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Cadmium, Zinc and Copper Accumulation in Limpets *(Patella vulgata)* from the Bristol Channel with Special Reference to Metallothioneins

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ABSTRACT: The study of metal concentrations in *Patella vulgata* caught from a polluted environment reveals a direct relationship between Cd concentration and body size and an inverse relationship in regard to Zn and Cu. Most of the Cd present in limpets with heavy Cd loads is bound to thioneins, but this is not the case for Zn and Cu. In young limpets, Cd-thioneins were not detected. All results indicate that during long-term Cd intoxication under field conditions, Cd deposition and increase in metallothioneins in limpets were directly linked. The induced production of metallothioneins may thus be considered as the main mechanism responsible for the cumulative absorption of Cd in limpets living in the Bristol Channel. Isolation and characterization of limpet metallothioneins were performed. They indicate that mollusc metallothioneins are very similar to those from vertebrates.

INTRODUCTION

In mammals most of the cadmium accumulated in tissues is bound to metallothioneins. These soluble proteins – characterized by a low molecular weight and a high cysteine and metal content – are synthesized in response to the administration of heavy metals (Kägi and Vallee, 1960, 1961; Piotrowski et al., 1973, 1974; Kägi et al., 1974; Chen et al., 1975; Olafson et al., 1979a).

The role of metallothioneins in the detoxication of Cd and Hg (Nordberg et al., 1971; Suda et al., 1974; Bouquegneau et al., 1975; Rugstad and Norseth, 1975) and in the control of Zn and Cu concentrations (Webb, 1972; Chen et al., 1974; Bremner and Davies, 1975; Richards and Cousins, 1975) has frequently been proposed in vertebrates. Few information however is available about the occurrence of these proteins in non-vertebrate organisms. In laboratory experiments the appearance of proteins similar to metallothioneins has been shown in molluscs (Noël-Lambot, 1976; Olafson, personal communication), crustaceans (Distèche, 1976; Olafson et al., 1979a) and blue alga (Maclean et al., 1972) in response to cadmium toxication. Some studies have also demonstrated the existence of such proteins in several species of marine molluscs living in polluted areas (Brown et al., 1977; Howard and Nickless, 1977; Noël-Lambot et al., 1978a; Talbot and Magee, 1978). The papers by Olafson et al. (1979b) and Overnell and Trewhalla (1979) on crustaceans, by Frankenne et al. (in press) on a mollusc, and by Olafson (1979c) on a blue-green alga charaterize for the first time unequivocally a protein isolated from an invertebrate or a prokaryotic organism as a metallothionein.

In this paper we describe the possible role of metallothioneins in the accumulation of Cd by the limpet *Patella vulgata* living in the polluted waters of the Bristol Channel (Somerset, England). Several studies have established that very high concentrations of heavy metals, principally cadmium, are found in those animals (Butterworth et al., 1972; Peden et al., 1973; Boyden, 1974; Stenner and Nickless, 1974; Shore et al., 1975). Moreover, we have previously shown that cadmium, in contrast to Zn and Cu, is principally bound to metallothioneins and that those proteins cannot be found in detectable amounts in limpets caught from unpolluted waters (Noël-Lambot et al., 1978a).

We report in this paper a detailed study of the intracellular distribution of cadmium versus the total concentration of the metal in limpets of various ages collected in the same region (Weston-super-Mare, Bristol Channel) and in limpets transferred to unpolluted sea water. We also give some characteristics of the metallothioneins of this species.

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MATERIAL AND METHODS

Limpets *Patella vulgata* were collected in May 1977 from the middle shore of Weston-super-Mare or Portishead (Somerset, England). The pollution levels of both localities are rather similar. From the studies of Butterworth et al. (1972) it appears that Cd concentration in water in this area of the Bristol Channel is about 5 ppb.

Some limpets were directly frozen whereas 70 living individuals were transferred to clean water. They were released on the rocky shore of STARESO (Station de Recherches sous-marines et océanographiques, Calvi, Corsica). The survival rate was very poor: after 80 d, only 1 individual could be recovered. Analyses were generally performed on whole soft tissue, but some specimens were dissected into two parts: foot (muscular tissue) and viscera.

Wet samples were analysed for Cd, Zn and Cu by atomic absorption spectrophotometry (Perkin-Elmer, Model 370A) after mineralization for 8 h at 80 °C in HNO_3 65 % (2.5 ml g^{-1} fresh tissue) and dilution.

