 mg/L on *Poecilia reticulata* (1) and 88.6 mg/mL on green sun fish (2). The chemical form of Cd was CdCl₂ · 2.5H₂O, 24 hr. However, these values represent acute toxicity. If fish take in trace amounts of Cd daily, Cd must accumulate in fish until absorption and excretion attain an equilibrium. But the behavior, chemical form, and physiological significance have not been clarified. A metallothionein (MT)-like protein has been isolated from copper rock fish (3) and goldfish (4),

and since then the characteristics of fish MT separated from eel (5), plaice (6), staghorn sculpin (7), carp (8,9), skipjack (10), rainbow trout (11), and gibel (12) were elucidated.

We studied the induction of MT in carp (*Cyprinus carpio*) exposed to Cd (13), separated MT from carp receiving IP injections of Cd and clarified amino acid compositions and physicochemical characteristics of these proteins (8,9). In addition we examined immunohistological localization of MT in carp organs (14), and the detoxification effect of MT to Cd toxicity (15). This paper summarizes some of the main points.

Experimental and Results

Induction of Cd-Binding Protein

Carp were exposed to 5.30 ppm Cd of in a total volume of 100 L in a polypropylene water tank. Two carp were killed by destruction of the medulla oblongata after 1/3, 1, 4, and 24 hr and 4, 15, and 31 days, and each organ (hepatopancreas, kidney, gill, gastrointestinal, spleen, bile, and muscle) were removed. All organs were washed with distilled water. The same weight samples thawed were homogenized in 3 volumes (v/w) of 10 mM Tris-HCl

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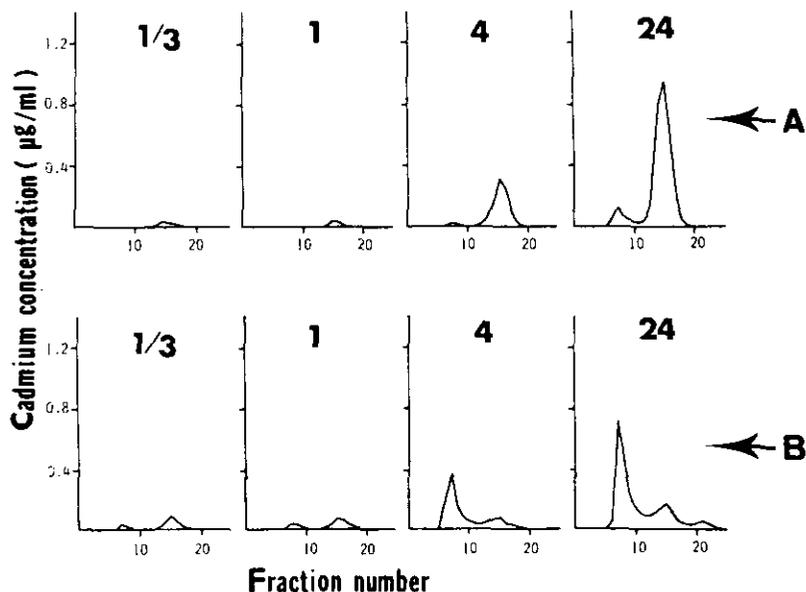


FIGURE 1. Time course of Cd patterns on Sephadex G-75 elution profiles of cytoplasmic solution in (A) hepatopancreas and (B) kidney of carp bred in 30 ppm Cd solution. Pairs of carp were sacrificed after 1/3, 1, 4, and 24 hr. From Kito et al. (13), reprinted with permission of Pergamon Press.

buffer, pH 7.4, using a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenates were ultracentrifuged at $105,000g$ for 60 min at $4^{\circ}C$ to remove nuclear and mitochondrial fractions. A 4-mL portion of each supernatant was applied to a column of Sephadex G-75 (1.8×46.5 cm) at $4^{\circ}C$ which had been equilibrated with the buffer that was used for homogenization. Metal concentrations in gel filtration fractions were measured with an atomic absorption spectrophotometer.

