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An Evaluation of Mercury-203 for Assessing the Induction of Metallothionein-Like Proteins in Mussels Exposed to Cadmium*

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Animals exposed to heavy metals often respond by synthesizing low molecular weight proteins called metallothioneins (Kagi & Nordberg 1979). These proteins are very rich in sulfhydryl groups which can bind heavy metal cations to varying degrees. Similar to the success which has been obtained in the field with mixed-function oxidases and organic compounds (Payne & Penrose 1975; Payne 1976; Kurelec et al. 1977; Stegeman 1980; Spies et al. 1982), metallothionein proteins have the potential to play a useful role in monitoring studies for biologically important levels of heavy metals (e.g. Klaverkamp et al. 1981). The most common method for the detection of metallothioneins utilizes gel chromatography to separate metallothioneins from high molecular weight cytosolic proteins. This method permits successful separation of the metallothionein fraction for further analysis or purification but is inadequate for large-scale screening studies in the laboratory or field. Thus, any method which would facilitate the detection of elevated metallothionein levels would be of interest in environmental studies.

Piotrowski et al. (1973) introduced a rapid method for the quantification of the metallothionein content of rat liver and kidney which can be adapted for use with relatively small amounts of tissue (less than 0.5 g). In this method, radioactive mercury was used to tag the metal-binding sulfhydryl groups and the metallothioneins were then separated from high molecular weight proteins by TCA precipitation. The purpose of the present study was to determine the sensitivity and feasibility of this method with cadmium-exposed marine mussels (Mytilus edulis). This mussel is very widely distributed, has been extensively studied for its ability to accumulate heavy metals (e.g. George 1980; Popham & D'Auria 1982; Lobel & Wright 1982; Lobel et al. 1982), and has been recommended as a global sentinel of marine pollution in the "mussel watch" programme (Goldberg 1976). Noel-Lambot (1976), using gel chromatography techniques, noted the induction of metallothionein-like proteins in mussels exposed to 5 ppb cadmium for 90 d.

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MATERIALS AND METHODS

Specimens of Mytilus edulis were obtained from two sites (Old Shop and Bellevue) on the Avalon Peninsula of southeastern Newfoundland and kept in tanks of running seawater at the Marine Sciences Research Laboratory, Memorial University. Mussels were exposed to cadmium according to the regime in Table 1. The water was not changed throughout the course of the experiments except in the case of GROUP C. The water in GROUP C was replaced with fresh seawater containing 8 ppb cadmium on days 7, 14, 21 and 34. The water temperature was 3-5°C and all animals remained alive and appeared to be siphoning normally throughout the experiments. In the cases of GROUPS A, B & C, fresh hepatopancreatic tissues were analyzed but in the cases of GROUPS D, E & F, tissues were stored at -80°C prior to analysis.

Table 1. Exposure of mussels to cadmium.

GROUP	Origin of mussels	Conc of cadmium ppb	Time of exposure d	Centrifuge speed g
A	Old Shop	400	34	16 000
B	Old Shop	400	29	105 000
C	Bellevue	8	36	16 000
D	Bellevue	1	13	16 000
E	Bellevue	10	13	16 000
F	Bellevue	100	13	16 000

The hepatopancreas was removed from each mussel and blotted dry on paper towel. The organs were then pooled into composite samples and wet weighed. The weighed samples were homogenized in 1.15% KCl (8 ml/g tissue) in a glass and teflon tissue homogenizer. Homogenates were then centrifuged at either 16 000 g or 105 000 g for 60 min (Table 1). The supernatant so obtained was called the initial supernatant and was tested for the presence of mercury-binding proteins or putative metallothioneins. Piotroswki et al. (1973) did not find it necessary to centrifuge the KCl homogenates of rat liver or kidney prior to analysis but in the present work with marine mussels, it was necessary to centrifuge the homogenates in order to obtain reasonable resolution between control and cadmium-exposed animals. It was also established in preliminary trials that centrifugation at 16 000 g for 60 min gave satisfactory results. This is an advantage for smaller laboratories since it precludes usage of an ultracententrifuge.

