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Evaluation of Metallothionein Measurement as a Biological Indicator of Stress from Cadmium in Brook Trout

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Abstract.—A modification of an established technique for quantifying metallothionein (MT) in mammals was used to evaluate the toxicological importance of MT as a biological indicator of stress from chronic cadmium toxicity in brook trout *Salvelinus fontinalis*. In a 30-d study, fish mortality was significantly increased but growth was not altered by exposure to 3.6 μg Cd/L or more. After chronic exposure to cadmium, both mortality and whole-body residues showed a dose–response relation over the exposure range of 3.6 to 60.6 μg Cd/L. Concentrations of MT were increased significantly in all exposures that resulted in significant mortality; however, they showed no dose–response relation to cadmium exposure, nor were they correlated with mortality or whole-body cadmium residues. Consequently, measurement of MT alone was not a useful indicator of cadmium toxicity in brook trout. A better biological indicator was the amount of free cadmium in liver tissue. Free cadmium (the difference between the amount of cadmium in the supernatant resulting from centrifugation at 100,000 × gravity and the amount bound to MT in an unsaturated condition) showed a dose-dependent increase with increasing cadmium exposure and was highly correlated with mortality and whole-body residues. Free cadmium concentrations were significantly elevated in all exposures to 3.6 μg Cd/L or greater. The presence of free cadmium in tissues of brook trout from all exposures suggests that MT was not saturated with cadmium before the appearance of pathological effects and thus conflicts with the "spillover" hypothesis. As judged by the results of our toxicity studies, the spillover hypothesis should be redefined as a continuum of toxic responses to varying balances between the relative abundance of metals present and their respective binding affinities for MT.

The protein metallothionein (MT) serves a homeostatic function for the essential metals copper and zinc, and also a detoxification function for metals such as cadmium and mercury (Kagi and Nordberg 1979). Metallothionein functions in these capacities because its abundant cysteine residues sequester bivalent cations. Metallothionein concentrations increase with exposure to metals such as cadmium, mercury, excess copper, and excess zinc (Noel-Lambot et al. 1978; Kito et al. 1982; McCarter et al. 1982). Other evidence shows that MT provides some protection against the toxic effects of these metals by sequestering and reducing the amounts of free metals in the tissues (Brown and Parsons 1978; Pruell and Engelhardt 1980).

Metallothionein measurements have been proposed as a biological indicator of metal stress in humans (Shaikh and Hirayama 1979). Inasmuch as MT is induced in both vertebrate and invertebrate aquatic organisms, Olafson et al. (1979) suggested that MT concentrations may be good measures of the extent of recent exposure of aquatic organisms to certain metals.

Closely associated with the detoxification function of MT is the "spillover" hypothesis first proposed by Winge et al. (1974) and advanced by Brown et al. (1977) and Brown and Parsons (1978). The hypothesis has been used to explain pathological effects in animals that have metals in protein fractions of tissues other than the protein fraction containing MT. The occurrence of pathological effects was considered a consequence of the failure of MT to be formed at rates equal to that of the influx of metals, or in sufficient quantity to bind the total metals present. Once the ability of MT to sequester metals was exceeded, the metals "spilled over" into other protein fractions and resulted in pathologic effects (Winge et al. 1974).

Metallothionein studies in fish, in general, have

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only identified the presence of this protein in fish exposed to metals in laboratory toxicity studies or field studies; the toxicological importance of its presence has not been examined. Several researchers have alluded to the toxicological importance of MT and the relation between MT and the spillover hypothesis in fish (Brown et al. 1977; Brown and Parsons 1978). Few investigators, however, have conducted studies in which mixtures of metals were used in attempts to clarify the relations between MT and the spillover hypothesis (McCarter et al. 1982; Roch et al. 1982; McCarter and Roch 1983). The importance of MT in fish for assessments of stress from metal contaminants has recently been reviewed (Hamilton and Mehrle 1986).

We previously conducted a cadmium injection study and a low-cadmium exposure study (Hamilton et al. 1987, this issue) with brook trout *Salvelinus fontinalis* to evaluate the cadmium-saturation method of Eaton and Toal (1982, 1983) for quantifying MT in fish. In the present study, we conducted a high-cadmium exposure study with brook trout to create a greater dose-response relation between cadmium and mortality and to further evaluate the cadmium-saturation technique. The objectives of our research were to evaluate the relation between MT and the responses of brook trout to cadmium exposure, as evidenced by such factors as mortality, whole-body residues, and growth; to assess the toxicological importance of the spillover hypothesis; and to evaluate the utility of MT measurements as biological indicators of metal contaminant stresses.

Methods

Brook trout were maintained at the National Fisheries Contaminant Research Center in well water (pH, 7.2; alkalinity, 237 mg/L; hardness, 272 mg/L; temperature, 15°C) and allowed to feed on Rangens® commercial diet ad libitum before and during the experiments.[2] The fish originated from eyed eggs obtained from Big Spring Trout Hatchery, Lewistown, Montana.