Figure 1 shows the elution profiles of cytoplasmic solution on Sephadex G-75 of Cd in hepatopancreas and kidney of carp bred in 30 ppm Cd solution. In hepato-

pancreas, a low molecular weight, Cd-binding protein (Fraction No. 14-16) (MTF) was observed 20 min after exposure and a Cd peak of a high molecular weight fraction (HMF) with a time lag (4 hr). The increase of Cd levels in MTF was intense from 4 to 24 hr after exposure. The first fraction corresponds to a molecular weight of 12,000 and the latter has a molecular weight of ca. 40,000. The levels of Cd in MTF showed a marked increase with time of exposure to Cd. In kidney, the Cd peak in both fractions was observed 20 min after exposure, and the level of Cd in HMF increased more than in MTF with time of exposure to Cd. In the gills, the Cd peak was

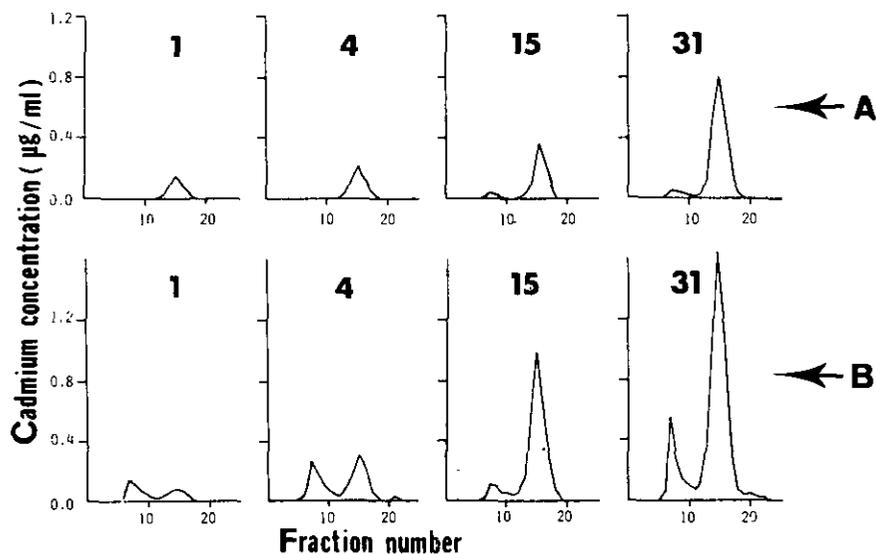


FIGURE 2. Time course of Cd patterns on Sephadex G-75 elution profiles of cytoplasmic solution in (A) hepatopancreas and (B) kidney of carp bred in 5 ppm Cd solution. Pairs of carp were sacrificed after 1, 4, 15, and 31 days. From Kito et al. (13), reprinted with permission of Pergamon Press.

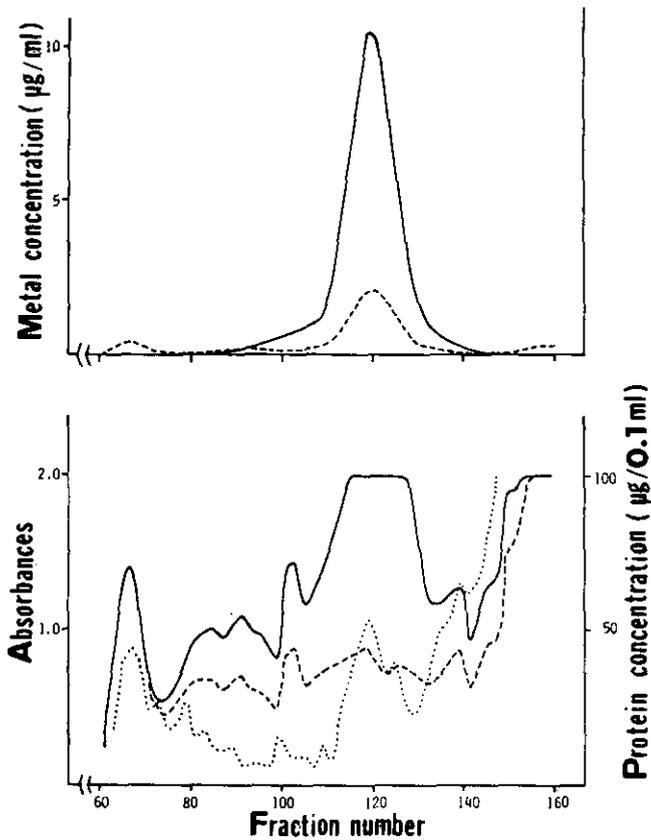


FIGURE 3. Separation by Sephadex G-75 chromatography of carp hepatopancreas: Column size: 5×70 cm, 1 fraction = 10 mL, buffer 10 mM Tris-HCl, pH 7.4, at 4°C (top) (—) Cd, (---) Zn; (bottom) (—) 254 nm, (---) 280 nm, (....) protein. From Kito et al. (13), reprinted with permission of Pergamon Press.

