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ENVIRONMENTAL CADMIUM IS NOT SEQUESTERED BY METALLOTHIONEIN IN RAINBOW TROUT

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Rainbow trout were exposed for up to 9 months to cadmium in their aquarium water at concentrations (from 9-54 µg/l) lethal to the majority of the fish. The metal accumulated only in liver, kidney and gills and was found in the cytosol in association with two low mol.wt. proteins that were not metallothioneins. However, zinc and copper thioneins were present in the same organs. By contrast, when cadmium was administered to the fish by intraperitoneal injection (in milligramme quantities) the metal was sequestered by metallothionein.

The toxic effects of cadmium are now well established (1,2) and in particular, studies on rainbow trout have shown that relatively low concentrations in aquaria (9µg cadmium/ 1.of water) can be lethal to a proportion of the fish within 5 days (3). We have shown previously that after varying periods of exposure to 9µg of cadmium/1, trout accumulated the metal in the gills, liver and kidney (4). It has been demonstrated by others that injecting cadmium intraperitoneally, at concentrations usually far in excess of those likely to be encountered in the environment, results in the metal binding to induced metallothionein, both in fish (5,6) and other species (7). Metallothionein is characterised by its low molecular weight (6-7,000), its lack of aromatic amino acids & histidine and a high (30%) content of cysteine residues that are involved in the binding of up to 7g. atoms of bivalent metal ions per mole as metal mercaptide complexes (8). As a result of its widespread distribution and its highly conserved structure, a role has been suggested for metallothionein in heavy metal detoxication (9) although alternative functions have been proposed (10,11). The data presented here argue against a detoxication role for metallothionein in

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fish exposed to cadmium in their environment. We describe two non-metallothionein proteins that sequester all of the hepatic cadmium in this species.

Materials and Methods

The rainbow trout, the conditions under which they were maintained and the technique used to administer cadmium to them in aquaria has been described previously (4).

The fish to which cadmium was administered by intraperitoneal injection (total of 2.8mg/kg over 12 days) were treated as described in (12).

After exposure to cadmium by either route, the trout were anaesthetized by immersion in a 0.01%(w/v) solution of 2.2.2. tricaine methane sulphonate. The livers were removed, perfused with 0.15M NaCl, and passed through a Fischer tissue press before being homogenized in 0.01M sodium phosphate buffer pH 7.4 containing 0.15M potassium chloride using a Dounce-type homogenizer. Kidney and gill tissues were rinsed in saline prior to homogenization by the same procedure. Homogenates were filtered through cheese-cloth and the resultant materials were centrifuged at 30,000g. for 40 min. The supernatants from this step were recentrifuged at 100,000g. for 120 min. to remove further particulate material and to ensure complete flotation of the lipid material present. The clear infranatants obtained after this step were removed carefully to avoid contamination by either pelleted or floating material and concentrated (usually 15 fold), by ultrafiltration in an Amicon stirred ultrafiltration cell fitted with either a PM30, PM10 or UM2 membrane. Usually more than 90% of the cadmium present in the filtered homogenates was retained in these concentrated extracts. They were clarified by centrifugation at 100,000g, for 120 min. before samples were applied to columns (4.8 x 102cm)of Sephadex G.75 equilibrated with 0.01M sodium phosphate buffer pH.7.4/0.15M KCl.

All subsequent concentration steps were carried out by ultrafiltration over a UM2 membrane and all dialyses employed Spectrapor 6 (nominal retenion of >1000 daltons) dialysis tubing (Raven Scientific Co. Haverhill, Suffolk).

Cadmium, zinc and copper were determined by atomic absorption spectrometry using a Varian AA275 instrument fitted with a background corrector.

Protein was determined using a micro-modification of the method of (13). Samples for amino acid analysis were hydrolysed in 6M-HCl in vacuo at 105°C for 24, 48 and 72h. Cysteine was determined as cysteic acid following oxidation of the protein samples with performic acid (14).

SDS gel electrophoresis and polyacrylamide gel electrophoresis in the presence of urea were carried out as described previously (15,16). Staining of gels was with Coomassie Blue (17).

