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Turnover rate of metallothionein and cadmium in *Mytilus edulis*

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The results demonstrate the first attempt to determine metallothionein turnover in the whole soft tissues of mussels *Mytilus edulis* exposed to cadmium. Half-lives for metallothionein and cadmium are 25 and 300 days, respectively. As metallothionein degrades the released cadmium induces further synthesis of the protein, to which the metal becomes resequestered. The slow metallothionein turnover rates (compared with mammals) and the lack of significant cadmium excretion testify to the relatively stable nature of the cadmium–metallothionein complex in these invertebrates and supports the view of a detoxifying role for metallothionein in the mussels.

Keywords: cadmium, detoxification, metallothionein, *Mytilus edulis*, turnover

Introduction

Metallothionein is a cysteine-rich heat-stable protein identified in the cytosol of different tissues and species of marine invertebrates. Functions attributed to this protein include regulation of the essential metals zinc and copper, and the detoxification of these and non-essential metals such as cadmium and mercury. Induction of metallothionein may consequently signify exposure to excessive concentrations of metal ions in cells and it has therefore been proposed that assays for metallothionein may be of value in monitoring the effects of metal contamination in aquatic organisms (Olafson *et al.* 1979, Engel & Roesijadi 1987, Bayne *et al.* 1988, Viarengo 1989).

Mussels of the genus *Mytilus* have often been chosen as indicator organisms for metal pollution because of their metal accumulating capacity and widespread distribution (Goldberg *et al.* 1978). Biochemical studies have also identified the presence of metal binding proteins, similar to metallothioneins, in several tissues of *M. edulis* and *M. galloprovincialis* (e.g. gills, hepatopancreas, digestive gland, mantle and kidney) following exposure to cadmium (Noel-Lambot 1976, George & Pirie 1979, Frankenne *et al.* 1980, Kohler & Riisgard 1982, Carpene *et al.* 1983, Nolan & Duke 1983a,b, Frazier *et al.* 1985, Viarengo *et al.* 1985b, Pavicic *et al.* 1987).

More recently, exposure of the mussels *M. edulis* and *M. galloprovincialis* to cadmium (400 μg $Cd\,l^{-1}$) has been shown to induce the synthesis of metallothionein. During exposure, metallothionein concentrations, measured by differential pulse polarography (Bebianno & Langston 1989), increased 3- to 4-fold compared with controls, supporting the view that metallothionein induction in mussels is a quantifiable biological response to sublethal levels of cadmium (Bebianno & Langston 1991, 1992). Metallothionein concentrations of 2–3 $mg\,g^{-1}$ (measured against rabbit metallothionein standards) have been proposed as being typical for mussels collected from sites free from cadmium pollution (Bebianno 1990, Bebianno & Langston 1991, 1992).

Before we can hope to use such assays in the field, however, it is important to have some estimate of turnover rates for metallothionein. This is necessary both in terms of clarifying the role of the protein in metal metabolism and more importantly in identifying the time-scales over which metallothionein levels respond to changes in contamination.

Turnover rates for metallothionein have been

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measured in some vertebrate tissues, notably in the liver of mouse (Olafson 1981) and rat (Chen *et al.* 1975, Feldman & Cousins, 1976, Shaikh & Smith 1976, Andersen *et al.* 1978, Bremner *et al.* 1978, Feldman *et al.* 1978, Cain & Griffiths 1984, Held & Hoekstra 1984), in the kidney of rats (Shaikh & Smith 1976, Bremner *et al.* 1978, Feldman *et al.* 1978), in the liver and kidney of the plaice *Pleuronectes platessa* (Overnell *et al* 1987), and in the liver of the coho salmon *Oncorhynchus kisutch* (McCarter & Roch 1984). In these organisms, the half-lives were determined after exposure to cadmium, copper or zinc. Individual half-lives for the isomers MT-I and MT-II have also been reported for mammals (Cain & Griffiths 1984). It is concluded from these studies that, in mammals, the turnover rates of metallothioneins induced by exposure to cadmium are slow by comparison with those induced by copper and zinc. To date, however, there is little direct information on the half-life of metallothionein in invertebrates.

