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Cadmium accumulation and detoxification in a Cd-resistant population of the oligochaete *Limnodrilus hoffmeisteri*

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It was reported earlier that the oligochaete *Limnodrilus hoffmeisteri* from metal-polluted Foundry Cove has evolved resistance to a combination of cadmium, nickel and cobalt (Klerks and Levinton, 1989). This paper addresses the possible physiological mechanisms by which this resistance is achieved. Exposing animals from both the control population and the resistant population to Foundry Cove sediment or to ^{109}Cd in water, shows that the resistance is not achieved by a reduced cadmium accumulation. HPLC gel-permeation of the cytosol obtained after the exposure to ^{109}Cd in water reveals that the resistant worms have significantly higher levels of a cadmium-binding, metallothionein-like protein than control worms. This elevated protein level is shown to be genetically determined and is proposed to contribute to the resistance of *L. hoffmeisteri* from Foundry Cove. In addition, electron microprobe analyses of Foundry Cove worms exposed to metal-rich sediment demonstrated the presence of Cd in granules. High levels of Cd were found in S-rich granules, possibly in the form of cadmium sulfide. These granules occurred both as individual ones and as large granular aggregates.

Key words: Resistance mechanisms; Cadmium; Accumulation; Detoxification; Metallothionein; Granules

INTRODUCTION

The benthic oligochaete *Limnodrilus hoffmeisteri* inhabiting cadmium-, nickel-, and cobalt-polluted Foundry Cove (located in NY on the Hudson River), has evolved a genetically-based resistance to a combination of these metals (Klerks and Levinton, 1989). For example, worms from Foundry Cove survived a 28-day exposure to sediment from Foundry Cove with highly elevated levels of Cd, Ni and Co, while none of the worms from the control site survived this exposure. Furthermore, laboratory-reared second generation offspring of Foundry Cove worms still exhibited

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this resistance to metal-rich sediment (Klerks and Levinton, 1989). This paper reports the results of experiments aimed at determining the physiological mechanism(s) responsible for this resistance.

It seems likely that the resistance in *L. hoffmeisteri* in Foundry Cove evolved mainly in response to the cadmium pollution at this site, rather than the nickel or cobalt pollution. This is based on the observation that these worms accumulated much more Cd than Ni, while no increased body burdens of Co were detected (Klerks, 1987). In addition, Cd is generally much more toxic than Ni (e.g. Babich et al., 1986; Khangarot and Ray, 1987). In this investigation into the mechanism(s) underlying the resistance in *L. hoffmeisteri* from Foundry Cove, we have therefore limited ourselves to the fate of the Cd in this worm. The possibility that resistance to nickel or resistance to the interactive effect of nickel and cadmium plays a role in the observed resistance, can however not be excluded. Especially since interactions in toxicity (both antagonistic and synergistic) between cadmium and nickel have been reported (Babich et al., 1986).

The highly elevated levels of Cd, Ni and (to a lesser extent) Co in the sediment of this tidal freshwater marsh on the Hudson River originated from a nearby situated Ni-Cd battery factory. Cd levels in excess of 100,000 μg Cd per g dry sediment have been reported (Knutson et al., 1987). Analysis of Cd levels in the macrobenthos of Foundry Cove demonstrated the bioavailability of the Cd to the macrobenthos (Klerks, 1987). Similarly, Hazen and Kneip (1980) and Hazen (1981) reported the Cd in Foundry Cove to be bioavailable to the local banded killifish (*Fundulus diaphanus*) and cattails (*Typha angustifolia*).

An increased resistance to a metal can be achieved by a reduced accumulation of the pollutant. This has been reported for an arsenite-resistant bacterial strain (Beppu and Arima, 1964), copper- and cadmium resistant algal strains (respectively: Hall et al., 1979; Bariaud et al., 1985), a zinc-resistant annelid population (Bryan and Hummerstone, 1973) and a methylmercury-resistant fish population (Weis et al., 1981). But several studies comparing the accumulation of metals in populations differing in resistance did not find reduced accumulations in the resistant populations (Bryan and Hummerstone, 1971; Brown, 1977; Kishinami and Widholm, 1986; Nagel and Voigt, 1989).

