Cadmium and Copper Metallothioneins in the American Lobster, *Homarus americanus*

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Lobsters were fed cadmium-rich oysters for 28 days, and the induction of cadmium metallothionein and its relation to concentrations of cadmium, copper, and zinc in the digestive gland and gills was determined. A portion of the tissues also was retained for determining the cytosolic distribution of these metals by gel filtration and ion-exchange chromatography. The digestive gland contained a majority of the cadmium, copper, and zinc, and both cadmium and zinc were actively accumulated from the oysters. Gel chromatography of the digestive gland cytosol showed that initially cadmium and zinc were bound to macromolecules with molecular weights of $> 70,000$, $\sim 45,000$ and < 5000, and for copper $> 70,000$, $10,000–7,000$, and < 5000. Therefore, only copper was bound to a protein with a molecular weight in the range of metallothionein (i.e., $10,000–7,000$). However, after feeding on cadmium-laden oysters for 28 days, both cadmium and copper were bound to the metallothioneinlike protein. Further purification of the cadmium/copper protein by ion-exchange chromatography showed that a large portion of the copper and all of the cadmium did not bind to DEAE–Sephacel. The induction of cadmium metallothionein in the digestive gland is correlated with tissue cadmium concentration. There is, however, a tissue threshold concentration of cadmium of 80 to 100 μg Cd/g wet weight required for induction. Coincident with the induction of the cadmium metallothionein was a cytosolic redistribution of copper. The distribution of zinc was not affected.

Crustaceans (*1–6*) and particularly the American lobster, *Homarus americanus* (*7,8*), concentrate metals, such as cadmium, copper, and zinc in their digestive glands (i.e., hepatopancreas). In addition to being a possible direct hazard to the organism, these metals also may be hazardous to organisms that consume digestive glands of crustaceans, including man. In the case of the lobster, such a situation already has occurred near Belledune Harbor, New Brunswick, Canada (*7,9*). Here, digestive glands from local animals were shown to contain up to 203.7 μg/g wet weight of cadmium (*9*). These levels were considered a health hazard to the local population, who consumed the digestive gland. A similar situation also has occurred for blue crabs, *Callinectes sapidus*, (*1,2*) from the Hudson River, where animals from Foundry Cove had cadmium concentrations in the digestive glands of up to 20 μg/g wet weight.

Because lobsters can accumulate large quantities of metals in the digestive gland, a physiologically active detoxification system must be present to sequester the accumulated metals, such as cadmium, copper, and zinc. It is, therefore, of interest to determine the chemical forms of these metals in the tissue. In our earlier research, we demonstrated that lobsters accumulate cadmium more efficiently from food than from water (*8,10*). Also the lobster has m etal-binding proteins in the diges-

tive gland cytosol that bind cadmium, copper, and zinc (*8,10*). An elution profile of the cytosol from the digestive gland of a lobster exposed to cadmium for 14 days showed that cadmium and zinc were bound to macromolecules with relative molecular weights M_r of $> 70,000$, $\sim 45,000$, and < 5000 (Fig. 1) (*8*). Unlike the metal-binding proteins in the blue crab digestive glands (*2,11*), only copper was bound to a protein that appeared to have the characteristics of metallothionein, i.e., low molecular weight $< 10,000$, high cysteine content, high affinity for cadmium, copper, or zinc, and low concentration of aromatic amino acids (*12*). Ray and White (*9*), however, demonstrated the presence of a 10,000 M_r cadmium-binding protein in lobsters from Belledune Harbor that had very high concentrations of cadmium in the digestive gland. Their analysis of the cadmium protein, however, was carried only as far as gel filtration.

Our current experiments were designed to determine if a cadmium metallothionein could be induced in lobsters and whether its induction could be related to the digestive gland concentration of cadmium, copper, or zinc.