observed in only the HMF, even after 24 hr. In carp exposed to 5 ppm Cd solution, the peaks were observed at a lower concentration but the pattern was the same.

Figure 2 shows the Cd patterns on Sephadex G-75 of elution profiles of cytoplasmic solution in hepatopancreas and kidney of carp bred in 5 ppm Cd solution. In hepatopancreas, the Cd peak in the HMF appeared after 15 days, and the level of Cd in MTF increased markedly with time. In kidney, the Cd concentrations in the HMF was higher than in the MTF after 1 day; however, it increased in MTF more than in HMF with time of exposure to 5 ppm Cd than in the previous case (Fig. 1). In gills and gastrointestinal tract, the Cd concentration in MTF increased with the duration of exposure, but its level was lower than that of hepatopancreas and kidney. To summarize, we found that low molecular weight Cd-binding protein is induced in each organ of carp exposed to Cd solution, and the pattern of appearance and the level differed with each organ as time progressed.

Purification of Metallothionein

Carp were administered 2 mg/kg Cd (as CdCl_2) IP daily for 6 days. Carp were killed by destruction of the medulla oblongata 24 hr after the last injection. The hepatopancreas and kidney were homogenized in 1.5 volumes (*v/w*) of 10 mM Tris-HCl buffer, pH 7.4, using a Potter-Elvehjem homogenizer. The homogenates were ultracentrifuged at $105,000g$ for 60 min at 4°C and the supernatants were applied to a column of Sephadex G-75 (5×70 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at 4°C . Major Cd-binding fractions were pooled and applied to a column of DEAE-Sephadex A-25 (3×40 cm) pre-equilibrated in same buffer (pH 7.4). The

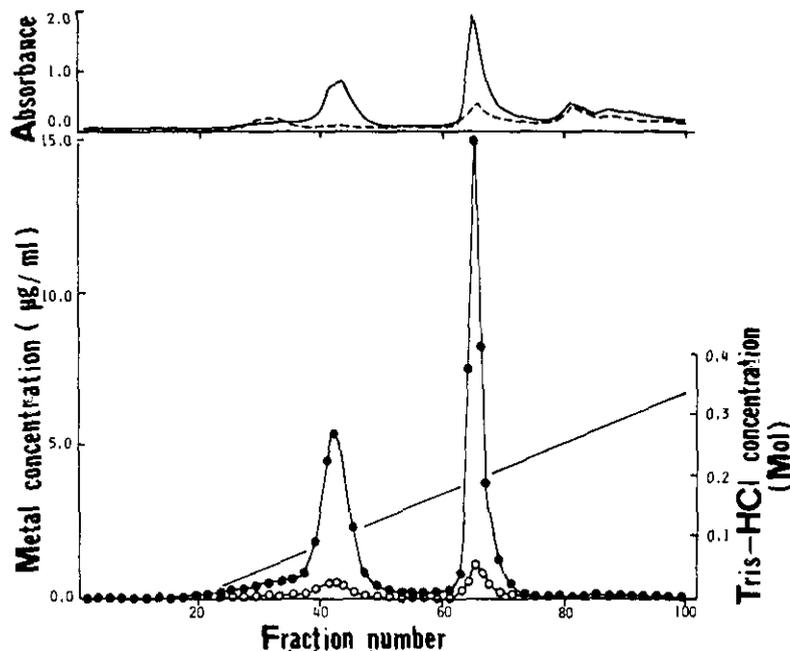


FIGURE 4. Separation on DEAE-Sephadex A-25 of MT fraction obtained by gel filtration on Sephadex G-75: Column size: 3×40 cm, 1 fraction = 10 mL, initial buffer, 10 mM Tris-HCl; final buffer, 350 mM Tris-HCl; pH 7.4 at 4°C . (●) Cd; (○) Zn; (—) 254 nm; (---) 280 nm.