Resistance differences can also be due to differences in metal detoxification. One possible mechanism involves the metallothioneins (MTs). MTs are low-molecular-weight cytosolic proteins rich in cysteine. They are induced by several types of factors, including exposure to such metals as cadmium, mercury, zinc, copper, gold, and silver (see review by Kägi and Schäffer, 1988). MTs have a high affinity for these metals and it is generally accepted that the binding of toxic metals to MTs constitutes a metal detoxification (though metal detoxification may not be MT's primary role). This detoxification is supported by several lines of evidence; an elevated metal resistance after an initial pre-exposure that resulted in elevated MT levels (e.g. Baer and Benson, 1987; Roesijadi and Fellingham, 1987), a protection against copper poison-

ing in yeast after the insertion of a monkey MT-gene (Thiele et al., 1986), and a reduced copper resistance in yeast after the deletion of the native MT-gene (Ecker et al., 1986). A relationship between genetically determined resistance differences and elevated levels of MT, MT-RNA or number of MT-genes has been demonstrated for mammalian cell lines (e.g. Beach and Palmiter, 1981; Enger et al., 1981; and Griffith et al., 1981) and a Cd-selected laboratory population of *Drosophila* (Otto et al., 1986). Though it has been reported that *Drosophila* strains derived from field-collected flies carrying an MT-gene duplication exhibited an increased Cu resistance (Maroni et al., 1987), there is no direct evidence that an increased detoxification of metals by MT has played a role in genetic adaptations to elevated metal levels in natural populations.

Another possible detoxification mechanism is the sequestering of metals in vesicles, lysosomes and other membrane-bound structures, as granules and intranuclear inclusions, or as insoluble metal precipitates (see reviews by Brown (1982) and George (1982)). A sequestering of a metal will constitute a detoxification if this process keeps the metal from interacting at sensitive sites in the organism. The only evidence for differences with respect to the sequestering of metals in organelles playing a role in genetic adaptations of animals to elevated metal levels, comes from a study reporting an elevated copper-content in 'granules' and 'dense spherical inclusions' in a copper-resistant population of the isopod crustacean *Asellus meridianus* (Brown, 1977).

The objective of this research was to determine the mechanism(s) underlying the metal resistance that has evolved in *L. hoffmeisteri* inhabiting Foundry Cove. This was approached by investigating the possibility that the resistant worms accumulate less cadmium than their sensitive conspecifics as well as the possibility that this resistance is achieved by an increased cadmium detoxification. Metal accumulation was determined after exposures to either cadmium-rich sediment or the radioisotope ^{109}Cd. For comparing cadmium detoxification, subcellular fractionation and gel-permeation of worms exposed to ^{109}Cd were used to quantify the amounts of cadmium that are bound to metallothionein-like proteins. In addition, electron microprobe analyses of metal-resistant worms exposed to cadmium-rich sediment were done to investigate the sequestering of cadmium in granules.

MATERIALS AND METHODS

Collection and culturing of animals

Limnodrilus hoffmeisteri (Annelida, Oligochaeta, Tubificidae) is a simultaneous hermaphrodite. The collection of these worms from Foundry Cove and the control area (South Cove; on the Hudson River approximately two kilometers south of Foundry Cove), the culturing in the laboratory, and the process by which second generation offspring of Foundry Cove worms were obtained, were all described by Klerks and Levinton (1989).

Cadmium accumulation from sediment

Cadmium accumulation in *L. hoffmeisteri* from Foundry Cove and the control area was determined by exposing these worms to sediment with different metal levels. This exposure was set up as a bioassay for a comparison of sediment toxicity among populations, as reported in Klerks and Levinton (1989). Sediment with different metal levels was obtained by collecting sediment from the control site, from different locations in cadmium-polluted Foundry Cove, and by combining sediment from the latter sites. All sediment was sieved to a <250 μm particle size, boiled and washed several times with GF/C (Glassfiber Filter, Whatman Grade GF/C) filtered Hudson River water. Three replicates of 10 worms were exposed to 6 different sediment metal levels (ranging from 15 to 34,000 μg Cd per g dry sediment), for 28 days (the duration of our sediment bioassays). Worms from the same batch as those used for the exposure were collected for metal analyses at the beginning of the experiment, as were the worms that survived the exposure (6 to 10 worms per replicate). These worms were kept in GF/C filtered Hudson River water for 2 days, to exclude gut contents. They were then rinsed with distilled water, pooled for each replicate, and frozen in acid-washed test tubes. Worms were later thawed, transferred to an acid-washed beaker together with the water in which they were frozen and the water used for several rinses of the test tubes. The beakers were then dried in an oven at 60°C, and dry weights were determined after equilibration at room temperature in the presence of a dessicant. Ultrex grade nitric acid (2 ml) was added to each sample in glass beakers, covered with watch glasses, refluxed for 2 to 4 h at 120°C, and evaporated to dryness. This was repeated twice, after which the sample volumes were brought to 5 ml with Ultrex nitric acid and distilled water. Cadmium concentrations were then determined with a Perkin Elmer 4000 graphite furnace atomic absorption spectrophotometer. Blanks were run concurrently with tissue samples. The use of our procedures on National Bureau of Standards Oyster Tissue resulted in values within the range specified for this reference material.