Methods and Materials

All lobsters used in these experiments were collected in Long Island Sound, Connecticut and air-shipped to Beaufort, NC. A group of 10 animals was maintained in a flowing water tank with a recirculating pump to maintain the temperature at 15°C and the salinity <

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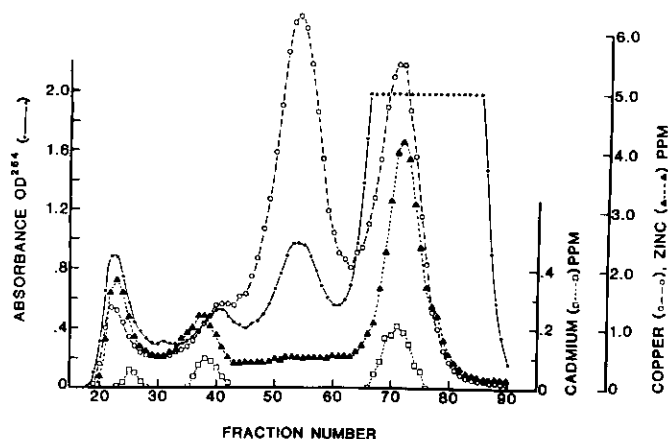†Duke University Marine Laboratory, Marine Biomedical Center, Beaufort, NC 28516.

FIGURE 1.   Representative Sephadex G-75 elution profile of the digestive gland cytosol from a lobster exposed to dissolved cadmium for 14 days. Modified from Engel and Brouwer (10).
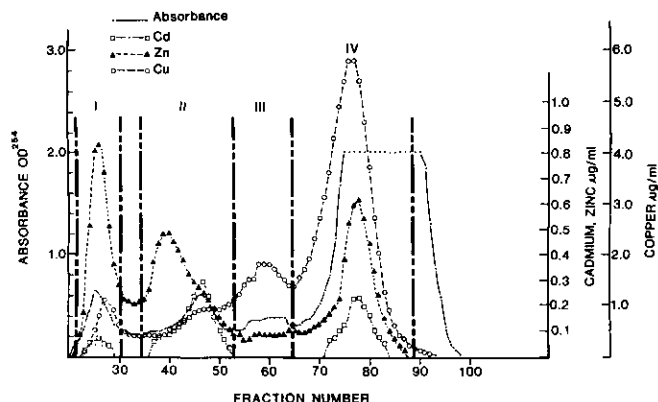

FIGURE 2.   Typical Sephadex G-75 elution profile of the cytosolic fraction of the digestive gland from an unexposed lobster. The four molecular weight regions represent portions of the elution profile where (cadmium, copper and zinc)-binding macromolecules are present: I (high molecular weight, > 70,000 M_r); II (intermediate molecular weight, 45,000–15,000 M_r); III (metallothionein region, 15,000–5,000 M_r); IV (very low molecular weight, < 5000 M_r). The metal-containing peak areas within these specified regions were integrated and used in calculations of percent abundance.

30‰. The lobsters were fed oysters that contained elevated concentrations of cadmium (i.e., cadmium, 88.5 ± 23; copper, 19 ± 2; and zinc 668 ± µg/g wet weight of whole oyster). The feeding was done en masse every other day for up to 28 days. Two or three individuals were sampled after 7, 14, 21, and 28 days of exposure.

The lobsters were killed by removing the carapace, and the digestive gland and gills were dissected out. Half of each tissue sample was used to determine total metal concentrations; the remainder was stored at −60°C before determining metal-binding proteins. Soluble metal-binding proteins were isolated from these tissues according to the modified procedure of Ridlington and Fowler (13). Tissues were homogenized in a Brinkman Polytron homogenizer at high speed in 1.5 volumes of 20 mM Tris buffer, pH 7.9, and 5 × 10^{-4} M phenylmethyl sulfonylfluoride (PMSF) at 4°C, and then centrifuged at 30,000g for 30 min at 4°C. The supernatant was heat-treated at 60°C for 10 min, cooled in ice to 0°C, and then centrifuged again at 30,000g for 30 min before being applied to a Sephadex G-75 gel filtration column (column 64 cm × 2.6 cm). The elutant was 20 mM Tris buffer, pH 7.9. The column was calibrated using molecular weight standards (blue dextran, M_r > 10^6; bovine serum albumin, M_r 6.7 × 10^4; ovalbumin, M_r 4.3 × 10^4; chymotrypsinogen A, M_r 2.5 × 10^4; and ribonuclease, M_r 1.37 × 10^4). For further purification, fractions in the 10,000 M_r region of the G-75 elution profile were pooled and applied directly to a DEAE–Sephacel ion-exchange column (2.6 × 15 cm). Proteins were eluted with a gradient generated from 500 mL 20 mM Tris and 500 mL 400 mM Tris both at pH 7.9. Fractions were analyzed for absorbance at 254 nm, and metals were measured by flame aspiration atomic absorption spectrophotometry.