This paper describes the first attempt to determine metallothionein turnover in the whole soft tissues of the mussel *M. edulis*. Experiments were devised to determine the half-life of metallothionein and associated metal (cadmium) in the soft tissues of *M. edulis, in vivo*, by following the incorporation of cadmium and [^{35}S]cysteine into the protein, and observing its subsequent loss.

Materials and methods

M. edulis (shell length 4–6 cm, mean dry weight 0.46 ± 0.11 g) were collected from Whitesand bay, south west England, and acclimated in aerated seawater, salinity 34‰, at 15 °C for 1 week prior to the experiments. Subsequently, groups of 15 animals were held in 5 l aquaria and exposed to a cadmium concentration of 400 $\mu g\,l^{-1}$ for 2 weeks. Control groups of mussels were maintained in clean seawater (37.2 ± 3.6 ng Cd l^{-1}). Water in each tank was changed twice weekly.

At the end of this period 2.5 μCi of L-[^{35}S]cysteine (Amersham International, Amersham, UK) was injected directly into the mantle cavity of control and cadmium-exposed mussels and the valves held closed with the use of elastic bands. Three hours after injection, the mussels were returned to the tanks from which they had been taken. Four days after injection the group exposed to cadmium was divided into two sub-groups; one was kept in the same cadmium concentration and the other was transferred to clean seawater ('detoxified').

Four control, four cadmium-exposed and four detoxified mussels were removed for analysis 4, 11, 18, 25 and 31 days after injection. Soft tissues were then separated from the shells, weighed and homogenized in three volumes of 0.02 M Tris–HCl (pH 8.6) buffer. Between 3 and 5 ml of the homogenate was centrifuged at 30 000 × *g* for 1 h at 4 °C and the supernatant (cytosol) was then separated from the pellet. A separate aliquot of the homogenate was weighed, dried and reweighed in order to determine the wet:dry weight ratio.

Measurement of radioisotope uptake and loss

The ^{35}S activity in the homogenate of the whole mussels was determined by digesting an aliquot of 0.2 mg of the homogenate overnight in 1 ml of tissue solubilizer (Optisolv, Wallac Pharmacia, Milton Keynes, UK). Subsequently 14 ml of liquid scintillant (Optiphase'MP', Wallac Pharmacia) was added and samples counted in a LKB Wallac 1215 Rackbeta II scintillation counter Pharmacia Biosystems Ltd, Milton Keynes, UK.

Aliquots of the cytosol (0.5–1 ml) were chromatographed at 4 °C on a Sephadex G-75 column (1.5 × 60 cm; Pharmacia Biotech Ltd, St Albans, UK) and eluted with 0.02 M Tris–HCl (pH 8.6) to separate the different molecular weight ^{35}S containing components. A detailed account of the elution of mussel metallothionein, in relation to other components, under similar conditions, is given elsewhere (Bebianno & Langston 1991). The ^{35}S activity of each column fraction was determined following the addition of 14 ml of liquid scintillant to 1 ml of each fraction. The ^{35}S activities were expressed as disintegrations per minute (d.p.m) for the original dry tissue weight (d.p.m. g^{-1}) and corrected for quenching, decay and background; counting efficiency was 98 ± 3%.

Cadmium analysis

Cadmium analysis, by atomic absorption spectrophotometry, was performed on acid-digested subsamples of the homogenate and cytosol of the whole soft tissues of *M. edulis*.

Results

Distribution of [^{35}S]cysteine among cytosolic components

After L-[^{35}S]cysteine was injected into the mantle cavity of the mussels most of the ^{35}S activity was detected in the cytosol of the whole soft tissue preparations (63% in controls, 70% in cadmium-exposed mussels).

