

## **The influence of dietary and waterborne zinc on heat-stable metal ligands in rainbow trout, *Salmo gairdneri* Richardson: quantification by $^{109}\text{Cd}$ radioassay and evaluation of the assay**

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The  $^{109}\text{Cd}$  binding assay of Eaton & Toal was critically evaluated and then used to assess the induction of cytosolic metal-binding ligands in rainbow trout exposed to Zn in the diet and/or in the water for 16 weeks. With purified rabbit Cd-Zn metallothionein (MT),  $^{109}\text{Cd}$  binding and total Cd recovery in the assay were linear up to 5  $\mu\text{g}$  of protein; gel chromatography revealed a single peak. With heat-denatured extracts of gill, liver and intestine from control and Cd- and Zn-injected trout,  $^{109}\text{Cd}$  binding was generally linear with sample size. Gel chromatography demonstrated that  $^{109}\text{Cd}$  was bound by a protein with the same apparent weight as MT (~11 000 daltons), but significant binding occurred also at three other regions [molecular weight (mol wt) > 70 000, 30 000 and < 3000]. In the dietary/waterborne Zn exposure, induced  $^{109}\text{Cd}$ -binding activity occurred not in the MT peak but in the low mol wt peak (< 3000). Activity in the gill rose in response to both dietary and waterborne Zn, but the liver did not respond. The maximum five-fold elevation in the gill was primarily a waterborne effect. In the intestine, the maximum rise was 25-fold due to both factors. The thresholds for induction were > 39  $\mu\text{g Zn l}^{-1}$  in water, and > 90  $\text{mg kg}^{-1}$  in the diet, but only when waterborne Zn was also high. There was no correlation between  $^{109}\text{Cd}$  binding and acid soluble thiol levels, which tended to decline at higher Zn exposures.

**Key words:** rainbow trout; zinc; metal ligands; metallothionein; diet.

### **I. INTRODUCTION**

Zinc is both an essential trace element and a potential toxicant to fish. Dietary Zn is normally the primary source (Pentreath, 1973; Merlini *et al.*, 1976; Willis & Sunda, 1984) but waterborne Zn can become significant if elevated (Milner, 1982) and/or when dietary supply is low (Spry *et al.*, 1988). Whole body [Zn] increases with increasing exposure (Spry *et al.*, 1988) but, at the cellular level, [Zn] appears to be tightly regulated (Williams, 1981, 1986). Zn turnover in fish is very slow. Half-lives for the largest compartment were close to 1 year (Pentreath, 1973; Willis & Jones, 1977). While bone and scales may be significant storage sites (Sauer & Watabe, 1984), soft tissues are undoubtedly also important, because Zn is quickly cleared to various tissues from the blood (D. J. Spry & C. M. Wood, unpubl. data). Little is known about this cellular metabolism.

Of the many cytosolic proteins which bind metals, metallothionein (MT, Kagi & Vallee, 1960) has received much attention for its putative role both in normal metabolism of trace elements and detoxification of non-essential, potentially toxic metals (Cherian & Goyer, 1978; Brady, 1982; Webb & Cain, 1982; Cousins, 1985). In fish, most of the emphasis has been in the latter area (reviewed by Klaverkamp

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*et al.*, 1984, and Hamilton & Merhle, 1986) using Cd, Cu, or Hg. However, Kito *et al.* (1982) demonstrated MT in carp exposed to  $10 \text{ mg Zn l}^{-1}$  for 14 days, and Bradley *et al.* (1985) found a doubling of heat-stable, sulphhydryl-rich protein in rainbow trout exposed to  $2100 \mu\text{g Zn l}^{-1}$  (0.3 of the LC50) for 5–20 days. Studies on MT induction in fish through dietary exposure to Zn are lacking.

Direct radioimmunoassays for MT (Vander Mallie & Garvey, 1979) are not widely available for fish. Indirect assays are based on the high sulphur content of MT (pulse polarography, Olafson & Sim, 1979; acid-soluble thiols, Wofford & Thomas, 1984) or the ability of the protein to bind certain cations (Hg, Zelazowski & Piotrowski, 1977; Cd, Eaton & Toal, 1982; Ag, Scheuhammer & Cherian, 1986). The  $^{109}\text{Cd}$  assay is both rapid and sensitive in mammalian systems. Recently, it has been partially validated in fish tissue (Hamilton *et al.*, 1987). However, molecular weight (mol wt) determinations of the ligands in the final assay have not been reported.

The first aim of our study was to further evaluate the  $^{109}\text{Cd}$  binding assay for use in trout tissue, in particular to determine the molecular weights of the ligands to which the radiolabel was bound. In fish, the specificity of metal-binding assays may be complicated by the presence of non-MT low mol wt proteins which also bind metals (Thomas *et al.*, 1983a,b; Pierson, 1985a,b; Kay *et al.*, 1986; Price-Haughey *et al.*, 1986). A second aim was to measure metal ligands in the gill, liver and intestine of rainbow trout which had been exposed to different levels of both dietary and waterborne Zn for 16 weeks (Spry *et al.*, 1988). The overall hypothesis tested was that gill ligands would correlate with the waterborne exposures, whereas the levels in the intestine would reflect the dietary exposure. The liver, on the other hand, should reflect the overall status of the fish and indicate the sum of dietary and waterborne uptake. We also measured acid-soluble thiols in these same tissues as an alternative assay for metal binding (Wofford & Thomas, 1984).

## II. MATERIALS AND METHODS

### EVALUATION OF THE ASSAY

#### *Performance*

The  $^{109}\text{Cd}$  radioassay was evaluated using purified Cd-Zn MT (Sigma M7641) or tissue extracts from Zn- or Cd-injected rainbow trout, *Salmo gairdneri* Richardson. The trout (120–340 g, from Spring Valley Trout Farm, Petersburg, Ontario) were held at McMaster University in flowing, charcoal-dechlorinated, Hamilton city tap water ([Ca] 0.9, [Na] 0.6, [Cl] 0.8 mmol  $\text{l}^{-1}$ , pH 8.1, temperature 6°C) and fed a pelleted commercial diet (Martin Feed Mills, Elmira, Ontario). The fish were given intraperitoneal injections with either Cd ( $1 \text{ mg kg}^{-1}$  body wt, as acetate) or Zn ( $10 \text{ mg kg}^{-1}$  body wt, as sulphate) in Cortland saline (Wolf, 1963) at 0 and 24 h and killed at 48 h. Tissues were quickly dissected on ice. Gills were trimmed from the arches, livers were removed whole and kept free from bile. The intestine from the pyloric sphincter to the beginning of the large intestine was gently purged with ice-cold saline, and external fat was removed. All tissues were homogenized in 4 ml  $\text{g}^{-1}$  of ice-cold buffer (30 mM Tris, pH 8.0, 150 mM KCl) with an homogenizer (Tissue-Tek; 30 s, medium speed). After centrifugation at 4500 rpm for 5 min to remove debris, a sample of supernatant was preserved for acid-soluble thiols (AST, see below). The remaining supernatant was heated in a boiling water bath for 2 min, cooled on ice and centrifuged at  $8000 \times g$  for 5 min. This heat-denatured supernatant (HDS) was transferred to clean centrifuge tubes and stored at  $-20^\circ\text{C}$  for up to 2 weeks until required for either the  $^{109}\text{Cd}$  radioassay or gel chromatography.

