Arch. Environ. Contam. Toxicol. 16, 95–101 (1987)

Archives of Environmental Contamination and Toxicology

Cadmium Kinetics in Freshwater Clams. III. Effects of Zinc on Uptake and Distribution of Cadmium in *Anodonta Cygnea*

J. Hemelraad, H. A. Kleinveld, A. M. de Roos, D. A. Holwerda, and D. I. Zandee

Laboratory of Chemical Animal Physiology, State University, Padualaan 8, 3508 TB Utrecht, The Netherlands

Abstract. Freshwater clams (*Anodonta cygnea*) were exposed to Cd^{2+} (25 μg/L) or to Cd^{2+} (25 μg/L) plus Zn^{2+} (2.5 mg/L). In the presence of zinc, uptake of cadmium in whole clam was halved. In organs such as the gills, mantle, and labial palps a still larger reduction of Cd accumulation occurred. By contrast, accumulation in midgut gland and kidney was hardly affected by the presence of zinc. It is shown that in the gills zinc competes with cadmium for metal binding sites both within the particulate fraction and the high-molecular weight fraction of the cytosol. Zinc probably did not induce an enlarged synthesis of specific metal-binding, metallothionein-like, proteins. In conclusion, zinc exerts antagonistic effects on uptake of cadmium by gills, and accelerates Cd transport from the gills towards the internal organs.

Zinc has an antagonistic and protective action in the uptake and toxic effects of cadmium. Pre-treatment of rats with Zn^{2+} reduced injurious effects of subsequently administered Cd^{2+} (Webb 1972). The protective action was assigned to Zn-induced synthesis of thionein that detoxifies Cd by firmly binding this metal. Also, the intestinal absorption of Cd^{2+} in cadmium-exposed calves was significantly reduced when the diet contained Zn^{2+} in amounts exceeding that of Cd^{2+} (Lamphere *et al.* 1984).

With respect to the uptake of cadmium by aquatic organisms, differing effects of zinc coexposure have been recorded. Fowler and Benayoun (1974) found no effect of zinc on the uptake of much lower concentrations of radioactive cadmium by *Mytilus galloprovincialis*. Also, uptake of cadmium in isolated gill of *Mytilus edulis* was not influenced by the presence of zinc or other metals (Carpené and George 1981). When *Mytilus edulis* were exposed to zinc, cadmium, or lead, either as the single metal or as a mixture, uptake of any individual metal was not affected by the presence of other metals (Phillips 1976). Investigating the uptake of cadmium in *Macoma baltica* McLeese and Ray (1984) stated that "the presence of zinc may or may not have altered Cd accumulation." By contrast, an inhibitory effect of zinc on the accumulation of cadmium has been reported for the bivalves *M. edulis* and *Mulinia lateralis* (Jackim *et al.* 1977), and the polychaetes *Nereis diversicolor* (Bryan and Hummerstone 1973) and *N. virens* (Ray *et al.* 1979). Antagonistic Cd-Zn interaction was also found for the estuarine teleost *Fundulus heteroclitus*, in the sense that comparatively low levels of cadmium inhibited uptake of zinc from the medium (Eisler and Gardner 1973). Differential effects of zinc on the accumulation of cadmium in the separate tissues of *Pandalus montagui* were observed by Ray *et al.* (1980), whereas in the shrimp *Callianassa australiensis* addition of zinc to the water increased the accumulation of cadmium (Ahsanullah *et al.* 1981).

For aquatic organisms, especially in the freshwater habitat, Zn interaction with uptake of cadmium may be of great significance, as these two metals are often disposed from zinc-producing plants in combination (RIWA 1983). In light of the controversial findings in the literature on the influence of zinc, and on the basis of our studies on Cd accumulation in *Anodonta* sp. (Hemelraad *et al.* 1986a, 1986b), an investigation was undertaken to study the effects of zinc on Cd kinetics in *Anodonta cygnea*. Total Cd uptake, as well as the metal distribution among the various tissues, and the subcellular and molecular localization were considered.