column was eluted with a linear gradient of 10 to 350 mM Tris-HCl buffer (pH 7.4). The two eluted Cd-binding fractions were pooled and concentrated under N₂ pressure using an Amicon UM-2 ultrafiltration membrane. The concentrated samples were applied to a column of Biogel P-10 (2.6 × 93 cm). The fractions having high Cd concentrations were collected, lyophilized, and desalted with Sephadex G-25. The final samples were lyophilized.

Figure 3 shows the Sephadex G-75 elution profiles of heated cytosolic fractions of hepatopancreas in carp administered Cd. The major Cd peak coincided with the elution volume of cytochrome c (molecular weight 12,400) and had $V_e/V_o = 1.8$. This fraction contained zinc and a little copper. It had a absorption maximum at 254 nm and an absorption minimum of 280 nm. The elution pattern of pooled fraction on DEAE-Sephadex A-25 is shown in Figure 4.

Two Cd peaks were observed. Both fractions had absorption maxima at 254 nm and showed absence of absorption at 280 nm. These fractions had the characteristics of MT; the first fraction was denoted MT-I, the second fraction, MT-II. From kidney (9), spleen (16), and gills (16) the presence of isometallothionein was found also. These samples were applied to a column of Biogel P-10, and a single Cd peak was observed in all cases. The purity of the final preparation was analyzed by using polyacrylamide disc gel electrophoresis. Purified MT-I and II gave a single protein band migrating towards the anode on polyacrylamide gel electrophoresis at pH 8.9.

The absorbance at 254 nm due to the Cd-mercaptide bond in hepatopancreas and kidney MT-II disappeared under acidic conditions. Amino acid compositions of carp hepatopancreas and kidney MT are given in Table 1.

These amino acid compositions were similar and indicated characteristics typical of MT with high cysteine contents (ca. 29–34%), but no aromatic amino acids (Tyr, Trp, Phe) or histidine. Arginine residues were absent from hepatopancreas MT-I and II, but some were present

in kidney MT-I and MT-II. The most abundant residues were glycine, serine, and lysine, and these levels were similar to those found in equine renal MT. The aspartic acid and threonine contents in carp MT were higher, but alanine was lower than in equine MT. From these results, we recognized a slight difference between carp and mammal MT. For MT-I and MT-II of each organ, the amino acid compositions were similar, but slightly different. The apparent molecular weight of carp hepatopancreas MT-II was estimated to be 9800 by gel chromatography on Sephadex G-75 following the method of Andrews (18). From the results of amino acid analysis, the molecular weights calculated were 6227 for MT-I and 6435 for MT-II. It is considered that the difference between two methods is due to the marked deviation of the molecular from the globular shape typical of that from mammals (19). The elemental spectra of carp hepatopancreas MT-I and II were measured by using analytical electron microscopy. Metal composition of hepatopancreas MT-I was Cd 5.5%, Cu 1.3%, Zn 1.0%; that for MT-II was Cd 11.8%, Cu 9.6%, Zn 1.0%, so these ratios were markedly different.

Immunohistological Localization of MT in Hepatopancreas and Kidney in Carp

Purified carp hepatopancreas MT-II (1 mg/mL saline) was emulsified with an equal volume of Freund complete adjuvant. New Zealand White rabbit (3 kg/body weight) was injected subcutaneously with a total of 6.0 mL of the emulsion at a rate of 1.0 mL/10 days. The rabbit was bled 14 days after the last injection, and antiserum was prepared. By using this antiserum, the immunological characteristics of hepatopancreas MT-I, MT-II, and kidney MT-II were studied by double diffusion test. Spur formation between carp hepatopancreas MT-I and -II was observed. Spur formation was observed between hepatopancreas MT-II and kidney MT-II, and also hepa-