Cadmium accumulation from ^{109}Cd in water

Worms collected from Foundry Cove and the control area, as well as second generation offspring of Foundry Cove worms born and raised in sediment from the control site, were exposed in plastic petri dishes to 8.9 μM ($=1$ mg/l) Cd (incl. ^{109}Cd) in reconstituted fresh water (pH 7.8–8.0, hardness 160–180 mg/l $CaCO_3$; ASTM, 1980) for 6 days. The addition of ^{109}Cd (New England Nuclear, reactor produced, as $CdCl_2$ in 0.5 M HCl with a specific activity of 65,490 kBq/mg Cd), resulted in a radioactivity of 22.2 kBq/ml in the exposure water. Surviving worms were pooled in three replicates for each group, and frozen at -80°C. Thawed samples were later homogenized in a Teflon/glass homogenizer (10 strokes) in a 50 mM Tris–HCl buffer (pH 7.4) using a 50:1 ratio of buffer to tissue weight. All samples were kept on ice

between manipulations. Cadmium concentrations of the homogenate were determined by gamma counting, using a Beckman 4000 gamma counter with a 3-inch sodium iodide crystal. Samples were counted for one minute at a counting efficiency of 25%.

Subcellular fractionation

The homogenate obtained in the previous step was separated by centrifugation into a 'debris' fraction (the 200 g pellet, which includes incompletely homogenized tissues and which was not included in further analyses), a particulate fraction (the pellets from several centrifugations, up to 100,000 g) and the cytosol (the 100,000 g supernatant). The pellets were resuspended in 0.5 ml of the homogenization buffer. Cd concentrations of the particulate fraction and the cytosol were determined by gamma counting. The cytosol samples were frozen at $-80°C$ after counting.

Gel-permeation

The cytosol samples from the previous step were separated by HPLC gel-permeation to compare the cytosolic Cd distributions. For this procedure the cytosol samples were first thawed and filtered through a 0.45 μm disposable filter assembly (Gelman, AcroLC13, HPLC certified). The HPLC gel-permeation separations were done on cytosol subsamples ranging from 465 to 700 μl and used a Toyo Soda (TSK-SW 3000, 600 × 7 mm) column and a 250 mM Tris-HCl running buffer (pH 7.4). For each sample, 65 fractions of 1 ml were collected and analyzed by gamma counting. The separation characteristics of the HPLC system were determined with proteins of known molecular weight.

Electron microprobe analyses

Fixed and embedded cross sections of Foundry Cove worms were studied with scanning electron microscopy (SEM) and electron probe microanalysis (EPMA), to investigate the physical and chemical nature of their Cd sequestering. Worms collected from Foundry Cove and kept in the laboratory in metal-rich Foundry Cove sediment (approximately 3500 μg Cd per g dry sediment) were removed from the sediment and kept in GF/C filtered Hudson River water for at least 12 h prior to fixing. The fixing and embedding procedure was modified from the one used by Ireland and Richards (1977). Fixation was accomplished by placing the worms in 5% glutaraldehyde (Sigma, grade I, specially purified) in a 100 mM phosphate buffer (pH 7.1) for two hours at room temperature. Fixed worms were then gradually dehydrated (20 min each at 20, 40, 60, 95, 100 and again 100% ethanol) and infiltrated with hard Spurr Low Viscosity Resin (Spurr, 1969) from Polaron (1 h each at 25, 50, 75, 100 and again 100% Spurr resin). Embedded samples were polymerized at 70°C. Thick

sections were made of the embedded worms, usually by cross sectioning a worm just posteriorly of the gonadal region (segments 10–12). Each section was highly polished and coated with an electrically conductive material. Initially this material was carbon, but poor stability of the sections under the electron beam led to the adoption of aluminum for this coating because of its much greater thermal conductivity.

Scanning electron images as well as qualitative and quantitative chemical analyses were obtained with a Cameca Camebax-Micro electron microprobe. The beam conditions were 15 kV and 10 nA. Backscattered electron (BSE) imaging was employed in order to identify density contrasts. Such contrasts would identify the location of tissues with high concentrations of heavy metals as well as the location of other high density structures consisting of non-hydrocarbon compounds. Quantitative microanalysis methods were then employed to determine the metal levels. To minimize damage during quantitative analyses the beam was rastered continuously over a 15 by 15 μm area. Cd, Ni, Ca, P, S, Fe and Cl were analyzed using 30 to 80 s counting times. The latter five elements were chosen since significant peaks were found for these elements in preliminary EDS (Energy Dispersive Spectrometer) analyses; the chlorine was assumed to be entirely introduced with the Spurr resin. Intensities were compared to that of natural and synthetic mineral standards and corrected concentrations were calculated by assuming that 99% of each analyzed volume consisted of C, H, and O (in the proportions characteristic for Spurr resin) and by employing a full ZAF matrix correction algorithm. For the Al coated sections, the Al concentration was determined initially and this value was subsequently added proportionally to the C, H, and O as one of the elements assumed to be present in the matrix at a constant concentration.