Materials and Methods

Animals

Freshwater clams, *Anodonta cygnea zellensis* Gmelin, were collected and kept in the laboratory as described earlier (Hemelraad *et al.* 1986a). At the start of the experiments, the clams had been in the laboratory for three weeks.

Exposure System

Clams were exposed to cadmium in glass aquaria to which tapwater and metal solution were supplied with pumps at rates of 9.6 L/hr and 25 mL/hr, respectively, without addition of food. Water quality parameters were the same as reported before (Hemelraad *et al.* 1986a). The exposure was started with about 150 animals, mean shell length 12.5 cm ± 1.5 (SD), in 250 L water. Cadmium was added as $CdCl_2 \cdot H_2O$ (Merck, no. 2011) at a final concentration at 25 μg/L (ppb).

Simultaneously, animals were exposed to Cd plus Zn. In this exposure system, water and metal solution were supplied with pumps at rates of 3.75 L/hr and 25 mL/hr, respectively. About 70 clams, mean shell length 11.9 cm ± 1.5 (SD) were kept in 85 L. Cadmium was added as $CdCl_2$ and zinc as $ZnCl_2$ (Fluka, no. 96470) at final concentrations of 25 ppb and 2.5 mg/L (ppm), respectively.

The exposures lasted from October 1984 until March 1985. The water concentrations of Cd and Zn were measured twice weekly. The actual Cd concentration amounted to 28 ppb ± 5 (SD) and 29 ppb ± 7 (SD) for the Cd and Cd plus Zn exposure, respectively. The final Zn concentration in the Cd plus Zn exposure amounted to 2.5 ppm ± 0.4 (SD).

Metal Analysis

At intervals of 2 or 4 weeks, four animals were examined individually for Cd in nine organs: labial palps, mantle, mantle-edge, foot, guts/gonads complex, kidney, adductor muscles, midgut gland and gills. The remainder, consisting of different tissues, is referred to as "rest fraction." Prior to dissection, the animals were held in unspiked tapwater for 24 hr, in order to eliminate adherent cadmium. No loss of accumulated cadmium was noted during this period. The excised organs were frozen (−20°C) overnight and lyophilized for 48 hr. The dry tissues were decomposed in 65% (w/w) nitric acid, using teflon (PTFE) bombs placed in a sandbath.

The Cd and Zn concentrations in tissue and water samples were determined by atomic absorption spectrophotometry. The instrument (Instrumentation Laboratory, type IL 451) was equipped with a deuterium lamp for background correction. Each sample was measured ten times (RSD < 5%).

Subcellular and Molecular Distribution

Gills, kidneys, and midgut glands of four animals were pooled and gently homogenized in 25 mM Tris-HCl buffer of pH 8.0, using successively an Ultra-Turrax homogenizer and a Potter tube. The homogenates were centrifuged (1 hr at 100,000 g) to obtain a particulate fraction and a cytosolic fraction. The latter was chromatographed on a Sephadex G-75 superfine column (Pharmacia), 1.4 × 60 cm, that was equilibrated and eluted (at 7 mL/hr) with 25 mM Tris-HCl buffer of pH 8.7 containing 100 mM NaCl. The fractionation procedure was carried out at 4°C. A calibrated 100-cm column was used for the estimation of molecular weights.

From the same four animals, haemolymph samples were taken from the ventricle with a syringe (2 mL/animal). The pooled haemolymph was centrifuged (10 min at 3000 g) to remove lymphocytes, and the plasma fraction was chromatographed on a Sephadex G-75 column, 1.4 × 115 cm, under the same conditions as mentioned above.

Cd and Zn concentrations of the cytosolic, plasma, and column fractions were measured without prior acid decomposition. The metal concentrations of the particulate fractions were determined after acid decomposition.