Table 1. Amino acid compositions of metallothioneins.^a

	Hepatopancreas		Kidney		Horse kidney
	MT-I	MT-II	MT-I	MT-II	
Lys	11.37 (7)	10.09 (6)	6.86	10.09	11.7 (7)
His	— (0)	— (0)	—	—	— (0)
Arg	— (0)	— (0)	1.80	0.81	1.9 (1)
Asp	8.47 (5)	9.10 (6)	6.97	7.53	5.0 (3)
Thr	7.10 (4)	8.47 (5)	6.16	9.48	1.8 (1)
Ser	11.23 (7)	10.13 (6)	7.97	11.78	11.9 (8)
Glu	3.11 (2)	3.50 (2)	4.72	4.29	4.7 (1)
Pro	4.89 (3)	4.16 (3)	5.03	3.85	2.9 (2)
Gly	10.64 (6)	11.13 (7)	11.50	11.51	9.2 (5)
Ala	5.82 (3)	5.59 (3)	5.58	6.05	11.6 (7)
Cys	34.24 (20)	31.90 (20)	30.64	29.39	33.9 (20)
Val	1.98 (1)	2.35 (1)	6.11	2.98	4.5 (3)
Met	1.01 (1)	1.68 (1)	1.44	0.50	1.5 (1)
Ile	0.11 (0)	0.99 (0)	2.50	0.70	— (0)
Leu	— (0)	— (0)	2.70	1.14	— (0)
Tyr	— (0)	— (0)	—	—	— (0)
Phe	— (0)	— (0)	—	—	— (0)
Trp	— (0)	— (0)	—	—	— (0)

^a In mole-%; numbers in parentheses are the number of amino acids.

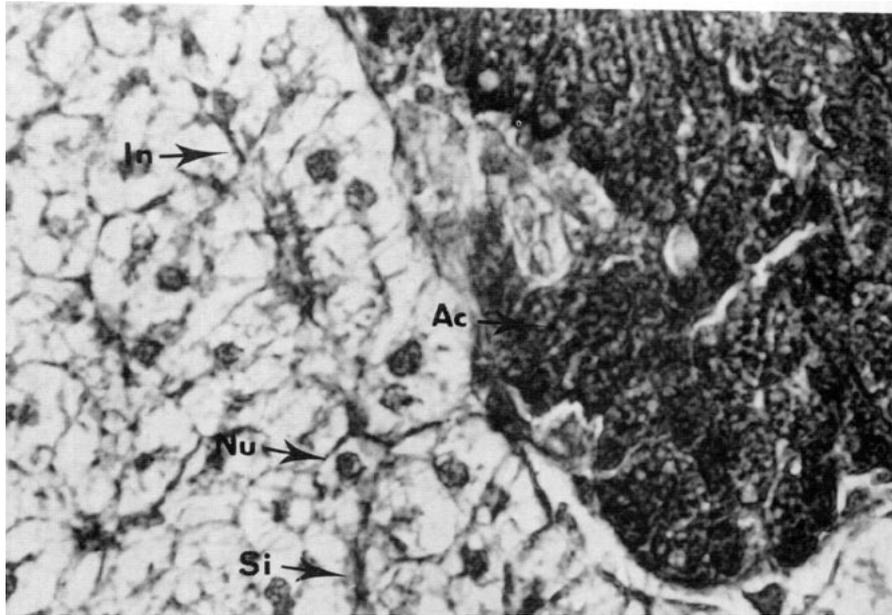


FIGURE 5. Immunohistological staining of hepatopancreas tissue of a carp injected with Cd for 3 days. Metallothionein was stained with horseradish peroxidase-IgG. The nuclei were lightly stained with methyl green. The nuclei in hepatocyte, sinusoids, intracellular space and acinar cells in pancreas tissue were strongly stained with labeled IgG. (Ac) acinar cells in pancreas tissue; (Nu) nuclei; (Si) sinusoids; (In) intracellular space. Magnification 320 \times .

topancreas MT-I and kidney MT-II. This phenomenon indicates a slight difference of antigenic determinant among them, possibly due to differences in metal compositions or amino acid sequences of these proteins. Tohyama et al. (20) and Vander Mallie et al. (21) suggested a high degree of structure similarity between rat hepatic Cd-MT-I and II from the result of double diffusion analy-

sis. Tohyama et al. reported that by RIA, rabbit anti-rat hepatic MT-II serum cross-reacted with various MTs of mammalian origin: rat hepatic MT-I, human renal MT-I and rabbit hepatic MT-II; the primary structures of mammalian MT are similar. However, this antiserum does not cross-react with crab hepatopancreas MT-I (22). We tested the cross-reaction between carp hepatopancreas