### Gel chromatography

Elution buffer (20 mM Tris, pH 8.0, 150 mM KCl, 0.02% sodium azide) was pumped through a  $1.5 \times 28$  cm Sephadex G-75 column at  $15 \text{ ml h}^{-1}$  and 2.5 or 5-ml fractions were collected. The columns were calibrated with blue dextran (2 000 000 mol wt), ovalbumin (43 000 mol wt), chymotrypsinogen A (25 000 mol wt), and cytochrome C (12 300 mol wt). Rabbit Cd-Zn MT (Sigma M7641) and Cd (as  $\text{CdCl}_2$ ) were also put through the column. Fractions were variously assayed for Zn or Cd by flame atomic absorption spectrophotometry,  $^{109}\text{Cd}$  by gamma activity (Chicago-Nuclear well-type counter, model 1084), or absorbance at 250 and 280 nm (Perkin-Elmer Lambda 3). Unlike other proteins, MT does not absorb at 280 nm, due to the absence of tryptophan and tyrosine, but does absorb strongly in the 250 nm region, due to the presence of the metal-thiolate bond.

### $^{109}\text{Cd}$ Binding

This assay (Onosaka *et al.*, 1978, as modified by Eaton & Toal, 1982) relies upon the binding of Cd to protein, and displacement of metals for which the ligand has equal or lower affinity. Stability constants for Cd are about three orders of magnitude larger than for Zn (Hamer, 1986). Cd binding is non-specific, as Cd binds to a large number of functional groups (Nieboer & Richardson, 1980). Some specificity is conferred, however, by the stability of MT to heat and/or acid. When the specific binding sites are saturated, any free or loosely-bound Cd is removed by the addition and subsequent heat precipitation of haemoglobin. The supernatant, now containing MT saturated with Cd/ $^{109}\text{Cd}$  is counted for radioactivity, and total Cd binding calculated from the known specific activity.

In the assay, 400  $\mu\text{l}$  of HDS, 400  $\mu\text{l}$  of homogenization buffer, and 100  $\mu\text{l}$  of Cd solution ( $1 \mu\text{Ci ml}^{-1}$ ,  $2.22 \text{ nmol Cd ml}^{-1}$ ) were mixed and incubated at room temperature for 30 min. Then, 100  $\mu\text{l}$  of 2% bovine haemoglobin (Hb, Type II, Sigma) was added, mixed, and the mixture heated for 2 min in a boiling water bath. After cooling on ice, the tubes were centrifuged at  $8000 \times g$  for 5 min. The Hb step was repeated once and 900  $\mu\text{l}$  of the final supernatant was counted for gamma activity. The Cd-binding activity was then calculated as:

$$\frac{\text{nmol Cd bound}}{\text{g wet tissue}} = \text{cpm} \times \frac{1}{\text{SA}} \times \frac{\text{total assay volume}}{\text{sample volume}} \times \text{tissue dilution},$$

where SA was the specific activity in  $\text{cpm nmol}^{-1} \text{ Cd}$ .

The linearity of the assay was evaluated with a dilution series of rabbit Cd-Zn MT (Sigma M7641:  $0.59 \mu\text{mol Cd}$ ,  $0.05 \mu\text{mol Zn g}^{-1}$  solid). The recovery of Cd from the added Cd-Zn MT was measured by atomic absorption analysis of the same dilution series (i) placed directly in the buffer alone, (ii) after the addition of Hb plus heating and centrifugation, and (iii) after the added MT was processed through the complete assay, which included the addition of  $^{109}\text{Cd}$ . These latter three steps assessed whether error occurred either due to stripping of Cd by Hb from binding sites (underestimation of binding), or due to incomplete removal of added free Cd (overestimation). The effect of changes in sample size was evaluated using HDS from gill and liver of Zn-injected fish. Sample sizes of HDS were varied from 50 to 800  $\mu\text{l}$ , and, by varying the amount of buffer, the total assay volume was kept constant. Gel chromatography of various assay supernatants was performed to estimate mol wt range of ligands for  $^{109}\text{Cd}$ .

As a check on day-to-day variability in the assay, a 'reference' HDS was constructed based on pooled supernatant from the Zn-injected fish, and stored in small batches at  $-20^\circ \text{C}$ . On each day of the assay, both this reference material and rabbit Cd-Zn MT standards were included in the measurements.

### ACID-SOLUBLE THIOLS

The thiol assay (Ellman, 1959) is also not specific for MT. It derives some specificity by acid precipitation of acid-labile cytosolic proteins (MT is acid-stable) but would also measure sulphhydryl groups on anything else not removed by the acid treatment (e.g. free cysteine and glutathione; Wofford & Thomas, 1984). In this assay, 100  $\mu\text{l}$  of supernatant from the crude homogenate was added to 100  $\mu\text{l}$  of 10% trichloroacetic acid and the

precipitate pelleted by centrifugation at  $8000 \times g$  for 5 min. This was then stored at  $-20^\circ\text{C}$  until analysis by the 5,5 dithionitrobenzoic acid (DTNB) method of Ellman (1959).

### EXPERIMENTAL EXPOSURE

Fingerling rainbow trout (3.5 g initial wt) were obtained from the same source and held at  $15^\circ\text{C}$  in the same water quality as adults at the Canada Centre for Inland Waters, Burlington, Ontario. The trout were exposed to various constant levels of Zn in the diet and/or in the water for 16 weeks. Full experimental details, including diet preparation, are given in Spry *et al.* (1988). In brief, the design was factorial, comprising a matrix of three dietary (1, 90 and  $590\text{ mg kg}^{-1}$  Zn as sulphate, in a semi-purified diet) and three waterborne concentrations (ambient = 7, 39 and  $148\text{ }\mu\text{g Zn l}^{-1}$  as sulphate). A fourth waterborne concentration ( $529\text{ }\mu\text{g Zn l}^{-1}$ ) was tested at the highest dietary Zn level ( $590\text{ mg Zn kg}^{-1}$ ), yielding a total of 10 treatment cells. After 16 weeks, three to nine fish were sampled from each cell, killed with MS-222, quick-frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until dissected. The assay protocols were thereafter identical to those used in the injection experiments. Due to extensive sampling for other physiological parameters (Spry *et al.*, 1988), only one fish was available at dietary  $[\text{Zn}] = 90\text{ mg kg}^{-1}$ , waterborne  $[\text{Zn}] = 7\text{ }\mu\text{g l}^{-1}$ .

### ANALYSIS OF THE DATA

Data have been expressed as means  $\pm 1$  S.E.M. ( $n$ ) where  $n$  represents the number of fish in a treatment. Simple comparisons used Student's two-tailed  $t$  test (unpaired) at  $P < 0.05$ . Multiple comparison tests were by Peritz's  $F$  test (Harper, 1984) with the experiment-wise error rate  $< 0.05$ .

