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Isolation of Heavy Metal Binding Proteins from Marine Vertebrates

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Abstract

Cadmium binding proteins have been isolated from liver homogenates of marine vertebrates by ultracentrifugation and gel filtration. Liver samples of the Atlantic grey seal *Halichoerus grypus* and the Pacific fur seal *Callorhinus ursinus* contain measurable quantities of cadmium binding protein. The copper rock fish *Sebastodes caurinus* showed an increase in hepatic cadmium binding protein on administration of $CdCl_2$, in agreement with the known inducible nature of the protein isolated from terrestrial animals. The apparent molecular weights of the isolated proteins were 9000 for the grey seal, 10,000 for the fur seal, and 11,000 for the copper rock fish, as determined by gel filtration.

Introduction

Much evidence has been compiled to implicate a low molecular weight protein from various tissues of terrestrial animals with a general heavy metal detoxication function (Pulido *et al.*, 1966; Nordberg, 1972; Winge and Rajagopalan, 1972; Piotrowski *et al.*, 1973). These proteins have been called metallothioneins, and were found by Pulido *et al.* (1966) to complex cadmium, zinc, mercury and copper. The latter group found as much as 8.9 gram atoms of metal per molecular weight of 10,500 in the human kidney protein.

Several studies have shown that cadmium, mercury and zinc induce the biosynthesis of metallothionein in animal tissues (Shaikh and Lucis, 1970; Webb, 1972; Winge and Rajagopalan, 1972; and Piotrowski *et al.*, 1973). Induction of such a protein would be an important criterion of a detoxication system which would be unneccessary in the absence of heavy metal.

Comparison of amino acid analyses for rabbit, rat and horse liver metallothionein shows remarkable conservation of composition, with half cystine values varying between 27 and 33% of the total number of residues (Winge and Rajagopalan, 1972). Conservation of composition should reflect conservation of primary sequence and a unique tertiary structure maintained throughout evolution (Hartley, 1970).

Recently, Maclean *et al.* (1972), working with the blue-green alga *Anacystis nidulans*, have demonstrated the presence of a low molecular weight protein which binds both cadmium and zinc. It thus appears that metallothionein proteins may be ubiquitous in the living world.

This preliminary study is the first result of an attempt to estimate the phylogenetic distribution and significance of metallothioneins in marine life. The animals chosen were representatives from the top of the food chain, and include the Atlantic grey seal *Halichoerus grypus*, the Pacific fur seal *Callorhinus ursinus*, and the copper rock fish *Sebastodes caurinus*.

Materials and Methods

The grey seal, *Halichoerus grypus*, liver sample was kindly donated by H.C. Freeman, Fisheries Research Board of Canada, Halifax, who obtained the animal from Forchu Bay, Cape Breton Island. Pacific fur-seal, *Callorhinus ursinus*, liver samples were a gift from Dr. M. Bigg, Fisheries Research Board of Canada, Pacific Biological Station, Nanaimo, B.C., and were obtained off the west coast of Vancouver Island. Copper rock fish, *Sebastodes caurinus*, were donated by the Vancouver Public Aquarium.

The isolation procedure used is similar to that developed by Shaikh and Lucis (1971), with certain modifications. Liver samples were thoroughly homogenized at 0°C in 3 volumes of isotonic saline, using a glass Potter Elvehjem homogenizer fitted with a motorized teflon pestle. The majority of the insoluble residue was separated by centrifugation at 27,000 x g for 10 min in a Sorval refrigerated centrifuge. The supernatant was retained while the pellet was washed with a small volume of saline. The resuspended pellet was centrifuged once again and the new supernatant combined with the initial supernatant fraction. This material was in turn centrifuged at 105,000 x g for 60 min in a Beckman Model L2-65B Ultracentrifuge. The final supernatant was then stored frozen or applied directly to a 5 x 100 cm column of Sephadex G-75 (Pharmacia Fine Chemicals, Dorval, Quebec) and eluted with 10 mM NH_4HCO_3 at 60 ml/h. Column effluent was monitored at 250 and 280 nm. Cadmium

determinations of fractions were performed directly on the samples without prior dilution or wet ashing. Aliquots of 5 to 10 µl were routinely charred at 400°C for 60 sec in the graphite rod of a Perkin Elmer 403 atomic absorption spectrophotometer fitted with an automatic background corrector. Errors in sampling were judged to be primarily associated with charring losses and estimated to be less than 10% for most samples. The cadmium-rich peak was pooled and stored in a lyophilized state. Further purification of the isolated grey seal metallothionein was carried out on a 2.5 x 175 cm Sephadex G-50 column eluted with 10 mM NH_4HCO_3 at 25 ml/h.