The metallothioneinlike proteins isolated by ion-exchange chromatography were concentrated on an Amicon UM-2 filter and dialyzed against distilled water. The protein was then lyophilized and analyzed for amino acid composition after performic acid oxidation (i.e., amino acid analysis done by Sequemat, Watertown, Mass.). Each of the G-75 elution profiles was divided into four

areas as shown in Figure 2. Each peak which contained cadmium, copper, or zinc was rounded by eye, and the percentage of the total area was determined for each peak by planimetry for each individual lobster.

Tissue samples were analyzed for cadmium, copper, and zinc by using standard atomic absorption spectrophotometric techniques. Samples were dried at 90°C, wet-ashed in concentrated HNO_3, and then diluted and analyzed by flame aspiration atomic absorption spectrophotometry.

The National Bureau of Standards Oyster Reference Material #1566 was used to calibrate the zinc, cadmium, and copper measurements. The certified concentrations in the Standard Reference Material were 852 ± 14 for zinc, 3.5 ± 0.4 for cadmium, and 63 ± 3.5 µg/g for copper (± refers to the 95% confidence interval around the mean value). Mean concentrations for 10 replicate aliquots of the standard measured in our laboratory were 843 ± 63 µg/g for zinc, 3.4 ± 0.4 µg/g for cadmium, and 60.2 ± 3.2 µg/g for copper. Our mean values, therefore, are not significantly different from the specified elemental concentrations.

Results and Discussion

The results of the present experiments with lobsters fed cadmium-laden oysters showed that individual lobsters accumulated both cadmium and zinc in the digestive gland and gills but that it was not necessarily a direct-time dependent process (Table 1). The lack of uniformity in the accumulation of these two metals was undoubtedly related to feeding and individual physiological differences among the lobsters. The concentrations of both metals in individual digestive glands increased by about seven to nine times relative to the initial concentrations in the first 7 days and then increased for the remainder of the experiment. Copper concentrations also increased over

Table 1. Concentrations of cadmium, copper, and zinc in the digestive glands and gills of individual lobsters fed on oysters laden with cadmium for 28 days.[a]

Days of feeding	Digestive gland			Gills		
	Cd	Cu	Zn	Cd	Cu	Zn
8	82[b]	1082	217	1.4	23	15
	24	401	40	0.9	52	15
14	104[b]	334	237	3.3	35	18
	72	937	140	1.2	22	14
21	83	1120	203	1.4	58	15
	198[b]	841	333	8.8	36	21
28	215	685	414	3.1	20	16
	236[b]	1339	746	2.7	12	14
	81	1064	168	1.4	34	13
Unfed (5)[c]	9.3 ± 1.5	559 ± 50	31 ± 1.3	1.0 ± 0.1	34 ± 3.0	12 ± 0.7

[a] All metal concentrations are in $\mu g/g$ wet weight of tissue.
[b] Lobster digestive glands used in chromatographic separations shown in Figure 4.
[c] These data are from five lobsters that were not fed an oyster diet. Mean $\pm$ SE

the period of exposure, but not as markedly (i.e., about a factor or two). In the gill tissue the concentrations of all three metals remained relatively constant with only a slight increase in cadmium. In this experiment, it is important to emphasize that even though cadmium is the primary metal of interest, the feeding on oysters that have naturally high levels of zinc caused an imbalance in zinc intake. This imbalance resulted in an increase in tissue zinc concentration. A similar result was noted previously in our research with lobsters (8). Also, in agreement with our previous experiments, no significant uptake of cadmium, copper, or zinc was observed in the abdominal muscle of the treated animals (8,10).

