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THE EFFECTIVENESS OF CADMIUM DETOXIFICATION BY SCORPIONFISH

The purpose of this study was to determine if measuring the effectiveness of trace metal binding by metallothionein is a useful indicator of the toxic effects of metal bioaccumulation. The protein metallothionein, found in the soluble portion of cell extracts (cytosol), has a strong binding affinity for both essential (zinc, copper) and nonessential (cadmium, mercury) trace metals (Kägi and Nordberg 1979). In laboratory exposures of animals to cadmium or mercury, toxic effects are usually not found so long as the accumulated metal is bound to metallothionein (Winge et al. 1973; Brown and Parsons 1978; Pruell and Englehardt 1980; Roesijadi et al. 1982). This finding, which indicates that metallothionein has a protective effect, has led to the formation of the "spillover" theory (Winge et al. 1973; Brown et al. 1977) that intracellular toxic effects of cadmium and mercury will not occur until the binding capacity of metallothionein is exceeded. At this point, metals spill over and bind to a site of toxic action, the cytosolic enzyme-containing (ENZ) pool. The ENZ pool can be easily separated from the metallothionein-containing (MT) pool of cytosol by gel filtration (Brown et al. 1982a), and the amount of spillover can be determined.

Although measurements of metal concentrations in the ENZ and MT pools of cytosol provide a better understanding of the significance of bioaccumulation to sea animals, much is still unknown about the specifics of metallothionein production and the relationship between the ENZ pool and toxic effects. This paper presents the trace metal chemistry results from an experiment in which scorpionfish were chronically exposed to sublethal levels of ionic cadmium. The work was intended to 1) confirm the protection against cadmium toxicity provided by metallothionein production, 2) determine the effects of cadmium accumulation on zinc and copper metabolism, and 3) better define the relationship between changes in ENZ-pool cadmium and toxic effects.

The cytosolic partitioning of cadmium was measured in four organs of

scorpionfish after 4 and 8 weeks of exposure to 0.1 or 1.0 mg Cd/L. As expected, we found that most of the accumulated cadmium was present in the MT pool of liver, kidney, intestine, and gill tissue. Increases were also observed in the amount of ENZ-pool cadmium in every tissue after 8 weeks, indicating that metallothionein does not provide 100 percent protection for the ENZ pool, even at the low bioaccumulation levels reached in this experiment. The cadmium binding effectiveness of metallothionein was lowest in the gills and intestine, where the highest concentrations of ENZ-Cd were found. Cadmium exposure was also shown to increase the cadmium content of the subcellular fraction containing cell organelles. These data indicate that other sites of toxic action may need to be measured to fully assess the impact of cadmium bioaccumulation.

MATERIALS AND METHODS

Scorpionfish (*Scorpaena guttata*) ranging in weight from 0.25 to 0.75 kg were captured from near Anacapa Island in June 1983. These fish were acclimated to laboratory conditions and fed pieces of anchovy for 4 weeks prior to the start of the cadmium experiment. The fish were randomly distributed to six 312- or 400-L aquaria (15 fish per aquarium). Two aquaria were used for each exposure level of 0 (control), 0.1, and 1.0 mg Cd/L. Cadmium was added to the seawater from a stock solution of CdCl₂. The water in each aquarium was changed weekly and received continuous aeration and biological and charcoal filtration. Weekly measurements of aquaria water quality indicated similar values for each exposure level. Mean water quality values for the experiment were as follows: dissolved oxygen, 6.1 mg/L; total ammonia, 0.23 mg/L; salinity, 34 ppt; pH, 7.42; and temperature, 14°C.

The concentration of cadmium in the exposure water was measured weekly and found to vary, probably because of loss to the biological filters. The weekly water changes and occasional additions of Cd stock to the water kept concentrations quite close to the nominal values. Mean Cd concentrations in the 0.1- and 1.0-mg Cd/L exposures were 0.09 and 1.1 mg Cd/L, respectively.