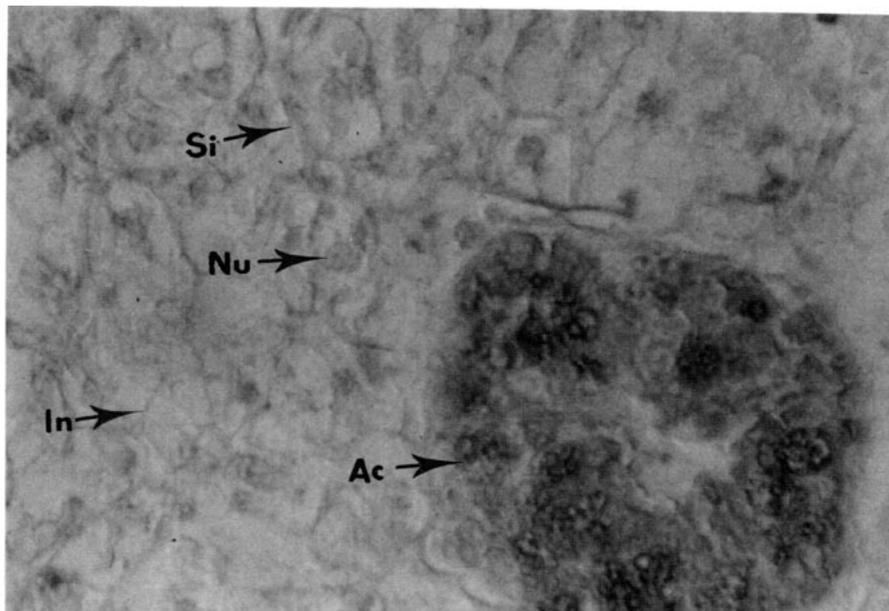


FIGURE 6. Immunohistological staining of hepatopancreas tissue of a noninjected carp. Metallothionein was stained with horseradish peroxidase-IgG. The nuclei were lightly stained with methyl green. The acinar cells in pancreas tissue were stained with labeled IgG, but other tissues were not stained with labeled IgG. (Ac) acinar cells in pancreas tissues; (Nu) nuclei; (Si) sinusoids; (In) intracellular space. Magnification 320 \times .

MT and pig kidney MT, and observed spur formation. It was considered that the antigenic determinant in carp MT is different from that in pig MT.

We investigated the distribution of MT in the tissues of hepatopancreas and kidney by immunohistological staining techniques. IgG was separated on a Protein A-Sepharose Cl-4B column and labeled with horseradish peroxidase. The slices of hepatopancreas and kidney from carp of the two groups, noninjected and Cd-injected (2 mg/kg Cd as CdCl₂, IP daily for 3 days) were prepared. The specimens were fixed with Bouin's fixative, dehydrated through a series of graded alcohols, and embedded in paraffin. MT in the slices reacted with the labeled IgG by the direct method. In the hepatopancreas of carp injected with CdCl₂, staining for MT was observed in nuclei of hepatocytes, sinusoids, intracellular space and acinar cells in pancreas tissue, the nuclei and acinar cells being stained markedly (Fig. 5). In a noninjected carp, the acinar cells in pancreas tissue were stained slightly, suggesting the presence of MT (Fig. 6). This result indicated that (Cu, Zn)-MT in hepatopancreas from a noninjected carp was present mainly in the acinar cells in pancreas tissue. Background staining was examined by using non-labeled IgG, and not found, so that staining with labeled IgG was not due to the nonspecific binding.

The presence of MT in nuclei of hepatocytes has been reported in rat (23,24) and our data in fish indicated the same result. Banerjee et al. (23) proposed a schematic model for the induced synthesis of MT. It may be suggested on the behavior and synthesis of MT in fish the same as mammals. Onosaka et al. (25) recognized the induction of Zn-MT in pancreas of rat administered Zn. We suggested that the acinar cells in pancreas were related to the production of MT.