The relationships between cadmium concentration and copper and zinc concentrations in the digestive gland are shown in Figure 3. In the copper/cadmium comparison there is significant, positive correlation between copper and cadmium concentrations in digestive glands ($r = 0.46$, $p < 0.05$), but the relationship is weak (Fig. 3A). There appears to be a threshold level at about 100 $\mu g/g$, above which additional cadmium tends to disrupt copper accumulation into the digestive gland. The linear relation between cadmium and zinc (Fig. 3B) is much stronger ($r = 0.83$, $p < 0.01$) and holds throughout the range of tissue cadmium concentrations. Such a relation would suggest a possible linkage between cadmium and zinc accumulation in the digestive gland of the lobsters.

The Sephadex G-75 elution profiles of the cytosols prepared from the digestive glands of exposed lobsters showed differences both with time of exposures and tissue cadmium concentration (Table 1 and Fig. 4). The cytosolic profile from an animal exposed 7 days (Fig. 4A) showed the distribution of metals that was similar to those that we have observed previously (8,10). As shown in Figure 1, the only metal bound to a protein in the metallothionein region, 10,000 M_r, was copper, whereas cadmium and zinc were bound to macromolecules with molecular weights $M_r > 70,000$, (75–125 mL), ~ 45,000 (220–280 mL), and < 5000 (> 300 mL). Also, the majority

of the copper was bound to a material of $M_r < 5000$. By 14 days (Fig. 4B) there was a suggestion of a cadmium-binding protein peak in the 10,000 M_r region of the profile. Also, there was a lack of copper in the material $M_r < 5000$. By the 21st and 28th day (Figs. 4C and 4D) of feeding, the amount of cadmium in the M_r 10,000 region of the elution profile was significant in both cases. From the data presented here there appears to be a linkage between cytosolic distribution of cadmium and copper, and to a much lesser extent between cadmium and zinc. This relation is much different from that observed in the whole digestive gland where zinc and cadmium concentrations were highly correlated (Fig. 3B).

Ion-exchange chromatography and subsequent amino acid analysis of the copper/cadmium binding protein showed that the protein had a cysteine content of 15%. A more extensive purification of this same protein showed it to be a mixture of isometallothioneins, with different cysteine contents. The full analysis of this protein will be discussed in a following paper (14).