## III. RESULTS

### EVALUATION OF THE ASSAY

#### *Performance*

The standard curve for the Cd binding assay [Fig. 1(a)] was linear up to  $1.25\text{ }\mu\text{g}$  of Cd-Zn MT, which corresponds to about 22% retention of total counts for the added  $^{109}\text{Cd}$ . Thereafter, the curve flattened gradually due to isotopic dilution of the  $^{109}\text{Cd}$  by the cold Cd in the rabbit standard (Eaton & Toal, 1982). Those authors showed that the curve was linear up to 100% retention of  $^{109}\text{Cd}$  counts when tissue from Zn-induced animals was used, so this was unlikely to be a problem with samples from our Zn-exposed fish.

The recovery of Cd from the added Cd-Zn MT [Fig. 1(b)] at various stages in the assay was linear up to the highest concentrations tested ( $5\text{ }\mu\text{g MT}$ ). While the Hb denaturation step removed a small fraction of the Cd, the complete assay actually elevated total Cd slightly due to incomplete removal of free Cd by the Hb. However, the overestimate was, at most, 20%, which may contribute to the reported discrepancy between this assay and the radioimmunoassay (Waalkes *et al.*, 1985). Interestingly, incomplete precipitation of the Hb by heat treatment, a potential source of error (Eaton & Toal, 1982; Eaton, 1985), was never observed in our hands.

Assays of HDS from fresh liver yielded final results independent of sample size over the range of  $50\text{--}800\text{ }\mu\text{l}$ . With gill tissue, however, at volumes  $< 400\text{ }\mu\text{l}$  ( $\sim 30\%$  retained counts), the apparent concentration of binding protein tended to decrease. Therefore, a sample size of  $400\text{ }\mu\text{l}$  was used for all tissues, except in the rare instance where further dilution was required to keep the response in the linear range of the standard curve (30–90% retained counts). The coefficient of variation (S.D./mean, expressed as %) for 22 replicate samples each of gill and liver tissue

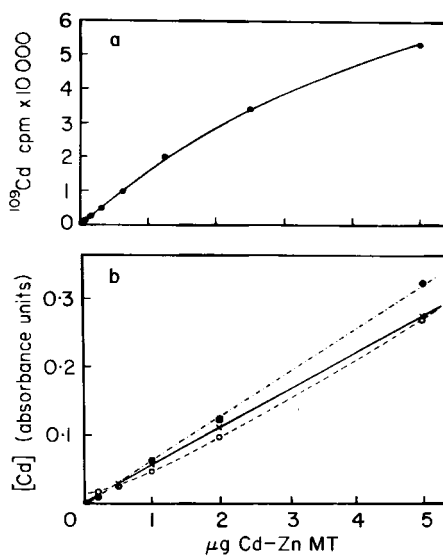


FIG. 1. (a) Calibration curve of  $^{109}\text{Cd}$  binding to rabbit Cd-Zn MT; the Y axis runs from 0 to 60 000 cpm. (b) Recovery of Cd-Zn MT, based on  $[\text{Cd}]$  after sequential steps in the  $^{109}\text{Cd}$  binding assay: (—X—), dilution with Tris buffer; (○), Hb addition and removal by heat precipitation; (●), the complete assay, consisting of dilution with buffer, addition of  $^{109}\text{Cd}$  followed by two additions and removal of Hb to remove excess Cd.

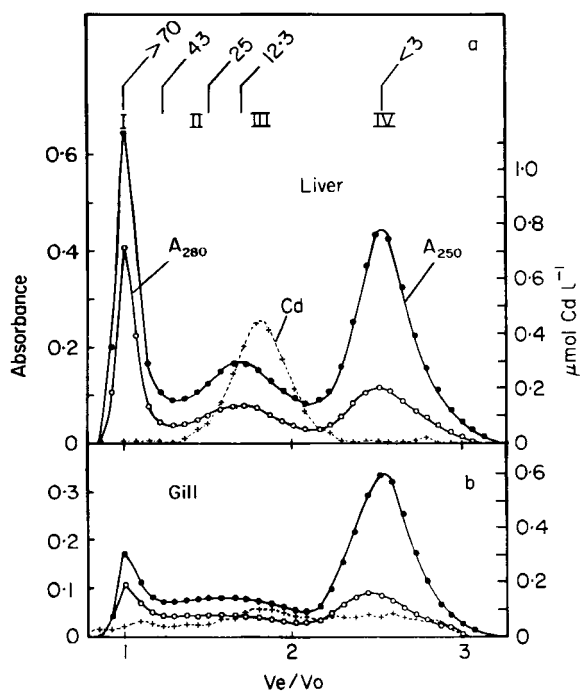


FIG. 2. Sephadex G-75 chromatograph of heat-denatured supernatant of homogenates of Cd-injected trout. (a) Liver. (b) Gill. (●),  $A_{250}$ ; (○),  $A_{280}$ ; (+),  $[\text{Cd}]$ . Peaks I-IV (see text) and position of molecular weight markers ( $\text{mol wt} \times 10^{-3}$ ) are shown.

TABLE I.  $^{109}\text{Cd}$ -binding activity in heat-denatured supernatant from control and Zn-injected trout (mean  $\pm$  S.E.M.  $n$ ), nmol Cd bound  $\text{g}^{-1}$  wet tissue)

	Control	Zn-injected
Gill	7.51† $\pm 2.12$ (7)	10.98† $\pm 3.83$ (7)
Liver	1.88* $\pm 0.14$ (7)	1.43* $\pm 0.11$ (10)
Intestine	13.57† $\pm 3.11$ (6)	16.31‡ $\pm 5.46$ (6)

\*, †Values with the same symbol superscript are not significantly different ( $P < 0.05$ ).

was  $< 5\%$ , spanning the range of 5–87% retained counts. The coefficient of variation of 'reference' HDS was 9.5%, whereas that for rabbit MT assayed simultaneously was only 1.8%, possibly due to its higher purity. In light of this overall uniformity, no adjustments were made for day to day variability in the assay.

#### $^{109}\text{Cd}$ -binding in Zn-injected trout

Assay of gill, liver and intestine HDS from control and Zn-injected trout revealed lowest concentrations of Cd-binding protein in the liver, and highest concentrations in the intestine (Table I). Cd-binding activity tended to increase non-significantly in the gill and intestine in response to Zn-injection. There were no changes in the Cd-injected trout.

The nature of these Cd ligands was investigated further. Heat denaturation removed much of the protein from the crude tissue homogenates. However, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE, not shown) revealed a profusion of bands, indicating that, qualitatively, traces of the proteins remained throughout the range 200 000–12 000 mol wt. Also, the liver HDS was frequently milky, presumably from glycogen, since this material precipitated with ethanol and gave a strong positive reaction with anthrone reagent. It eluted in the void volume when chromatographed, but did not bind  $^{109}\text{Cd}$ . Intestine was the most difficult tissue from which to recover a clear HDS. Careful removal of the external fat was essential; even so, in a significant number of cases, it was necessary to heat the homogenates twice to precipitate a firm pellet.

