Characterization Studies on the Cadmium-Binding Proteins from Two Species of New Zealand Oysters

by Monica Nordberg,† Iris Nuottaniemi,*‡ M. George Cherian,*‡ Gunnar F. Nordberg,* Tord Kjellström,§ and Justine S. Garvey¶

Two different types of New Zealand oysters—*Ostrea lutaria* (OL) and *Crassostrea glomerata* (CG)—contained different concentrations of zinc, copper, and cadmium. OL oysters had 5.3 µg Cd/g, 3.4 µg Cu/g, 100 µg Zn/g; CG oysters had 1.4 µg Cd/g and 936 µg Zn/g. Both kinds of oysters were shown by gel filtration (G-75) to contain cadmium and zinc in fractions corresponding to a high molecular weight protein (corresponding to the size of albumin or larger) which was heat labile. OL oysters contained cadmium in fractions corresponding to a molecular weight of approximately 6500. The cadmium-binding protein in these fractions was heat-stable. This protein contained no detectable amounts of zinc and was not present in the CG oysters. Further purification by gel filtration (G-50) was performed to obtain a purer protein fraction. Isoelectric focusing of the protein obtained by G-50 filtration showed one main fraction of protein with a pI ~ 5.9 at ~ 13°C. CG oysters contained cadmium and zinc in a polypeptide with low molecular weight (MW 1000).

The cadmium-binding oyster proteins are minimally reactive in a competitive binding radioimmunoassay in comparison to the reactivity of a typical vertebrate metallothionein; the proteins may be metallothioneins, but, if so, they do not exhibit the principal determinants characteristic of vertebrate metallothioneins.

Introduction

The major source of environmental exposure to cadmium in the general population is food, with little contribution from air and water (1). Cadmium is present mainly in shellfish, beef liver and kidney, certain vegetables, and cereal crops. The concentration of cadmium in these foods can vary depending on their source. The increases in use of sewage sludge for soil treatment and phosphate fertilizers are probably the main reasons for the increased content of cadmium in soil (1). In combination with soil acidity this can give rise to an increase in content of cadmium in food and therefore in intake of cadmium in humans. In order to evaluate the extent to which cadmium represents a threat to the environment in general and to human health in particular, a number

of factors should be considered. Some of these factors involve the form of cadmium in the food, its absorption from the gastrointestinal tract, tissue distribution and retention, and also interaction with other metals.

It is generally considered that renal tubular damage is the critical effect of cadmium in humans after long-term exposure to high amounts of cadmium (1,2). In animal studies the critical concentration for such damage may vary from 10 to 200 µg Cd/g kidney cortex (wet weight), depending upon exposure pattern and the form of cadmium administered. Therefore, it is important to study various factors influencing the movement and deposition of cadmium in the kidney in order to prevent cadmium-induced renal disease. The biocomplexes of cadmium are particularly important in determining toxicity and metabolism because of specific binding of Cd to metallothionein (3,4) a low molecular weight protein with high content of cysteine. A selective and rapid deposition of cadmium in kidney has been shown in animals given cadmium as metallothionein intravenously (5). A polymer of cadmium-metallothionein has a longer biological half-time in blood than both cadmium bound to nonpolymerized metallothionein and cadmium administered as soluble salts (6). Although involved in the pathogenesis of cadmium-induced renal damage, metallothionein may

---

*Department of Environmental Medicine, Umeå University, S-901 87 Umeå, Sweden.

†Present address: Department of Environmental Hygiene, Karolinska Institute, S-104 01 Stockholm, Sweden.

‡On sabbatical leave from Department of Pathology, Health Sciences Centre, London, Canada, N6A, 5C1.

§Department of Community Health and General Practise, University of Auckland, Auckland, New Zealand.

¶Department of Biology, Syracuse University, Syracuse, New York 13210, U.S.A.

also play a protective role against cadmium toxicity (7) under certain experimental conditions.