Copper rock fish were administered $CdCl_2$ (1 mg/ml) in isotonic saline intramuscularly in the caudal peduncle every third day. The low-dose-schedule fish (B) received an increasing dose from 0.25 to 2.0 mg/kg body weight in 3 injections; the high-dose-schedule fish (C) received an increasing dose from 1.1 to 10.0 mg/kg in 4 injections; the control (A) received equivalent volumes of isotonic saline. All rock fish were killed 3 days after the final injection.

Results

The elution profile of the separated cytoplasmic components from a 27 g liver sample of a 3-year old grey seal is shown in Fig. 1. Optical absorption of collected fractions was monitored at both 250 and 280 nm, since the metallothioneins isolated to date contain no aromatic residues but do absorb substantially at 250 nm (Winge and Rajagopalan, 1972). This phenomenon, according to Pulido *et al.* (1966), can be attributed to a charge transfer transition of cadmium mercaptide bonds. Together with molecular weight data, this property serves as a useful means of differentiating metallothioneins from other proteins. From Fig. 1 it is evident that a cadmium binding protein is resolved, free of contamination from any major protein fraction. There is clearly a pronounced cadmium concentration superimposed directly over a 250 nm absorption maximum. It will be subsequently shown that the elution position of this peak corresponds to that observed for metallothioneins. On the other hand, the first peak eluted in Fig. 1 corresponds to large molecular weight proteins, while the second peak eluting before the cadmium binding protein corresponds to the elution position of haemoglobin.

In an attempt to further purify the isolated grey seal metallothionein, lyophilized protein was subjected to Sephadex G-50 gel filtration. The resultant profile is shown in Fig. 2. The cadmium binding peak isolated from the Sephadex G-75 column is apparently quite heterogeneous, being composed of at least 3 cadmium binding fractions. Clearly this procedure is inadequate for complete resolution of the components, but serves to indicate the complexity of the protein fraction isolated from Sephadex G-75.

The yield of the grey seal metallothionein complex from Sephadex G-75 was 0.07 mg/g of liver on a wet-weight basis. A similar isolation procedure using 24 g of Pacific fur seal liver resulted in 0.09 mg/g of liver. The cadmium binding protein from the latter animal exhibited all the properties of metallothioneins isolated from the grey seal, with the exception that cadmium concentrations in the peak fraction of the fur seal profile were nearly an order of magnitude larger.

Fig. 3 illustrates the result of administration of $CdCl_2$ to the copper rock fish *Sebastodes caurinus*. Profile A in Fig. 3 represents a control specimen which was not administered the heavy metal, while B and C represent elution profiles produced with tissue obtained from experimental animals which received final $CdCl_2$ doses of 2 and 10 mg/kg body weight, respectively. The latter

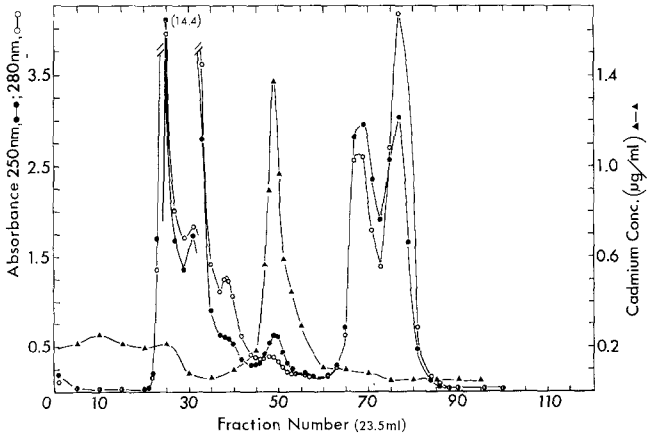

Fig. 1. *Halichoerus grypus*. Sephadex G-75 gel filtration of grey seal liver homogenate after centrifugation at 105,000 x g

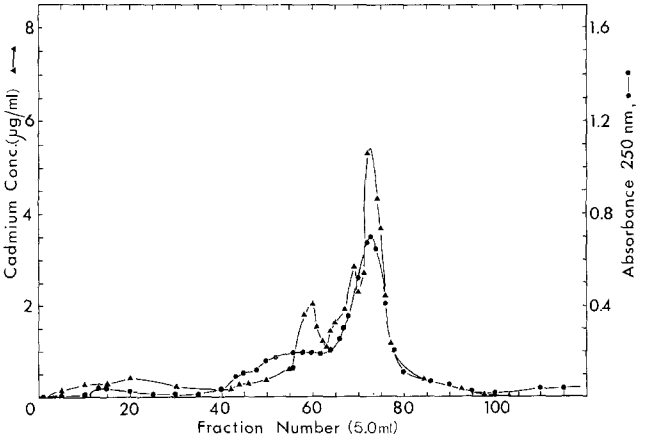

Fig. 2. *Halichoerus grypus*. Sephadex G-50 gel filtration of cadmium binding fraction from Sephadex G-75 separation of grey seal liver homogenate