Typical elution profiles showing the distribution of the ^{35}S-labeled amino acid in the cytosol of mussels, sampled 25 days after injection, are shown in Figure 1. It would seem that little of the L-[^{35}]cysteine remains as free amino acid (fractions 25–30), most being incorporated as protein. In control mussels there is a small peak, amounting to 30% of the cytosolic ^{35}S, which elutes with high molecular weight compounds, presumed to be proteins (fractions 10–15, molecular weight > 75 000). The activity of the ^{35}S in the metallothionein pool of controls

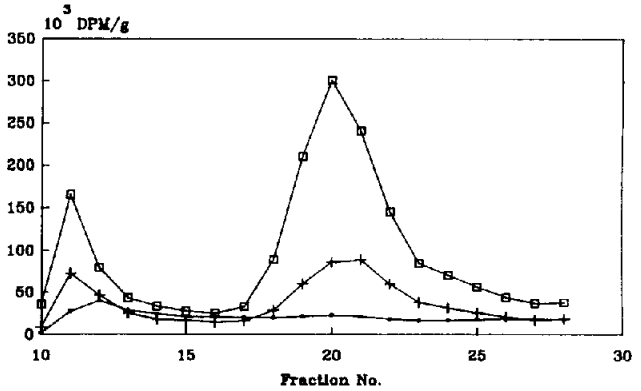

Figure 1. Chromatographic elution profiles of the ^{35}S activity in the cytosol of control (●), cadmium-exposed (□) and cadmium-detoxified mussels (+), 25 days after injection with [^{35}S]cysteine.

(centered on fraction 20) was extremely low (5% of total cytosolic ^{35}S). However, in cadmium-exposed and cadmium-detoxified mussels, 40% of the ^{35}S was detected in association with metallothionein, clearly at significantly higher levels than in controls (ANOVA, $P < 0.05$).

The proportion of isotope in the high molecular weight protein pool and very low molecular weight fractions (fractions 30–54) in both controls and cadmium treated mussels was not significantly affected by cadmium exposure.

Turnover of [^{35}S]cysteine

The ^{35}S activity in the pooled metallothionein-containing fractions (fractions 17–28) of controls, cadmium-exposed and cadmium-detoxified mussels are plotted against time in Figure 2. The results confirm earlier findings, based on polarographic measurements, that cadmium stimulates the induction of metallothionein (Bebianno & Langston 1991). In both cadmium-exposed and cadmium-detoxified mussels [^{35}S]cysteine incorporation into metallothionein increased in the first 2 weeks after injection. In cadmium-exposed mussels the activity continued to increase but at the end of the experiment (day 32) it appeared that a steady state had been reached (Figure 2). This also confirms earlier results, using electrochemical techniques to measure metallothionein, that production of metallothionein reaches steady state after 1 month (Bebianno & Langston 1991). In detoxified mussels (transferred to clean seawater), however, there was an apparent slow net loss in activity, beginning 18 days after injection with [^{35}S]cysteine. The calculated half-life was approximately 25 days. No significant change was detected in controls (Figure 2).

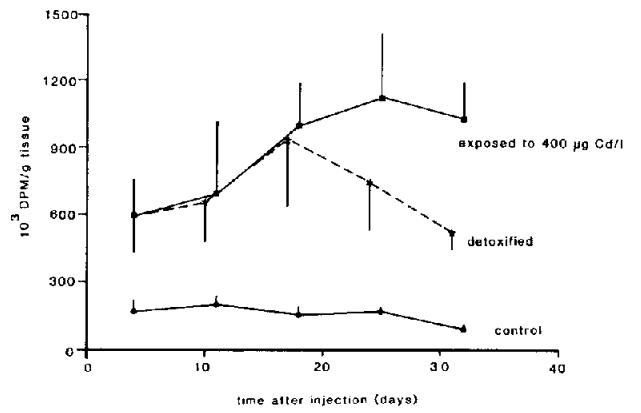

Figure 2. [^{35}S]cysteine (d.p.m. g^{-1}) incorporation into and loss from the metallothionein pool from control, cadmium-exposed and cadmium-detoxified mussels. Each point represents mean pooled counts of the ^{35}S activity in the metallothionein-containing fractions (fractions 17–28 from Figure 1). Vertical bars represent one-half the standard deviation of the mean ($n = 4$).