Two ml aliquots of initial supernatant were placed in disposable polypropylene tubes. Each tube was then treated with 0.2 ml of a solution of mercury (as mercuric chloride labelled with mercury-203) in distilled water. The amount of mercury added to each tube varied according to the nature of the experiment. Upon mixing, the tubes

were permitted to stand for 15 min and 0.5 ml of 10% trichloroacetic acid (TCA) was then added to each tube to precipitate the high molecular weight proteins. After standing for at least 10 min, the tubes were centrifuged at 4 000 g for 5 min to obtain the TCA supernatant. At this point, all of the mercury in each tube was distributed between one of two fractions: the soluble TCA supernatant and the pellet which contained the higher molecular weight proteins. In order to determine the relative amount of mercury in each of these two fractions, one ml of TCA supernatant was removed from each tube for gamma scintillation counting. Standards with known amounts of mercury were prepared using 2 ml of distilled water in place of 2 ml of initial supernatant but were otherwise given the same treatment as the samples. Because there was no initial supernatant in the standards, TCA addition did not cause any precipitation and it can be assumed that all of the added mercury was found in the TCA supernatant. For each sample of initial supernatant, it was possible to calculate the percentage of mercury that remained in the supernatant after TCA precipitation.

The above procedure was also used to assess metallothionein induction in gill tissues but no significant differences were observed between cadmium-exposed and control mussels.

RESULTS AND DISCUSSION

The rationale for this test was noted by Piotroswki et al. (1973) and Kotsonis & Klaassen (1977) in their studies with rat liver tissues. Briefly, mercury has a strong tendency to bind to sulfhydryl groups (e.g. cysteinyl residues) and under the present test conditions will bind preferentially to metallothionein or metallothionein-like proteins. If enough mercury is added to the initial supernatant to "saturate" most of the sites available on the low molecular weight metal-binding proteins, mercury will then bind to higher molecular weight cytosolic proteins (e.g. enzymes). When the cytosolic sulfhydryl groups are saturated, the excess mercury remains in solution probably as unreacted mercury. The addition of TCA to the reaction mixture precipitates the higher molecular weight proteins leaving the lower molecular weight proteins in solution. Thus, the amount or proportion of mercury remaining in the TCA supernatant becomes a simple indicator of the presence of metal-binding proteins. This is illustrated in Fig. 1 which shows the effect of adding increasing quantities of mercury to the initial supernatant of mussels exposed to 400 ppb cadmium for 34 d (GROUP A mussels). When low levels of mercury are added (e.g. 150 nmoles/g), a high proportion of the metal is found in the TCA supernatant bound to metallothioneins (or similar low molecular weight compounds with a high affinity for mercury). When higher levels of mercury are added (e.g. 1200 nmoles/g), much of this mercury is precipitated with high molecular weight proteins and does not appear in the TCA supernatant. Finally, if an excess of mercury is added (e.g. 2000+ nmoles/g), much of this appears in the TCA

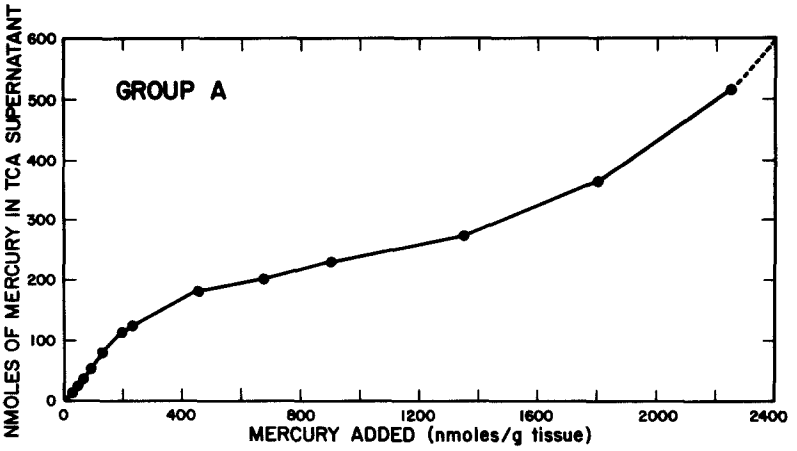

Fig. 1. Relationship between the amount of mercury added to the initial supernatant and the amount of mercury appearing in the TCA supernatant. GROUP A.