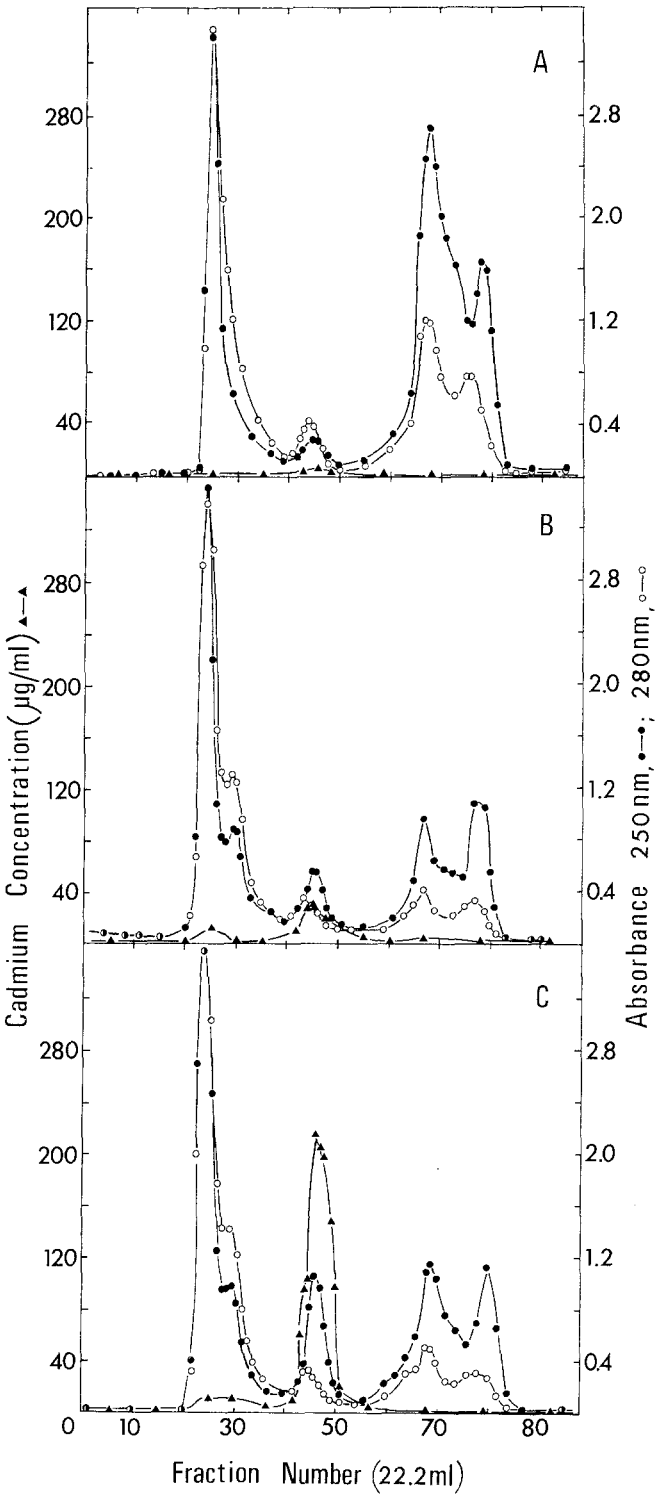

Fig. 3. *Sebastodes caurinus*. Sephadex G-75 gel filtration of copper rock fish liver homogenate after centrifugation at 105,000 x g. (A) control; (B) and (C): experimentals which received 2 and 10 mg/kg $CdCl_2$, respectively, as final doses