Uptake and elimination of cadmium in M. edulis

Figure 3 shows total and cytosolic cadmium concentrations in the whole soft tissues of the mussels exposed to 400 μg $Cd l^{-1}$ and subsequently transferred to clean seawater (detoxified). The results demonstrate that cadmium accumulated rapidly in the whole soft tissues of the mussels during the uptake phase and reached a total concentration of 286 $\mu g\, g^{-1}$ dry weight after 19 days. Much of the accumulated cadmium (235 $\mu g\, g^{-1}$) was present in the cytosol. These are equivalent to uptake rates of 13.9 $\mu g\, g^{-1}\, day^{-1}$ ($P < 0.001$) and 10.9 $\mu g\, g^{-1}\, day^{-1}$

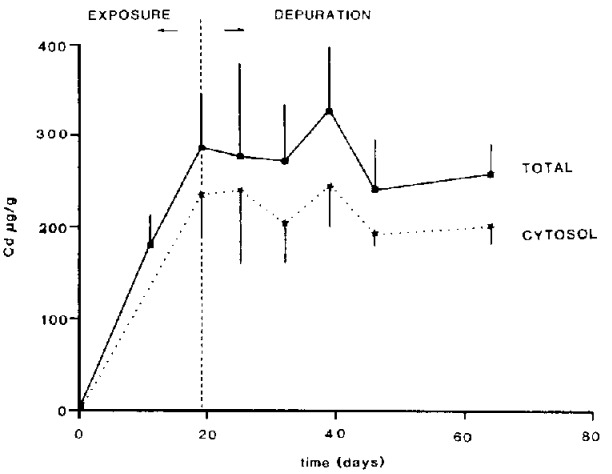

Figure 3. Total (—) and cytosolic (· · ·) cadmium concentrations in the whole soft tissues of mussels exposed to cadmium (400 $\mu g\, l^{-1}$) for 19 days and detoxified for 42 days. Vertical bars represent one-half the standard deviation of the mean.

($P < 0.001$) for total and cytosolic cadmium, respectively.

Following transfer of mussels to clean seawater, loss of cadmium from the soft tissues was slight, though not significant ($P > 0.05$) (Figure 3), despite the concomitant turnover of metallothionein indicated in Figure 2. Thus, after 2 months in clean seawater the mussels still contained extremely high cadmium levels when compared with controls (100-fold). The calculated half-life of cadmium in the whole soft tissues was approximately 300 days.

Discussion

Viarengo *et al.* (1980) have previously demonstrated that exposure to copper stimulates the assimilation of ^{35}S from L-[^{35}]cysteine into metallothionein in the gills of *M. galloprovincialis*. Similarly, mercury exposure leads to enhanced uptake of ^{35}S associated with metallothionein in the gills of *M. edulis* (Roesijadi *et al.* 1982). The increased incorporation of L-[^{35}S]cysteine into the metallothionein pool in cadmium-exposed mussels, described in the current experiment, confirms earlier electrochemical studies which indicated that cadmium also induces synthesis of this metal-binding protein (Bebianno 1990, Bebianno & Langston 1991). Furthermore the 3.5-fold increase in [^{35}S]cysteine activity incorporated into the metallothionein pool of cadmium-exposed mussels (19 days), relative to controls, is of similar magnitude to the induction of metallothionein, measured by differential pulse polarography, in identically exposed mussels (Bebianno & Langston 1991).

Following its initial assimilation, [^{35}S]cysteine associated with the metallothionein-containing fraction of mussels continuously treated with cadmium was maintained at elevated levels throughout the experiment (Figure 2). For those mussels initially exposed to cadmium and subsequently transferred to clean seawater, the decrease in [^{35}S]cysteine from the metallothionein pool only becomes detectable after 2 weeks, reflecting the slow turnover of these proteins. A metallothionein half-life of 25 days was calculated.

This value represents the first attempt to calculate the half-life of metallothionein in marine invertebrates following cadmium exposure. However there are previous observations that zinc–metallothionein in *M. edulis* has a shorter half-life than zinc in tertiary lysosomes (half-life of about 60 days) (George & Pirie 1979, George & Viarengo 1985).