Statistical analyses

Differences in cadmium concentrations were analyzed by Analysis of Variance (ANOVA) and a posteriori pairwise comparisons (Scheffé's F-test) using the Statview 512+ computer program (Abacus Concepts, 1986). The standard error of differences between the mean cadmium accumulations at two different times, was computed according to Sokal and Rohlf (1981).

RESULTS

In the experiment addressing the accumulation of Cd from metal-rich Foundry Cove sediment, the Cd concentrations of *L. hoffmeisteri* did not differ significantly among the groups exposed to sediment with Cd levels ranging from 5,400 to 34,000 μg Cd per g dry sediment (see Figure 1; $P > 0.05$ in an ANOVA). This was the case for the worms from the control area as well as the ones from Foundry Cove. The data for these groups were therefore pooled for comparing the Cd accumulation from metal-rich sediment by the worms from Foundry Cove to that of their conspecifics

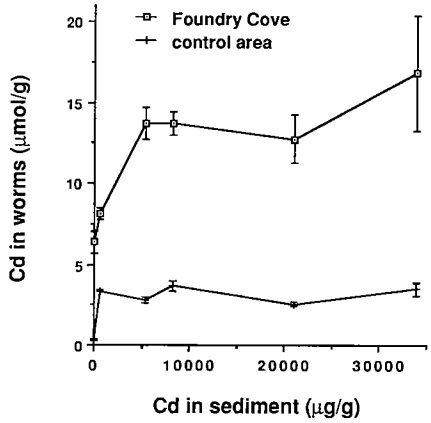

Fig. 1. Cadmium concentrations after a 28-day exposure to sediment with different metal levels, in *L. hoffmeisteri* from Foundry Cove and the control area. Values are mean $\pm$ SE_{mean} ($n=3$), in μmol Cd per g dry weight.

from the control area. Figure 2 shows the Cd concentrations of the worms before and after the 28 day exposure, as well as the Cd accumulation. These data do not show any evidence for a reduced Cd accumulation in the Foundry Cove worms. This

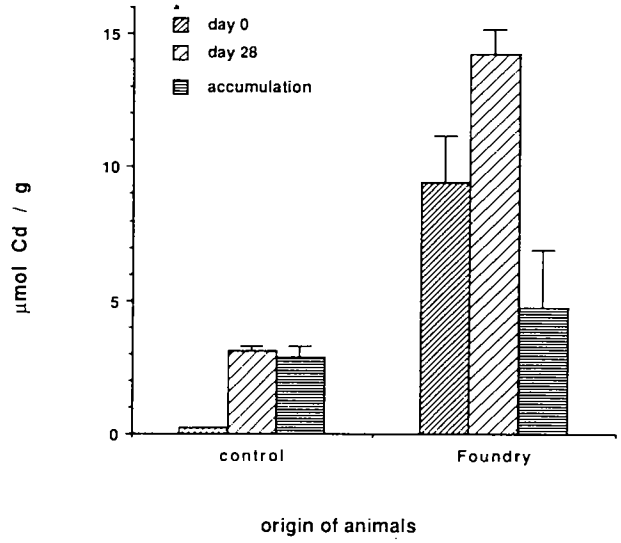

Fig. 2. Cadmium accumulation during a 28 day exposure to Cd-rich sediment (ranging from 5,400 to 34,000 μg Cd per g dry sediment) and Cd concentrations before and after this exposure, in *L. hoffmeisteri* from Foundry Cove and the control area. Values for day 0 and day 28 are mean $\pm$ SE_{mean} (respectively $n=3$ and $n=12$), while accumulation values are mean $\pm$ $SE_{difference\ between\ means\ of\ day\ 0\ and\ day\ 28}$, in μmol Cd per g dry weight.

TABLE I

CADMIUM ACCUMULATION AND SUBCELLULAR DISTRIBUTION IN *L. HOFFMEISTERI* EXPOSED TO 8.9 μM Cd IN WATER FOR 6 DAYS.[a]

	c	F	F_{off}	Scheffé
Homogenate	1,010 ± 157	2,232 ± 182	2,040 ± 46	$c < F_{off} = F$
Particulate	594 ± 98	1,184 ± 142	1,251 ± 83	$c < F = F_{off}$
Cytosol	218 ± 10	1,161 ± 238	522 ± 50	$c < F_{off} < F$
HMW[b]	26 ± 5	64 ± 7	26 ± 3	$c = F_{off} < F$
MT[c]	55 ± 4	415 ± 76	229 ± 13	$c < F_{off} = F$
LMW[d]	96 ± 14	388 ± 59	208 ± 26	$c < F_{off} = F$