#### Gel chromatography

Molecular weights of the species present in the HDS were estimated by gel chromatography. The rabbit Cd–Zn MT standard eluted at a  $V_e/V_0$  (fraction containing the highest concentration/fraction with the highest concentration of

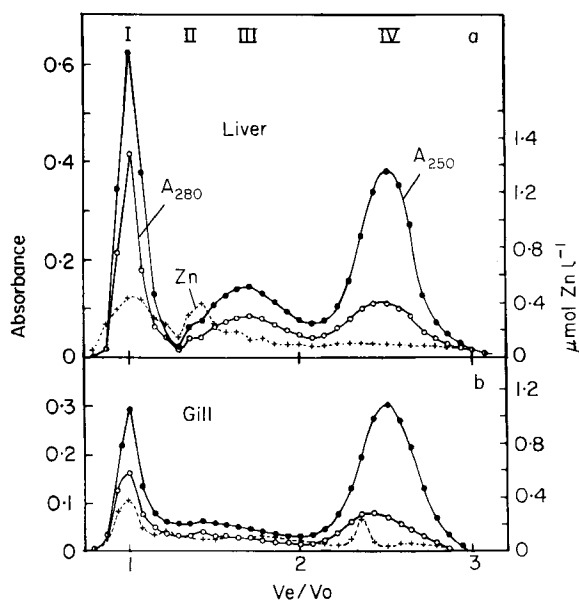


FIG. 3. Sephadex G-75 chromatogram of heat-denatured supernatant of homogenates of Zn-injected trout. (a) Liver. (b) Gill. (●),  $A_{250}$ ; (○),  $A_{280}$ ; (+), [Zn].

blue dextran, the void volume marker) of 1.75–1.85. This peak was characterized by its absorbance at 250 nm and a lack of absorbance at 280 nm. Coincident with the protein peak was a Cd peak. In contrast, free Cd (added as  $\text{CdCl}_2$ ) eluted at  $V_e/V_0 > 2.5$ . When HDS was applied to the column, two or three absorbance peaks were always noted (Figs 2, 3). The 250 nm peak was always higher than the 280 nm peak, and both were always present together, indicating the presence of other proteins, as previously seen with SDS-PAGE. The peaks and their  $V_e/V_0$  values were: peak I, 1.0, the void volume, all proteins heavier than 70 000 mol wt; peak II (not always present), 1.4, 30 000 mol wt; peak III, 1.8, 11 000 mol wt (the rabbit Cd–Zn MT peak); peak IV,  $> 2.5$ ,  $< 3000$  mol wt. Liver tissue from Cd-injected trout showed a large Cd peak III [Fig. 2(a)] whereas gill tissue had a smaller peak III, but a generally higher baseline [Fig. 2(b)]. Zn-injected fish showed no Zn in peak III; instead, liver showed Zn in peaks I and II [Fig. 3(a)] whereas, in gill, the significant peaks were I and IV [Fig. 3(b)]. The chromatography was repeated three times for Zn and twice for Cd, with only slight quantitative variation amongst fish; the  $V_e/V_0$  values were always the same.

Gel chromatography of the supernatants from the  $^{109}\text{Cd}$ -binding assays revealed the same four absorbance peaks, but not in all tissues. Heat-stable liver proteins from Zn-injected trout showed  $^{109}\text{Cd}$ -binding only at peak III [Fig. 4(a), (b)]. Thus although there were measurable amounts of amino acids, peptides or low mol wt proteins in the HDS, as shown by the absorbance, at  $V_e/V_0 > 2.5$  (peak IV), they did not bind  $^{109}\text{Cd}$ . In contrast to liver, gill tissue showed some  $^{109}\text{Cd}$ -binding at all four peaks [Fig. 4(b)]. The actual amount bound (area under the curve) was similar for peaks I and II. The contribution of peak III, the presumed Mt peak, varied from 46 to 86%. The intestine of Zn-injected trout showed the same pattern, but

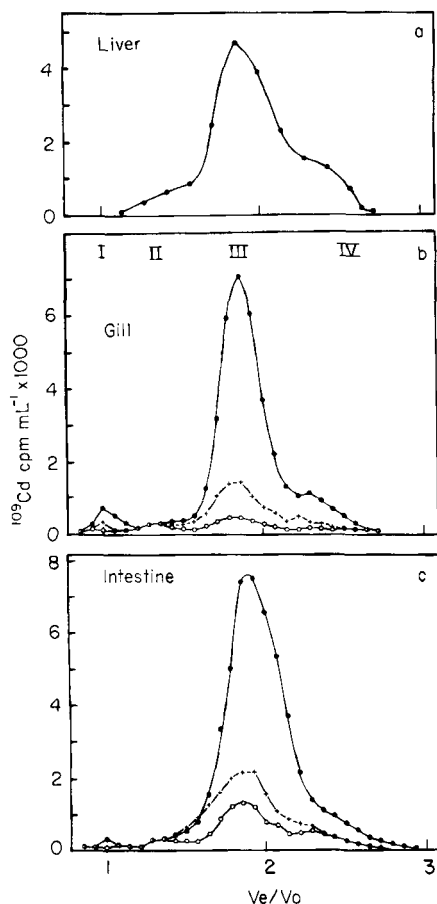


FIG. 4. Sephadex G-75 chromatograms of the supernatant from the  $^{109}\text{Cd}$ -binding assay of Zn-injected rainbow trout. (a) Liver tissue. (b) Gill tissue, showing profiles from high (●), medium (+) and low (○) total  $^{109}\text{Cd}$  binding. (c) Intestinal tissue; key as for (b).

peak III consistently bound  $>80\%$  of the total counts. Peak I was attenuated, while peaks II and IV were unchanged [Fig. 4(c)]. Patterns in control and Cd-injected trout were similar to those in Zn-injected trout for all tissues.

To ensure that the  $^{109}\text{Cd}$ -binding, in the gill sample especially, was not being relocated by the Hb and heating procedures, chromatography of assay supernatant from gill of a Zn-injected trout was compared with the same HDS which had simply been incubated with  $^{109}\text{Cd}$  but not Hb-treated. There are several important points to note here (Fig. 5). First, the profiles before and after the Hb step were very similar. Second, all the  $^{109}\text{Cd}$  added originally was bound to proteins in the HDS, since there was no difference in counts in peak IV (where free Cd would elute) in contrast to other areas. Third, the total area under the curve of the  $^{109}\text{Cd}$  incubation alone was nearly two-fold greater than after Hb treatment, although peak III contained 83% of total counts in both cases. This showed that the haemoglobin step removed loosely bound  $^{109}\text{Cd}$  from all the proteins in solution but did not alter the distribution of  $^{109}\text{Cd}$  counts.



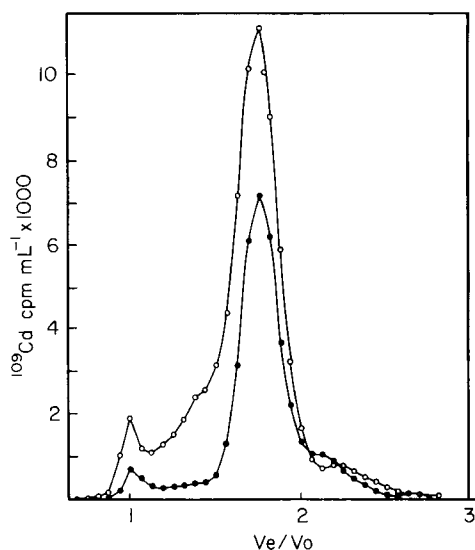


FIG. 5. Sephadex G-75 chromatogram to show  $^{109}\text{Cd}$ -binding of heat-denatured supernatant incubated with  $^{109}\text{Cd}$  (○), compared to the same sample after haemoglobin addition and denaturation (●), i.e. the complete assay.