In order to study the fraction of Cd bound to metallothioneins, extraction of water soluble constituents was performed. Limpets were homogenized in three volumes of 0.5M sucrose by means of an Ultra-Turrax homogenizer and then centrifuged at 35 000 g for 1 h at 4 °C. The pellet obtained after a first centrifugation was resuspended in one volume of 0.5M sucrose and centrifuged again under the same conditions. Both supernatants were pooled before further fractionation. Metal concentrations in pellets and supernatants of the homogenates were determined by atomic absorption spectrophotometry after mineralization in HNO_3. Extraction efficiency improved only very little by additional stages of homogeneization and centrifugation (Table 1), by use of a Potter homogenizer, or of an ultrasonic device.

Table 1. *Patella vulgata*. Cd, Zn and Cu content of successive supernatants obtained by 5-step extraction of limpet samples from Bristol Channel (Portishead). Samples were homogenized with an Ultra-Turrax homogenizer and then centrifuged. At each step, the pellet was resuspended in 0.5 M sucrose, homogenized and centrifuged again

Number of centrifugations	Metal content in successive supernatants expressed in % of total metal in the 5 supernatants; mean ± standard error (n = 4)		
	Cd	Zn	Cu
1	87.2 ± 1.5	73.5 ± 5.6	85.8 ± 1.5
2	7.6 ± 1.0	10.6 ± 1.8	8.1 ± 1.1
3	2.7 ± 0.3	8.4 ± 2.8	4.2 ± 0.5
4	1.4 ± 0.2	4.8 ± 0.7	1.4 ± 0.3
5	0.9 ± 0.2	3.1 ± 0.4	< 1

The supernatants corresponding to a two-step extraction were pooled and filtered on a LKB Ultrogel AcA 54 column (2.6 × 33.5 cm) equilibrated against ammonium formate 0.01M pH 7.4, with a flow rate of 30 ml h^{-1}. The fractions (5 ml) were directly analysed for metal content by atomic absorption spectrophotometry.

The relative concentrations of metallothioneins were estimated from the metal content (sum of Cd, Zn and Cu, in g-atoms) of the chromatographic fraction containing the metallothioneins (MT fraction, Fig. 2). However, contrary to metallothioneins from liver of vertebrates, limpet metallothioneins contain only trace amounts of Zn and Cu, hence both metals are omitted in the calculation. This method of estimation of metallothionein concentration, assuming saturation of the metal-binding sites, was described in detail and discussed in a previous paper (Noël-Lambot et al., 1978b). Other recent results obtained in our laboratory confirm its validity.

Characterization of metallothioneins of *Patella vulgata* was performed on limpets collected from unpolluted sea water (Cap Gris-Nez, Pas-de-Calais, France) and then intoxicated in the laboratory for 60 d in sea water containing 0.5 ppm of Cd ($CdCl_2$). Supernatant obtained from 13 individuals after homogenization and centrifugation as described above was fractionated with acetone. Acetone cooled at −30 °C was added dropwise, under magnetic stirring, to give a concentration of 45 %. After 30 min, the mixture was centrifuged and the pellet discarded. The concentration of acetone of the supernatant was then raised to 80 %. After 45 min, the supernatant was discarded. The precipitate was dissolved in a minimum volume of 0.05M NH_4HCO_3 and immediately applied on a 5 × 50 cm column of Ultrogel AcA 54, eluted with the same buffer. The 250 nm absorbing fractions corresponding to the elution characteristics of metallothioneins were pooled, concentrated by ultrafiltration on a UM2 membrane (Amicon Corporation) and equilibrated in 0.015M Tris-HCl (pH 8.5) on a 2 × 20 cm column of Sephadex G-25.

This material was then applied on a 1.5 × 30 cm column of DEAE cellulose (Whatmann DE52) equilibrated against the same buffer. The column was further eluted with a linear gradient of 0–0.6M NaCl produced by a Varigrad (Büchler) device. The different fractions resolved by this chromatography were concentrated, desalted and concentrated again. They were studied by polyacrylamide gel slab electrophoresis according to the method of Perrie and Perry (1970). The Cd content of the various proteins separated by electrophoresis was determined by analysing gel slices. Note that these metal analyses must necessarily be

performed on unstained gels. These gel slices were solubilised in 50 % H_2O_2 at 50 °C during 48 h.