Results and Discussion

When rainbow trout were exposed as described in Materials & Methods to cadmium in their water at concentrations between 9 and 54µg/l for 8 weeks, the metal was found in increasing amounts in the liver (Fig.1). Trout that had been maintained in the presence of 9µg cadmium/l for up to 9 months, accumulated the metal progressively in the liver (Fig.1). The significance of this hepatic accumulation of cadmium is emphasised by the observation that with continuous

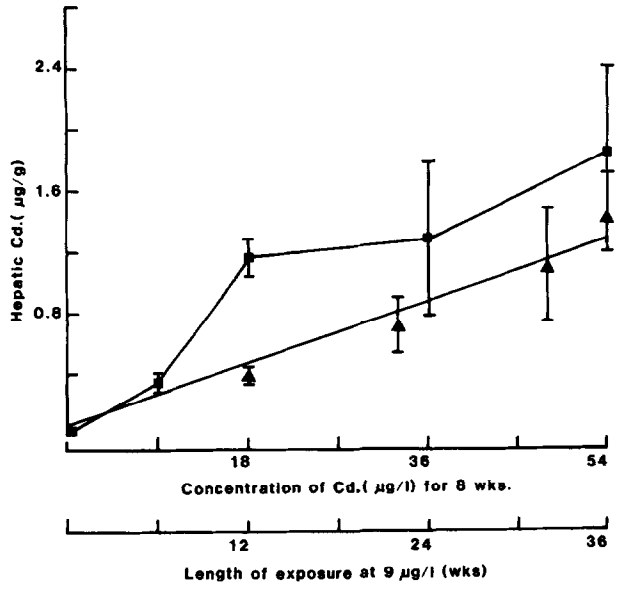

Figure 1.

Hepatic accumulation of cadmium in trout exposed to the metal in their aquarium water

Trout were exposed to cadmium at various concentrations (9-54µg/l) in their aquarium water for 8 weeks. The hepatic cadmium concentrations (µg/g wet weight, ■-■) following exposure are shown together with those for trout exposed to 9µg/l of the metal for different periods (2-36 weeks)(▲-▲).

exposure to 9µg of cadmium/l, a constant proportion (approximately 30%) of the increasing total body load of the metal was present in the liver at all times of exposure between 2 and 36 weeks. As we have shown previously (4), virtually all of the remaining body cadmium is accounted for in the kidney and gills.

When trout were injected with cadmium via the intraperitoneal route, the liver contained between 20 and 80 times more cadmium than was accumulated by the livers of fish exposed to cadmium in their aquarium water. More than 90% of the total body load in injected fish was present in the liver. Thus, it was considered of primary importance to compare the nature of cadmium sequestration in the livers of injected fish and those exposed to the metal in their aquarium water.

Extracts of the liver material were prepared as described in Materials and Methods from fish exposed to cadmium either by injection or via the environmental route and chromatographed on columns of Sephadex G.75. The elution profiles as shown in Fig.2 were obtained consistently (5 independent experiments). In both

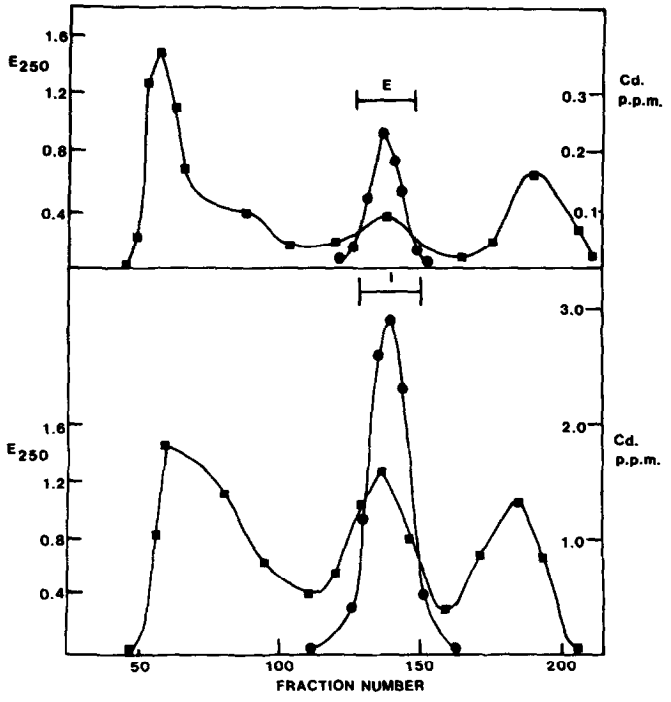

Figure 2.

Gel filtration of concentrated hepatic extracts from trout exposed to cadmium either in their aquarium water or by intraperitoneal injection

Concentrated hepatic extracts were prepared from trout exposed to cadmium via their aquarium water (upper panel) or by intraperitoneal injection (lower panel) and chromatographed on columns of Sephadex G-75 (4.8 x 102 cm) equilibrated in 0.01M sodium phosphate buffer, pH 7.4/0.15M KCl.

■ – ■ = E_{250} ●–● = Cd. Fractions were pooled as shown to give E and I for ion-exchange chromatography.

cases, the cadmium was eluted from the columns at a position consistent with the metal being bound in a macromolecular form of approximately 10,000 daltons. Fractions were pooled as shown (Fig.2). Pool I from the injected trout contained much higher amounts of cadmium and had a much greater E_{250}/E_{280} ratio than the material in pool E from the fish exposed via the environmental route. Both pools were also found to contain zinc.