Statistical Analysis

Corresponding samples in the time courses of Cd accumulation in the Cd, and Cd plus Zn exposure (Figure 1) were analyzed for significance of difference by Student's t-test, with d.f. corrected for unequal sample variance. Difference of organ distribution (Figure 2) between the two exposures was analyzed with the non-parametric sign test. In the time course of subcellular distribution (Figure 3), control, unexposed samples were treated with Dixon's test for outliers against the collection of samples in the steady state phase (three out of four courses).

Results

Accumulation of Cd in Total Organism and Separate Organs

Cadmium accumulations in whole animal for the Cd, and Cd plus Zn exposure are shown in Figure 1A. The relatively large deviation between individual animals does not admit a definite conclusion about linearity or non-linearity of accumulation during exposure to cadmium alone. As observed earlier (Hemelraad *et al.* 1986a), the pattern suggests that Cd accumulation levels off after about six weeks, followed by an increase of the rate of uptake after eight weeks and an ultimate plateau of the Cd level after about twelve weeks. In the Cd plus Zn exposure, Cd accumulation deviated from the onset of exposure. The accumulation rate was about one-half that of exposure to cadmium alone, and accumulation proceeded more linearly. In both exposures, mortality was negligible and no weight loss observed.

In gills, a stronger divergence was observed between the two conditions (Figure 1B). A much larger accumulation was found in Cd-exposed animals. Comparable, but smaller, divergences were found for guts/gonads complex and muscle tissue. For midgut gland and—to a lesser extent—kidney,

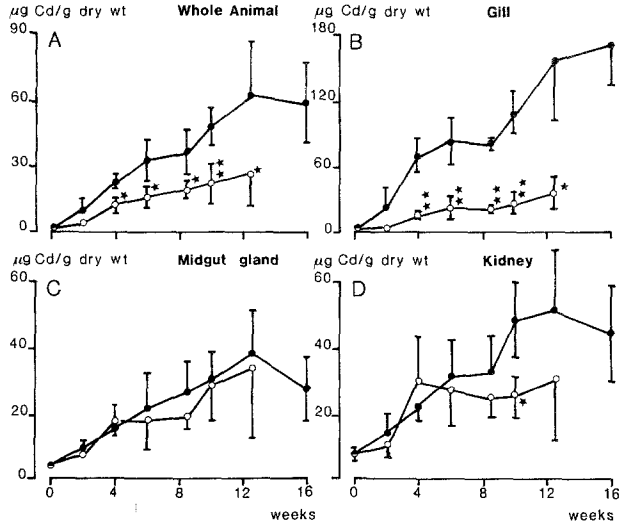

Fig. 1. Cd concentrations *vs* exposure time in whole body and some organs of *A. cygnea*, exposed to 25 ppb Cd (-●-) or 25 ppb Cd + 2.5 ppm Zn (-○-). Mean ± SD of four animals; significant differences between Cd and Cd plus Zn groups at each exposure time are denoted by * ($p < 0.05$) or ** ($p < 0.01$)

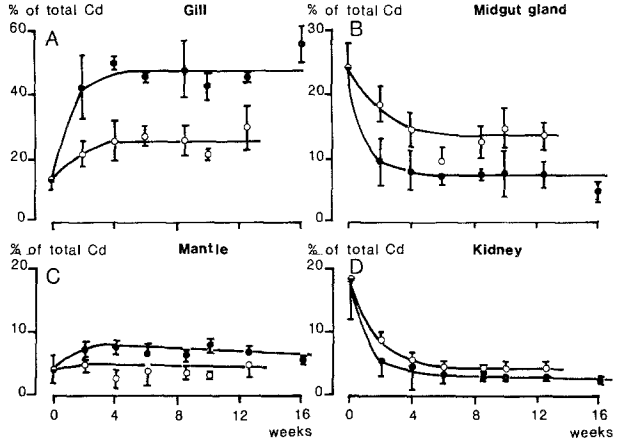

Fig. 2. Distribution of Cd (as % of total body burden) *vs* exposure time in four organs of *A. cygnea*, exposed to 25 ppb Cd (-●-) or to 25 ppb Cd plus 2.5 ppm Zn (-○-). Mean ± SD of four animals

the effect of Zn coexposure on Cd accumulation was considerably smaller (Figures 1, C and D). The same was true for mantle-edge and rest fraction.