## EXPERIMENTAL EXPOSURE

### $^{109}\text{Cd}$ -binding in trout exposed to dietary and waterborne zinc

After 16 weeks exposure to various Zn levels in the water and in the diet, juvenile trout showed significant induction of metal binding proteins in both gill (Fig. 6) and intestine (Fig. 7) but not liver.

Over the lower, more normal range of  $[\text{Zn}]$  in the diet ( $1\text{--}90\text{ mg Zn kg}^{-1}$ ) and in the water ( $7\text{--}39\text{ }\mu\text{g Zn l}^{-1}$ ), there was no change in  $^{109}\text{Cd}$ -binding in the gill (points a, d, b, e in Fig. 6). However, ligand activity showed increases at both the higher dietary  $[\text{Zn}] = 590\text{ mg kg}^{-1}$ , and at the higher water  $[\text{Zn}] = 148\text{ }\mu\text{g l}^{-1}$ , alone and in combination (points g, h, i, f, c in Fig. 6). There was a further large increase at waterborne  $[\text{Zn}] = 529\text{ }\mu\text{g l}^{-1}$  dietary  $[\text{Zn}] = 590\text{ mg kg}^{-1}$  (point j in Fig. 6). The maximum overall increase was about five-fold.

$^{109}\text{Cd}$  binding by intestine showed more striking changes with Zn exposure (Fig. 7). There was an apparent plateau over much of the exposure grid where treatment had no significant effect (points a–g in Fig. 7). However, at high combinations (points h, i, j in Fig. 7) there was an interaction between dietary and waterborne Zn which magnified  $^{109}\text{Cd}$ -binding considerably above that expected by simple additive effects. In fact, maximum induction was about 25-fold. Thus, not only did dietary  $[\text{Zn}]$  affect intestinal  $^{109}\text{Cd}$ -binding, a reasonable finding related to exposure, but waterborne Zn had an important effect as well.

The results for liver tissue (Table II) showed no significant differences or trends. Most of the values were  $<2\text{ nmol Cd g}^{-1}$  wet tissue with occasional high values which accounted for the very high variability. Thus, neither very high dietary nor waterborne  $[\text{Zn}]$  induced significant metal binding species in the liver.

Gel chromatography of HDS from the diet/waterborne study revealed a very different pattern from the injection study. Gill tissue showed low counts in peak I

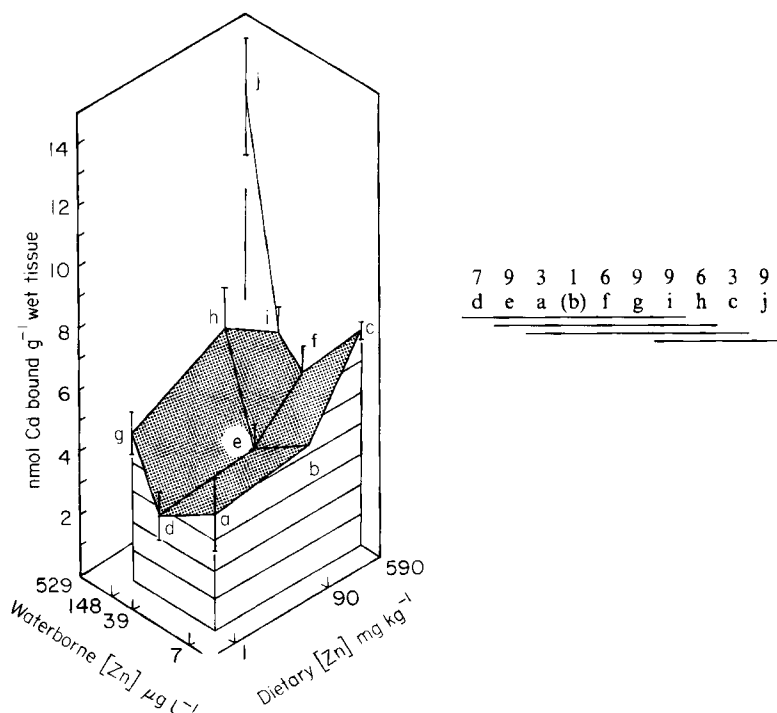


FIG. 6.  $^{109}\text{Cd}$ -binding ( $\text{nmol Cd bound g}^{-1}$  wet tissue, means  $\pm$  S.E.M.) by gill tissue from rainbow trout exposed to different levels of zinc in the diet and in the water. Means, represented by letter, underlined by the same line were not significantly different.  $n$  values are given above each letter. The letter b, though ranked here for comparative purposes, was not included in the analysis, since  $n = 1$ .

and significant counts in peak II. By far the largest number of counts were in peak IV, however, and only one out of three trout showed any counts at all in peak III [Fig. 8(a)]. Liver tissue showed the same profile [Fig. 8(b)]. In intestine, Cd-binding in peak IV dwarfed peaks I and II which were actually similar in area to those for gill tissue [Fig. 8(c)]. There was no Cd-binding in peak III. Thus, in contrast to the injection study, the metal ligands induced occurred mainly in the low mol wt fraction ( $< 3000$ ).

#### *Acid-soluble thiols in trout exposed to dietary and waterborne zinc*

AST showed a fundamentally different pattern from  $^{109}\text{Cd}$ -binding in response to dietary and waterborne exposure. Rather than increasing, there was a general trend for AST in gills to decrease as waterborne Zn increased, an effect which was seen most prominently at the highest dietary level (Fig. 9). Thus, the lowest AST concentration was seen in the highest waterborne ( $529 \mu\text{g Zn l}^{-1}$ ) and highest dietary ( $590 \text{ mg Zn kg}^{-1}$ ) treatment (Fig. 9, point j). Liver AST showed some significant differences but no well-defined trends (Table III). Whereas the gill and liver tissue had the same range of concentration of AST ( $0.1\text{--}0.5 \mu\text{mol thiol g}^{-1}$  wet tissue), intestine was characterized by levels nearly 10-fold higher (Table III, Fig. 9). There was also greater variability, and no significant differences or trends were apparent, in the intestine.