Amino acid analyses of the proteins isolated by ion exchange chromatography were also performed: lyophilyzed samples were hydrolysed under vacuum at 107 °C during 24 h in constant boiling HCl. In order to determine the cystein content as cysteic acid, the samples were oxidized with performic acid before hydrolysis according to Hirs (1956). Analyses were performed with a Beckman amino acid analyser Model 120B.

RESULTS

Cd, Zn and Cu Concentrations in Limpets

From Figure 1 it appears that Cd concentrations in limpets collected from Weston-super-Mare vary in the range of several ppm to more than 100 ppm (wet weight). These values are quite high compared to those of individuals from unpolluted areas (generally below 1 ppm w.wt., Noël-Lambot et al., 1978b). On the other hand, Zn and Cu concentrations in limpets from Weston are only a little above normal.

Close relationship exists between metal concentration and body size. This relationship differs, depending on the metal: Cd tissue concentration is directly proportional to body size, but for Zn and Cu, inverse relationships are observed. In Figure 1, body size is expressed as length of the shell, but similar relationships are observed when metal concentrations are plotted against wet body weight.

Table 2 shows that viscera have higher concentrations in Cd and Cu than foot muscles. Cd concentration in muscular tissue is nevertheless considerable (see 'Discussion').

Table 2. *Patella vulgata*. Concentrations of Cd, Zn and Cu in foot and viscera of limpets (shell length 30 mm) caught off Portishead (Bristol Channel). Mean concentrations (n = 6) ± standard error, expressed as ppm in wet flesh

Tissue	Cd	Zn	Cu
Foot	38.6 ± 6.3	56.5 ± 6.3	2.7 ± 0.4
Viscera	78.3 ± 11.5	60.5 ± 4.9	8.1 ± 1.8

Cd is very slowly lost from the tissues when limpets are exposed to unpolluted water (see 'Material and Methods'). Eighty days after transfer to clean sea water, the test limpet (shell length : 36 mm) still contained 75 ppm Cd – a value quite similar to that before transfer (Fig. 1).

Distribution of Cadmium Among Different Fractions of Homogenates

Figure 2 illustrates the chromatographic profile of the soluble fraction from whole soft parts from large limpets collected from the Bristol Channel. The Cd

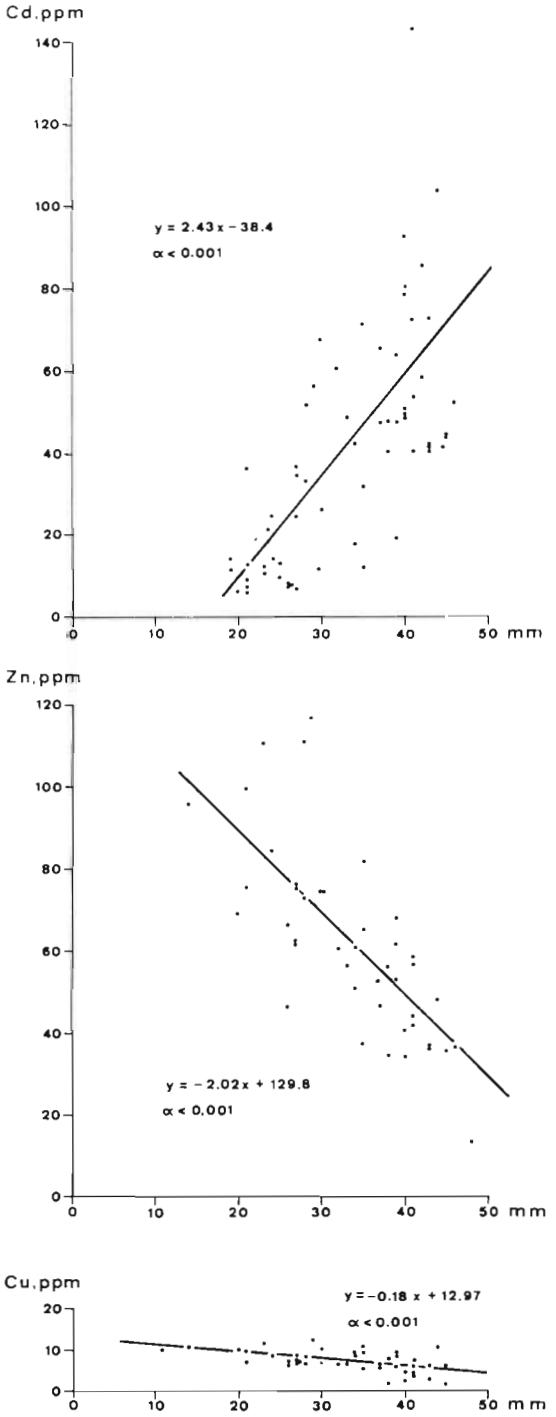

Fig. 1. *Patella vulgata*. Relation between metal concentration in soft tissue (ppm, w.wt.) and shell length for Cd, Zn and Cu in limpets collected from Weston-super-Mare