A chronic toxicity study was conducted with brook trout (average weight, 5 g; age, 2–3 months) continuously exposed to concentrations of 0, 3.6, 7.6, 15.7, 30.0, and 60.6 μg Cd/L as cadmium chloride for 30 d in an intermittent-flow diluter (Mount and Brungs 1967). Cadmium chloride dissolved in pure water, and a mixture of sulfuric acid and nitric acid (3:1, volume : volume), were metered into the diluter with an automatic pipette. The cadmium was added to the toxicant mixing chambers of the diluter and the acid mixture was added to the flow-splitting chambers, where both were thoroughly mixed prior to delivery by glass tubes to duplicated exposure aquaria. The cadmium and acid mixture were combined with reconstituted low-alkalinity–low-hardness water from a water softener–reverse osmosis–deionizer water treatment system to simulate acidified lake conditions in the northeastern USA. The experimental design, including measurement of water quality variables, was described by Hamilton et al. (1987). Water quality variables measured during the study approximated: pH, 6.1; alkalinity, 2.5 mg/L; hardness, 14.5 mg/L; conductivity, 40 μs/cm; oxygen, >8.5 mg/L; temperature, 12°C.

We placed 25 fish in each of 12 exposure aquaria and acclimated them first to soft water over a 48-h period and then to pH 6.1 over an additional 48-h period before cadmium was added to the exposure water. Mortality was recorded daily and dead fish were removed from the aquaria.

After 30 d of exposure, one to three pooled samples of six fish each were randomly sampled from each duplicate concentration of cadmium. Fish were anesthetized with tricaine and weighed (grams). Livers and kidneys were removed and weighed individually, then combined into pooled samples and homogenized in 14 mL ice-cold 0.01M tris–HCl buffer (pH 8.1) with 0.01% sodium azide. Homogenates were heat-denatured, centrifuged, and quantified for cadmium content as described by Hamilton et al. (1987). Four fractions were employed: 10,000 and 100,000 × gravity supernatants, unsaturated MT (amount of cadmium bound to MT in an unsaturated condition), and saturated MT (amount of cadmium bound to MT in a cadmium-saturated condition). Briefly, saturated MT was quantitated indirectly by the method of Eaton and Toal (1982, 1983) and involved the saturation of MT with excess cadmium, removal of non-MT-bound cadmium with a 2% hemoglobin solution, and subsequent purge of the hemoglobin by heat denaturation and centrifugation. The amount of cadmium remaining in solution after this procedure was quantified by atomic absorption; the amount of MT was calculated by assuming 112.4 ng Cd/nmol Cd, 7 nmol Cd/nmol MT, and 1 nmol MT/6,000 ng MT. Un-

[2] Reference to trade names, commercial products, or manufacturers does not imply or constitute government endorsement or recommendation for use.

saturated MT was quantitated in a similar manner, except that the binding sites on MT were not saturated with excess cadmium and a 1% hemoglobin solution was used in the removal of non-MT-bound cadmium. The difference between the amount of cadmium in the 100,000 × gravity supernatant and the amount bound to unsaturated MT was termed "free cadmium."

In addition to the fish sampled for liver and kidney material, three fish were randomly sampled from each concentration for determination of individual whole-body cadmium residues. Fish were minced, weighed, digested in concentrated nitric acid for 48 h at 70°C, and analyzed in duplicate for cadmium by atomic absorption with a graphite furnace technique.

Statistical analysis.—Statistical analyses were performed with Statistical Analysis System programs. The percent mortalities of fish in the experiment were analyzed by one-way analysis of variance on arcsine-transformed values. Toxicant effects on various cadmium-containing tissue fractions (10,000 and 100,000 × gravity supernatants, and unsaturated and saturated MT) and whole-body cadmium residues were also determined by one-way analysis of variance. These variables were first examined by regression analysis of their means, standard deviations, and variances to determine if transformation was needed to normalize data before statistical analysis. Logarithmic transformations were applied to all cadmium-containing tissue fractions and whole-body residues before analysis because correlations (r) between means, standard deviations, and variances were greater than 0.40. Treatment means were compared by multiple-means-comparison tests (least significant difference; Snedecor and Cochran 1967). Simple linear regression was used to analyze the relation between cadmium exposure concentrations and mortality, whole-body residues, and various cadmium-containing tissue fractions.

Relations among various cadmium-containing fractions, whole-body residues, mortality, and cadmium exposure concentrations were analyzed with individual aquaria as the experimental units. Various cadmium-containing fractions and whole-body residues, as well as mortality, were measured for different fish taken from individual aquaria; thus the common denominator for the comparison of these data was the individual aquarium.

The specific relation among mortality, whole-body residues, and MT concentrations in liver tissue of fish was calculated by a quadratic (second-degree) polynomial and fitted to a quadratic response surface (Cochran and Cox 1957). The maximum value of mortality on the fitted surface was calculated by differentiating with respect to whole-body residues and MT concentrations to define a stationary value. Linear terms were removed from the quadratic polynomial by translation of axes, and the cross-product term was removed by rotation of axes. The identified surface was an ellipse.

TABLE 1.—Percent mortality of brook trout exposed to cadmium. Asterisks denote significant differences from control values ($P \leq 0.05$); $N = 50$.

Exposure concentrations (μg Cd/L)	Days of exposure		
	7	15	30
0	0	2.0	2.0
3.6	4.4*	6.6	19.9*
7.6	10.3*	18.2*	40.4*
15.7	33.9*	38.9*	51.0*
30.0	26.8*	31.2*	46.2*
60.6	30.6*	38.8*	56.8*

One- and two-variable predictive models of mortality were calculated by stepwise regression with whole-body residues, MT concentrations in liver tissue, free-cadmium concentrations, and cadmium-exposure concentrations.