[a] Worms were obtained from the control area (c), Foundry Cove (F), or were second generation offspring of the Foundry Cove worms (F_{off}). Values are mean ± S.E. ($n = 3$), in nmol Cd per g wet tissue. The last column shows the results of a posteriori pairwise comparisons (Scheffé's *F*-test) on log-transformed data (a significant effect of origin was found for all variables in an ANOVA, at $P < 0.01$). [b] HMW = high-molecular-weight pool; [c] MT = metallothionein pool; [d] LMW = low-molecular-weight pool.

result did not seem to be biased by differences in growth rates during the exposure among the animals from the two populations, since the same result was obtained when comparing the total amounts of Cd that were accumulated rather than concentrations (data not shown).

Similarly, a reduced cadmium accumulation in worms from the metal-resistant population was not found either after the 6 day exposure to Cd in water (Table I, 'homogenate'). In fact, the animals from Foundry Cove accumulated significantly more Cd (approximately twice as much) than their conspecifics from the control population. This increased Cd accumulation also occurred in the Foundry Cove offspring that had never been exposed to elevated metal levels prior to this experiment.

The higher Cd accumulation by the two groups of Foundry Cove worms was also reflected in the cytosol levels. The worms collected from Foundry Cove accumulated significantly more Cd in the cytosol than their offspring, which in turn accumulated more Cd in this fraction than the control area worms (Table I). The Cd distributions within the cytosol of the three groups of worms (Fig. 3) revealed that much Cd was associated with a protein with an apparent molecular weight of 16,000 (as determined from the elution volumes of proteins with known molecular weights). This apparent molecular weight and its high affinity for Cd are consistent with this protein being a metallothionein (MT). We will use the term MT-like protein in the remainder of this paper, since further characterization of this protein is required for an unambiguous classification as an MT.

For a more general quantification of the amounts of Cd associated with components of the cytosol, the individual cytosolic fractions were pooled. Fractions 11–20 (see Fig. 3) were pooled into a high-molecular-weight (HMW) pool (mol. wt. > 23,000), fractions 21–26 into an MT-pool (mol. wt. 23,000–5,000) and fractions

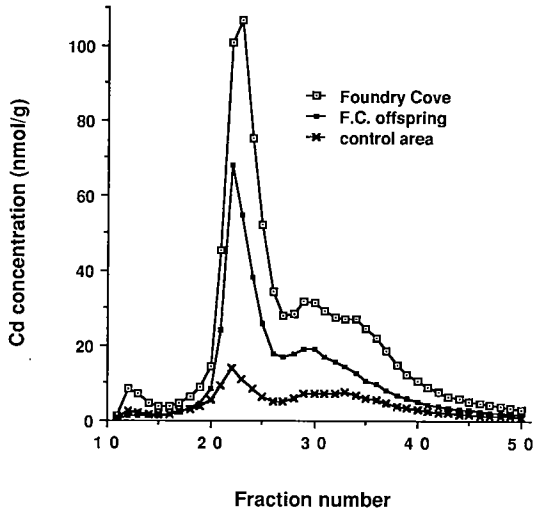

Fig. 3. Cadmium accumulation in cytosolic fractions of *L. hoffmeisteri* separated by HPLC gel-permeation. Worms were obtained from the control area. Foundry Cove, or were second generation offspring of the Foundry Cove worms and were exposed to 8.9 μM Cd in water for 6 days. Values are means ($n=3$), adjusted for animal wet weight.

27–50 into a low-molecular-weight (LMW) pool (mol. wt. < 5,000). Both groups of Foundry Cove worms had significantly more Cd in both the MT and LMW pools than the control area worms (Table I). This increased binding of Cd to an MT-like protein in the Foundry Cove worms was consistent with their elevated resistance. However, these data did not show any evidence that the increased binding to MT in Foundry Cove worms resulted in lower Cd levels in other subcellular components. We therefore decided to look at the possibility that other detoxification mechanisms, such as the sequestering of Cd in granules, contributed to the elevated resistance of these worms.

All three groups of worms had high Cd levels in the particulate subcellular pool, after the exposure to Cd in solution (Table I). The control area worms had significantly less Cd in this pool than the worms from Foundry Cove. This pattern followed the resistance differences (Klerks and Levinton, 1989), suggesting that sequestering of Cd in the particulate fraction might play a role in the resistance.

Our SEM-EPMA data show that in *L. hoffmeisteri* from Foundry Cove, Cd is also sequestered in granules. BSE imaging showed the presence of two distinct types of granules: a lower BSE intensity (lower density) type and a higher BSE intensity (higher density) type (Fig. 4A). The high density granules were found in most body tissues (especially the chloragocytes, the body wall and the gut wall) while the low density granules were found only in the chloragog tissue surrounding the gut.