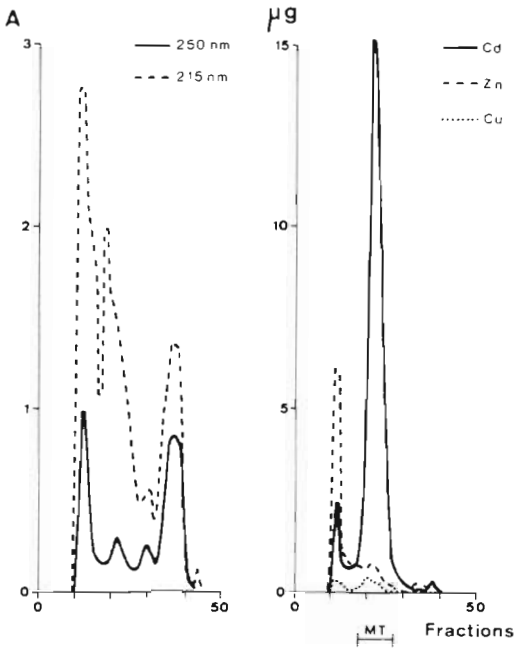

Fig. 2. *Patella vulgata*. Elution profiles on AcA 54 column (2.6 × 33.5 cm; fraction volume: 5 ml) of water soluble fraction extracted from whole soft tissues of limpets from Portishead (shell length: 38 – 42 mm). Metal concentrations in elution fractions expressed in μg per fraction and per 1.2 g of tissue

peak in the lowest molecular weight region corresponds to metallothioneins. This fraction, called MT fraction, coincides with a 250 nm absorbance peak. It only contains small amounts of Zn and Cu.

Such chromatograms were used to estimate the amount of Cd bound by metallothioneins. This value was obtained by adding up the Cd contents of the elution fractions corresponding to the MT peak. Cd present in fractions eluted before the MT fraction was called 'Cd bound to high molecular weight proteins' (HMWP). Note that in fractions eluted after MT fraction, Cd concentrations fall below the detection limit, with the result that the sum of Cd bound to HMWP and of Cd bound to MT corresponds to the total Cd content of the water soluble extract.

Limpets of various size were studied in this way. Results are presented in Figure 3. There is a sharp correlation between total accumulated Cd and the Cd attached to metallothioneins. Moreover in limpets with minimum Cd loads (Cd concentration below 13 ppm), the metal is not detected in the MT fraction of the chromatograms. A significant part of the total Cd is always present in the pellet. Cd concentration in this fraction also increases with the total Cd load. On the other hand, the amount of Cd bound to soluble proteins of high molecular weight increases very slightly as compared to cadmium bound to metallothioneins.

Intracellular distribution of Cd is, therefore, quite different in small individuals, thus having a low Cd concentration, and in large individuals with a high Cd concentration. In small limpets, Cd is almost exclusively stored at the level of pellet and soluble proteins of high molecular weight; in large ones, Cd bound to metallothioneins represents about 85 % of soluble Cd which corresponds to approximately 50 % of total Cd.

As shown in Figure 4, metallothioneins can be detected both in viscera and in foot muscle. In both tissues, about 80 % of soluble Cd in present in the MT fraction.

A comparison of Figure 5 with Figure 2 shows that intracellular Cd distribution does not change when limpets are exposed for a long time to unpolluted sea water. The largest part of Cd in tissues thus persists as Cd-thionein.