To further analyze the relationship between the induction of cadmium metallothionein and the tissue concentration of cadmium, the G-75 elution profiles were integrated, and the percentages of the total cytosolic metal under each peak were determined. The percentages of cadmium and copper under peak III (the metallothionein region) and copper under peak IV (the very low molecular weight material) (see Fig. 2) were calculated and related to the total cadmium concentration in the digestive gland. The relationship between total cadmium concentration and the percent in the metallothionein region of the chromatograms reinforces the hypothesis that a threshold cadmium concentration is necessary before cytosolic cadmium is bound to metallothionein (Fig. 5). The apparent threshold for the induction of a cadmium/copper metallothionein appears to be between 80 and 100 $\mu g/g$ total tissue cadmium. When a similar relation is plotted for the percentage of copper under the metallothionein peak, a similar sigmoidal re-
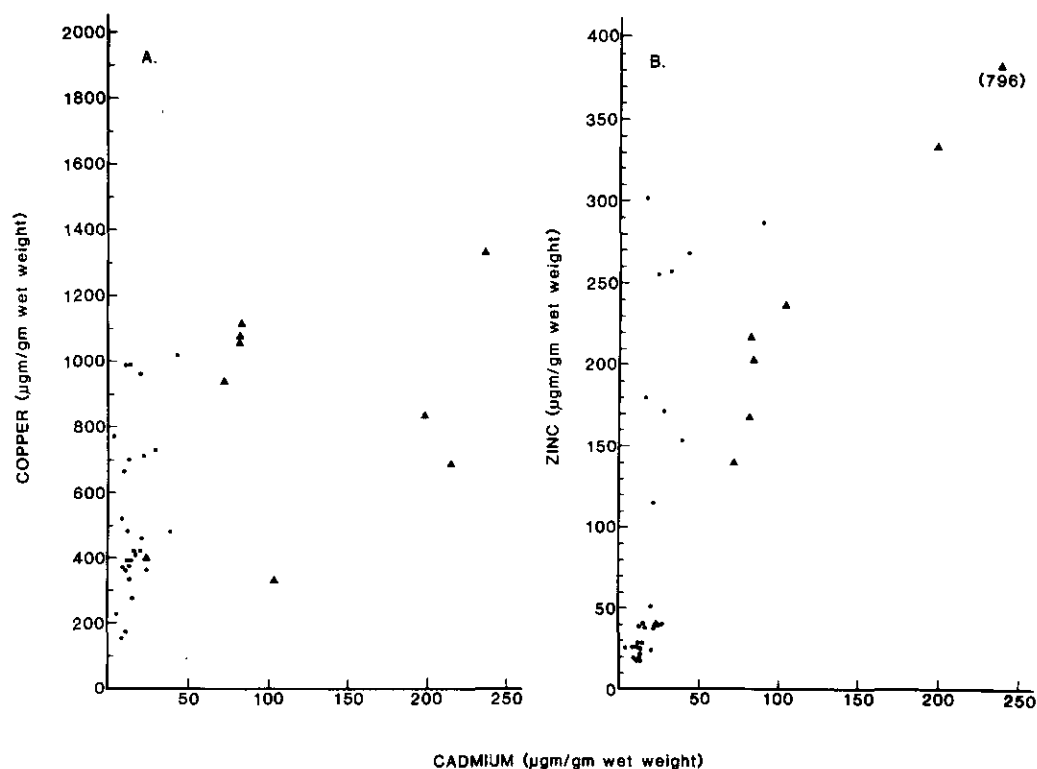
FIGURE 3.   Relation between total concentrations of (A) cadmium and copper or (B) zinc in digestive gland for lobsters (▲) from this series of experiments and (●) from all lobsters examined in previous experiments. Each point represents a single observation.
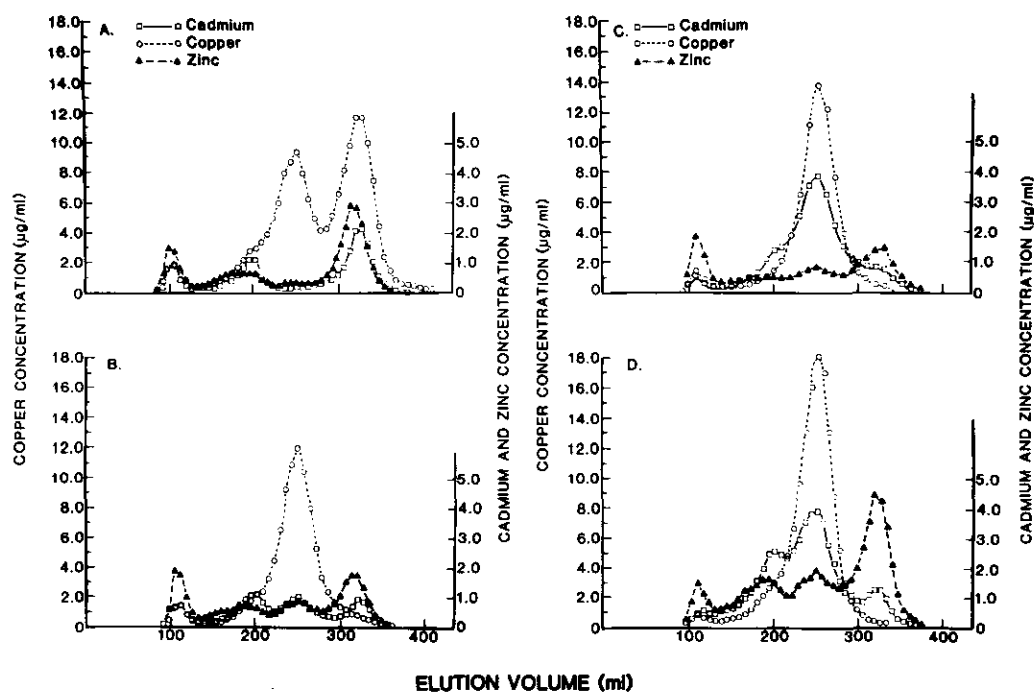