Pools E and I were concentrated separately by ultrafiltration over UM2 membranes and aliquots (containing 1-2µg of cadmium) were heated at 80°C for 1 minute as described by Vander Mallie & Garvey (18) in their protocol for cadmium-thionein purification. Chromatography on Sephadex G-15 of both heated

and unheated aliquots from pool I resulted in all of the applied cadmium, E_{280} and E_{250} absorbing materials being eluted at the void volume of the column. A similar result was obtained with an unheated sample from pool E. By contrast, when the heated aliquot from pool E was chromatographed, while all of the E_{280} and E_{250} absorbing materials were eluted at the void volume as before, the cadmium emerged at the total volume of the column (recovery=100%). Thus, the macromolecules binding cadmium in the hepatic cytosol of fish exposed to the metal via their environment appeared to be distinct in character from those observed with animals injected with the metal. Elution of cadmium from Sephadex G-75 at a position consistent with its binding to a protein of low mol.wt. has sometimes been considered as indicative of the presence of cadmium-thionein. The above results suggest that such findings must be interpreted with caution.

After chromatography on Sephadex G-75, conventional purification procedures for metallothioneins employ ion exchange chromatography on DEAE-cellulose as the next step, usually with the resin equilibrated in 0.02M Tris-HCl buffer pH 7.4 or 8.6 (18,5). However, when a sample of pool E was dialysed against 20mM Tris-HCl buffer, pH 7.4 in Spectrapor 6 tubing, approximately 50% of the cadmium and 17% of the protein (i.e. Lowry positive material)were lost through the dialysis membrane. Less than 3% of the cadmium and 15% of the protein were lost on dialysis of Pool I against this buffer. By contrast, virtually quantitative recoveries of Cd were obtained with both types of extract when they were dialysed against buffered isotonic saline. However, when the two pools were applied to DEAE-cellulose equilibrated in isotonic saline, no binding to the matrix was observed so that it was necessary to lower the ionic strength of the two pools by dialysis against 0.02M Tris-HCl buffer, pH 7.4 before application of the dialysates to separate columns of DEAE-cellulose. Representative profiles of the elution patterns obtained on application of a 20-100mM Tris-HCl gradient, pH 7.4 are shown in Fig.3.

The cadmium in the fractions from fish exposed to the dissolved metal (Fig 3a) was resolved into two peaks (E1 and E2) which were eluted at buffer concentrations of 30 and 65mM respectively. These in turn were distinct from the

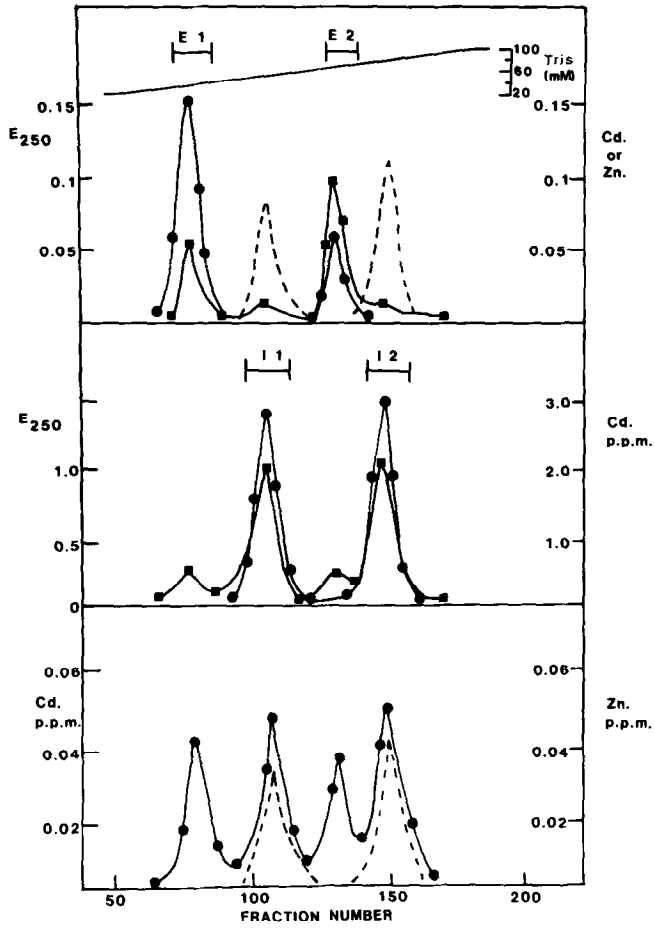

Figure 3.