Preliminary studies (8,9) suggest that, upon oral exposure, a portion of cadmium-metallothionein may be absorbed intact (10) from the gastrointestinal tract in experimental animals and deposited mainly in the kidney, the critical organ in long-term cadmium toxicity. Therefore, it may be important to characterize the dietary forms of cadmium. It has been reported that oysters from certain areas of New Zealand have a very high content of cadmium (11). This may be a useful source for characterization of naturally occurring dietary cadmium. The fishermen who consume large numbers of these oysters can be considered as one of the highest cadmium intake groups in the world and are being studied to evaluate the effects of increased dietary cadmium intake (12). The characterization of the form of cadmium in the oysters is essential for interpreting the results of the epidemiological study. There have been several reports on the form of cadmium in shellfish (13–17). However, most of these studies have been performed after exposure of collected shellfish to different concentrations of cadmium. Some data on concentration of naturally occurring cadmium in shellfish are available for Pacific oysters (18). Low molecular weight cadmium-binding proteins have been reported in American oysters (13), and it has been suggested that these proteins have an amino acid composition different from metallothionein (MT). Although metallothionein from other crustaceans displays a similar amino acid composition as mammalian MT, they differ in the nature of their metal clusters (19). It was reported that MT-1 from *Scylla serrata* contains two distinct three-metal divalent clusters, unlike mammalian MT which contains one three-metal and one four-metal cluster.

The present investigation was undertaken to study the binding of metals (Cd, Zn, and Cu) in New Zealand oysters containing different amounts of these metals (20).

Experimental

Material and Methods

Oysters were collected from two different areas of New Zealand, Foveaux Strait (*Ostrea lutaria*, type OL) and Waiheke Island (*Crassostrea glomerata*, type CG). The oysters were immediately frozen in dry ice and hexane, packed, and stored at −70°C. They were transported in dry ice to Umeå, Sweden, where they arrived in a frozen condition with dry ice. The study was split into pilot and main studies. All chemicals used in this study were of high analytical grade. Glass-distilled, deionized water was used in order to avoid any contamination in metal analyses. In order to determine the total concentration of metals, the oysters were hydrolyzed in concentrated nitric acid (Merck, Darmstadt, Germany) overnight at room temperature and then heated in a water bath after addition of a few drops of 30% hydrogen peroxide (Merck, Darmstadt, Germany). These samples were analyzed for cadmium and zinc in an atomic absorption spectrophotometer (Varian AA-875, Varian Techtron Pty. Ltd., Aus-

tralia) using an air-acetylene flame. Standards were prepared from $ZnSO_4 \cdot 7H_2O$ (Merck, Darmstadt, Germany), $CdCl_2 \cdot 2.5\ H_2O$ (Mallinckrodt AR) and metallic copper powder (PA). The background cadmium, copper, and zinc levels in the nitric acid were also tested.

Pilot Study

Two oysters (about 14 g), partly thawed, of each type were homogenized (50%) in an Ultraturrax (Janke and Kunkel KG, Staufen i. Breis o., Germany), in 0.25 M sucrose (BO Chemicals LTD, AnalaR) (Merck, Darmstadt, Germany), 0.1 M Tris-HCl, pH 8.6, containing 10^{-3}M PMSF (Phenylmethylfluorosulfonate, Boehringer Mannheim GmbH, Germany), a protease inhibitor. The homogenates were divided into two parts. One portion was centrifuged at 105,000g for 1 hr in a Beckman L5 ultracentrifuge and the other portion was heated at +80°C for 2 min in a water bath before centrifugation (105,000g for 1 hr). These steps were performed at +4°C. The supernatants were fractionated on a calibrated Sephadex G-75 (Superfine, Pharmacia Fine Chemicals, Uppsala, Sweden) column (25 × 390 mm) and 5-mL fractions were collected in an LKB Ultrarac (LKB-Instruments, Stockholm). The column was eluted with a buffer consisting of 0.01 M Tris-HCl, 0.05 M NaCl, pH 8.0, at a flow rate of 16 mL/hr. The elution profile was monitored at 254 nm in an LKB UV-cord II (LKB-Instruments, Stockholm). All the fractions were analyzed for cadmium and zinc by atomic absorption spectrophotometer. All the analyses were repeated at least twice.