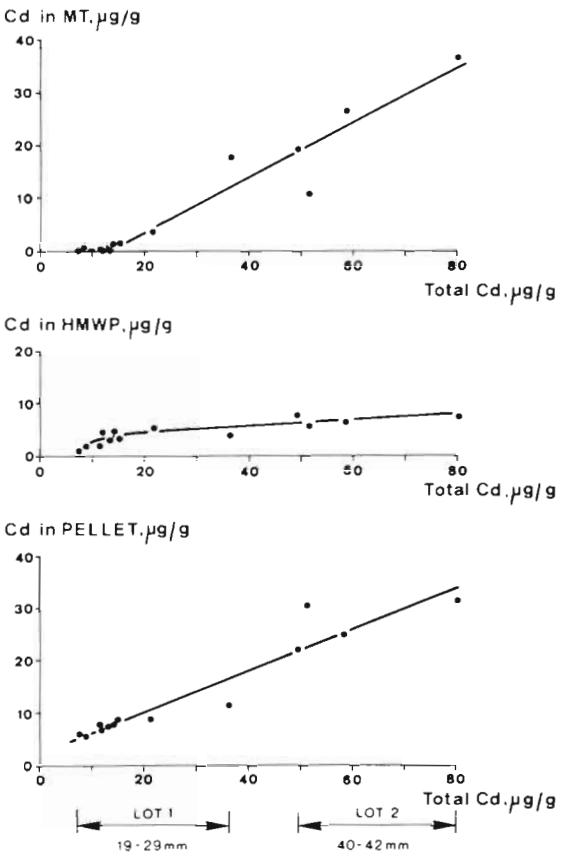

Fig. 3. *Patella vulgata*. Relation between Cd concentration in whole soft parts of limpets collected from Weston-super-Mare and concentrations of Cd associated with (1) metallothioneins (MT); (2) high-molecular weight proteins (HMWP) – both isolated by gel filtration of the supernatant of the homogenate; (3) the centrifugation pellet of this homogenate. All concentrations expressed in μg Cd g^{-1} wet tissue. Based on shell length, limpets were classed into two lots

Characterization of Limpet Cd-Thioneins

As already pointed out, this work was performed on animals toxicated under laboratory conditions. After 60

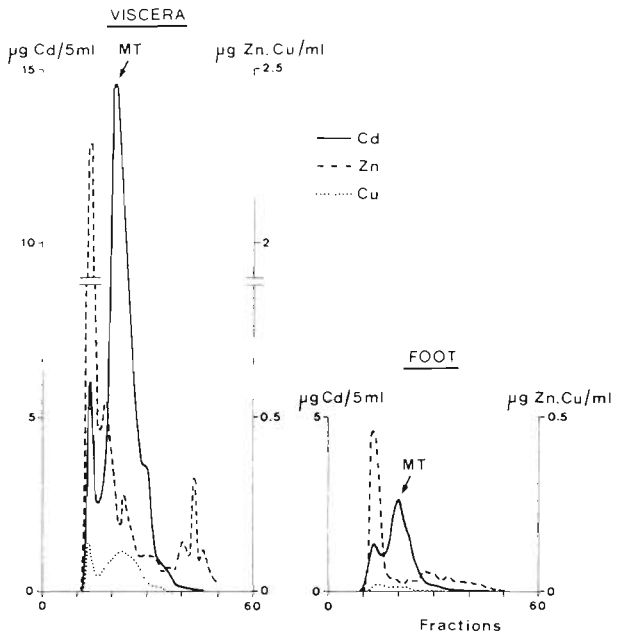

Fig. 4. *Patella vulgata*. Elution profiles on AcA 54 column (2.6 × 33.5 cm; fraction volume: 5 ml) of water soluble fraction extracted from viscera and foot of limpets from Portishead. Metal concentrations in elution fractions expressed in µg 1 ml^{-1} or 5 ml^{-1} and per 1.2 g of tissue

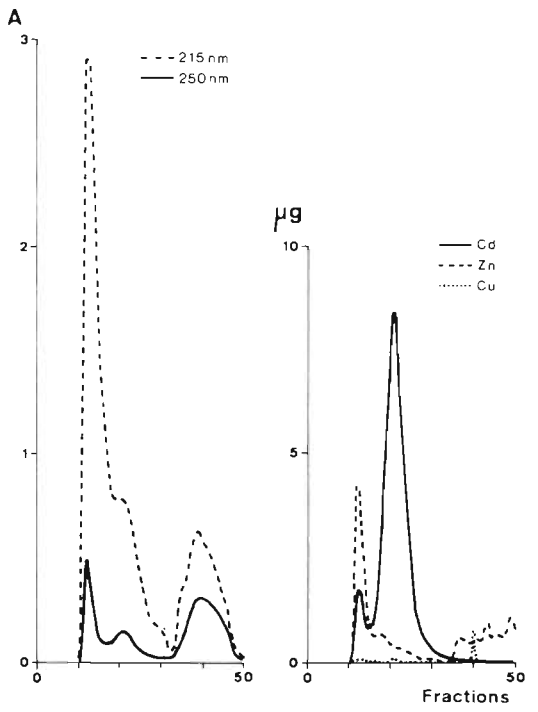

Fig. 5. *Patella vulgata*. Elution profiles on AcA 54 column (2.6 × 33.5 cm; fraction volume: 5 ml) of soluble fraction of one individual collected from Bristol Channel and then transferred for 80 d to unpolluted sea water. Metal concentrations in elution fractions expressed in µg per fraction and per 1.2 g of tissue