The turnover of metallothionein thus appears to be extremely slow in these marine invertebrates when compared with some vertebrates. In rats metallothionein turnover, following cadmium exposure, is extremely rapid, and the protein has a half-life of 3–4 days in the liver and 4–5 days in the kidney (Chen *et al.* 1975, Shaikh & Smith 1976, Feldman *et al.* 1978, Held & Hoekstra 1984). However, in mice a half-life of 32 days has been reported for the liver (Shaikh & Smith 1976, Feldman *et al.* 1978). The half-lives of metallothionein induced by zinc in the liver and kidney of the plaice *Pleuronectes platessa* ranged from 22 to 32 days (Overnell *et al.* 1987). These results suggest therefore that synthesis and turnover of metallothionein progresses at potentially higher rates in more highly evolved organisms such as mammals.

Our experimental study of metallothionein metabolism in *M. edulis* also reveals that, although the turnover of metallothionein in molluscs may be relatively slow, it is nevertheless much faster then suggested by the loss of associated cadmium. The release of cadmium from the metallothionein pool, and thus in whole mussels, was indeed extremely slow with an estimated half-life of approximately 300 days. This was similar to the calculated half-life for cadmium (300 days) for the kidney of *M. edulis* following exposure to 100 μg $Cd\,l^{-1}$ (George & Pirie 1979). However, Scholz (1980) reported a shorter half-life for cadmium ranging from 14 to 29 days for *M. edulis* exposed to a range of cadmium concentrations (10–100 μg $Cd\,l^{-1}$) and a value of 70 days was calculated for the clam *Macoma balthica* (Langston & Zhou 1987a). Cadmium turnover may therefore display a high degree of variability among bivalve molluscs and even among individual tissues. The estimated half-life for cadmium of 35 days in the gills of the oyster, *Crassostrea virginica*, for example, is much shorter that in whole cadmium-exposed oysters, transferred to clean seawater, where the half-life was 150 days (Roesijadi & Klerks 1989). It was suggested by these authors that cadmium was transported out of the gills to internal tissues where the cadmium turnover rate was considerably slower. A similar phenomenon has also been described in gastropod molluscs (Langston & Zhou 1987b).

In molluscs, cadmium is commonly accumulated in the digestive gland and in the kidney, notably in the residual bodies of lysosomes where it is probably bound to the peroxidated matrix (George 1982, George & Viarengo 1985, Viarengo *et al.* 1985a). Although residual bodies are excreted there is no evidence that significant amounts of cadmium are lost in this manner. Viarengo *et al.* (1985b) detected an overall 50% decrease in cadmium levels, during 4

months of detoxification, from the digestive gland and gills of *M. galloprovincialis* previously exposed to 200 μg $Cd\,l^{-1}$ for 28 days. Cadmium bound to metallothionein, however, increased during the first phase of the detoxification period and decreased only slowly thereafter. The tendency for cadmium to be retained in association with metallothionein was seen as a means of preventing the toxic metal from disrupting the normal biochemical processes of the tissues. In the depuration period (2 months) used in the current experiment, less than 20% of the cadmium was released from *M. edulis*, with much of the remaining cadmium being bound to metallothionein. It is likely therefore that re-sequestration of cadmium occurs during turnover of metallothionein. A similar effect has been reported for cadmium-induced metallothioneins in rats, where, in contrast to relatively rapid turnover of the protein, cadmium exhibited a very long half-life, suggesting that the metal was reincorporated by freshly synthesised metallothionein (Andersen *et al.* 1978, Feldman & Cousins 1976, Bremner *et al.* 1978).

The relative turnover of metallothionein and cadmium in *M. edulis* described this paper is consistent with the concept of a 'closed cycle' of cadmium-binding metallothioneins proposed by other workers (Viarengo *et al.* 1985b, Viarengo 1989). Thus, these proteins may be compartmentalized in the lysosomes (George & Pirie 1979) and, on hydrolysis, release most of the cadmium back to the cytosol. There it may continue to stimulate the production of, and eventually re-bind to, the newly resynthesized metallothionein.

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