Results

Mortality increased rapidly during cadmium exposures. It was significantly higher than in the control at all cadmium concentrations after 7 d of exposure and, with one exception, remained so after longer exposures (Table 1). Mortality showed a dose–response relation to cadmium exposure concentration. Fish growth, as measured by weight, was not affected by cadmium exposure (Table 2).

Tissue Distribution of Cadmium

Cadmium concentrations in the 10,000 × gravity supernatant were about 10–36 times higher in liver tissue, and about 2–5 times higher in kidney tissue, of exposed fish after 30 d of exposure than in controls (Table 2). Cadmium concentrations in liver and kidney were not dose dependent in fish exposed to concentrations of 3.6–15.7 μg Cd/L, but increased with dose in exposures to 30.0 and 60.6 μg Cd/L. Thus, cadmium uptake by liver and kidney seemed to shift from dose independence to dose dependence at exposures above 16 μg Cd/L. Concentrations of MT in liver, but not kidney, were significantly elevated at all cadmium exposures and coincided with increased mortality.

TABLE 2.—Means and (in parentheses) SDs of cadmium concentrations in various liver and kidney fractions and weight of brook trout exposed to cadmium for 30 d. Asterisks denote significant differences from control values ($P \leq 0.05$); MT = metallothionein.

Exposure concentration (μg Cd/L)	Cadmium-containing fraction					MT (μg/g tissue)	Fish weight (g)	Number of pooled samples
	Supernatant (10,000 × gravity) (ng Cd/g tissue)	Supernatant (100,000 × gravity) (ng Cd/g tissue)	Free cadmium (ng Cd/g tissue)	Unsaturated MT (ng Cd/g tissue)	Saturated MT (ng Cd/g tissue)			
Liver fractions								
0	106	113	101	12	460	3.5	6.6	6
	(43)	(41)	(39)	(11)	(70)	(0.5)	(0.9)	
3.6	1,088*	908*	742*	166*	2,062*	15.7*	6.8	5
	(238)	(191)	(153)	(55)	(664)	(5.1)	(1.0)	
7.6	1,261*	898*	714*	184*	3,031*	23.1*	6.3	4
	(194)	(126)	(157)	(93)	(1,589)	(12.1)	(0.5)	
15.7	1,313*	1,113*	1,001*	113*	1,305*	9.9*	6.2	4
	(207)	(594)	(553)	(62)	(711)	(5.4)	(0.6)	
30.0	1,884*	1,628*	1,282*	346*	3,042*	23.2*	6.3	4
	(405)	(299)	(244)	(62)	(327)	(2.5)	(0.6)	
60.6	3,590*	3,204*	2,857*	347*	2,128*	16.2*	5.8	3
	(281)	(228)	(81)	(257)	(1,360)	(10.4)	(0.4)	
Kidney fractions								
0	141	69	59	10	1,003	7.6	6.6	6
	(69)	(23)	(21)	(3)	(136)	(1.0)	(0.9)	
3.6	336*	231*	170*	61*	812	6.2	6.8	5
	(82)	(52)	(54)	(18)	(321)	(2.4)	(1.0)	
7.6	344*	183*	132*	52*	1,044	8.0	6.3	4
	(97)	(58)	(44)	(15)	(266)	(2.0)	(0.5)	
15.7	392*	225*	186*	39*	949	7.2	6.2	4
	(131)	(121)	(115)	(9)	(84)	(0.6)	(0.6)	
30.0	505*	337*	260*	77*	1,048	8.0	6.3	4
	(41)	(51)	(51)	(29)	(84)	(0.6)	(0.6)	
60.6	742*	543*	461*	82*	1,246	9.5	5.8	3
	(89)	(104)	(88)	(19)	(104)	(0.8)	(0.4)	

However, MT concentratioins in the liver did not show a dose–response relation to increasing cadmium exposure concentration. The quantity of cadmium bound to unsaturated MT in liver and kidney was significantly increased above control concentrations at all exposures but was not highly correlated with cadmium exposure concentrations (Table 3). This decreased response was due partly to lower-than-expected cadmium concentrations in unsaturated MT in liver and kidney of fish exposed to 15.7 and 60.6 μg Cd/L (Table 2).

Concentrations of free cadmium were significantly increased in all cadmium concentrations at which significant mortality occurred (Table 2). Free-cadmium concentrations in liver and kidney showed a close dose–response relation to cadmium exposure concentrations (Table 3). Moreover, free-cadmium concentrations were closely correlated with mortality and whole-body residues (Table 3).

Whole-body residues of cadmium increased significantly in fish at all exposures and showed a dose-dependent response in the 3.6–30.0 μg Cd/L exposures, and then plateaued (Table 4). Whole-body residues were highly correlated with cadmium exposure concentrations and mortality after 30 d of exposure (Table 3). They were also highly correlated with cadmium concentrations in the 100,000 × gravity supernatant of liver and kidney but not with MT concentrations in either tissue (Table 3). Likewise, mortality was well correlated with cadmium concentration in the 100,000 × gravity supernatant of liver and kidney but not with MT concentrations (Table 3).

Mortality and Cadmium Distribution

The mortality response in this study was evaluated by stepwise regression analysis to determine the best one-variable and two-variable models. All cadmium measurements in liver were evalu-

TABLE 3.—Correlation coefficients (r) among cadmium concentrations in various liver and kidney fractions, metallothionein (MT) quantities, cadmium exposures, whole-body cadmium residues, and mortality of brook trout exposed to cadmium for 30 d.