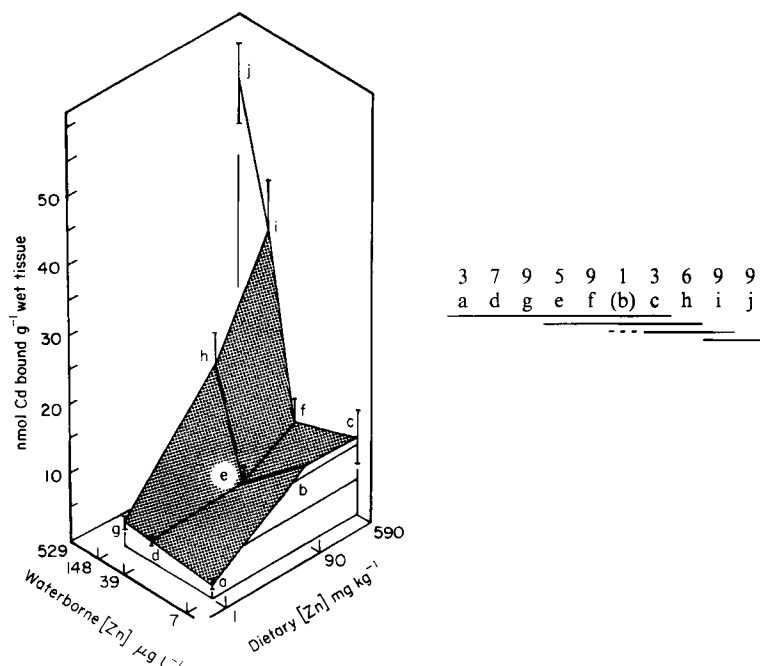


FIG. 7.  $^{109}\text{Cd}$ -binding ( $\text{nmol Cd bound g}^{-1}$  wet tissue, means  $\pm$  S.E.M.) by intestine from rainbow trout exposed to different levels of zinc in the diet and in the water. Key as Fig. 6.

## DISCUSSION

### EVALUATION OF THE ASSAY

#### Performance

The requirements for an assay include specificity, sensitivity, accuracy, reproducibility and linearity. The  $^{109}\text{Cd}$ -binding assay was reported to fulfil these requirements even when used with semi-purified homogenate from rat (Eaton & Toal, 1982) or brook trout, *Salvelinus fontinalis* Mitchill, (Hamilton *et al.*, 1987). However, neither of these groups of authors performed mol wt determinations on the assay. We therefore further evaluated this assay for use with rainbow trout.

When purified rabbit Cd-Zn MT was used, the assay was linear with increasing concentration, based on measurement of total Cd. Error due to incomplete removal of added free Cd by haemoglobin was at most an overestimate of <20%. When HDS was used, the assay was linear as long as the sample size was kept at 400  $\mu\text{l}$  or above. Reproducibility of replicates was good even when retained counts were low. The coefficient of variation for Cd-Zn MT standards on different days was excellent (1.8%) and comparable to that reported by Eaton & Toal (1982). That of 'reference' trout HDS on different days was higher (9.5%) but none-the-less satisfactory.

#### Gel chromatography

Heat-denaturation of crude homogenate purified it considerably, but significant amounts of protein still remained. Cd-binding by these proteins occurred in four

TABLE II.  $^{109}\text{Cd}$ -binding activity in heat-denatured supernatants of liver tissue from trout exposed to combinations of dietary and waterborne zinc. Values are nmol Cd bound  $\text{g}^{-1}$  wet tissue; means  $\pm$  S.E.M ( $n$ ). There were no significant differences

Waterborne [Zn] $\mu\text{g l}^{-1}$	Dietary [Zn] $\text{mg Zn kg}^{-1}$ dry diet		
	1	90	590
7	1.53		4.62
	$\pm 0.62$ (3)	—	$\pm 3.45$ (3)
39	2.08	1.17	7.35
	$\pm 0.75$ (6)	$\pm 0.46$ (9)	$\pm 2.63$ (7)
148	2.67	3.93	3.62
	$\pm 1.09$ (9)	$\pm 1.67$ (6)	$\pm 0.89$ (9)
529			5.41
			$\pm 1.59$ (9)

major peaks, with only peak III corresponding to a MT-like protein: total  $^{109}\text{Cd}$ -binding by the other three peaks did not change much, but, on a percentage basis, when peak III was low, resulted in overestimation of MT-like protein by as much as 54%. Eaton & Toal (1982) showed only one peak of  $^{109}\text{Cd}$ -binding potential, but they assayed gel filtration fractions from  $100\,000 \times \text{g}$  rat liver cytosol, and did not chromatograph supernatant from the assay itself. Chellman *et al.* (1985) did, however, chromatograph supernatant from  $^{109}\text{Cd}$ -binding assays of testes from Cd-injected mice. They found significant  $^{109}\text{Cd}$ -binding peaks at 30 000, 14 500 (MT), and  $< 3000$  mol wt; these results corresponded to our peaks II, III and IV, respectively, and differed from ours only in the lack of a  $> 70\,000$  mol wt peak (peak I). Waalkes *et al.* (1985) found that two metal-binding assays (Hg or Cd) overestimated MT concentration in rats when compared with radioimmunoassay; they speculated that low mol wt peptides were the cause. High mol wt species must also be considered. Other potential causes of overestimation (Eaton & Toal, 1982; Eaton, 1985) were small (incomplete removal of free  $^{109}\text{Cd}$ ,  $< 20\%$ ) or did not occur (incomplete precipitation of haemoglobin) in the present study.

In summary, while the  $^{109}\text{Cd}$ -binding assay appears to detect MT satisfactorily, it also responds to at least three other groups (based on mol wt) of heat-stable, metal ligands in trout tissues. Thus, the most serious overestimation of MT concentration will occur where there is little or no induction and MT levels are low, as was also shown by Nolan & Shaikh (1986). While no attempt was made in the present study to further separate or characterize these three other groups of

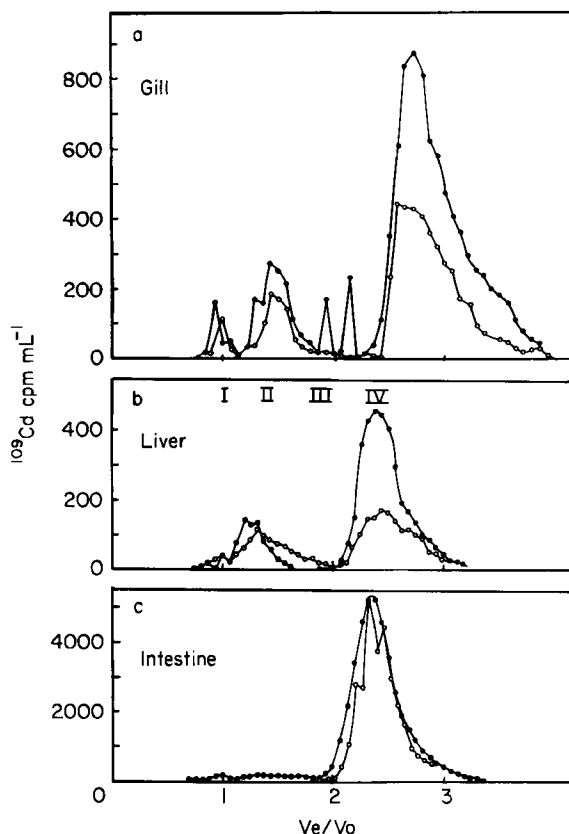


FIG. 8. Sephadex G-75 chromatogram of supernatants from the  $^{109}\text{Cd}$ -binding assay from tissues of trout exposed to different levels of zinc in the water and in the diet. (a) Gills ( $\circ$ ), water  $148\ \mu\text{g Zn l}^{-1}$ , diet  $1\ \mu\text{g Zn g}^{-1}$ ; ( $\bullet$ ), 529, 590. (b) Liver ( $\circ$ ), 148, 590; ( $\bullet$ ), 529, 590. (c) Intestine ( $\circ$ ), 529, 590; ( $\bullet$ ), 148, 529, from individual rainbow trout. Note the change in scale in panel (c).

metal ligands, future work with more sophisticated techniques such as gradient ultracentrifugation, ultrafiltration etc. may be informative. Whatever the identity of the other three fractions, they certainly have the ability to avidly bind  $^{109}\text{Cd}$  under the conditions of the assay. Several other studies have also reported the presence of non-MT metal-binding proteins in rainbow trout. Thomas *et al.* (1983a,b) and Kay *et al.* (1986) described the induction of two non-MT proteins with mol wts very similar to true MT in Cd-exposed trout. Pierson (1985a,b) induced a 17 000 mol wt protein by Zn-injection of trout. Some method of visualizing mol wt ranges of  $^{109}\text{Cd}$ -binding proteins is clearly essential with this assay (Chellman *et al.*, 1985).