Subsamples of scorpionfish were dissected after 0 (preexposure), 4, 8, and 16 weeks of exposure. Seven fish from the entire population were sampled at 0 weeks. Seven or six fish were sampled from each exposure level at 4, 8, and 16 weeks. Fish were first anesthetized with carbonic acid; a blood sample was then taken from a severed gill arch with a heparinized syringe. The body cavity of each fish was then opened and the internal organs removed. Samples of liver, kidney, intestine, gill, and muscle tissues were preserved in 10% buffered

formalin for histological examination. Additional portions of these tissues were placed in plastic vials and stored at -80°C for analysis of cytosolic metal distribution and enzyme activity and for other biochemical measurements.

Scorpionfish liver, kidney, intestine, and gill samples were homogenized in cold Tris buffer (0.05 M; pH 8.0) and ultracentrifuged at $100,000 \times g$ to produce cytosol extracts. These cytosols were then separated into fractions according to molecular size using Sephadex G-75 gel filtration. Fractions corresponding to a high molecular weight ($>20,000$ daltons) ENZ-pool, a medium molecular weight (3,000–20,000 daltons) MT-pool, and a low molecular weight ($<3,000$ daltons) glutathione-containing (GSH) pool were composited and analyzed for their cadmium, copper, and zinc content. The pellet resulting from ultracentrifugation was resuspended in homogenization buffer and stored at -80°C for analysis of metal content. Protein content of the cytosol samples was determined using the Coomassie Blue assay (Bradford 1976).

Statistical analyses of the data were performed using the SAS package of computer programs (SAS Institute, Inc., 1982). The significance of changes in ENZ- or MT-pool cadmium was tested using one- and two-way analyses of variance (ANOVA) on log-transformed data. Simple linear regressions and analyses of covariance were used to examine the relationship between MT- and ENZ-Cd concentrations. Spearman's nonparametric correlation coefficients were calculated to examine the nature of the relationship between changes in cytosolic pool Cd and changes in pool Cu or Zn.

RESULTS

Cytosolic Distribution of Cadmium

Trace metal analyses of the tissue cytosolic pools of scorpionfish exposed to cadmium for 4 and 8 weeks were completed. Tissues from the preexposure and 16-week groups have not yet been analyzed. The concentrations of Cd, Cu, and Zn in the ENZ and MT pools of liver, kidney, intestine, and gills are shown in Figures 1 and 2. Metal levels in the GSH pools of these fish have not been reported because these concentrations did not represent an additional site of metal binding, but instead reflected a small fraction of MT-pool metal that was not completely resolved by the chromatography procedure.

Increases of Cd in the MT pools of exposed scorpionfish in relation to controls were observed in all tissues at both 4 and 8 weeks (Figures 1 and 2). MT-pool Cd concentrations were highest in fish exposed to 1.0