Cadmium-containing fraction or MT	Cadmium exposure concentration	Whole-body residue	Mortality
Liver			
Supernatant[a]	0.97	0.85	0.75
Free cadmium	0.97	0.83	0.74
Unsaturated MT	0.62	0.66	0.52
MT quantity	0.17	0.31	0.31
Kidney			
Supernatant[a]	0.94	0.89	0.69
Free cadmium	0.95	0.88	0.67
Unsaturated MT	0.74	0.82	0.65
MT quantity	0.60	0.52	0.33
Whole-body residue	0.89		0.81

[a] 100,000 × gravity supernatant.

ated in the analysis and included cadmium concentrations in supernatants collected after centrifugation at 10,000 and 100,000 × gravity, unsaturated and saturated MT, free cadmium, and whole-body residues. Stepwise regression analysis showed that measurements of MT concentrations in liver were not useful predictors of mortality. When a one-variable regression model was used, saturated MT explained only 15% ($r^2 = 0.15$) of the mortality response. With addition of the squared value of saturated MT to the model (squaring incorporates quadratic responses of the data in the regression model), the two-variable model of mortality explained only 33% ($R^2 = 0.33$) of the mortality. For unsaturated MT, r^2 was 0.30 for the one-variable regression model and R^2 was 0.46 for the two-variable model. The two best one-variable regression models of mortality were for free-cadmium concentration ($r^2 = 0.50$) and whole-body residue ($r^2 = 0.52$). The predictive equations were

$$\text{mortality} = 0.0002(\text{free Cd}) + 0.3650;$$
$$\text{mortality} = 0.00007(\text{whole-body residues}) + 0.3325.$$

Addition of the squared value of the respective variable improved the models, and yielded R^2 values of 0.81 for the two-variable regression model with free-cadmium concentrations and 0.80 for the model with whole-body residues. The predictive equations were

$$\text{mortality} = 0.0009(\text{free Cd}) + [-0.0000002(\text{free Cd})]^2 + 0.0607;$$

TABLE 4.—Whole-body residues of cadmium in three brook trout exposed to cadmium for 30 d. All values for fish exposed to cadmium were significantly higher than the control value ($P \leq 0.05$).

Exposure concentration (μg Cd/L)	Residue (μg Cd/g tissue) (Mean ± 1 SD)
0	35 ± 2
3.6	144 ± 19
7.6	282 ± 105
15.7	406 ± 324
30.0	752 ± 715
60.6	751 ± 293

$$\text{mortality} = 0.003(\text{whole-body residue}) + [0.000003(\text{whole-body residue})]^2 + 0.0626.$$

The identification of free cadmium and whole-body residues as the best variables for predicting mortality was not surprising because of their strong relation to cadmium exposure concentrations.

When the relation between mortality, whole-body residues, and MT concentrations in liver of fish was evaluated by quadratic polynomial, the fitted response surface was an ellipse (Figure 1). As judged from the fitted response surface, if MT concentrations were maintained at 4.4 μg/g tissue (which was very close to MT concentrations in control fish) and whole-body residues were elevated to 609 μg Cd/g tissue, mortality should be 90%. These values fell within the ranges of values in the study but it may be biologically unrealistic for all three conditions to occur in the same organism. Moreover, MT concentrations were three to six times greater in exposures when high whole-body residues were present but mortality was less than 60%. Thus, elevated MT concentrations coincided with reduced mortality compared with the value derived from the quadratic polynomial and fitted surface, even though high whole-body residues were present.

Discussion

Growth is the culmination of many biochemical processes, and toxicant-induced changes in metabolic pathways or in macromolecules involved in growth, such as RNA or DNA, should occur before changes occur in growth. Stroganov (1967) reported that cadmium did not affect RNA or DNA in tissue of common carp *Cyprinus carpio* exposed to 10 mg Cd/L for 21 d, although the exposure was lethal. Rombough and Garside (1982) showed, however, that exposure to cadmium reduced growth in alevins of Atlantic salmon *Salmo salar,* probably as a result of interfer-

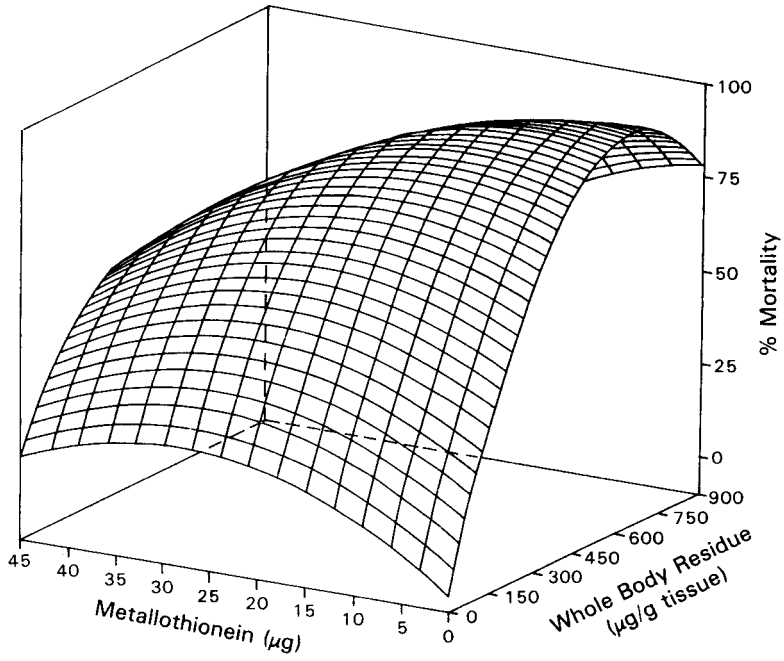

FIGURE 1.—Fitted response surface (quadratic polynomial) of the relation between mortality, whole-body cadmium residues, and metallothionein concentration in liver of brook trout exposed to cadmium for 30 d.