Rat blood was centrifuged at 20,000 rpm (microcentrifuge 1555L O. Dich, AB Ninolab Upplands Väsby, Sweden) in order to separate erythrocytes from plasma. The erythrocytes were hemolyzed with distilled water, and the hemoglobin purified by another centrifugation. The rat hemoglobin solutions were mixed with 105,000g supernatant of the OL oysters extracted as above. The mixture was heated at +80°C for 2 min in a water bath before centrifugation (105,000g for 1 hr). This supernatant was then fractionated on a Sephadex G-75 column.

Main Study

In order to prepare a large quantity of cadmium-binding protein for further characterization studies, 69 g of OL oysters were homogenized in buffer with protease inhibitor, ultracentrifuged (105,000g, 1 hr) and then fractionated on a column (50 × 700 mm) packed with Sephadex G-75 (superfine) as described previously. All fractions were analyzed for cadmium, zinc, and copper. Absorption was measured at 250 and 280 nm on a spectrophotometer (Model 25, Beckman Instruments, USA).

Samples (10, 1.0, 0.1, 0.01, and 0.001 μL aliquots) from the different protein peaks of the G-75 Sephadex chromatography were assayed in a competitive binding radioimmunoassay (RIA) for metallothionein (21, 22).

The indicated low molecular weight fractions (Fig. 2) were pooled and concentrated on an Amicon cell with a UM-2 filter. They were further separated on a column
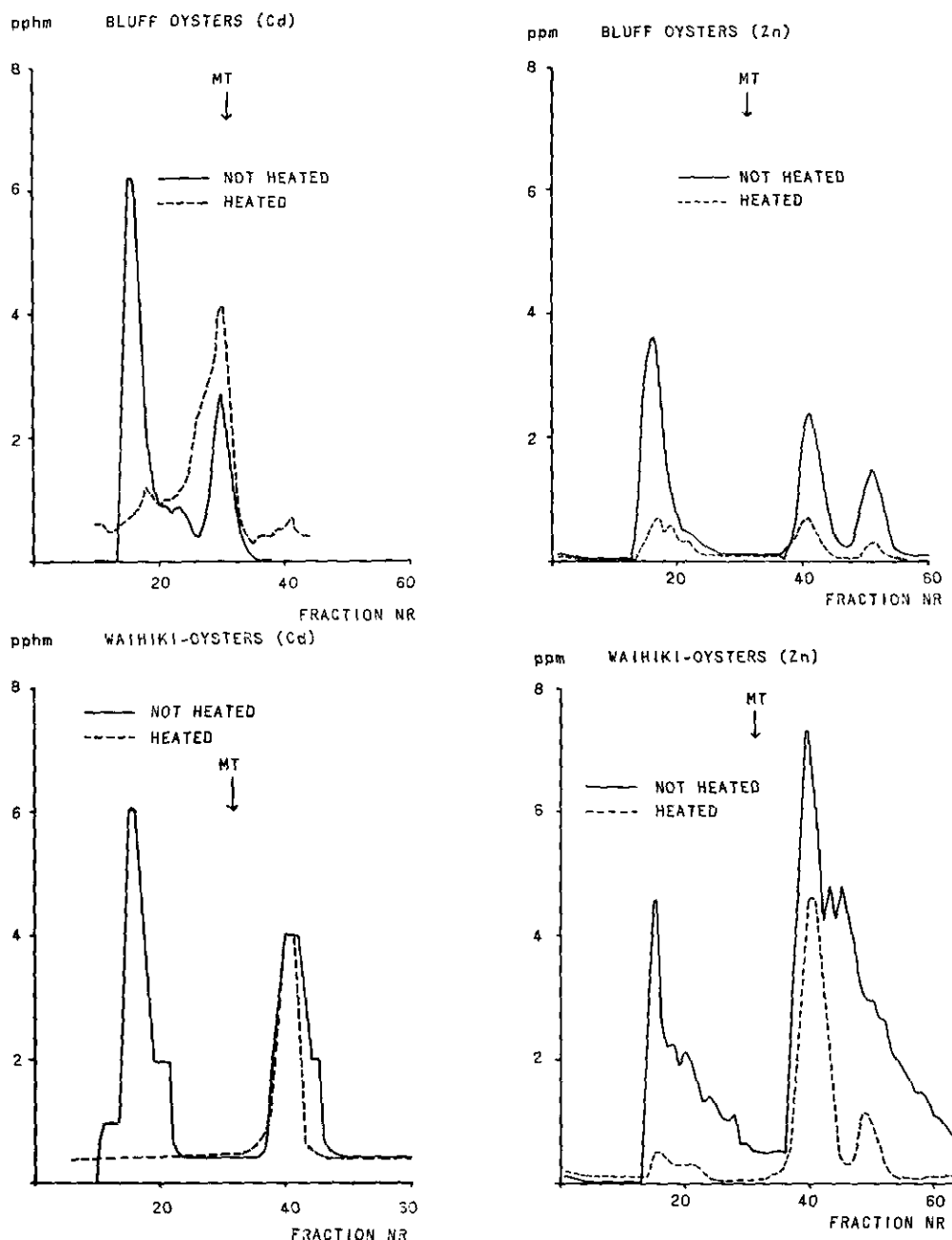
FIGURE 1. Gel chromatography on Sephadex G-75 of (A) dredge oysters *Ostrea lutaria*, OL (Bluff) (A) and (B) rock oysters *Crassostrea glomerata*, CG (Waiheke). The column dimensions were 25 × 390 mm. Elution with 0.01 M Tris-HCl in 0.05 M NaCl, pH 8.0, at a flow rate of 16 ml/hr (pilot study): (——) not heated; (--) heated.