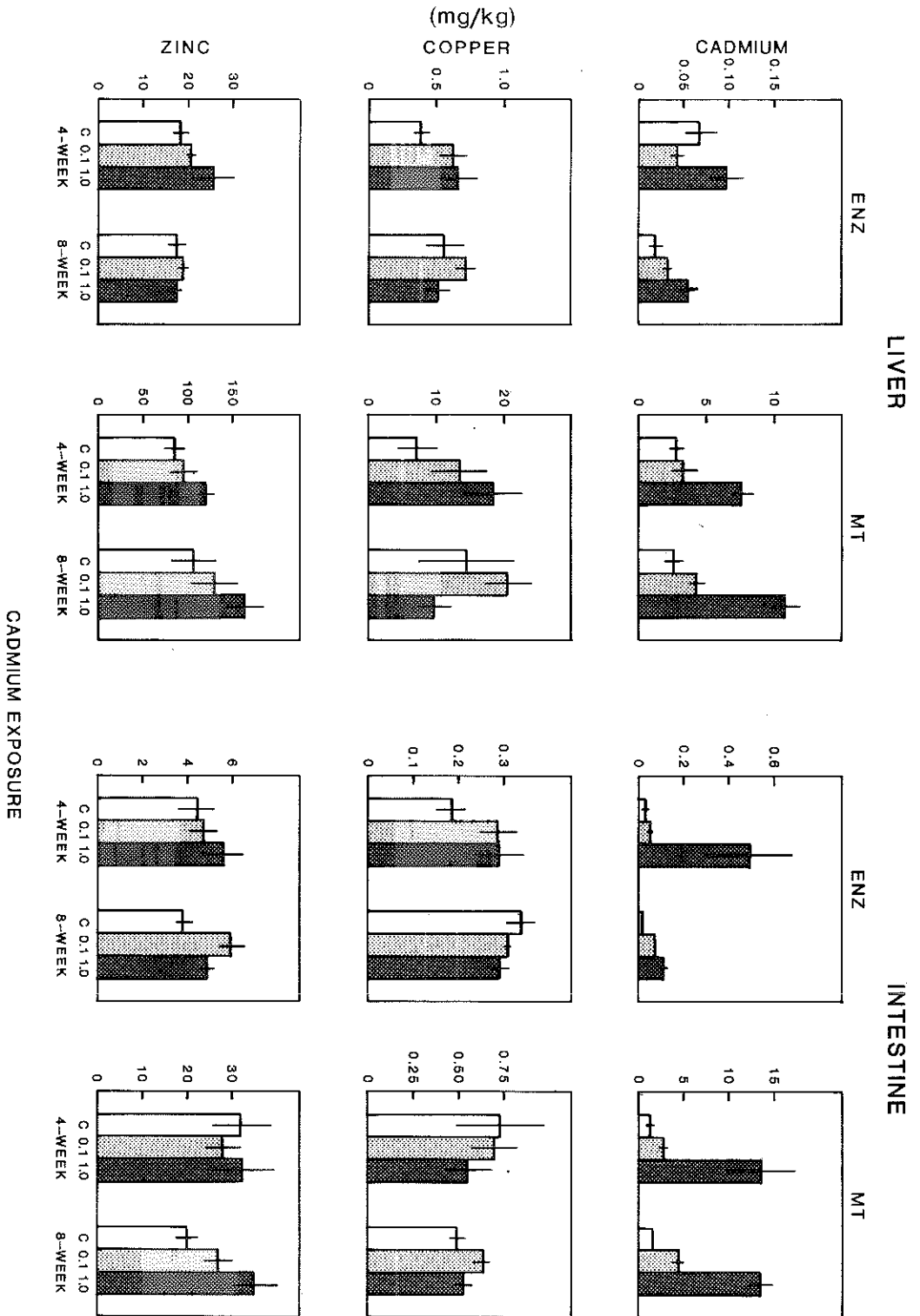


Figure 1. Cd, Cu, and Zn concentrations (mean \pm 1 standard error (n = 7)) in liver and intestine ENZ and MT pools of scorpionfish exposed to cadmium. Fish were exposed to 0 (control), 0.1, or 1.0 mg Cd/L for 4 and 8 weeks. The intestine showed greater increases in ENZ-Cd with exposure than did liver. MT-Zn usually increased at higher MT-Cd levels. Note the scales are different.