ence with normal assimilation of yolk proteins, and also delayed yolk absorption. Such delay was also reported in young bluegills *Lepomis macrochirus* exposed to cadmium (Eaton 1974). Exposure to near-lethal concentrations of cadmium did not interfere with metabolically important enzyme activities in several tissues of brown trout *Salmo trutta* and rainbow trout *Salmo gairdneri* (Roberts et al. 1979), mummichog *Fundulus heteroclitus* (Jackim et al. 1970), or winter flounder *Pseudopleuronectes americanus* (Gould 1975). The most profound effect of cadmium exposure is on the kidney; additional toxic effects occur in the nervous system (Shukla and Singhal 1984). Consequently, it was not surprising that we observed no effects on growth.

Overall, our study showed that concentrations as low as 3.6 µg Cd/L induced MT formation in liver, but not in kidney, after 30 d of exposure. Although MT was measured as a stress-induced response in other investigations of cadmium exposure to fish (Noel-Lambot et al. 1978; Reichert et al. 1979; Kito et al. 1982), sublethal effects such as whole-body residues and mortality were not reported; consequently, exposure concentration and duration are the only variables with which exposure effects can be compared. In the three studies cited, cadmium exposure resulted in increased MT concentrations in liver, kidney, and gill tissues, and in the gastrointestinal tract. The lowest cadmium exposure reported to induce MT formation was in common carp exposed to 13 µg Cd/L for 180 d by Kito et al. (1982). Their results and ours indicated that exposure to low concentrations of cadmium can increase MT concentrations in fish.

Assessment of the Spillover Hypothesis

In our study, the amount of free cadmium in liver and kidney represented about 85 and 79%, respectively, of the cadmium in the 100,000 × gravity supernatant. This free cadmium was probably bound to nonmetallothionein proteins or enzymes and, therefore, exerted a sublethal influence. Correspondingly, about 15% of the cadmium in the liver and 21% in the kidney was present in the unsaturated MT fraction, compared with the amount in the 100,000 × gravity supernatant. Concentrations of cadmium in the unsaturated MT fraction were low even though the quantitation of saturated MT indicated that a large binding capacity was potentially available. The cadmium-binding potential of MT was 16 times greater in liver and 31 times greater in kidney than the amount of cadmium bound to unsaturated MT. The large amount of free cadmium in liver and kidney and the correspondingly small amount of cadmium bound to unsaturated MT indicated that

MT did not bind all the cadmium present or remove it from potential biological availability to proteins and enzymes.

Measurement of saturated MT showed that metallothionein's potential cadmium-binding capacity was about three times greater in liver and about six times greater in kidney than the amount of cadmium present in the respective 100,000 × gravity supernatants. Only in the 60.6-μg Cd/L exposure did the amount of cadmium in the 100,000 × gravity supernatant exceed the potential binding capacity of MT.

The large discrepancy between the potential binding capacity of MT in liver and kidney and the amount of cadmium bound to it in an unsaturated condition suggested the presence of substantial amounts of metals other than cadmium. This deduction arises in part because MT has not been isolated with free sulfhydryl groups, which means that metals are bound to all available sites on MT (Kagi and Nordberg 1979). Copper and zinc are the most likely metals that would compete with cadmium for binding sites on MT, because one function of MT is the homeostatic storage of these essential metals. Copper and zinc are actively taken up through gills or by intestinal absorption in fish because they are essential elements in many metalloproteins. In the present study, copper and zinc were available for uptake by fish primarily from the diet; they were also available for uptake from exposure water, although only at concentrations less than 10 μg/L. In a study reported by Hamilton (1985), in which brook trout were exposed to low concentrations of cadmium, concentrations of copper and zinc in the 100,000 × gravity supernatant of liver were 50–100 times higher than cadmium, taken individually, and 130–160 times higher when combined. In this same study, the cadmium-binding potential of MT was 112 times greater in liver than the amount of cadmium bound to unsaturated MT (Hamilton et al. 1987). About 8% of the cadmium in the liver was bound to unsaturated MT, whereas about 92% was present as free cadmium, i.e., nonmetallothionein-bound.

Brown and Parsons (1978) reported that MT concentrations were not increased in chum salmon *Oncorhynchus keta* exposed to 5 μg Hg/L for 62 d; however, the ratio of copper plus zinc to mercury in the liver exceeded 300:1, which probably prevented mercury from stimulating increases in MT concentration. Copper and zinc had a competitive advantage for MT binding sites due to their greater abundance, even though their binding affinity was lower than that of mercury. Roch and McCarter (1984) reported that MT concentrations in liver increased up to sixfold in chinook salmon *Oncorhynchus tshawytscha* exposed to a metal mixture (Zn:Cu:Cd ratio of 400:20:1) for 21 weeks; the ratio of copper plus zinc to cadmium in the liver was 86:1. Although exposure to excess copper and zinc probably played the major role in inducing thionein synthesis and MT formation, the relatively low ratio of copper plus zinc to cadmium compared to the more than 300:1 ratio mentioned above probably increased the importance of cadmium in stimulation of MT formation.