packed with Sephadex G-50 (superfine), dimensions 26 × 381 mm.

Fractions containing cadmium from gel chromatography on Sephadex G-50 (Fig. 3) were pooled, concentrated, and washed before further characterization by isoelectric focusing. The concentrated protein solution contained 5.6 μg cadmium, 2.8 μg zinc, and 3.7 μg copper.

The isoelectric focusing was performed as described previously by Nordberg (4). The protein solution was applied in a 110-mL column equipped with a double cooling jacket (LKB-Produkter, Stockholm) by using a sucrose gradient (0–50 %, w/v).

The focusing was performed at 13°C and with a power less than 1.4 W. The contents of the column were collected in 2.5 mL fractions. These were analyzed for cadmium and zinc content.

Detailed information on isolation procedure is provided on figure legends. The recoveries of metals from the columns were more than 95%.

Results

The partial characterization of the cadmium and zinc binding ligands showed differences in two different types
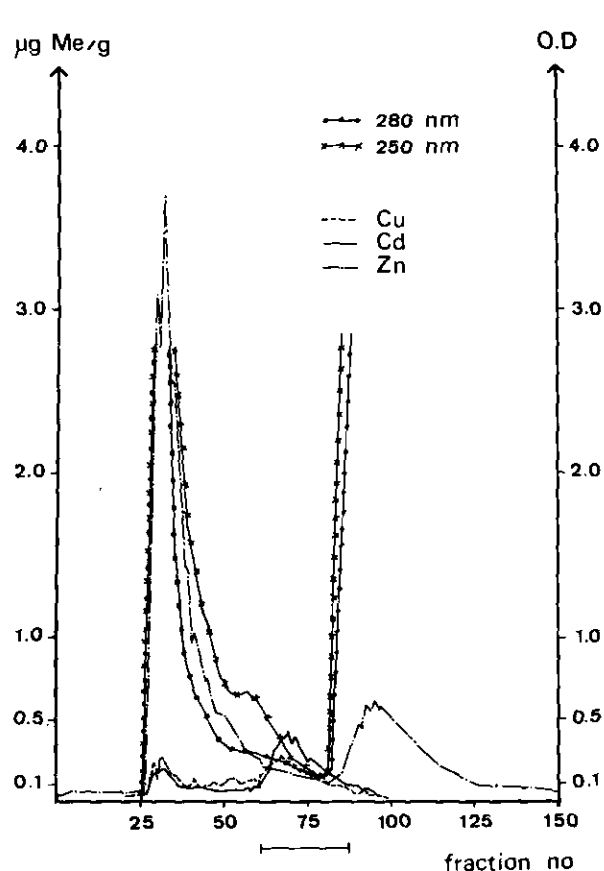
FIGURE 2.   Gel chromatography on Sephadex G-75 of OL oysters. The column dimensions were 700 × 50 mm. Elution with 0.01 M Tris HCl in 0.05 M NaCl, pH 8, at a flow rate of 56 ml/hr. Volume of fractions: 15 mL. Samples of fractions 27, 50, 70, and 90 were taken for radioimmunological metallothionein analysis. The indicated fractions were pooled, concentrated on an Amicon cell with UM-2 filter, and taken for further chromatographic and isoelectric focusing studies.