In the kidney slices prepared from noninjected and Cd-injected carp, the MT staining was observed in the epithelium of the neck segment, proximal convoluted segment II, and the distal convoluted segment, but not in the glomeruli. It was proved that (Cu, Zn)-MT also exists in the kidney of a noninjected carp. In fact, the natural occurrence of MT has been found in eel (5), staghorn sculpin (7), crab (26), and lobster (27). We found the

presence of MT binding with Cd, Zn, or Cu in some fish captured in the Nagara River and breeding ponds (13).

Protection by MT against Cadmium Toxicity

Groups of 15 carp were maintained in tap water (group A), 1 ppm Cd solution (group B) or 5 ppm Zn solution (group C) for 14 days and then transferred into 15 ppm Cd solution for 18 hr. The solutions were changed at 24 hr intervals during pre-exposure and 4 hr intervals during 15 ppm Cd exposure in order to avoid suffocation. Results of survival ratio in 15 ppm Cd solution following prior exposure to low concentrations of metals is shown in Figure 7.

All carp in group A died after 15 hr, and those in group B died after 20 hr. One carp in group C lived for 26 hr. This provided evidence that Cd tolerance to high concentrations of Cd was improved by prior exposure to low concentrations of metal. This experiment was repeated three times and similar results were obtained in each case.

After pre-exposure and 15 ppm Cd exposure, three carp from each group were killed, and the cytoplasmic solution of each dissected organ was prepared by ultracentrifugation. Aliquots of those were applied to a column of Sephadex G-75 (1.8 × 46.5 cm). Cd and Zn concentrations in the eluted fractions were analyzed.

The increase of Cd content in HMF and MTF from prior exposure to 15 ppm Cd solution is given in Table 2.

In the hepatopancreas, the MT contents increased in the order: group C > group B > group A. The increase of Cd contents in HMF showed a higher value than that in MTF for group A. In the kidney the same trend was observed. It was proved that thionein was induced by prior exposure to a low concentration of Cd or Zn, and Cd was bound to the induced thionein during exposure to the high concentration of Cd. In the gills, the increase of Cd in MTF was low in any group, but in group A that of Cd in HMF was high. This may be related to Cd toxicity. We examined the binding capacity of Cd to HMF and MTF in the hepatopancreas. Cd was added *in vitro*

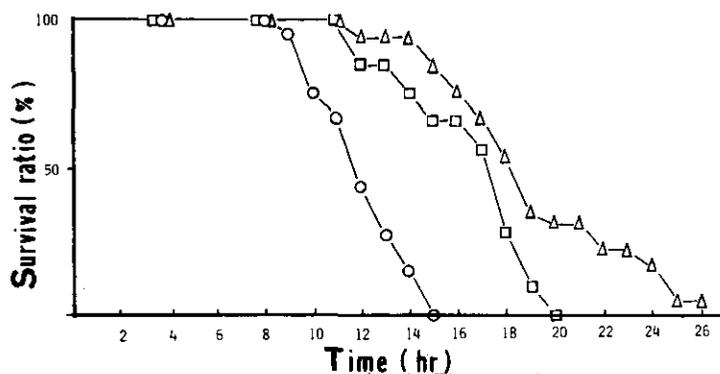


FIGURE 7. Survival ratio of carp kept in 15 ppm Cd solution following prior exposure: (○) tap water group; (□) 1 ppm Cd group; (△) 5 ppm Zn group. From Kito et al. (13), reprinted with permission of Pergamon Press.

Table 2. Increase of cadmium concentrations in metallothionein (MTF) and high molecular fractions (HMF) after exposure to 15 ppm cadmium solution for 18 hr following prior exposure to tap water, 1 ppm cadmium or 5 ppm zinc solutions for 14 days.

Organ	Fraction	Cd concentration, $\mu\text{g}/2\text{ g}$ of organ		
		Tap water (group A)	1 ppm Cd (group B)	5 ppm Zn (group C)
Hepatopancreas	MTF	5.62	8.23	13.32
	HMF	6.62	1.06	2.93
Kidney	MTF	3.31	15.30	33.29
	HMF	7.86	3.18	4.13
Gill	MTF	1.62	1.57	1.76
	HMF	8.30	1.37	1.90

to the supernatants of the hepatopancreas after prior exposure. The levels of Cd bound to HMF and MTF are shown in Figure 8.