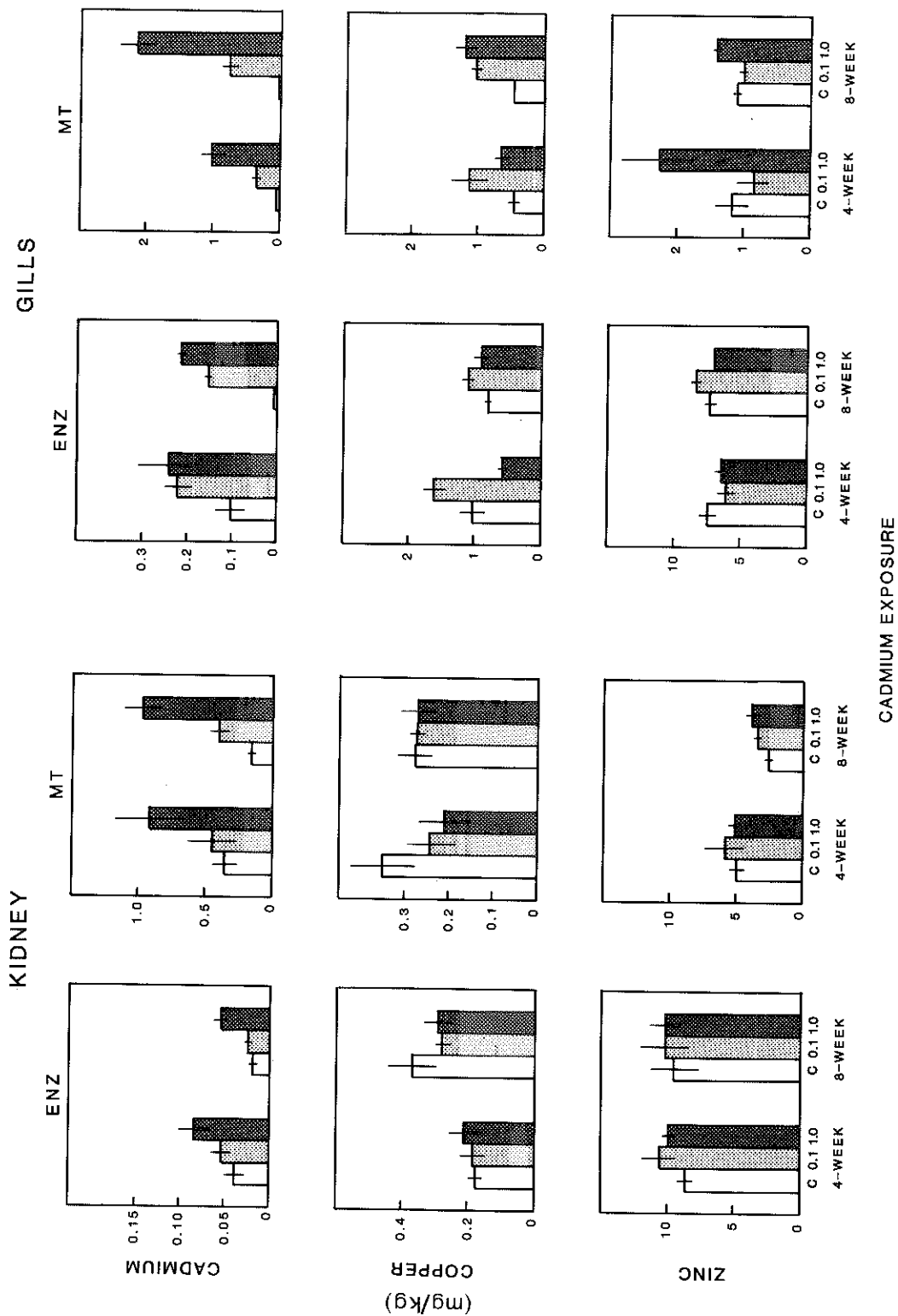


Figure 2. Cd, Cu, and Zn concentrations (mean \pm 1 standard error (n = 7)) in kidney and gill ENZ and MT pools of scorpionfish after cadmium exposure. Fish were exposed to 0 (control), 0.1, or 1.0 mg Cd/L for 4 and 8 weeks. The gills showed the greatest increase in ENZ-Cd and MT-Cd, in relation to controls. Note the scales are different.

mg Cd/L. Little change in MT-Cd concentration between 4 and 8 weeks was evident in kidney and intestine samples. An increase of approximately 50% in livers of fish exposed to 1.0 mg Cd/L was observed at 8 weeks. The greatest increases in MT-Cd were found in the gills, where concentrations at 8 weeks were increased by 200 and 100% in the 0.1- and 1.0 mg-Cd/L exposures, respectively (Figure 2).