FIGURE 3.   Gel chromatography on Sephadex G-50 of pooled fractions from Fig. 2. Column dimensions were 381 × 26 mm. Elution with 0.01 M Tris-HCl in 0.05 M NaCl, pH 8, at a flow rate of 10 ml/hr. Volume of fractions: 5 mL. The indicated fractions were pooled and concentrated, washed and taken for isoelectric focusing.

of oysters from New Zealand. The OL oysters contained 5.3 μg Cd/g, 3.4 μg Cu/g, and 100 μg Zn/g wet weight and the CG oysters 1.4 μg Cd/g and 936 μg Zn/g.

Pilot Study

In both types of oysters, a large proportion of the recovered cadmium was eluted in the void volume of Sephadex G-75 columns, suggesting the presence of cadmium-binding proteins with a molecular weight higher than albumin (Fig. 1). This fraction also contained bound zinc and was heat-labile. Most of the high molecular weight, metal-binding protein fractions were denatured by heating at +80°C for 2 min. In addition to this, both types of oysters contained a low molecular weight cadmium-binding ligand. However, the low molecular weight cadmium-binding ligand in CG oysters (Fig. 1B) with low cadmium concentrations was different from that in OL oysters with high cadmium concentrations. The low molecular weight, cadmium-binding ligand in OL oysters (Fig. 1A) was eluted at the same position as metallothionein (molecular weight ~ 6500). This fraction was heat-stable. No bound zinc was detected in this fraction. On
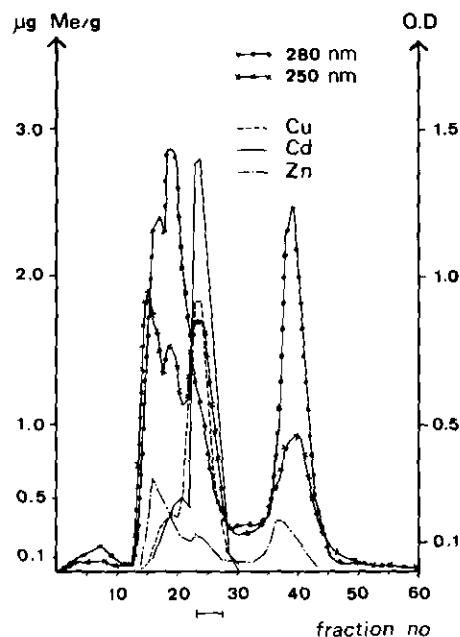
the other hand, CG oysters did not have any zinc- or cadmium-binding ligand in the same position as metallothionein but contained zinc and cadmium in a very low molecular weight fraction (<1000).

The fractionation of OL oyster supernatant on G-75 Sephadex after treatment with rat hemoglobin showed that the major cadmium-binding ligand in these oysters was eluted in the same positon as metallothionein. These results indicate that the heat-stable protein had a higher affinity for cadmium than hemoglobin.

Main Study

Figure 2 gives the elution profile of oyster supernatant fractions on a preparative scale. This study was performed on the basis of data obtained in the pilot study. All fractions were analyzed for cadmium, zinc, and copper content. As seen in Figure 2, cadmium was mainly recovered in fractions 62-88 corresponding to the molecular size of metallothionein, while zinc was bound mainly to molecular species eluting at lower or higher volumes.