Comparison of the curves for relative Cd burden in the organs (Figures 2, A–D) shows that the presence of zinc significantly changed Cd distribution. Whereas gills of animals exposed to cadmium alone eventually accomodated half of total cadmium, in case of Zn coexposure only 25% of total body cadmium was present in gills. By contrast, the relative Cd burden of midgut gland was almost twice as high in (Cd + Zn)-exposed animals as in Cd-exposed

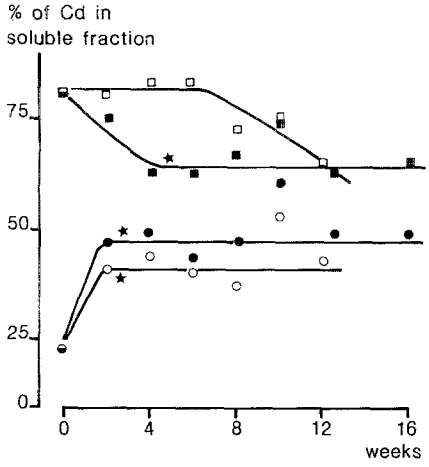

Fig. 3. Subcellular distribution of Cd *vs* exposure time, in gills (-●-, -○-) and kidney (-■-, -□-) of animals exposed to Cd alone (closed symbols) or to Cd plus Zn (open symbols). Pooled organs of four animals; * denotes constant level that differs significantly ($p < 0.05$) from the unexposed control

animals. For either condition of exposure, a steady state of Cd distribution was reached after four to six weeks.

Subcellular Cd Distribution

Coexposure with zinc exerted differential effects on the subcellular Cd distribution in gills and kidney (Figure 3). Whereas, in gills, the distribution between the particulate and soluble fraction significantly changed on exposure to either condition, the difference between Cd-exposed and (Cd + Zn)-exposed animals was small. By contrast, the two curves for kidney deviate considerably. During exposure to cadmium, Cd percentage in the soluble fraction decreased to a constant level that was reached after four weeks, but in the presence of zinc there was no change of subcellular Cd distribution until the seventh week of exposure. Thereafter, cadmium in the soluble fraction decreased relatively. By the end of exposure, after twelve weeks, Cd distribution was the same under either condition.

Although the subcellular distribution of zinc did not change on exposure (Table 1), neither to cadmium alone nor to cadmium plus zinc, it could be argued that a strong increase of absolute Zn concentration in the kidney particulate fraction would inhibit cadmium to be bound to this fraction. It is, however, not clear why this effect would not appear in gills that have no less than ninety nine percent of total zinc in the particulate fraction.

Table 1. Subcellular distribution of zinc: Percentage of zinc bound to the particulate fraction in gills and kidney, during exposure of *A. cygnea* to Cd or to Cd plus Zn[a]

Exposure time (weeks)	Gills		Kidney	
	Cd	Cd + Zn	Cd	Cd + Zn
0	99.0		83.1	
2	98.7	98.5	86.0	86.5
4	98.9	98.0	84.7	69.5
6	99.1	97.6	89.5	78.7
8	99.2	99.0	86.0	88.7
10	99.2	98.4	87.9	93.3
12		99.0		88.2
12½	98.7		86.2	
		$\bar{x}_{2\to12½} \pm$ SD		
	99.0 ± 0.2	98.4 ± 0.6	86.7 ± 1.7	84.2 ± 8.6

[a] Pooled samples of four animals

Molecular Distribution of Cytosolic Cd

In Figure 4A, the elution profile is given for gel filtration of the cytosolic fraction of gills, after 12 weeks of Cd exposure. Cadmium was recovered in two protein fractions, with virtually no metal present in the low-molecular weight range. In the first peak (P_1), cadmium is associated with high-molecular weight proteins. The second Cd peak (P_2) was accompanied by an increase of the absorbance ratio A_{250}/A_{280}. Cytosolic fractions of midgut gland and kidney showed similar elution profiles. The increase of the A_{250}/A_{280} ratio, together with observed apparent molecular weights of 9 kD for gill and midgut gland, and 11 kD for kidney, suggests that P_2 might comprise metallothionein-like proteins. The P_2 fraction was practically devoid of zinc. The latter metal was associated almost exclusively with the high-molecular weight peak.