Although most of the cytosolic cadmium in the tissues of exposed scorpionfish was bound to metallothionein, some of this cadmium escaped detoxification by MT and was associated with the ENZ pool (Figures 1 and 2). ANOVA tests on these data indicated that ENZ-Cd concentrations were significantly increased by cadmium exposure in all four scorpionfish tissues after 8 weeks of exposure to 1.0 mg Cd/L (Table 1). At 4 weeks, only the intestine and kidney ENZ-Cd concentrations of fish exposed to 1.0 mg Cd/L were significantly higher than the controls. Exposure to 0.1 mg Cd/L produced elevated ENZ-Cd concentrations only in the gills of fish exposed for 8 weeks.

The two-way ANOVA results (Table 1) indicate a significant effect of exposure time on the ENZ-Cd concentrations in each tissue: ENZ-Cd tended to decrease between the 4- and 8-week sampling times. This trend was present at each exposure level, but can be most easily seen in the control ENZ-Cd concentrations at each timepoint. There was a

Table 1. Summary of analysis of variance (ANOVA) results on ENZ-Cd changes in scorpionfish exposed to cadmium. Values indicate the probabilities (significance levels) for cadmium or time effects. Both cadmium exposure and time of sampling influenced ENZ-Cd levels in each of the four tissues examined.

	Two-Way ANOVA			One-Way ANOVA	
	Cd	Time	Cd-Time Interaction	Cd 4-Week Exposure	Cd 8-Week Exposure
Liver	0.002	0.001	0.202	0.043 ^a	0.003 ^b
Kidney	0.003	0.001	0.908	0.055 ^b	0.001 ^b
Intestine	0.001	0.043	0.013	0.004 ^b	0.037 ^b
Gill	0.001	0.057	0.330	0.085	0.001 ^c

^a1.0 mg Cd/L ENZ-Cd >0.1 mg Cd/L ENZ-Cd, but not control ENZ-Cd.

^b1.0 mg Cd/L >control.

^c1.0 and 0.1 mg Cd/L >control.

Table 2. Summary of regression analyses examining the linear dependance of ENZ-Cd on MT-Cd concentration in cadmium-exposed scorpionfish. A significant relationship was found in all cases, except for intestine after 8 weeks of exposure.

	4-Week Exposure				8-Week Exposure				Equality of Slopes ^c
	r ^{2a}	Slope	Beta ^b	Signifi- cance of Slope	r ²	Slope	Beta	Signifi- cance of Slope	
Liver	0.43	0.009	0.658	0.001	0.76	0.005	0.874	0.001	0.063
Kidney	0.26	0.035	0.509	0.018	0.56	0.044	0.750	0.001	0.637
Intestine	0.59	0.033	0.767	0.001	0.01	0.001	0.087	0.708	0.001
Gill	0.18	0.114	0.423	0.056	0.66	0.086	0.812	0.001	0.585

^a Coefficient of determination (percent of ENZ-Cd change accounted for by MT-Cd).

^b Standardized regression coefficient.

^c Significance of time-slope interaction from analysis of covariance.

significant interaction between exposure level and time for intestine cytosol ENZ-Cd concentration (Table 1), indicating that the nature of the response of ENZ-Cd to exposure level in this tissue was different for each time period.

A significant correlation between ENZ-Cd and MT-Cd concentration was found for all tissues at 4 weeks and for all tissues except intestine at 8 weeks. These results indicate that, even with low levels of MT-Cd accumulation, there are increased ENZ-Cd levels. The relationship between ENZ- and MT-Cd levels was further explored by fitting a linear regression model to the data (Table 2). Significant regressions were obtained for all tissues except for 8-week intestines. Examination of the coefficients of determination (r²) for these regressions indicated that between 18 and 76% of the changes in ENZ-Cd could be accounted for by changes in MT-Cd concentration. The magnitude of the change in ENZ-Cd concentration with change in MT-Cd is indicated by the regression slopes. Analysis of covariance on these data showed that there were no differences in the slopes between 4 and 8 weeks in liver, kidney, or gill tissue (Table 2). Comparisons of the slopes between different tissues can be made by examining the standardized slopes (beta coefficients). These values were similar between tissues, ranging from 0.423 (4-week gills) to 0.874 (8-week liver).