The competitive binding RIA of samples of various elution volumes from the G-75 Sephadex chromatography of OL oysters demonstrated that all the samples exhibited but slight reactivity in comparison to that exhibited by a typical vertebrate MT (in this case, a 50/50 composite of rat MT-1 and rat MT-2). The logit–log regression expressing the response of the ^{125}I-labeled reference antigen (rat MT-1) was $Z = 4.1524 - 0.6714\,\hat{Q}$; $r^2 = 0.992$. $Z = \log([100Y/(1 - Y)]$, where Y is fraction of labeled MT bound by the antibody; $Q = \log X$, where X is picograms competing MT. For sample aliquots in the range 0.001 to 1 μL, all samples were characterized by responses in the region beyond $Y = 0.8$ (on the terminal
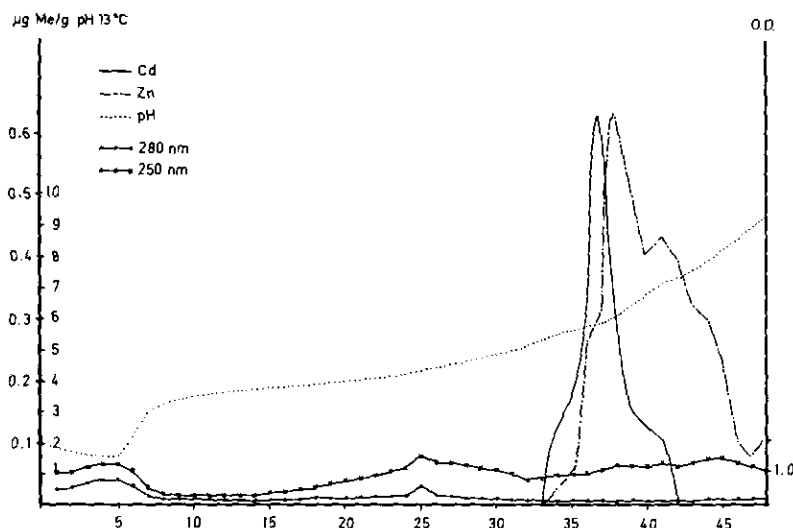
FIGURE 4.   Isoelectric focusing of pooled fractions indicated in Fig. 3 was carried out by use of sucrose gradient 0–50 % (w/v) used were: In the less dense 0.2 mL, pH 3.5–5; 0.2 mL, pH 4–6; and 0.2 mL; pH 6–8; and dense 2 mL pH 3.5–5.

sigmoid part of the typical response curve). The regression (standard curve) for the rat MT-1 and rat MT-2 was developed to characterize the central linear response region between $Y = 0.3$ and $Y = 0.8$ where quantitation is most accurate. Four of the samples were quantifiable at a sample volume of 10 μL. Three of these were from fractions identified as containing components in the high molecular weight region (in excess of 70,000) or components in the very low molecular weight region (less than 3000). Their nonreactivity in the RIA is as expected. Two samples were identified as containing components of approximately the molecular weight of MT (6500). Sample 1 is estimated to contain 0.6 μg Cd/mL or approximately 6 μg MT/mL. The 10 μL aliquot then contains circa 9.2 pmole MT. The experimental Z value was 2.4452. If this Cd-BP were a metallothionein, then the regression indicates the aliquot contains 337 pg MT or 0.052 pmole MT. The ratio 9.2/0.052 = 177 is then a measure of the factor by which a vertebrate MT is more reactive in the RIA than is the Cd-BP of sample 1. Sample 2 is estimated to contain 0.4 μg Cd/mL or approximately 4 μg MT/mL. The 10 μL aliquot then contains circa 6.2 pmole MT. The experimental Y value was circa $Y = 1.0$ (all aliquot volumes of sample 2 were at or in excess of this value). Sample 2 is then essentially nonreactive in the RIA. In sum, the Cd-BP does not possess the same principal antigenic determinants typical of vertebrate MTs (23,24). This does not imply that the Cd-BP is not a MT; the latter point will be clarified later when the primary structure, particularly the cysteine sequence is determined.

The Cu- and Cd-containing fractions obtained after gel chromatography G-75 and subsequently on G-50 Sephadex (Fig. 3) were further characterized by isoelectric focusing. Cadmium (Fig. 4) was found to be bound to a protein appearing at a pH of 5.9 at +13°C. Due to the low protein concentration no distinct protein peak was observed but a small peak at absorption 250 to 280 nm was observed in fractions 24–26. This peak

was a background peak identified also in a separate gradient without an applied protein sample containing only ampholytes. Copper was impossible to quantitate, as the copper concentration in the fractions was just below detection limit.