Hamilton (1985) showed that MT concentrations in liver were significantly increased twofold in fish at ratios of copper plus zinc to cadmium of 150:1. This ratio is intermediate between the ratios of Roch and McCarter (1984) and Brown and Parsons (1978). Although different species and different metal stresses were used in these studies, the relation between the ratios and the relative change in MT concentrations is suggestive. At low ratios of 86:1, MT concentrations in exposed fish were six times higher than in controls; at intermediate ratios of 150:1, MT concentrations were two times higher than in controls; and at high ratios of over 300:1, no increase in MT was observed. This relation suggests that the ratio of copper plus zinc to cadmium or mercury indicates the detoxification effort of MT toward removing toxic metals. Simkiss and Taylor (1981) proposed a similar approach to using ratios of metal contaminants to essential metals in aquatic animals to assess contamination of the environment with metals. However, their approach was not specific to a metalloprotein such as MT but, rather, was concerned with the general accumulation of metals.

Although cadmium was the stimulus causing increased MT concentrations in our study, the natural concentrations of copper and zinc in the liver probably prevented cadmium from having a greater effect on MT concentration. Establishment of an equilibrium among cadmium, copper, and zinc in the various protein fractions of the liver must have favored copper and zinc because of their greater abundance, and counterbalanced the greater binding affinity of MT for cadmium than for zinc. After establishment of an equilibrium, the final metal content of MT may reflect only the relative binding affinities of MT and the abundance of the metals present (Brady et al. 1979).

Several investigators have reported that exposure of aquatic organisms to cadmium results in a mixture of metals bound to MT even under near-

lethal concentrations. Coombs (1975) reported that cadmium was not exclusively bound to MT in plaice *Pleuronectes platessa* exposed to 2 mg Cd/L for 28 d, but was distributed among other metalloproteins of the liver. Similar results have been reported for European eels *Anguilla anguilla* (Noel-Lambot et al. 1978), the mussel *Mytilus edulis* (Talbot and Magee 1978), threespine sticklebacks *Gasterosteus aculeatus* (Woodworth and Pascoe 1983), a dogfish *Scyliorhinus canicula* (Hidalgo et al. 1985), and rainbow trout (Olsson and Haux 1985). Results of these studies and ours indicate that cadmium binds to nonmetallothionein and MT fractions of liver and kidney of aquatic organisms that are exposed to a wide range of cadmium concentrations. Consequently, toxic effects of cadmium should occur as a continuum of stress responses from mild to severe and increase with increases in the amount of free cadmium (nonmetallothionein-bound) in tissues.

The spillover hypothesis states that pathological effects occur when MT becomes saturated by a metal (Winge et al. 1974), but the hypothesis does not account for the interrelations among the abundances of various metals or their relative binding affinities for MT. Results of our present study and Hamilton's (1985) study conflict with the spillover hypothesis because we demonstrated that exposure of fish to lethal concentrations of cadmium in water resulted in toxic effects regardless of the saturation state of MT. Our experiments also showed that a large potential cadmium-binding capacity was present in MT in the liver of fish exposed to all cadmium concentrations except 60.6 μg Cd/L. Results of the present study further showed that a large potential cadmium-binding capacity was present in the kidney tissue of fish.

On the basis of the preceding discussion, we believe that the spillover hypothesis should be redefined. Exposure to low concentrations of cadmium results in the binding of some cadmium to MT, but competition for binding sites by copper and zinc displaces most cadmium to other cellular components, resulting in toxic effects. At higher exposure concentrations, cadmium should bind to a greater extent to MT, but competition from copper and zinc for binding sites displaces proportionally larger quantities of cadmium, i.e., free cadmium, to other cellular components, thus resulting in increased toxic effects. Consequently, the relatively greater binding of cadmium to cellular components other than MT should result in toxic responses that depend on varying equilibria between the relative abundances of metals present and their respective binding affinities for MT.

"Spillover" is an unfortunate misnomer for describing the hypothesis linking the occurrence of pathological effects in animals stressed by metals to the inability of MT to sequester the metal stressor. The term spillover implies that a substance was briefly contained but overflowed its container because of additions of the substance. Use of this term implies that MT briefly binds a metal stressor such as cadmium, and then allows the metal to be displaced by additional metals. The flaw in this logic is that the metals are probably not bound to MT initially because there is competition for binding sites on MT, the intensity of which depends on two factors: the abundance of the competing metals, and the binding affinity of the metals for MT. Our results indicated that a metal stressor such as cadmium binds to MT only in proportion to its competitive advantage, which is small in the presence of abundant copper and zinc. Metallothionein is not exclusive for a specific metal except in extreme conditions such as those imposed by intraperitoneal injection (Day et al. 1984; Hamilton et al. 1987). Perhaps a more appropriate term for the redefined hypothesis would be the "metal-binding" hypothesis.