Changes in Copper and Zinc

The relationship of changes in cytosolic cadmium to Cu and Zn changes

was examined by calculating Spearman nonparametric correlation coefficients. A significant positive relationship between changes in MT-Cd and MT-Zn was found in most cases (Table 3). Significant positive correlations between MT-Cd and MT-Cu were found only in two cases (4-week kidney and 8-week gills). No significant correlations between changes in ENZ-Cd and ENZ-Cu or -Zn were found.

Metal Concentrations in Cell Organelles

The relative significance of other sites of metal accumulation within the cell was investigated by analyzing the metal content of the 100,000 x g pellet (containing cell organelles) from the 8-week liver samples (Table 4). The Cd concentration of these pellet samples increased with greater cadmium exposure, as did cytosol Cd concentrations. As a result, the percentage of cadmium in the pellet remained similar between exposure levels (18-15%). The percentage of copper found in the pellet material was also similar between exposure levels (19-14%). The percentage of zinc in the pellet showed a decreasing trend with exposure level, ranging from 17% in the controls to 11% in fish exposed to 1.0 mg Cd/L.

Table 3. Correlation of changes in MT-Cu or MT-Zn with changes in MT Cd. A positive correlation was usually found between MT-Cd and MT-Zn, but rarely with MT-Cu.

	MT-Zn				MT-Cu			
	4-Week Exposure		8-Week Exposure		4-Week Exposure		8-Week Exposure	
	r ^a	Significance ^b	r	Significance	r	Significance	r	Significance
Liver	0.436	0.048	0.588	0.005	0.338	0.134	0.000	1.000
Kidney	0.736	0.001	0.621	0.003	0.425	0.055	-0.040	0.862
Intestine	0.254	0.265	0.582	0.006	-0.082	0.724	0.378	0.091
Gill	0.403	0.070	0.335	0.138	0.170	0.461	0.538	0.012

^aSpearman correlation coefficient.

^bProbability that no correlation is present.

Blood Concentrations

The cytosol from scorpionfish liver, gill, and kidney contained blood which was trapped within the tissues at the time of dissection. The influence of this blood on the cytosolic pool metal values was examined by analyzing a whole blood sample from each cadmium exposure level at 16 weeks. These blood samples were treated the same as any other tissue for homogenization, centrifugation, and G-75 chromatography. The Cd, Cu, and Zn concentrations of the ENZ and MT pools from these blood samples are shown in Table 5. The concentrations of Cu and Zn in the MT pools of blood were less than those in the ENZ pool,

Table 4. Concentrations of Cd, Cu, and Zn in 100,000 x g pellet and cytosol of scorpionfish livers following 8-week exposure to cadmium. Values are mean \pm standard error (n = 6 for control and 7 for 0.1- and 1.0-mg/L exposure levels). These results show that the cadmium exposures, as it did in cytosol.

Exposure	Metal Concentration (mg/kg)								
	Cadmium			Copper			Zinc		
	Pellet	Cytosol ^a	%Pellet ^b	Pellet	Cytosol	%Pellet	Pellet	Cytosol	% Pellet
Control	0.59 \pm 0.28	2.62 \pm 0.68	18 \pm 2.5	3.3 \pm 1.8	15.7 \pm 8.8	19 \pm 2.7	26.6 \pm 8.0	126 \pm 31	17 \pm 1.6
0.1 mg/L	1.05 \pm 0.25	4.44 \pm 0.52	18 \pm 2.1	3.4 \pm 0.5	21.4 \pm 3.4	14 \pm 1.6	24.7 \pm 5.0	15 \pm 27	14 \pm 0.8
1.0 mg/L	1.93 \pm 0.28	11.1 \pm 1.0	15 \pm 1.8	1.6 \pm 0.4	10.3 \pm 2.5	16 \pm 3.0	23.7 \pm 4.8	183 \pm 21	11 \pm 1.1

^aSum of metal content of ENZ, MT, and GSH pools.