#### $^{109}\text{Cd}$ -binding in Zn-injected trout

The levels of  $^{109}\text{Cd}$ -binding in livers from control trout were similar to values for rat liver given by Eaton & Toal (1982), but lower than other literature values, whereas values for trout intestine agreed well with literature values for rats (Nolan & Shaikh, 1986). Although increases in  $^{109}\text{Cd}$ -binding were not significant in Cd- and Zn-injected trout, Eaton & Toal (1982) and Waalkes *et al.* (1985) indicated

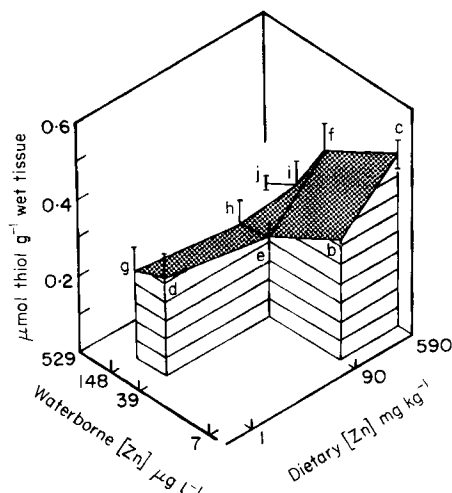


FIG. 9. Acid-soluble thiols in gill tissue from rainbow trout exposed to different levels of zinc in the diet and in the water. Key statistics as for Fig. 6. Only point c was significantly different.

>20-fold induction of MT in rats. The reason for this difference is not clear, but fish seem generally less responsive than mammals (see below). The isolation procedure appeared adequate based upon mol wt estimates of the  $^{109}\text{Cd}$ -binding protein and recoveries of rabbit MT. Since trout were considerably colder ( $6^\circ\text{C}$ ) than rats ( $38^\circ\text{C}$ ), longer induction times might be required. Hamilton *et al.* (1987) measured an increase in  $^{109}\text{Cd}$ -binding in brook trout injected with a similar dose of Cd over 5 days, as opposed to 2 days in our study. Ley *et al.* (1983) characterized MT from rainbow trout injected with Zn using a similar protocol to ours, but did not report MT levels. In rainbow trout exposed to waterborne Zn, there was a doubling of MT-like protein after 5 days at  $15^\circ\text{C}$  (Bradley *et al.*, 1985), while Zn-injection induced significant, non-MT, Zn-binding protein after 4 days at  $13\text{--}20^\circ\text{C}$  (Pierson, 1985a,b).

## EXPERIMENTAL EXPOSURE

### $^{109}\text{Cd}$ -binding in trout exposed to dietary and waterborne zinc

In the present study we have shown the induction of high levels of metal ligands in trout chronically exposed (16 weeks) to Zn from dietary and waterborne sources. While effects of waterborne exposure, generally at much higher levels, have been reported previously (e.g. Kito *et al.*, 1982; Bradley *et al.*, 1985), this is the first report of induction in trout through dietary exposure, by Zn or any other metal.

The identity of metal ligand(s) detected by the  $^{109}\text{Cd}$  assay in these dietary/waterborne-exposed trout, based on mol wt, did not appear to be MT. In contrast to the situation seen in the injected trout where most of the  $^{109}\text{Cd}$  binding was in peak III (the putative MT peak), the contribution of peak III was negligible in the environmentally-exposed fish. Peak II contributed a minor amount, while most was associated with peak IV. Therefore, most of the  $^{109}\text{Cd}$  was bound to species having an apparent mol wt  $<3000$ . This peak was not due to free Cd, since haemoglobin typically removed  $>98\%$  of Cd added. It was probably not due to

TABLE III. Acid-soluble thiol concentrations in liver and intestine from rainbow trout exposed to combinations of dietary and waterborne zinc. Values are  $\mu\text{mol thiol g}^{-1}$  wet tissue, means  $\pm$  S.E.M. ( $n$ )

Waterborne [Zn] $\mu\text{g l}^{-1}$	Dietary [Zn] $\text{mg Zn kg}^{-1}$ dry diet		
	Liver		
	1	90	590
7	—	0.186 (1)	0.201*,† $\pm 0.049$ (3)
39	0.139*,† $\pm 0.025$ (9)	0.090* $\pm 0.009$ (8)	0.301† $\pm 0.051$ (6)
148	0.123* $\pm 0.023$ (9)	0.106* $\pm 0.034$ (6)	0.183*,† $\pm 0.050$ (9)
529			0.142*,† $\pm 0.015$ (9)
	Intestine		
	1	90	590
7	—	2.505 (1)	1.775 $\pm 0.004$ (3)
39	2.147 $\pm 0.436$ (9)	4.170 $\pm 0.543$ (9)	3.540 $\pm 0.892$ (6)
148	3.617 $\pm 0.565$ (9)	3.281 $\pm 0.845$ (6)	2.515 $\pm 0.272$ (9)
529			2.936 $\pm 0.550$ (9)

\*,†Values with the same symbol were not significantly different ( $P > 0.05$ ). There were no significant differences in intestine.

glutathione, which would also elute in this peak, because Cd did not bind to glutathione in bile from striped mullet, *Mugil cephalus* L., (Wofford & Thomas, 1984). The possibility exists that the metal ligand was a metabolite or breakdown product of MT (Cousins *et al.*, 1978; Nolan & Shaikh, 1986), because a single

cleavage of MT (true mol wt = 6000–7000) into two subunits would bring it into this range. However, the complete lack of correlation (indeed opposite trend) of AST with  $^{109}\text{Cd}$ -binding activity argues against this, as well as against glutathione, and raises the possibility that a non-MT binding protein was induced. The identity of the small metal ligand(s) therefore remains unknown.

Identity notwithstanding, there was clear induction in gill and intestine. Our initial hypothesis that induction in a tissue would reflect the route of exposure was not confirmed. Thus, while the gill demonstrated a plateau over the waterborne range of  $7\text{--}39\ \mu\text{g Zn l}^{-1}$  and dietary range  $1\text{--}90\ \text{mg Zn kg}^{-1}$  (Fig. 6), there were increases associated with both high waterborne and higher dietary levels. Nevertheless, the largest increase at  $529\ \mu\text{g Zn l}^{-1}$  appeared solely attributable to the waterborne exposure. This five-fold increase in gill from lowest to highest exceeds the 2.5-fold increase shown by Bradley *et al.* (1985) for trout in response to a much higher waterborne Zn exposure.