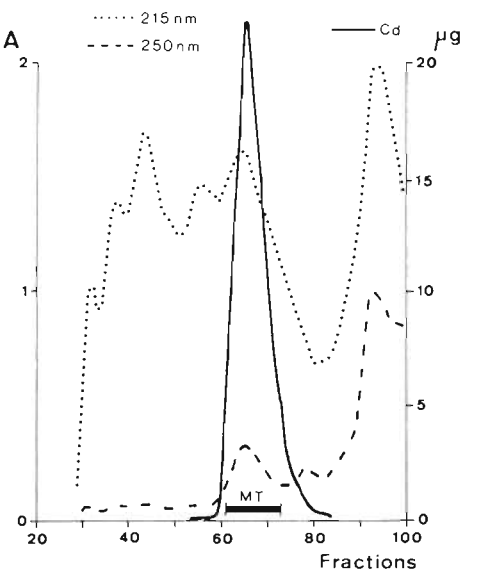

Fig. 6. *Patella vulgata*. Elution profiles on AcA 54 column (5 × 50 cm; fraction volume: 10 ml) of the 45–80 % acetonic fraction from limpets experimentally toxicated with Cd. MT: metallothioneins containing fractions. Extract prepared from 21.7 g tissue

Table 3. *Patella vulgata*. Amino acid composition of metallothioneins

Amino acid	Number of residues (%)□ MTa	MTb
Lysine	8.8	8.6
Histidine	1.0	1.4
Arginine	0.8	0.9
Aspartic acid	11.2	12.0
Threonine	7.2	6.8
Serine	8.8	8.5
Glutamic acid	7.7	8.5
Proline	3.8	4.1
Glycine	11.0	10.6
Alanine	8.7	8.9
△ Cysteine (1/2)	21.0	20.0
Valine	3.2	2.7
○ Methionine	< 0.5	< 0.5
Isoleucine	1.8	1.5
Leucine	2.6	2.7
Tyrosine	1.8	1.8
Phenylalanine	1.0	1.0
▶ Tryptophan	–	–

□ 24 h hydrolysis.
△ Determined as cysteic acid.
○ Determined as methionine sulfone.
▶ Not determined.

d in sea water containing 0.5 ppm Cd, the Cd in limpets reached about 30 ppm.

Figure 6 shows the AcA 54 elution profile of the 45–80 % acetonic fraction. Two main peaks of absorb-

ance at 215 nm are observed, one in the 6000–10 000 Daltons range, the second corresponding to lower molecular-weight molecules. Acetone fractionation thus eliminates most of the high molecular-weight proteins. For the first peak, 250 nm as well as the Cd distribution follow the profile of 215 nm. It was therefore designated as MT in Figure 6.

As shown by polyacrylamide gel electrophoresis (Fig. 7), this MT fraction is heterogeneous. The two major constituents are characterized by fast migration and high Cd amounts. Therefore, they probably correspond to metallothioneins. They can be partially resolved by ion exchange chromatography (Fig. 8).

Amino acid analysis reveals that both components are very similar to one another (Table 3). This amino acid composition is typical of metallothioneins: a high content in cysteine and very low amounts of histidine, arginine and aromatic amino acids.