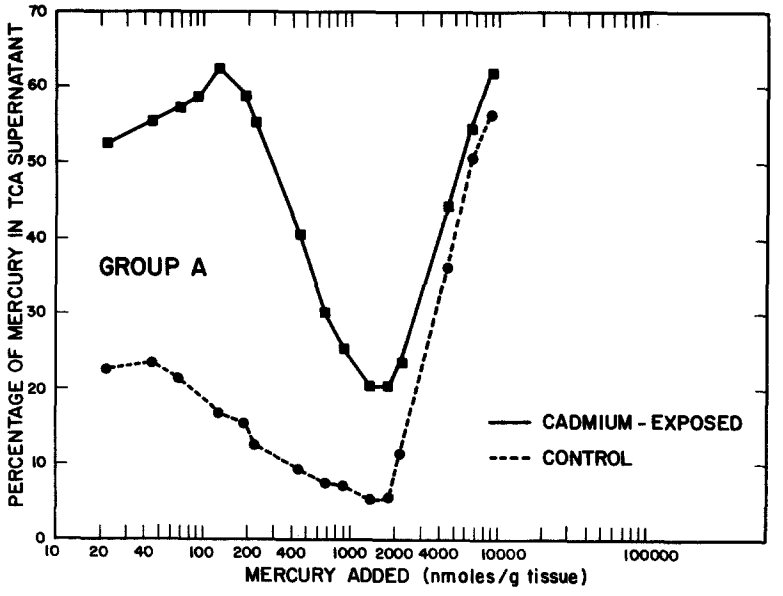

Fig. 2. Relationship between the amount of mercury added to the initial supernatant and the percentage of added mercury appearing in the TCA supernatant. GROUP A.

supernatant as unreacted mercury. The curve in Fig. 1 can thus be seen to consist of three sections: a steep initial section (mercury binding preferentially to low molecular weight proteins), a flatter middle section (mercury also binding to higher molecular weight proteins) and a final steep section (unreacted mercury in supernatant). Figure 1 is similar to comparable graphs presented by Piotworski et al. (1973) and Kotsonis & Klaassen (1977) in their studies with rats injected with cadmium.

The data in Fig. 1 can be more dramatically illustrated by considering the percentage of mercury appearing in the TCA supernatant (Fig. 2). Because of the wide range of values used in this experiment, a semi-log scale was used. When small amounts of mercury were added, as much as 60% of the mercury appeared in the TCA supernatant of the cadmium-exposed mussels but only about 20% in the case of controls. Similar results are shown in Fig. 3 where the homogenate was spun at 105 000 g instead of 16 000 g for 60 min. In this respect, it is worth noting that the use of an ultracentrifuge did not confer any marked advantage. The mussels presented in Figs. 2 and 3 were exposed to 400 ppb cadmium which is much higher than would be found in the environment. Some of the highest values found in the environment have been around 40 ppb and it is of interest that the World Health Organization limit for cadmium in drinking water is 10 ppb (Fassett 1980). Hence, in order to see whether the present test was capable of detecting metallothionein induction at more realistic levels of cadmium exposure, mussels were exposed to 8 ppb for 36 d. The results of this are shown in Fig. 4. As expected, the percentage mercury in the TCA supernatant is less for GROUP C mussels than for GROUP A or GROUP B mussels. Nonetheless, the data clearly demonstrates an increase in the percentage mercury in mussels exposed to very low levels of cadmium when compared with unexposed control mussels. This indicates that the test is quite sensitive and has potential for use in laboratory screening programs as well as in the field.

It would be tedious and time-consuming to construct a curve for each tissue homogenate and both Piotrowski et al. (1973) and Kotsonis & Klaassen (1977) chose a single standard value of mercury to add to each sample. They determined the point at which all the metallothionein was bound to mercury but at which no excess mercury was present in the TCA supernatant. At this point, they reasoned that all of the mercury in the supernatant was bound to metallothionein and would provide a quantitative assay for metallothionein. Standard curves for this metallothionein assay were constructed using pure metallothionein collected from previous experiments. Using gel chromatography, they verified that all of the mercury appearing in the TCA supernatant was bound to metallothionein. The single standard value of mercury addition used in the rat study was 3490 nmoles per gram tissue (700 micrograms/g). Examination of Figs. 1 to 4 shows that this value would be unsuitable for the present work with mussels since this would represent a gross excess of mercury. One reason for this

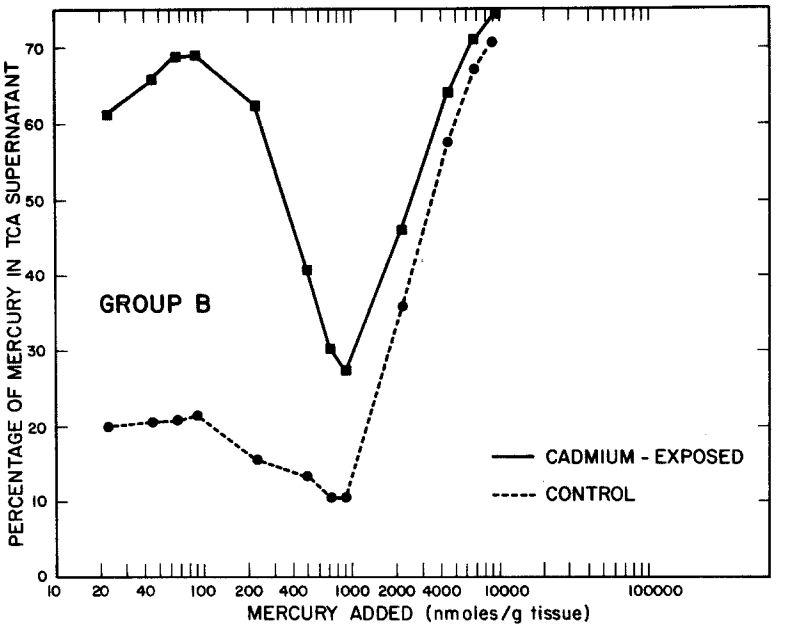

Fig. 3. Relationship between the amount of mercury added to the initial supernatant and the percentage of added mercury appearing in the TCA supernatant. GROUP B.