$^{109}\text{Cd}$ -binding in intestine, unexpectedly, did not change with dietary exposure ( $1\text{--}590\ \text{mg Zn kg}^{-1}$ ) when waterborne Zn was low ( $7\text{--}39\ \mu\text{g Zn l}^{-1}$ ) (Fig. 7). Only when waterborne Zn reached  $148\ \mu\text{g Zn l}^{-1}$  did intestine reflect dietary loading, indicating a strong interaction between dietary and waterborne Zn, which is discussed below. This may be explained in part by routes of excretion of Zn. The 25-fold increase in metal ligand activity in response to combined dietary and waterborne exposure is higher than any previously described for fish (Hamilton & Mehrle, 1986).

The small response shown by liver, contrary to our initial hypothesis, was surprising, since this has been the tissue of choice for MT induction in nearly all studies. The most common form of administration, however, has been by acute injection. Studies which have examined metal ligands in fish have used environmental exposures only rarely. Roch *et al.* (1982) sampled rainbow trout along a natural metal gradient, and found that liver MT rose 4.6-fold. At the most contaminated site, Zn : Cu : Cd was  $170 : 9 : 0.7\ \mu\text{g l}^{-1}$ , yet liver MT bound only Cu and Cd; Zn in liver homogenates was invariant, while Cu and Cd reflected the exposure. They suggested that, in the presence of those three metals, MT synthesis was ineffective in detoxifying Zn. With a similar mixture, the threshold for induction of hepatic MT was indexed to  $40\ \mu\text{g l}^{-1} [\text{Zn}]$  in soft water. The role played by the Cu and Cd in the mixture was not examined (Roch *et al.*, 1986). Bradley *et al.* (1985) found 2.5-fold elevations in heat-stable, sulphhydryl-rich protein in livers of rainbow trout exposed to  $2100\ \mu\text{g Zn l}^{-1}$  for 5–20 days. In contrast, Davies (1985) found increased Zn in Sephadex G-75 fractions of liver cytosol from trout exposed for 96 h to  $360\ \mu\text{g l}^{-1}$  (70% of the 96 h LC50). However, the Zn occurred in the 20 900–25 100 mol wt range, not in the MT range. Following 60 h in clean water, a peak occurred at 4300 mol wt. Thus, while induction of liver MT by Zn alone is equivocal, these results do indicate that trout liver is much less responsive than mammalian liver, and its role in detoxification remains unclear.

#### *Acid-soluble thiols in trout exposed to dietary and waterborne zinc*

In contrast to  $^{109}\text{Cd}$ -binding activity, AST tended to fall in gills of fish exposed to high environmental levels of Zn, and showed no clear trends in liver and intestine. The three-fold increases in liver AST in Cd-exposed mullet (Wofford & Thomas, 1984) were comprised of both increases in glutathione and a residual



fraction (putative MT) while cysteine remained unchanged. Thus the lack of response of AST (liver and intestine), or trends contrary to what was expected (gills), could reflect the sum of at least three dynamic factors. In order for significant MT synthesis to occur, other elements of the AST pool would have had to decrease. Glutathione was the largest component in control trout (Laurén & McDonald, 1987). It is unlikely that glutathione levels would decrease much, given the short half-life of glutathione and the fact that it is usually only depressed following acute oxidative stress (Reed & Beatty, 1980).

#### THE ROLE OF METAL LIGANDS

Induction of  $^{109}\text{Cd}$ -binding was highest in trout exposed to the highest combinations of dietary and waterborne Zn. It is significant that these fish suffered neither mortality nor any demonstrable pathology (Spry *et al.*, 1988) but it is uncertain whether the observed elevation in metal binding activity was protective or merely correlative.

The functional role of inducible metal-binding proteins remains controversial. Suggestions include both the normal metabolism of metals and protection against toxic effects (Brady, 1982; Webb & Cain, 1982; Cousins, 1985). The longest standing hypothesis (the 'spill-over' hypothesis, Winge *et al.*, 1973; Brown & Parsons, 1978) is that the protein serves a protective role by binding tightly certain metals, thereby preventing pathological effects of the free metal on high mol wt cytosolic components such as enzymes or nucleic acids. McCarter *et al.* (1982) and Roch *et al.* (1982) have disputed this hypothesis, showing parallel accumulations of Cd and Cu in both the MT and high mol wt fractions. Furthermore, in water polluted with high levels of Zn, and only small amounts of Cu and Cd, only Cu and Cd were associated with liver MT (Roch *et al.*, 1982). As pointed out by Hamilton & Merhle (1986), sublethal pathologies associated with induction of high levels of MT, which would clearly support the spill-over hypothesis, remain to be demonstrated in fish. The only measures of acclimation have been increased tolerance in lethality bioassays, a response at least one step removed from the spill-over hypothesis.

The very high levels of induced metal ligand seen in the intestine in the present study may indicate an excretory role and/or uptake blockade role. Increases in  $^{109}\text{Cd}$ -binding activity due to diet were not significant when waterborne [Zn] was low. The dramatic increase when waterborne [Zn] was high, however, indicates an additional source of Zn to the intestinal mucosa. Drinking of the medium has been precluded (Spry *et al.*, 1988). We suggest that serosal-mucosal flux of parenteral Zn (Cousins, 1985) is the cause. In this case, the parenteral route was via the gills. Further, an excretory role is more probable than reduced uptake, since Shears & Fletcher (1984) induced MT in the intestine of winter flounder, *Pseudopleuronectes americanus*, but found that it neither inhibited nor enhanced Zn uptake. Induction of MT or other cellular metal ligands would thus provide a mechanism whereby fish could regulate net loading from both the food and the water. Measurements of the whole body Zn in trout from the final sample day of the present study (Spry *et al.*, 1988) indicated that whole body Zn increased with increasing exposure concentration, but that the increase tended to be linear whereas the exposure was logarithmic. Both mechanisms, decreased uptake and increased excretion, may occur.

In summary, the  $^{109}\text{Cd}$ -binding assay proved sufficiently sensitive and precise to demonstrate the induction of heat-stable metal ligands in gills and intestine of rainbow trout exposed to elevated levels of Zn in the water and in the diet. The identity of these ligands remain uncertain, since mol wt determinations (Sephadex G-75 filtration) did not correspond with MT. Further, there was no corresponding increase in AST concentrations as would be anticipated with MT induction. By far the greatest induction was shown by the intestine (25-fold) in fish exposed to combined high waterborne and high dietary Zn. While these increases are modest for mammals, they constitute the highest levels of induction yet described for fish. Thresholds for induction were  $>39\ \mu\text{g Zn l}^{-1}$  in moderately hard water and  $>90\ \text{mg Zn kg}^{-1}$  in the diet, but only when waterborne [Zn] was also high. The threshold for waterborne induction would be expected to alter somewhat with changes in water hardness, i.e. [Ca], given the competitive nature of Ca/Zn uptake (Wright, 1980; Spry & Wood, 1989). The waterborne threshold is close to the existing water quality objective of  $30\ \mu\text{g Zn l}^{-1}$  in Canadian and U.S. boundary waters (IJC, 1976) and the dietary threshold is similar to that of most hatchery diets (Spry *et al.*, 1988).

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