The corresponding result from the Cd plus Zn exposure is shown in Figure 4B. Zinc was elevated in P_1, while cadmium was decreased in this fraction, in favour of an increased content of cadmium in P_2. This picture was consistently observed after four weeks of exposure (Figure 5). The percentage of cadmium in the MT-like fraction fluctuated around 35 for gills of Cd-exposed animals. In the Cd plus Zn exposure, the proportion of cadmium in this fraction increased to a constant level of more than 70 percent.

Haemolymph

Cadmium levels in haemolymph were measured in pooled samples from four animals. It is, therefore, not possible to evaluate the significance of the noticeable time course in the Cd exposure (Figure 6).

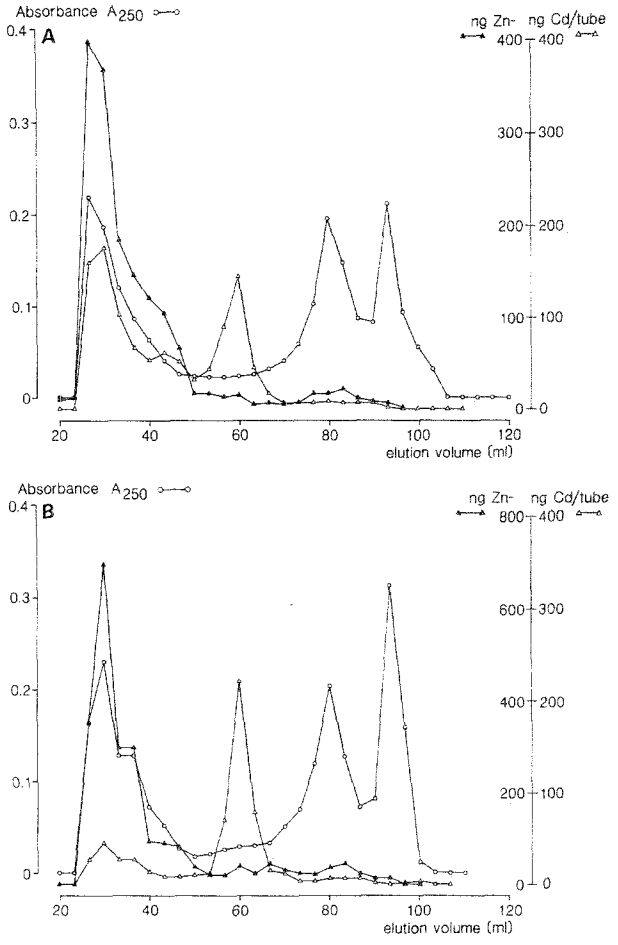

Fig. 4. Sephadex G-75 elution profile of gill soluble fraction of *A. cygnea*, exposed to 25 ppb Cd for 12 weeks (**A**) or to 25 ppb Cd + 2.5 ppm Zn for 12½ weeks (**B**); –○– A_{250}, –△– Cd, –▲– Zn

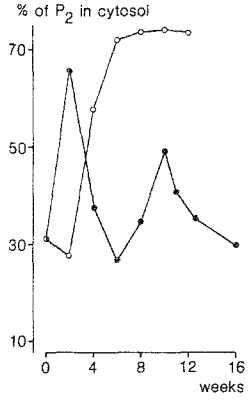

Fig. 5. Percentage of Cd bound to P_2 in the gill cytosolic fraction during exposure to 25 ppb Cd (–●–), or 25 ppb Cd + 2.5 ppm Zn (–○–). Pooled organs of four animals