Metallothionein as an Indicator of Metal Stress

Development of biological indicator techniques to monitor the effects of environmental contaminants on fish is a relatively new field of research on aquatic contaminants; advantages and disadvantages of the techniques were reviewed by Mehrle and Mayer (1980). The objective of developing a biological monitoring tool is to use it to assess the effect of chemical contaminants on the health and well-being of aquatic organisms in laboratory and field investigations. Assessment of the utility of a biological indicator is based on the relation between the metabolic whole-animal responses and time–dose responses of the measurements.

In our experiments, MT concentrations in liver were significantly elevated above concentrations in control fish only in exposures that caused significant mortality. From this observation, one might conclude that MT concentrations in liver were related to an important whole-animal response, i.e., mortality, and, therefore, would be a useful biological monitoring tool. However, MT concentrations showed an "all or nothing" response in liver and no significant response in kidney. Both the poor relation between MT and mortality or whole-body residues and the lack of a dose-dependent response in MT concentrations, suggested that measurement of MT in liver or kid-

ney as a biological monitoring tool, by itself, is probably not worthwhile.

Although we showed that the measurement of MT may not be a sound indicator of early, mild exposure to a metal where a dose-dependent response is an important factor, MT concentrations may be a good indicator of long-term, severe exposure in fish populations. If, during an environmental monitoring process, MT concentrations in liver were found to be significantly elevated in a fish population relative to those in a control population, one could conclude that a metal stress was present. A more far-reaching conclusion would be that if MT were significantly elevated, mortality was probably occurring in the population as a result of the metal stress.

Perhaps a more useful biological indicator of cadmium toxicity would be the measurement of free-cadmium (nonmetallothionein-bound) concentrations in liver. The strong dose-dependent response shown by free cadmium and the strong relation with important whole-animal responses such as mortality and whole-body residues fulfill the criteria of a good biological monitoring tool for assessing cadmium toxicity.

Another useful biological indicator of cadmium toxicity in fish might be the determination of the ratio of copper plus zinc to cadmium in unsaturated MT. This ratio would indicate the prominence of cadmium on MT and the magnitude of the ratio would indicate the severity of the metal stress from cadmium.

As judged by our research, the monitoring of aquatic populations in habitats contaminated with cadmium should include isolation and measurement of various cadmium-containing protein fractions in liver. Comparison of these measurements would indicate the intoxification state from exposure to cadmium. Further research on MT in fish with mercury as the metal stressor might indicate that a similar approach would be useful in determining the intoxification state in populations exposed to mercury contamination.

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References

Brady, F. O., M. Panemangalore, F. A. Day, A. M. Fiskin, and G. Peterson. 1979. In vivo and ex vivo induction of rat liver metallothionein. Pages 261–271 *in* J. H. R. Kagi and M. Nordberg, editors. Metallothionein. Birkhauser Verlag, Basel, Switzerland.

Brown, D. A., C. A. Bawden, K. W. Chatel, and T. R. Parsons. 1977. The wildlife community of Iona Island jetty, Vancouver, B.C., and heavy-metal pollution effects. Environmental Conservation 4:213–216.

Brown, D. A., and T. R. Parsons. 1978. Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (*Oncorhynchus keta*) exposed to mercury in a controlled ecosystem. Journal of the Fisheries Research Board of Canada 35:880–884.

Cochran, W. G., and G. M. Cox. 1957. Experimental designs, 2nd edition. Wiley, New York.

Coombs, T. L. 1975. The significance of multielement analyses in metal pollution studies. Pages 187–195 *in* A. D. McIntyre and C. F. Mills, editors. Ecological toxicology research: effects of heavy metal and organohalogen compounds. Plenum, New York.

Day, F. A., A. E. Funk, and F. O. Brady. 1984. In vivo and ex vivo displacement of zinc from metallothionein by cadmium and by mercury. Chemico-Biological Interactions 50:159–174.

Eaton, D. L., and B. F. Toal. 1982. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Toxicology and Applied Pharmacology 66:134–142.

Eaton, D. L., and B. F. Toal. 1983. A simplified method for quantitating metallothionein in biological tissues. Science of the Total Environment 28:375–384.

Eaton, J. G. 1974. Chronic cadmium toxicity to the bluegill (*Lepomis macrochirus* Rafinesque). Transactions of the American Fisheries Society 103:729–735.

Gould, E. 1975. Alteration of enzymes in winter flounder, *Pseudopleuronectes americanus*, exposed to sublethal amounts of cadmium chloride. Pages 209–224 *in* F. J. Vernberg, A. Calabrese, F. P. Thurberg, and W. B. Vernberg, editors. Physiological responses of marine biota to pollutants. Academic Press, New York.

Hamilton, S. J. 1985. Metallothionein in brook trout (*Salvelinus fontinalis*) as a biological indicator of inorganic chemical contaminant stress. Doctoral dissertation. University of Missouri, Columbia.

Hamilton, S. J., and P. M. Mehrle. 1986. Metallothionein in fish: review of its importance in assessing stress from metal contaminants. Transactions of the American Fisheries Society 115:596–609.

Hamilton, S. J., P. M. Mehrle, and J. R. Jones. 1987. Cadmium-saturation technique for measuring metallothionein in brook trout. Transactions of the American Fisheries Society 116:541–550.

Hidalgo, J., L. Tort, and R. Flos. 1985. Cd-, Zn-, Cu-binding protein in the elasmobranch *Scyliorhinus canicula*. Comparative Biochemistry and Physiology C, Comparative Pharmacology 81:159–165.