Discussion

Results from the present study showed differences in cadmium binding in two different species of oysters collected from two different areas of New Zealand. Cadmium in dredge oysters (Ostrea lutaria, OL) was bound to a protein similar to metallothionein in its molecular size, heat stability, and high cadmium affinity. The pI (5.9) is higher than previously observed and reported for mammalian metallothioneins. The most MT-like fractions from the Sephadex G-75 separations (samples 1 and 2) are minimally reactive (or essentially nonreactive) in the RIA. Amino acid composition, as well as sequence, is another criteria needed for a final decision whether this cadmium-binding protein is metallothionein or should be named differently.

In rock oysters (Crassostrea glomerata, CG) no such metal-binding protein could be detected, which indicates a possible difference in the metabolism and binding of cadmium in the two kinds of oysters. A probable reason for this may be the difference in environmental levels of cadmium for the two kinds of oysters. Since humans who consume the OL oysters (from the Bluff area) with high concentration of cadmium did not have an expected high concentration of cadmium in blood (12), it has been suggested that there is a different handling of cadmium from these oysters (due to the specific protein binding) compared to cadmium in most other food stuffs (20). Cadmium in the OL oysters was shown to be partly bound to a low molecular weight protein. Cadmium bound to such a low molecular weight protein might be taken up intact in blood, in a similar way as demonstrated for metallothionein in animals (8). Similarly to metallothi-

onein-bound cadmium it may be quickly transported to the kidney (5) and possibly contribute less to blood cadmium levels than albumin-bound cadmium, which is the normal binding form of cadmium in blood.

An additional possibility to be considered is that the oyster protein might still be metallothionein, but that nonmammalian metallothionein possesses different biological properties than mammalian metallothionein and might be excreted quickly in urine. Some nonmammalian metallothioneins have been shown to contain two clusters each with three divalent metals instead on one three- and one four-metal cluster found in mammalian metallothioneins (25). It is interesting to speculate on possible differences in the metabolism of cadmium due to the different chemical forms which are ingested. In this regard, it could very well be that cadmium-metallothionein containing a four-metal cluster is more rapidly taken up by the kidney. It might therefore be more toxic to animals than invertebrate MT, which contains only the three metal clusters. This needs, however, further investigation.

Defense mechanisms against metal toxicity are of importance (26) in environmental health. No data are available on the toxicity of metallothionein with differing metal clusters and thus differing in metal content per molecule of protein. This should be of great interest in further investigations of the toxicity of cadmium. The significance of the different metal-binding ligands in the two different types of oysters from New Zealand is not clear. However, the presence of a low molecular weight metal-binding protein with some properties similar to metallothionein in OL oysters (but not in CG oysters) is interesting in view of its potential role of detoxification in cadmium toxicity. The difference in absorption and tissue distribution of cadmium from these various forms in oysters needs further studies in order to evaluate the risk of high cadmium intake via food and subsequent health effects in humans.