After an initial increase during the first four weeks, Cd level decreased strongly for the next four weeks. This course might reflect the reduction of Cd accumulation rate in gills by that time (Figure

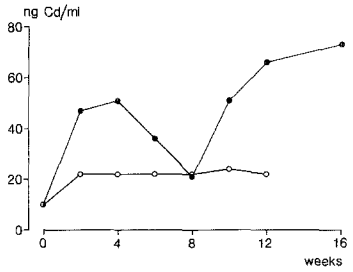

Fig. 6. Cd concentrations *vs* exposure time in haemolymph of *A. cygnea*, exposed to 25 ppb Cd (–●–), or 25 ppb + 2.5 ppm Zn (–○–). Pooled haemolymph fraction of four animals

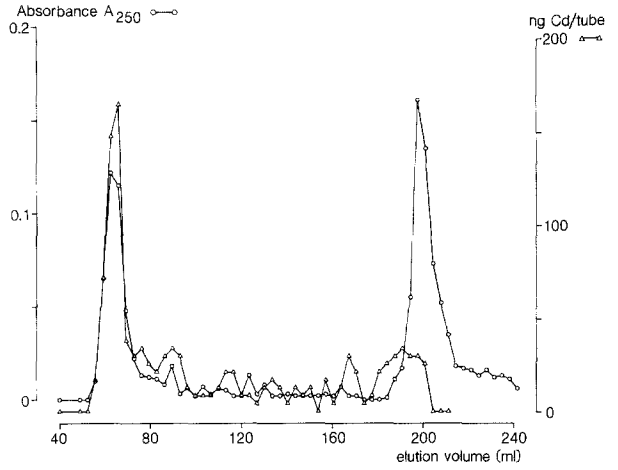

Fig. 7. Sephadex G-75 elution profile of pooled haemolymph (four animals) of *A. cygnea*, exposed to 25 ppb Cd for 12 weeks; –○– A_{250}, –△– Cd

1B) on the one hand, and the more or less continuous accumulation in internal organs such as midgut gland (Figure 1C) on the other. After eight weeks, Cd level in haemolymph increased again strongly, concomitantly with restarted accumulation in gills. In the mixed exposure, a constant level of 20 ppb was reached after two weeks. Apparently, after this period, zinc has induced a Cd release from the gills in equilibrium with Cd uptake from haemolymph by internal organs.

At all sampling times, under either condition of exposure, cadmium was found mainly (>95%) in the plasma fraction. The elution profile of plasma on Sephadex G-75 is shown in Figure 7. Cadmium was recovered only in the void volume peak (P_1); a specific Cd-binding protein (MT) was not visible. This pattern was independent of exposure time and condition. Zinc was also recovered in the P_1 fraction (not shown in the Figure). Cadmium and zinc of the lymphocyte fraction, disintegrated by sonication, emerged in the P_1 peak and, to a lesser extent, in a protein fraction with a molecular weight smaller than the P_2 fraction of gills and other organs.

Discussion

The presence of zinc reduced the accumulation of cadmium in whole animals to about one half the value for Cd exposure alone. The reduction factor was constant during the entire period of twelve weeks. A similar reduction of Cd accumulation by a hundred-fold excess of zinc has been observed for the marine bivalve *Mulinia lateralis*, whereas a still larger effect of Zn exposure occurred in *Mytilus edulis* (Jackim *et al.* 1977).

A remarkable effect of zinc was found on the Cd accumlation of the separate organs. Whereas in gills of *A. cygnea*, over the entire period of exposure, Cd concentration was decreased to about 25%, virtually no effect of zinc was observed on the Cd amount of midgut gland. In the kidney, a reduced Cd accumulation occurred only after about eight weeks of exposure. The effect of zinc on the organ distribution of cadmium is clearly illustrated by the courses of relative organ burden (Figure 2). A steady state of Cd distribution among the organs is reached after about six weeks. By that time, the gills contained 50% of the body burden in the exposure to cadmium alone. In the Cd plus Zn exposure, this value was reduced to 25%. On the other hand, the relative Cd burden in midgut gland had nearly doubled, from 7 to 12.5%. A comparable effect of zinc, when added in ten-fold excess over cadmium, has been reported for Cd uptake in the shrimp *Pandalus montagui* (Ray *et al.* 1980): Cd uptake in whole animal was decreased by 13%, but in hepatopancreas Cd concentration was increased by more than 40%.