^bPellet concentration/pellet + cytosol concentration.

a reversal of the general pattern seen in the other tissues. The cadmium content of the ENZ and MT pools was negligible when compared to concentrations in liver, kidney, intestine, or gill tissue (Figures 1 and 2). The copper concentrations in blood ENZ and MT pools were relatively low, being similar only to kidney concentrations. The ENZ-Zn level of blood was relatively high, with concentrations similar to or greater than those for the other four scorpionfish tissues. Blood MT-Zn concentrations were much lower than the MT concentration of all other tissues except the gills.

DISCUSSION

Influence of Exposure Type on Cytosolic Cadmium Distribution

The exposure of scorpionfish to 1.0 mg Cd/L for up to 8 weeks resulted in increased cadmium concentrations in both the MT and ENZ pools of all tissues examined. Comparison of these data to the results of an acute (96-hour) exposure of scorpionfish to 25 and 50 mg Cd/L (Brown et al. 1984) reveals important differences in cytosolic cadmium distribution patterns. In the acute experiment, MT-Cd concentration was greatest in the liver (60 mg/kg) and the highest ENZ-Cd concentration was found in the kidney (8 mg/kg). These data indicate large concentration changes in these organs due to cadmium uptake from the blood. In the chronic experiment reported here, much lower MT- and ENZ-Cd concentrations were found, especially in the internal organs. The highest MT-Cd concentration in these fish was found in the intestine (13 mg/kg), with the highest ENZ-Cd levels occurring in the intestine (0.5 mg/kg) and gills (0.2 mg/kg). As both of these

Table 5. Cd, Cu, and Zn concentrations in the ENZ and MT pools of scorpionfish whole blood after 16 weeks' exposure to cadmium. The highest concentrations of metal were found for ENZ-Zn, which had levels greater than most other scorpionfish tissues.

Exposure	Metal Concentration in Pool (mg/kg)					
	Cadmium		Copper		Zinc	
	ENZ	MT	ENZ	MT	ENZ	MT
Control	0.006	0.013	0.793	0.252	22.3	1.43
0.1 mg Cd/L	0.011	0.043	1.13	0.156	21.2	1.11
1.0 mg Cd/L	ND ^a	0.007	0.885	0.159	13.4	0.630

^a Not detectable.

tissues were in direct contact with the cadmium-spiked seawater, metal uptake from the water, rather than the blood, seems to have had the greatest influence on cytosolic cadmium distribution in the present experiment.

Although the acute and chronic experiments resulted in different ENZ-Cd concentrations, both of these experiments showed that the least amount of spillover occurred in the liver. This finding indicates that, in studies in fish from the sea, the examination of tissues other than the liver may provide the most sensitive measure of cadmium spillover.

Efficiency of Metallothionein Binding

The changes in cytosolic cadmium distribution reported here indicate that metallothionein will not provide complete protection from cadmium toxicity even though its total cadmium binding capacity has not been exceeded. The regression analysis results indicate that ENZ-Cd usually increased whenever MT-Cd levels increased (Table 2), suggesting the presence of a binding equilibrium between these two cytosolic pools. Additional evidence that the absolute binding capacity of metallothionein was not exceeded in this experiment is provided from the MT-Zn measurements. Increases in MT-Cd were often accompanied by increases in MT-Zn (Table 3). This relationship is a common response to chronic cadmium exposure and indicates an increased rate of metallothionein synthesis (Cousins 1983). The MT-Zn data also point out an abundance of potential cadmium binding sites through displacement of this bound zinc (Leber and Miya 1976). These results

indicate that, while increased metallothionein synthesis may reduce the toxicity of Cd, even low levels of bioaccumulation will result in metal binding to the ENZ pool and possibly to other sensitive sites within the cell.