In all groups, the level of Cd binding to MTF increased at first, but it reached a limiting value, and that of Cd binding to HMF increased. This shows that the affinity of MTF to Cd is stronger than that of HMF. In group C, the saturation level of Cd bound to MTF was larger than that of the other groups. Presumably, the content of MT in group C was larger than that in the others, so the larger amount of Cd was captured by MT. These results support the "spillover" theory (28), that is, Cd and Hg will spill over from MT to the enzyme-containing pool when the binding capacity of MT is exceeded. The pattern of accumulation of Cd in carp kidney at the early stage, however, does not coincide to the spillover theory (Fig. 1).

In addition the substitution of Zn by Cd in MTF *in vitro* was examined. Cd was added *in vitro* to the supernatants of hepatopancreas of each group after prior exposure, followed by gel chromatography (Sephadex G-75), and the metal contents in MTF were analyzed. In

all groups, the amount of Cd in MTF increased and that of Zn correspondingly decreased. Presumably, Zn in MTF was replaced by Cd.

Conclusion

Two Cd-binding proteins were isolated from hepatopancreas and kidney of carp administered 2 mg/kg Cd as CdCl₂ daily for 6 days. We identified these Cd-binding proteins as MT, and clarified some characteristics of these isoproteins.

The results of the immunohistological study proved by uptake of trace amounts of Zn and Cu from water that noninjected fish have (Zn,Cu)-metallothionein. The MT contents increased in fish organs (especially hepatopancreas and kidney) on successive exposure to Cd, and Zn bound to MT was replaced by Cd. Cd levels in fish were in excess of the binding capacity of MT, so Cd bound to HMF too. We suggest that this binding causes Cd toxicity.

Finally, we found the development of tolerance to Cd toxicity after exposure to a lower concentration of Cd and

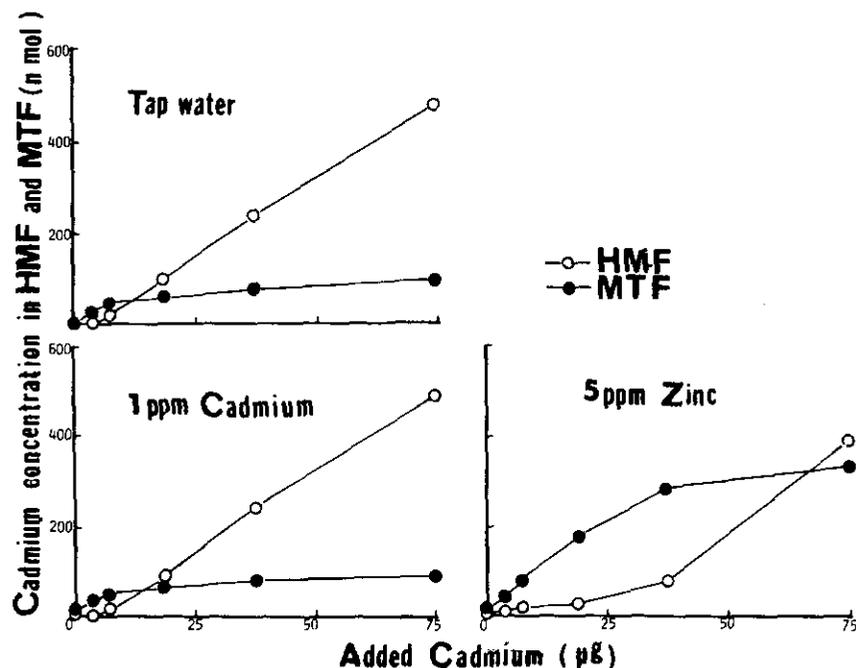


FIGURE 8. Binding of added Cd to cytoplasmic solution of hepatopancreas of carp kept in tap water, 1 ppm Cd, and 5 ppm Zn solutions for 14 days: (○) HMF; (●) MTF. From Kito et al. (18), reprinted with permission of Pergamon Press.

Zn; this phenomenon was related to the increased content of induced MT.

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