Both types of granules were found to be generally about 1 μm in diameter. This made quantitative analysis of the granules difficult since specimen instability under

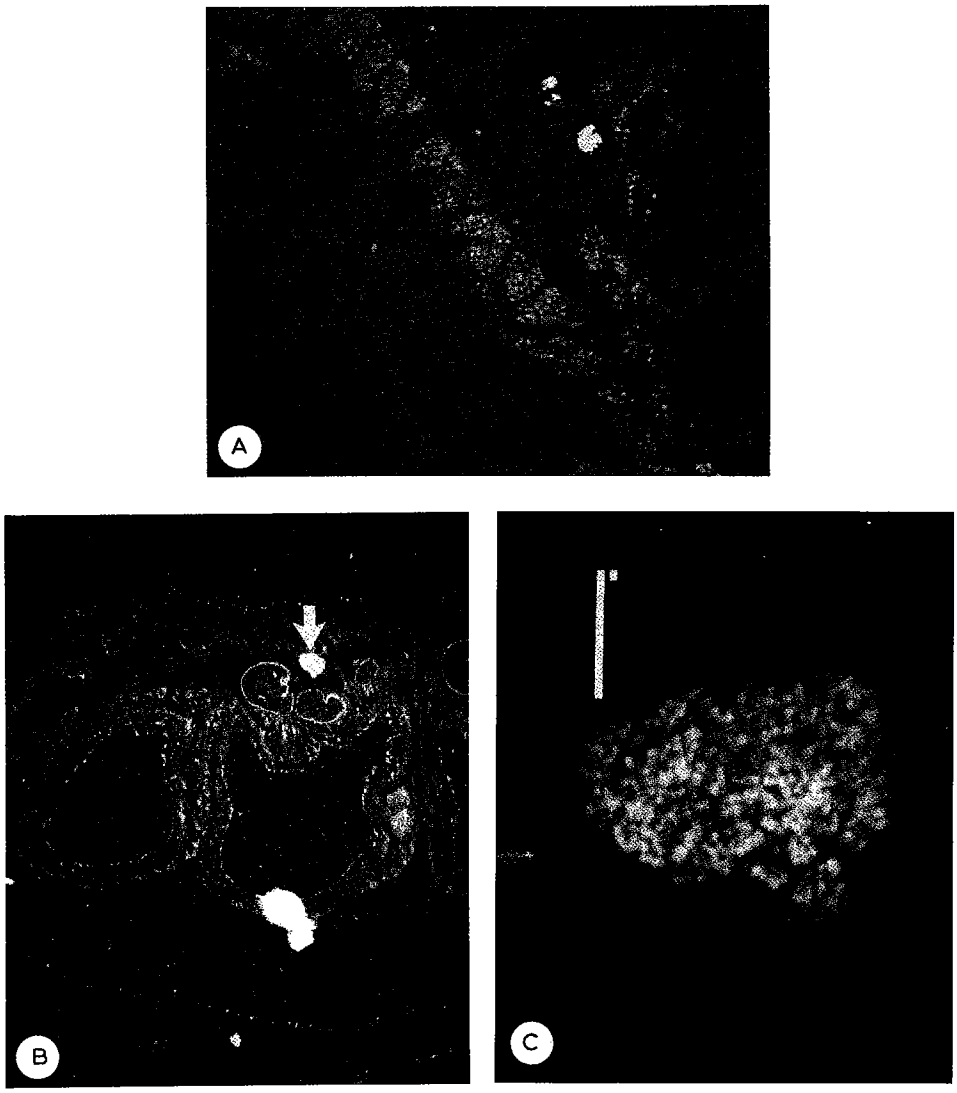

Fig. 4. Backscattered electron images of *L. hoffmeisteri* from Foundry Cove exposed to Cd-rich sediment. A: A cross-section, showing a portion of the chloragog tissue containing Ca- and P-rich granules as well as the brighter Cd- and S-rich granules (scale bar: 100 μm). B: An oblique section, showing the distribution of Cd- and S-rich granules. The arrow indicates a granular aggregate. The horizontal dimension of the photograph represents 400 μm. C: The granular aggregate of Fig. 4B at higher magnification (scale bar: 10 μm).

the beam had forced us to spread out the beam over a 15 μm diameter area. Positive identification of the primary constituents of the high density granules was made possible by the occasional occurrence of much larger (up to 30 μm) examples (Fig. 4B).

Upon close examination these large granules were found to be aggregates of the 1 μm granules (Fig. 4C). Analyses of these large granules therefore represented major amounts of granule material and minor amounts of their matrix. These analyses showed major amounts of only Cd and S and these were consistently present in a 1:1 ratio.