From the foregoing, it is concluded that zinc exerts a dual effect on Cd kinetics. First, zinc antagonizes uptake of cadmium by freshwater clams; second, ingested cadmium is transported to internal organs more rapidly when zinc is present in the water in excess. It is, therefore, proposed that Zn interaction with cadmium occurs at membrane transport and also on the intracellular level. Surprisingly, these effects are not reflected by a substantially altered subcellular Cd distribution in gills. Indeed, a large difference exists between unexposed and exposed animals with respect to the subcellular Cd location (*i.e.* particulate *vs* soluble), but the difference between the two exposed groups is small (Figure 3). The latter is also true for the location of zinc, but for this metal exposed clams were not different from unexposed clams.

A strong influence of Zn coexposure was ob-

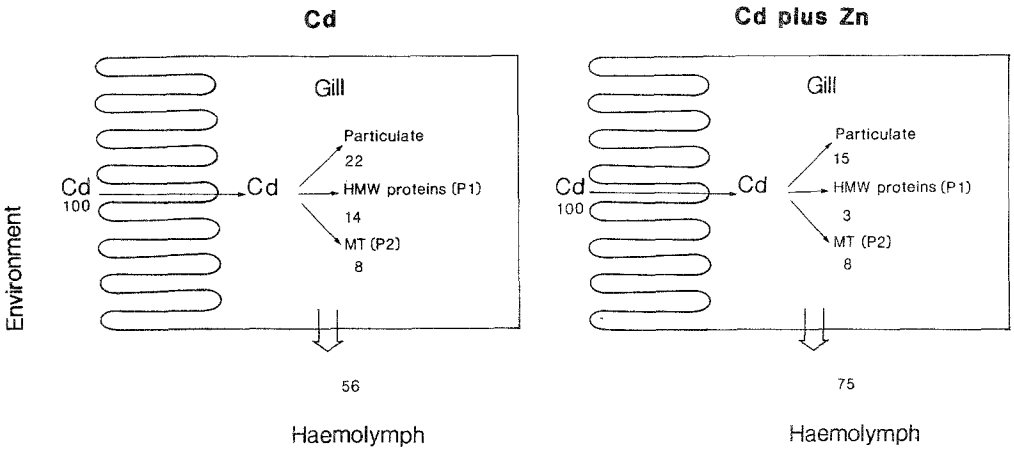

Fig. 8. Destination of Cd, ingested in the gills during the steady state phase of Cd exposure and Cd plus Zn exposure, respectively. Numbers represent percentage of Cd in the different compartments or flows

served with respect to the molecular Cd distribution in gill cytosol. After four weeks of exposure to cadmium alone, one third of cytosolic cadmium in gills was present in the metallothionein (MT) region, and two thirds were bound to high-molecular weight (HMW) proteins. In the steady state phase of the metal mixture (after six weeks), a distribution of 75% in the MT region against 25% for the HMW fraction was found. The effect of ingested zinc in gills could be attributed to an enhanced, Zn-induced, synthesis of specific, metal-binding protein that would sequester a relatively larger part of the ingested cadmium. However, on the basis of the data of Figures 2A, 3 and 5, we have calculated a flow scheme (Figure 8) for the steady state dynamics of cadmium in gills. This, necessarily rough, model presupposes that a) during the exposure phase depuration of cadmium is negligible, and b) Cd ingestion takes place predominantly via the gills.