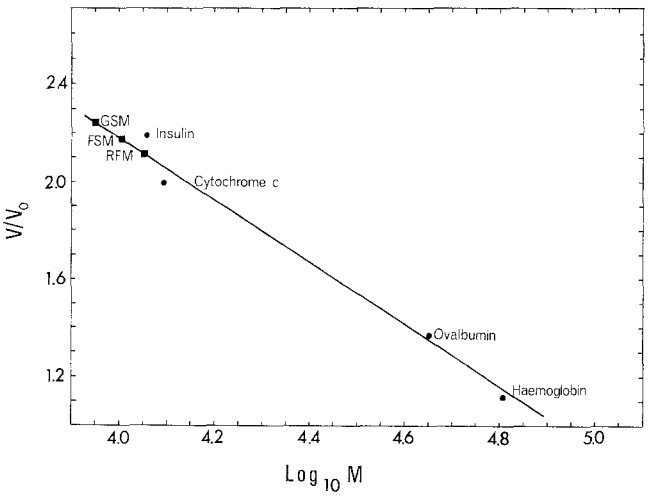

Fig. 4. Determination of molecular weight of cadmium binding proteins by gel filtration. Relationship between elution volume V/V_O, and molecular weight of standard proteins. Isolated metallothionein complexes demonstrated apparant molecular weights as follows: grey seal, *Halichoerus grypus*, metallothionein (GSM) = 9000; rock fish, *Sebastodes caurinus*, metallothionein (RFM) = 11,000; fur seal, *Callorhinus ursinus*, metallothionein (FSM) = 10,000

two profiles indicate an apparent increase in 250 nm absorption known to be associated with an increase in cadmium-bound metallothionein. This is similar to the results obtained earlier by Winge and Rajagopalan (1972), working with rats, and has been ascribed to induction of protein synthesis by this group and others using incorporation of labelled amino acid as a criterion of induction (Shaikh and Lucis, 1970; Webb, 1972).

In order to estimate the molecular weights of the cadmium binding proteins isolated from these marine forms, a Sephadex G-75 column was calibrated with known standards, and elution positions of the isolated proteins compared with those of the standards. The result is shown graphically in Fig. 4. Extrapolated apparent molecular weights were as follows: grey seal metallothionein, 9000; fur seal metallothionein, 10,000; copper rock fish metallothionein, 11,000. These values are in good agreement with published molecular weights for terrestrial vertebrate metallothioneins (Pulido *et al.*, 1966).

Discussion

The present results have indicated the presence of metallothioneins in two marine mammals and a teleost. Amounts of metallothionein isolated from marine animals are similar to those isolated from terrestrial laboratory animals. Piotrowski *et al.* (1973) reported 0.07 to 0.20 mg/g of metallothionein in the livers of non-induced rats, where-

as the grey seal and the Pacific fur seal had 0.07 and 0.09 mg/g respectively. It should be emphasized that mass determinations are subject to an array of errors encountered during isolation procedures and are, at best, only useful in relative assessments of metallothionein levels. This criticism is particularly relevant when amounts of isolated material are in the low milligram range. It is for this reason that values were not available for the copper rock fish. Since this protein has no known enzymatic function, it is necessary that a radioisotope assay procedure be established to allow for accurate determinations of metallothionein levels in the future.

It is interesting that the cadmium levels in the 20-year old fur seal are greatly elevated over the values observed in the grey seal. While these data could reflect differences in environmental exposure to cadmium or a species-specific phenomenon, it is also possible that cadmium accumulation in the liver will be found to be a function of age - in analogy with mercury (Bigg and MacAskie, 1972). This phenomenon may occur by low level induction or in the absence of induction, by replacement of Zn^{2+} by Cd^{2+} in available protein. No firm conclusion is warranted, however, without a larger sampling and a more accurate means of assessing metallothionein concentrations in non-induced tissues.

The heterogeneity of grey seal metallothionein observed in Fig. 2 is quite likely due to isoproteins, as observed by Pulido *et al.* (1966) with human metallothionein and Nordberg *et al.* (1972) with rabbit metallothionein. Both groups separated the major metal binding components and, by amino acid analysis, demonstrated that they were isoproteins. A similar separation and analysis of metallothioneins from marine organisms is now in progress in this laboratory.

With respect to the copper rock fish data, although these results are highly indicative of an induction phenomenon, further confirmatory work is necessary to clearly denote an inductive process. However, this result is analogous to those obtained by several other groups studying terrestrial animals. Shaikh and Lucis (1970) using cystine-^{14}C and Webb (1972) using leucine-^{14}C have clearly shown that rat liver metallothionein is induced in the presence of cadmium chloride.

Data presented here demonstrate the presence of metallothioneins in two marine mammals and a teleost. Work is presently in progress to elucidate the presence or absence of these proteins in lower trophic levels of the marine environment. Studies are also being conducted towards establishing a rapid radioisotope assay procedure to be used during kinetic studies with plankton. Such an assay procedure would provide a ready means of estimating metallothionein levels in organisms collected from the environment as well as those organisms exposed to non-lethal levels of heavy metals in controlled ecosystem enclosures. It is also hoped that such an assay would be of considerable utility in demonstrating a correlation of enhanced tolerance to heavy metals with induction of metallothionein. Similarly, an attempt to correlate saturated binding capacity and observed toxic levels would be greatly facilitated. Although it is clearly possible that metallothioneins may be involved in a heavy metal metabolic function yet to be defined, positive correlations in the above experiments would strongly support the proposed detoxification function of metallothioneins.

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