A series of analyses of the various body tissues – specifically avoiding the high density granules – showed that only the chloragog tissue contained significant concentrations of Cd (10 to 80 μmol/g) and an association with the low density granules was proposed. A series of analyses of only chloragog tissues were examined for correlations among the analyzed elements. Calculated correlation coefficients (for $n=15$) show a strong correlation of Ca with P ($r=0.85$), a moderate correlation of Fe with P ($r=0.76$), a somewhat weaker correlation of Cd and S with P ($r=0.65$ and $r=0.68$ respectively) and a strong correlation of Cd with S ($r=0.92$). The sum of Ca and Fe shows a stronger correlation with P ($r=0.91$) than Ca or Fe alone, indicating that both Ca and Fe are components of the same compound. Adding Cd to the sum of Ca and Fe does not significantly change the correlation with P. This may indicate that Cd is not a component of the Ca-Fe-P compound. It may also reflect the generally lower concentration of Cd (the average ratio of Fe:Ca is 0.37, while the average ratio of Cd to Ca is 0.07). The average ratio of P to Ca + Fe is 1.16. Nickel was not found in detectable concentrations (>100 μg/g) in any tissue.

DISCUSSION

Our data show that the Cd-resistance in *L. hoffmeisteri* from Foundry Cove is not due to a reduced accumulation of the metal. If anything, it appears that worms from this population accumulate more Cd than their metal-sensitive counterparts. Similar findings have been reported for metal-resistant populations of algae (Nagel and Voigt, 1989), annelids (Bryan and Hummerstone, 1971), crustaceans (Brown, 1977) and for tobacco plant cell cultures (Kishinami and Widholm, 1986). Our results agree with Jamieson (1981) who predicted that metal excretion is probably not an important strategy implemented by aquatic oligochaetes in dealing with metal toxicity, because of the absence of dorsal pores (which would allow excretion of coelomic fluid) in these worms. It appears that the elevated metal accumulation in the Foundry Cove worms is, similarly to the resistance, genetically determined; offspring of the Foundry Cove worms also accumulated more Cd than their conspecifics from the control area.

Since the Cd-resistant worms of Foundry Cove accumulated more Cd than their Cd-sensitive counterparts, other mechanisms must be responsible for the resistance. A reduced sensitivity at a target site for the cadmium is one possible mechanism. Such a reduced sensitivity is known to be responsible for some occurrences of insecticide resistance (Price and Chapman, 1987; Price, 1988) and is implied for a Cd-resistant algal strain (Nagel and Voigt, 1989). However, we did not attempt to compare the sensitivity of target sites among the populations of *L. hoffmeisteri*, because of the

extreme difficulties in identifying such sites. We did however investigate the possibility that the increased resistance in the Foundry Cove worms was due to an increased capacity to detoxify the cadmium.

After being exposed to cadmium, *L. hoffmeisteri* from Foundry Cove had much higher levels of a Cd-binding protein than their conspecifics from the control area. Preliminary results indicate this protein to be an MT, though the possibility that it is not an MT can presently not be excluded. The presence of an MT-like protein in these oligochaetes is consistent with the ubiquitous distribution of MT (Kägi and Kojima, 1987), and with other reports of their occurrence in oligochaetes (Suzuki et al., 1980, Yamamura et al., 1981; Morgan et al., 1989). The higher levels of the proposed MT-like protein in the worms from metal-polluted Foundry Cove relative to the ones from the control site, could have been entirely due to the cadmium pre-exposure of the Foundry Cove worms in the field; the pre-exposure having resulted in the induction of the MT-like protein. Yet the elevated level of an MT-like protein was also observed in the second generation offspring of the Foundry Cove worms that had no previous history of exposure to Cd. This demonstrated that the elevated MT level had a genetic component in common with the resistance differences, indicating that this increased level of an MT-like protein could be responsible for the increased resistance in the Foundry Cove worms. It should be noted though that our data did not reveal that this elevated level of an MT-like protein in Foundry Cove worms resulted in lower Cd burdens in other subcellular compartments. Nor was it possible to demonstrate a reduction in the amount of Cd that is exerting critical toxic effects, which would have required the identification of such effects. The direct connection between the increased level of an MT-like protein in *L. hoffmeisteri* from Foundry Cove and its elevated Cd resistance therefore has to remain tentative. Moreover, our experimental procedures did not permit a distinction between an elevated basal MT level, an increased rate of MT induction, or an increased stability of the MT as a basis for the elevated level of an MT-like protein in the Foundry Cove worms. A relationship between elevated MT levels and increased resistance agrees with the detoxification role of MT, and with previous reports of such a correlation between MT levels and resistance (Beach and Palmiter, 1981; Enger et al., 1981; Griffith et al., 1981; Otto et al., 1986; Maroni et al., 1987). The presence of genetically determined differences in levels of an MT-like protein among populations from different sites, is an important finding. Genetically based differences in MT levels have been found among mammalian cell lines (e.g. Beach and Palmiter, 1981; Enger et al., 1981; and Griffith et al., 1981) and laboratory-selected strains of *Drosophila* (Otto et al., 1986), but have not been reported previously for natural populations of animals. The presence of such genetically based differences is likely to have other effects in addition to its influence on Cd-resistance, for example on the metabolism of such essential metals as copper and zinc.