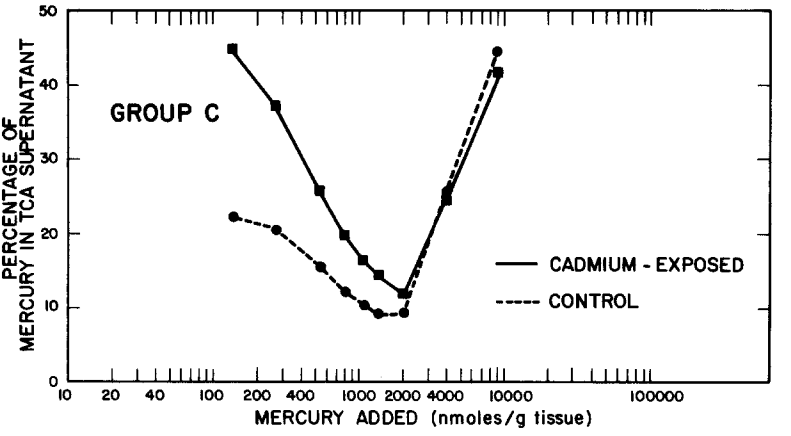

Fig. 4. Relationship between the amount of mercury added to the initial supernatant and the percentage of added mercury appearing in the TCA supernatant. GROUP C.

discrepancy is that the mussel homogenates were centrifuged but the rat homogenates were not. Also, it may be that mussel tissue has a lesser capacity to bind mercury. Mammalian metallothionein has a cysteine content of 33 residue percent (Kagi & Nordberg 1979) but cadmium-induced mussel metallothionein has been variously reported to have a cysteine content of 25.5 residue percent (Frankenne et al. 1980), 11.2 to 20 residue percent (George et al. 1979) and 9 residue percent (Marshall & Talbot 1979). Using the criteria mentioned above for rat studies, it appeared that a suitable standard dose of mercury for use with mussels would be 1346 nmoles mercury per g tissue (60 micrograms of mercury per 2 ml of initial supernatant). This value was chosen from the flatter middle portion of Fig. 1. This amount of mercury was used to test GROUPS D, E and F for metallothionein induction (Table 1).

Table 2. Percentage of mercury in TCA supernatant after addition of 1346 nmoles Hg/g tissue (60 micrograms per 2 ml of initial supernatant). Mussels were exposed to various concentrations of cadmium for 13 d.

GROUP	Conc of cadmium ppb	Mean percent	(s.e.)
Control	0	7.07	(0.41)
D	1	7.92	(0.42)
E	10	9.20*	(0.69)
F	100	9.95**	(0.46)

Significantly different from control at (p=0.03)* or (p=0.002)** using t-test.

It can be seen that both the group exposed to 10 ppb and the group exposed to 100 ppb for 13 d showed an increase in mercury in the TCA supernatant. The percentage mercury in the TCA supernatant is indicated to be related to the degree of exposure to cadmium in each mussel group.

It has been reported that neither cadmium, zinc nor lead will bind to sulfhydryl groups at the pH of the TCA supernatant (about 1.75) whereas mercury binds readily at this pH (Fuhr & Rabinstein 1973). This means that mercury will not face competition from these three metals in this assay. However, preliminary results in this laboratory (unpublished data) suggest that if copper ions are added to the initial supernatant before analysis (4-40 micrograms Cu per 2 ml), the amount of mercury appearing in the TCA supernatant is substantially reduced. This might indicate that copper can displace mercury from metallothionein under the conditions of the test. If so, then it will not be possible to use the assay in the presence of high levels of copper. This problem was not encountered with other metal ions examined at similar levels including aluminum, cadmium, lead, manganese and zinc.

A radioisotopic assay employing mercury-203 was demonstrated to be a useful adjunct to present methods of metallothionein detection in mussels. Such a simple technique would appear to be particularly useful for large scale studies and where only small quantities of tissue are available. The sensitivity of the method has been demonstrated with cadmium, a heavy metal of prime environmental concern, and in view of the usage of mussels in chemical monitoring programs it would be presently of interest to assess the induction potential of other metals and metal mixtures.

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