FIGURE 4.   Sephadex G-75 elution profiles of the cytosolic distributions of cadmium, copper, and zinc from digestive glands of lobsters fed cadmium-laden oysters for (A) 7 days, (B) 14 days, (C) 21 days, and (D) 28 days. Each individual tissue selected had the highest cadmium concentration for the specified sampling time and total metal concentrations shown in Table 1.
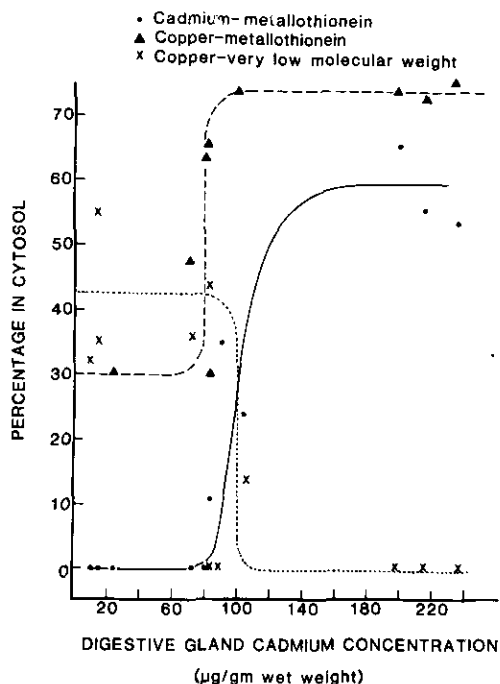
FIGURE 5. Relationship between the percentages of cytosolic cadmium and copper metallothionein and copper in the very low molecular weight fraction and the total cadmium concentration in digestive glands of cadmium-exposed lobsters. The metallothionein area is the (III) region and the very low molecular weight area is the (IV) region of the G-75 elution profile (see Fig. 2).

lationship emerges. The primary difference between the cadmium and copper metallothioneins is that there is an ambient level of copper metallothionein in all lobsters examined (i.e., ~ 35% of cytosolic copper), and the increases in the percentages of bound copper indicate that there is an increase of *de novo* synthesis of metallothionein induced by cadmium. When the percentages of copper in peak IV are related to tissue cadmium concentration, the amount of copper in the very low molecular weight fraction decreased with increased cadmium. Such a relationship indicated that the accumulation of cadmium in the digestive glands of these lobsters triggered a cytosolic redistribution of copper in the tissue. Even though copper concentrations in the digestive glands of lobsters that had highest cadmium concentrations were highly variable, i.e., 334–1339 μg/g (Table 1), percentages of copper metallothionein were high, and no significant amounts of copper were observed in the very low molecular weight fractions of the elution profiles. Throughout the feeding period the zinc concentrations in the digestive glands of lobsters tended to increase (Table 1), but the cytosolic distribution of the metal did not change markedly. It appears, therefore, that the only interactions among the three metals in the cytosol involve cadmium and copper.

The results of this investigation have shown that the concentrations of cadmium and zinc in the individual digestive glands of fed lobsters increased throughout the experiment but were extremely variable, and that the induction of a cadmium-containing metallothionein was

dependent upon the tissue concentration of cadmium. In other investigations with crustaceans, it was assumed that the induction of cadmium metallothionein was immediate (4,15,16), as was shown to occur in the gills of the blue crab (11). In many of these other investigations, however, injection was used as the mode of exposure rather than having the metal incorporated into food or dissolved in water. In our investigations, where food was the vehicle of exposure, there was a threshold concentration of tissue cadmium that was required before cadmium metallothionein was synthesized. The threshold effect may have been partially due to the naturally high concentrations of copper in the digestive gland (Table 1). In all of the lobsters that we have used (8,10), the concentrations of copper in the digestive glands have always been high and extremely variable, < 200 to 1000 μg copper per gram wet weight of tissue, which may indicate elevated copper concentrations in food and/or water at their site of collection, Long Island Sound. It is our hypothesis that food is the source of copper, since our previous experiments showed very poor accumulation of metal from water (8). The possibility that copper concentrations are elevated in the digestive glands is supported by measurements recently made of the metal contents of five lobsters from New Brunswick, Canada. While the cadmium concentrations were higher than those for Long Island Sound lobsters, the mean copper concentration in the digestive glands was 132 ± 54 μg/g wet weight, which is much lower. A possibility, therefore, exists that the induction-threshold phenomenon observed in our lobsters may have been caused by cadmium–copper competition for sites on newly synthesized metallothionein molecules. Before such a hypothesis can be substantiated, further information on lobsters with low copper concentrations in the digestive gland must be collected.

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