REFERENCES

1. Friberg, L., Piscator, M., Nordberg, G. F., and Kjellström, T. Cadmium in the Environment, 2nd ed. CRC Press, Cleveland, Ohio, 1974.
2. Nordberg, G. F., Kjellström, T., and Nordberg, M. Kinetics and metabolism. In: Cadmium and Health (L. Friberg, C.-G. Elinder, G. F. Nordberg, and T. Kjellström, Eds.), CRC Press, Boca Raton, FL, 1984, in press.
3. Kägi, J. H. R., and Nordberg, M. Metallothionein. Birkhäuser Verlag, Basel-Boston-Stuttgart, 1979.
4. Nordberg, M. General aspects of cadmium: transport, uptake and metabolism by the kidney. Environ. Health Perspect. 54: 13–20 (1984).
5. Nordberg, M., and Nordberg, G. F. Distribution of metallothionein-bound cadmium and cadmium chloride in mice: preliminary studies. Environ. Health Perspect. 12: 103–108 (1975).
6. Cherian, M. G., Nuottaniemi, I., and Nordberg, M. Comparison of tissue distribution of cadmium in mice intravenously injected with metallothionein and a polymer of metallothionein. Toxicology 26: 161–171 (1983).
7. Nordberg, G. F. Effects of acute and chronic cadmium exposure on the testicles of mice. With special reference to protective effects of metallothionein. Environ. Physiol. Biochem. 1: 171–187 (1971).
8. Cherian, M. G., Goyer, R. A.,, and Valberg, L. S. Gastrointestinal absorption and organ distribution of oral cadmium chloride and cadmium-metallothionein in mice. J. Toxicol. Environ. Health 4: 861–868 (1978).
9. Cherian, M. G. Metabolism of orally administered cadmium-metallothionein. Environ. Health Perspect. 28: 127–130 (1979).
10. Cherian, M. G. Absorption and tissue distribution of cadmium in mice after chronic feeding with cadmium chloride and cadmium-metallothionein. Bull. Environ. Contamin. Toxicol. 30: 33–36 (1983).
11. Nielsen, S. A. Cadmium in New Zealand dredge oysters: geographic distribution. Int. J. Environ. Anal. Chem. 4: 1–7 (1975).
12. Sharma, R. P., Kjellström, T., and McKenzie, J. Cadmium in blood and urine among smokers and non-smokers with high cadmium intake via food. Toxicology 29: 163–171 (1983).
13. Ridlington, J. W., and Fowler, B. A. Isolation and partial characterization of a cadmium-binding protein from American oyster. (Crassostrea virginia). Chem. Biol. Interact. 25: 127–138 (1979).
14. Ridlington, J. W., Chapman, D. C., Goeger, D. E., and Whanger, P. D. Metallothionein and Cu-chelatin: characterization of metal-binding proteins from tissues of four marine animals. Comp. Biochem. Physiol. 70B: 93–104 (1981).
15. George, S. G., Carpene, E., Coombs, T. L., Overnell, J., and Youngson, A. Characterization of cadmium-binding proteins from mussels, Mytilus edulis (L), exposed to cadmium. Biochem. Biophys. Acta 580: 225–233 (1979).
16. Frankenne, F., Noel-Lambot, F., and Disteche, A. Isolation and characterization of metallothionein from cadmium-loaded mussel Mytilus edulis. Comp. Biochem. Physiol. 66C: 179–182 (1980).
17. Carmichael, N. G., and Fowler, B. A. Cadmium accumulation and toxicity in the kidney of the Bay Scallop Argopecten irradians. Marine Biol. 65: 35–43 (1981).
18. Wong, M. H., Choy, C. K., Lau, W. M., and Cheung, Y. H. Heavy-metal contamination of the Pacific oysters (Crassostera gigas) cultured in Deep Bay, Hong Kong Environ. Res. 25: 302–309 (1981).
19. Otvos, J. D., Olofson, R. W., and Armitage, I. M. Structure of an invertebrate metallothionein form Scylla serrata. J. Biol. Chem. 257: 2427–2431 (1982).
20. Nordberg, M., Cherian, M. G., and Kjellström, T. Defence mechanisms against metal toxicity and their potential importance for risk assessments with particular reference to the importance of various binding forms in food stuff. KHM Technical Report No. 55, Swedish State Power Board S-16287 Vällingby, Sweden.
21. Vander Mallie, R. J., and Garvey, J. S. Radioimmunoassay of metallothioneins. J. Biol. Chem. 254: 8416–8421 (1979).
22. Garvey, J. S., Vander Mallie, R. J., and Chang, C. C. Radioimmunoassay of metallothioneins. Methods Enzymol. 84: 1212–138 (1982).
23. Winge, D. R., and Garvey, J. S. Antigenicity of metallothionein. Proc. Nat. Acad. Sci. (U.S.) 80: 2472–2476 (1983).
24. Garvey, J. S. Metallothionein: structure/antigenicity and detection/quantitation in normal physiological fluids. Environ. Health Perspect. 54: 117–127 (1984).
25. Briggs, R. W., and Armitage J. M. Evidence for site-selective metal binding in calf liver metallothionein. J. Biol. Chem. 257: 1259–1262 (1981).
26. Cherian, M. G., and Nordberg, M. Cellular adaptation in metal toxicity and metallothionein. Minire. Toxicol. 28: 1–15 (1983).