Although the amount of spillover of cadmium into the ENZ pool was much smaller in this experiment than was observed in the acute experiment, the increases of ENZ-Cd seen in the present study are significant because 1) the pool contains many enzymes that can be adversely affected by cadmium binding and 2) the spillover theory predicts that altered enzyme function and other molecular effects will occur in affected tissues. Changes in blood chemistry and enzyme activity were found in some cases (Bay et al., this volume), but these molecular and physiological changes could not always be correlated with degree of spillover. Moreover, histological examination of the scorpionfish tissues discussed here did not reveal harmful effects due to cadmium exposures (Perkins and Rosenthal, this volume).

Effects of Time

A complete understanding of the mechanism for the ENZ-Cd changes found in this study is complicated by the presence of temporal effects. Presently, we have no explanation for the reductions in ENZ-Cd concentration between the 4- and 8-week sampling times in both control and exposed fish (Figures 1 and 2). No correlations of ENZ-Cd change with changes in ENZ-Cu or ENZ-Zn were found, ruling out a general reduction of ENZ-metal concentration, the displacement of Cd by Cu or Zn, or calculation errors. These temporal changes complicate the interpretation of the ENZ-Cd increases observed in the liver at 8-weeks. Even though the 1.0-mg Cd/L exposure resulted in elevated ENZ-pool concentrations (suggesting toxicity), these levels were below those found in the 4-week controls (suggesting no effect). These data indicate the dynamic nature of cytosolic metal distributions and the need for a more thorough examination of time-related effects.

Cell Organelle Concentrations

Measurements of the amount of cadmium associated with the 100,000 x g pellet fraction of liver tissue indicate that this portion of the cell may be an important site of metal detoxification or toxic action. Pellet cadmium concentrations increased in cadmium-exposed fish, suggesting the binding of metal to the mitochondria and endoplasmic reticulum. These cellular organelles contain many enzymes and have been shown to be affected by cadmium exposure (Yoshida et al. 1976; Sato et al. 1978). Although up to 18% of the total cell cadmium was present in these pellet samples, this metal may not all be bound to sensitive sites, but may represent a detoxified form. Investigators have reported the occurrence of cadmium in membrane vesicles (Marshall and Talbot 1979),

preventing cadmium interactions with sensitive macromolecules. The measurements of pellet metal content in this study may be overestimates of the actual values because of contamination from cytosol trapped within the pellet. This contamination was estimated to account for up to 30% of the measured values for cadmium. Additional centrifugation steps need to be incorporated into the tissue preparation process to reduce this contamination before more accurate measurements can be made.

Blood Concentrations

The blood tissue was also shown to be a potentially important site of cytosolic metal binding. The patterns of Cd, Cu, and Zn concentration in the ENZ- and MT-pools of blood were very different from those in other tissues. Thus, the use of blood samples as an indicator of the metal detoxification status of other tissues is inappropriate. Knowledge of the metal content of the blood is important, however, in assessing the true metal concentrations of other tissues containing substantial amounts of blood. Houston and McCarty (1978) estimated that a majority of the protein content of gill and kidney homogenates came from blood trapped within the tissue. For scorpionfish, blood contamination had an insignificant effect on the cadmium content of tissue cytosols but probably substantially increased ENZ-Zn, ENZ-Cu, MT-Zn, and MT-Cu concentrations of gill and kidney samples. We are currently attempting to measure the amount of blood contamination in scorpionfish tissue cytosol so that changes in the concentrations of these metals can be accurately estimated.

CONCLUSIONS

The results from this experiment indicate that the criteria for determining the occurrence of cadmium spillover into the ENZ pool must be carefully defined. It is apparent that some amount of ENZ-pool Cd will be present in a tissue due to certain equilibrium processes with metallothionein and should therefore not be interpreted as a failure of that tissue's detoxification system. The amount of spillover resulting from this experiment appeared to be near or below a threshold level for toxicity. Additional experiments utilizing other exposure levels and more sensitive measures of toxicity are needed to determine this threshold level and to better define the range of natural variation in ENZ-Cd concentration.

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