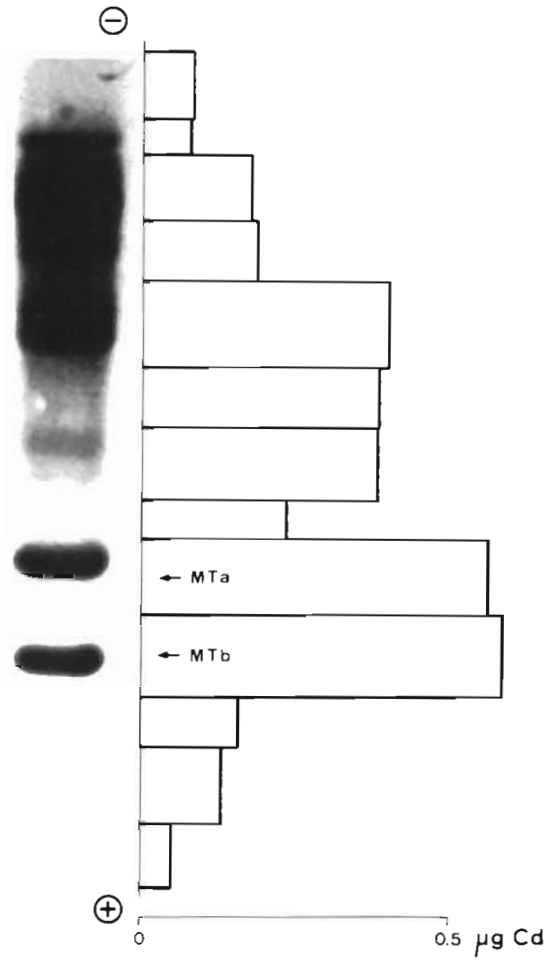

Fig. 7. *Patella vulgata*. Study by polyacrylamide gel electrophoresis of MT fraction isolated by filtration on Ultrogel AcA 54 (see Fig. 6); right: cadmium content of various gel slices (in µg per slice)

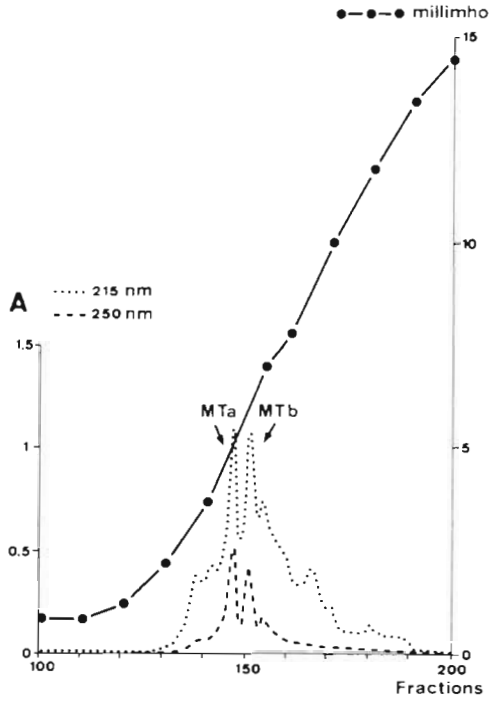

Fig. 8. *Patella vulgata*. Study by ion exchange chromatography (DEAE-cellulose, fraction volume: 4 ml) of MT fraction isolated by filtration on Ultrogel AcA 54 (see Fig. 6)

Both metallothioneins bind high amounts of cadmium and a little copper, but zinc seems to be absent. The ratio cysteine/metal is 2.3 (Table 4).

Table 4. *Patella vulgata*. Metal content of metallothioneins (number of metallic ions per 30 cysteinyl residues)

	Cd	Zn	Cu	Total	Cysteine metal
MTa	12.4	0	0.9	13.3	2.3
MTb	12.0	0	1.0	13.0	2.3

DISCUSSION AND CONCLUSIONS

From a practical point of view, our results concerning the relation between size of *Patella vulgata* and Cd, Zn and Cu concentrations suggest that the use of organisms as indicators of pollution may present some problems. Indeed, differences in metal tissue concentrations between populations of the same species may reflect real differences in environmental concentrations, but they also may be due to differences in body size. Moreover, environmental variables such as salinity, temperature, position of the organism in the water column, season, coexistence of several metals and presence of chelating agents also may influence

the uptake of metals by organisms (Eisler and Gardner, 1973; Vernberg et al., 1974; Phillips, 1976; George and Coombs, 1977; George et al., 1978). It is thus essential to take all these variables into account when biological indicators are used to monitor environmental contamination by trace metals.

Similar relations as those described here between metal content and body size were reported previously by Boyden (1974) for *Patella vulgata* collected from Portishead, Bristol Channel. This work, like ours, indicates that Cd displays a relationship opposite to Zn and Cu (Fig. 1), suggesting quite different metabolic pathways of these elements.

Concerning the increase of Cd concentration with body weight, Boyden (1974) concluded: '. . . this relationship can best be explained as being due to removal of this element from body circulation and accumulation within specific tissue, possibly as a result of some exceptional affinity.'