As noted from Figure 8, a roughly constant portion of the ingested cadmium is bound as "metallothionein." It is, therefore, improbable that the Zn effect is exerted through an increased synthesis of specific, metal-binding protein. Rather one could imagine an enhanced release of cadmium from the gills caused by the competitive binding of zinc to the particulate and HMW fraction. Indeed, gill zinc is very predominantly associated with the particulate fraction, independent of exposure condition. Within the cytosolic fraction virtually all zinc is retained in the HMW region (Figures 4, A and B).

It must be noted that metal concentrations of the medium, applied in this study, are more than one order of magnitude higher than those present in contaminated fresh waters. Indeed, the experimental levels in the medium are acutely toxic to most freshwater species. By contrast, molluscs are remarkably resistant to high levels of heavy metals in the surroundings. If, however, the protective action of zinc against Cd ingestion solely depends on the Zn/Cd ratio, and not so—or less—on absolute metal concentrations, it might be concluded that the observation of Zn antagonism has an ecologically significant worth. Definite proof of the latter awaits further experimentation under application of considerably lower metal concentration.

References

Ahsanullah M, Negilski DS, Mobley MC (1981) Toxicity of zinc, cadmium and copper to the shrimp *Callianassa australiensis*. III. Accumulation of metals. Mar Biol 64:311–316

Bryan GW, Hummerstone LG (1973) Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentrations of zinc and cadmium. J Mar Biol Assoc U.K. 53:839–857

Carpené E, George SG (1981) Absorption of cadmium by gills of *Mytilus edulis* (L.). Molec Physiol 1:23–34

Eisler R, Gardner GR (1973) Acute toxicology to an estuarine teleost of mixtures of cadmium, copper and zinc salts. J Fish Biol 5:131–142

Fowler SW, Benayoun G (1974) Experimental studies on cadmium flux through marine biota. In: Comparative studies of food and environmental contamination IAEA-SM 175/10. International Atomic Energy Agency, Vienna, pp 159–178

Hemelraad J, Holwerda DA, Zandee DI (1986a) Cadmium kinetics in freshwater clams. I. The pattern of cadmium accumulation in *Anodonta cygnea*. Arch Environ Contam Toxicol 15:1–7

Hemelraad J, Holwerda DA, Teerds KJ, Herwig HJ, Zandee DI (1986b) Cadmium kinetics in freshwater clams. II. A comparative study of cadmium uptake and cellular distribution in the Unionidae *Anodonta cygnea, Anodonta anatina* and *Unio pictorum*. Arch Environ Contam Toxicol 15:9–21

Jackim E, Morrison G, Steele R (1977) Effects of environmental

factors on radiocadmium uptake by four species of marine bivalves. Mar Biol 40:303–308

Lamphere DN, Dorn CR, Reddy CS, Meyer AW (1984) Reduced cadmium body burden in cadmium-exposed calves fed supplemental zinc. Environ Res 33:119–129

McLeese DW, Ray S (1984) Uptake and excretion of cadmium, CdEDTA and zinc by *Macoma balthica*. Bull Environ Contam Toxicol 32:85–92

Phillips DJH (1976) The common mussle *Mytilus edulis* as an indicator of pollultion by zinc, cadmium, lead and copper. I. Effects of environmental variables on uptake of metals. Mar Biol 38:59–69

Ray S, McLeese DW, Pezzack D (1979) Chelation and interelemental effects on the bioaccumulation of heavy metals by marine invertebrates. In: Proc Int Conf, Management and Control of Heavy Metals in the Environment. CEP Consultants, Edinburg, pp 35–38

Ray S, McLeese DW, Waiwood BA, Pezzack D (1980) Disposition of cadmium and zinc in *Pandalus montagui*. Arch Environ Contam Toxicol 9:675–681

RIWA: Rijncommissie Waterleidingbedrijven (1983) Annual report of the cooperating Rhine and Meuse Water Supply Companies in the Netherlands and Belgium, Amsterdam (in Dutch)

Webb M (1972) Protection by zinc against toxicity. Biochem Pharmacol 21:2767–2771

Manuscript received March 20, 1986 and in revised form June 23, 1986.