Jackim, E., J. M. Hamlin, and S. Sonis. 1970. Effects of metal poisoning on five liver enzymes in the killifish (*Fundulus heteroclitus*). Journal of the Fisheries Research Board of Canada 27:383–390.

Kagi, J. H. R., and M. Nordberg, editors. 1979. Metallothionein. Birkhauser Verlag, Basel, Switzerland.

Kito, H., T. Tazawa, Y. Ose, T. Sato, and T. Ishikawa. 1982. Protection by metallothionein against cadmium toxicity. Comparative Biochemistry and Physiology C, Comparative Pharmacology 73:135–139.

McCarter, J. A., A. T. Matheson, M. Roch, R. W. Olafson, and J. T. Buckley. 1982. Chronic exposure of coho salmon to sublethal concentrations of copper. II. Distribution of copper between high- and low-molecular-weight proteins in liver cytosol and the possible role of metallothionein in detoxification. Comparative Biochemistry and Physiology C, Comparative Pharmacology 72:21–26.

McCarter, J. A., and M. Roch. 1983. Hepatic metallothionein and resistance to copper in juvenile coho salmon. Comparative Biochemistry and Physiology C, Comparative Pharmacology 74:133–137.

Mehrle, P. M., and F. L. Mayer. 1980. Clinical tests in aquatic toxicology: state of the art. Environmental Health Perspectives 34:139–143.

Mount, D. I., and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Research 1:21–29.

Noel-Lambot, F., C. Gerday, and A. Disteche. 1978. Distribution of Cd, Zn and Cu in liver and gills of the eel *Anguilla anguilla* with special reference to metallothioneins. Comparative Biochemistry and Physiology C, Comparative Pharmacology 61:177–187.

Olafson, R. W., A. Kearns, and R. G. Sim. 1979. Heavy metal induction of metallothionein synthesis in the hepatopancreas of the crab *Scylla serrata*. Comparative Biochemistry and Physiology B, Comparative Biochemistry 62:417–424.

Olsson, P.-E., and C. Haux. 1985. Rainbow trout metallothionein. Inorganica Chimica Acta 107:67–71.

Pruell, R. J., and F. R. Engelhardt. 1980. Liver cadmium uptake, catalase inhibition and cadmium thionein production in the killifish (*Fundulus heteroclitus*) induced by experimental cadmium exposure. Marine Environmental Research 3:101–111.

Reichert, W. L., D. A. Federighi, and D. C. Malins. 1979. Uptake and metabolism of lead and cadmium in coho salmon (*Oncorhynchus kisutch*). Comparative Biochemistry and Physiolgy C, Comparative Pharmacology 63:229–234.

Roberts, K. S., A. Cryer, J. Kay, J. F. de L. G. Solbe, J. R. Wharfe, and W. R. Simpson. 1979. The effects of exposure to sub-lethal concentrations of cadmium on enzyme activities and accumulation of the metal in tissues and organs of rainbow and brown trout (*Salmo gairdneri,* Richardson and *Salmo trutta fario* L.). Comparative Biochemistry and Physiology C, Comparative Pharmacology 62:135–140.

Roch, M., and J. A. McCarter. 1984. Metallothionein induction, growth, and survival of chinook salmon exposed to zinc, copper, and cadmium. Bulletin of Environmental Contamination and Toxicology 32: 478–485.

Roch, M., J. A. McCarter, A. T. Matheson, M. J. R. Clark, and R. W. Olafson. 1982. Hepatic metallothionein in rainbow trout (*Salmo gairdneri*) as an indicator of metal pollution in the Campbell River system. Canadian Journal of Fisheries and Aquatic Sciences 39:1596–1601.

Rombough, P. J., and E. T. Garside. 1982. Cadmium toxicity and accumulation in eggs and alevins of Atlantic salmon *Salmo salar*. Canadian Journal of Zoology 60:2006–2014.

Shaikh, Z. A., and K. Hirayama. 1979. Metallothionein in the extracellular fluids as an index of cadmium toxicity. Environmental Health Perspectives 28:267–271.

Shukla, G. S., and R. L. Singhal. 1984. The present status of biological effects of toxic metals in the environment: lead, cadmium, and manganese. Canadian Journal of Physiology and Pharmacology 62: 1015–1031.

Simkiss, K., and M. Taylor. 1981. Cellular mechanisms of metal ion detoxification and some new indices of pollution. Aquatic Toxicology (Amsterdam) 1:279–290.

Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods, 6th edition. Iowa State University Press, Ames.

Stroganov, N. S. 1967. Role of environment in the plastic metabolism of fish. Pages 35–47 *in* G. S. Karzinkin, editor. Metabolism and biochemistry of fishes. Indian National Scientific Documentation Centre, New Delhi.

Talbot, V., and R. J. Magee. 1978. Naturally-occurring heavy metal binding proteins in invertebrates. Archives of Environmental Contamination and Toxicology 7:73–81.

Winge, D., J. Krasno, and A. V. Colucci. 1974. Cadmium accumulation in rat liver: correlation between bound metal and pathology. Pages 500–502 *in* W. G. Hoekstra, J. W. Suttie, H. E. Ganther, and W. Mertz, editors. Trace element metabolism in animals, volume 2. University Park Press, Baltimore.

Woodworth, J., and D. Pascoe. 1983. Induction of cadmium-binding protein in the three-spined stickleback. Aquatic Toxicology (Amsterdam) 3:141–148.

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