Our results with Cd-thioneins confirm and allow to better understand this interpretation. Metallothioneins may be considered as the specific Cd-binding compound responsible for the unusually high levels of Cd in large limpets. First of all it must be pointed out that the shell length of *Patella vulgata* constitutes a good indicator for age. Several studies describe the rate of growth of the shell of this species (Russell, 1909; Orton, 1928; Fischer-Piette, 1941). They show that shell growth varies considerably with environmental factors, but that in the same population shell-length classes may be considered as age classes. Maximum Cd concentrations are recorded in the oldest individuals, i.e., in limpets exposed for a long time to water pollution. From the above-mentioned studies, it may be estimated that the largest individuals we have studied are 2 to 3 years old.

Figure 3 shows that the amount of Cd bound to metallothioneins is much higher in old individuals (Lot 2) than in young ones (Lot 1). The increase in MT-bound Cd with increasing total Cd load undoubtedly indicates an increase in metallothionein concentration (see section 'Material and Methods': 'Estimation of metallothionein content'). In the same way, absence of Cd, Zn and Cu in the MT chromatographic fraction may be considered as an indication of the real absence of these proteins. Scanning at 520 nm of stained polyacrylamide gels of the MT chromatographic peaks, as well as determination of the cysteine content of these peaks, provides additional evidence of higher metallothionein concentrations in old limpets than in young ones. These observations are compatible with experimental results concerning molluscs and fish showing a direct relationship between the metallothionein content and the time of exposure to Cd (Noël-Lambot, 1979). It is evident that increase in the amounts of metallothioneins with time corresponds to an equivalent increase of Cd-binding sites and thus to a more and more extensive capacity of Cd storage.

It can also be observed from Figure 3 that metallothioneins appear in the organism at a critical level of cadmium load. In individuals collected from Weston-super-Mare, this critical level in the tissues is approximately 13 ppm but some of our unpublished results seem to indicate that, in the same species, this level might vary – for example according to the environmental Cd concentration.

It further appears from Figure 3 that when the critical total Cd concentration for metallothionein induction is reached, Cd bound to high-mol.wt. proteins hardly increases any more as the total Cd concentration rises. It thus may be considered that metallothioneins only appear when the high-mol.wt. soluble proteins reach a certain level of saturation by cadmium and that, from then on, Cd accumulation in the cytosol almost exclusively occurs at the level of metallothioneins.

The separate study of foot and viscera provides also interesting informations. It is surprising to observe in limpet muscular tissue relatively high levels of Cd bound to metallothioneins. This is quite different from observations made in vertebrates where muscular tissue never reaches concentrations higher than 1 or 2 ppm wet weight, even in the case of drastic Cd intoxication (Noël-Lambot and Bouquegneau, 1977). Moreover, muscle is to our knowledge the only tissue in which the existence of metallothioneins has never been reported.

Once again, high Cd concentrations in tissue and abundance of metallothioneins seem to be closely linked. This relation between concentrations of cadmium and metallothioneins, already observed in whole individuals collected from the same area (Weston-super-Mare) but differing in Cd concentrations (Fig. 3), also pertains to limpets caught from two distinct areas with different Cd pollution levels (Noël-Lambot et al., 1978a).

When toxication ends, metallothioneins persist in the tissues although the cause of their formation has disappeared. The persistence of Cd-thioneins may, however, be linked to a continuous important turnover of apoprotein, as shown by Chen et al. (1975) in mammals.

The characterization of low-molecular weight Cd-binding proteins isolated from Cd-toxicated limpets confirms that they belong to the group of metallothioneins. The cysteine/metal ratio is a little lower than that reported for mammals, but it is very similar to the value we found for eel metallothioneins (unpublished). At least two metallothioneins – very similar in amino acid and metal composition but with different electrical charges – occur in limpets. As in mammals, these

probably correspond to isoproteins (Kojima and Kägi, 1978).

In conclusion, it may be considered that the ability to synthesize metallothioneins provides extensive possibilities of Cd accumulation to living organisms. This ability partly explains why Cd concentrations in limpets living in Cd-polluted waters are directly related to age. Induced metallothionein production and its persistence after toxication allows a better understanding of the wellknown process of cumulative Cd absorption during long-term Cd toxication and of the very long half life of this metal. Metallothioneins thus act as a trap for Cd.

Moreover, binding of Cd to metallothioneins very likely constitutes a mechanism of Cd detoxication. This may explain how limpets can tolerate such high Cd levels in their tissues. It is felt that the fate of Cd discharged into the environment greatly depends on the presence or absence of active metallothioneins. This is one more argument to study these proteins within the framework of ecotoxicological research.

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