Since both groups of Foundry Cove worms also had higher levels of Cd in the LMW fraction than the control area worms, it is also possible that a LMW-ligand

played a role in the resistance of the Foundry Cove worms. However, our chromatography data did not show a well defined LMW peak. Moreover, the existence of LMW metal-detoxification ligands is not well established. Results by Frazier and George (1983) suggest the detoxification of Cd by a LMW ligand. This is contradicted by results from Sanders and Jenkins (1984), which show that the accumulation of copper in this pool correlates positively with Cu toxicity.

Our results showed the presence of two types of granules in *L. hoffmeisteri* from Foundry Cove. These two types have different densities as shown by their BSE intensities.

Microanalysis results indicate that the higher density granules consist primarily of Cd and S. The 1:1 ratio of Cd to S in these granules indicates that they may consist of cadmium sulfide. Such a precipitation as a sulfide has been reported for copper-resistant yeasts (Ashida, 1965). But our data can not rule out other possibilities, such as an organic complex as was reported for Cu-rich granules in barnacles (Walker, 1977) and amphipods (Icely and Nott, 1980). High density, sulfur- and metal-rich granules have been found in many organisms, usually as copper-containing granules (see review by Brown, 1982). The presence of high levels of Cd and S in granules within the chloragog tissue of oligochaete agrees with a study by Morgan and Morris (1982) where such granules ('cadmosomes') were reported for two species of earthworms collected from soils contaminated by metal-mining.

The fact that the Cd-S granules were found in several tissues other than the chloragog tissue, including the gut-wall, points to the possibility that these granules are involved in the excretion of cadmium via the gut. Similarly, preliminary evidence of the excretion of such granules (in that case Cu- and S-rich granules) has been reported for an amphipod from a metal-polluted site (Icely and Nott, 1980). However, as pointed out earlier, an increased Cd excretion does not appear to be a mechanism underlying the resistance of the Foundry Cove worms.

The occurrence of large aggregates of Cd- and S-rich granules is not widely reported in the literature. Granules in that size-range have been described (Walker, 1977; Doyle et al., 1978; Brown, 1977). However, we are aware of only one other study describing the presence of aggregates of S- and metal-rich granules: aggregates of Cu- and S-rich granules in an amphipod from a Cu-contaminated site (Icely and Nott, 1980).

The lower density granules seemed to consist primarily of Ca, Fe and P. It is proposed that these elements are present as a precipitated inorganic phosphate, although we could not determine an exact chemical formula. The presence of Ca- and P-containing granules in Cd-exposed *L. hoffmeisteri* is consistent with the observation that such granules are common intracellular granules in animals (George and Pirie, 1980), while the presence of an inorganic phosphate is in line with the occurrence of inorganic zinc phosphate in P-rich granules in barnacles (Walker et al., 1975). Our analyses indicated an association of Cd and S with these granules. It is possible that the Cd is a component of these low-density granules (e.g. as a mixed Ca–Cd–Fe phos-

phate), though other explanations could account for the observed correlations between the Cd, S and Ca concentrations.

An investigation has been initiated to determine whether the metal-sensitive worms do not have this ability to sequester Cd as non-toxic granules, or are less efficient at it. Preliminary data point to the latter case. Definitive characterization of the role of granular sequestering in the observed variation in Cd resistance will require quantification of the Cd in granules of Cd-exposed *L. hoffmeisteri* from Foundry Cove and the control area.

In conclusion, the resistance to Cd in *L. hoffmeisteri* from Foundry Cove is not achieved by a reduced accumulation of the metal. These worms had much higher levels of a cadmium-binding protein than their Cd-sensitive conspecifics, after being exposed to Cd. This indicates that this protein, proposed here to be a metallothionein, plays a role in the resistance. In addition, the resistant worms sequester Cd in individual granules and granular aggregates which consist primarily of Cd and S and may be cadmium sulfide. The processes leading to precipitation of these granules appear to take place in the chloragog tissue, where the presence of inorganic phosphate granules might be functionally related to these processes. It is not clear at this point what role this sequestering plays in the documented variations in metal resistance among populations of *L. hoffmeisteri.*

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