Ion-exchange chromatography of E and I

Samples of the E (upper panel = a) and I (middle panel = b) pools from gel filtration (Fig.2) were chromatographed on columns of DEAE-cellulose (1.2x14cm) equilibrated in 20mM Tris-HCl buffer, pH 7.4. A linear gradient of Tris-HCl buffer (20-100mM) was used for desorption. ●-● = Cd, ■ - ■ = E_{250} and - - - = Zn. E1 and E2 (upper) and I1 and I2 (middle) were pooled as described in the text and aliquots of all four were mixed, dialysed and rechromatographed (bottom panel = c) on a fresh DEAE-cellulose column.

two peaks of zinc which were obtained, eluting at 45 and 80mM buffer respectively. When the material from the livers of injected fish was chromatographed (Fig.3b), two peaks of cadmium (I1 and I2) were eluted once more but at buffer concentrations of 45 and 80mM respectively, corresponding to the elution positions of the zinc in the environmentally-exposed trout.

Samples from E1,E2,I1 and I2 were mixed, dialysed against 20mM Tris-HCl buffer, pH 7.4 and rechromatographed on DEAE-cellulose (Fig.3c). Four peaks of

cadmium were observed, two of which eluted at the same buffer concentrations as had been previously determined for zinc elution in Fig.3a. All of the peaks were eluted at ionic strengths identical to those observed in the original, separate fractionations (Figs.3a & 3b). Only I1 and I2 (Fig.3b) had the elevated E_{250}/E_{280} ratio characteristic of cadmium-metallothionein. Results similar to those in Fig.3a were obtained in all cases with livers from fish exposed to cadmium at concentrations up to 54µg/l of water for different periods of time. When the chromatography on DEAE-cellulose was carried out in Tris buffer, pH 8.6, identical elution profiles were observed.

Fractions were pooled (Figs.3a & b) as shown to give E1, E2, I1 and I2 and each pool was freeze-dried, redissolved in 0.3M ammonium chloride and the pH was adjusted to 5.5. The pools were applied separately to columns of thiopropyl-agarose,an affinity resin that has been used for purification of metallothionein (19). E1 and E2 were not retained by the column whereas, under identical conditions, both pools of material from the cadmium-injected trout (I1 and I2) were adsorbed to the matrix and were eluted only by mercaptoethanol (0.15M) in 0.02M Tris-HCl buffer, pH 7.4 containing 1M NaCl (19). Aliquots of I1 and I2 as obtained after this purification step on thiopropyl-agarose were analysed by urea/polyacrylamide gel electrophoresis and each was shown to contain only one band with R_F values of 0.16 (I1) and 0.18 (I2) respectively. Amino acid analysis of I1 and I2 indicated that both proteins contained high amounts of cysteine (22%) and only one residue each of tyrosine, phenylalanine and histidine per mole - features usually considered as typical of the isomeric forms of metallothionein. By contrast, E1 and E2 contained much less cysteine (<5% and 3% respectively) and were further distinguised from the metallothioneins by their relatively higher contents of hydrophobic and aromatic amino acids. SDS-polyacrylamide gel electrophoresis indicated that E1 had an apparent mol.wt. of 12,500 while E2 migrated as a species of 14,000 daltons (both consistent with the elution positions observed on G-75 (Fig.1.)

Analysis of the metal content of E1,E2,I1 and I2 (after the thiopropyl-agarose chromatography) revealed that while I1 and I2 contained substantial amounts

of copper and zinc in addition to the cadmium, E1 and E2 contained cadmium as the only metal present.

Analysis of the cadmium-binding proteins in the gill and kidney tissues from the same trout by chromatography on G-75, DEAE-cellulose and thiopropyl-agarose, produced identical elution profiles to those described above for the liver.

Thus, the series of features reported here distinguishes between the sequestration of injected cadmium and that entering from the environment via the fish's aqueous medium. Under all of the conditions of environmental dosage (at concentrations high enough to kill the trout eventually) and at all times of exposure, no cadmium was ever observed bound to metallothionein-like proteins despite the fact that these proteins appeared to be present in association with zinc in the tissues (Fig.3a). The cadmium appeared to be sequestered by two, distinct, low molecular weight acidic proteins present in liver, gill and kidney cytosols.

A final confirmatory distinction between the proteins binding environmentally-applied cadmium and those responsible for sequestration of the much higher injected amounts was obtained with a competitive radioimmunoassay. The environmental cadmium-binding proteins did not compete with purified rat metallothionein for rat-metallothionein antibody whereas the proteins to which injected cadmium was bound in the trout livers, were able to compete partially under the same conditions (Cryer,A., Kay, J. & Garvey, J.S., unpublished observations).

It is not known whether an analogus situation to that reported here for fish exists in higher mammals. However, recently, it has been shown (20) that the majority of the cadmium in the soluble fraction of the lungs of rabbits exposed to aerosols of cadmium chloride was bound to low molecular weight, non-metallothionein proteins.

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