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Interaction of trace metals Zn, Cd, and Mn, with Ca concretions in the gills of freshwater unionid mussels

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The interaction of Zn, Mn, and Cd with Ca concretions in the gills of unionid mussels was investigated. Raising blood levels of these ions to twice their normal values for a week does not result in significant deposition of these metals into gill concretions. The interaction of Zn with the concretions was examined in more detail both *in vivo* and *in vitro*. Isolated concretions bind Zn as rapidly as Ca. However short-term *in vivo* labelling studies using radiolabelled Zn and Ca verify that Zn is not added to concretions while Ca is being rapidly accumulated. These studies demonstrate that calcium concretions from the gills of unionid mussels do not indiscriminately bind divalent cations, but precisely regulate the species of divalent cation incorporated. This is further evidenced by the lack of relationship between blood or pond water cation levels and the levels of specific cations found in the concretions. Since the concretions are mobilized during the reproductive period, and serve as a source of glochidial calcium, it would be disadvantageous for these concretions to bind divalent cations for detoxification purposes.

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L'interaction entre le Zn, le Mn et le Cd d'une part et les concrétions de Ca présentes sur les branchies des moules unionidés a fait l'objet d'une étude. L'augmentation des concentrations sanguines de ces ions jusqu'au double de leurs valeurs normales durant 1 semaine ne suffit pas à entraîner le dépôt de ces métaux sur les concrétions branchiales. L'interaction entre le Zn et les concrétions a été examinée plus en détail *in vivo* et *in vitro*. Des concrétions isolées se lient au Zn aussi rapidement qu'au Ca. Cependant, des études de marquage à court terme *in vivo* au moyen de Zn et de Ca radioactifs ont démontré que le Zn ne se dépose pas sur les concrétions lorsqu'il y a une accumulation rapide de Ca. Ces travaux démontrent que les concrétions de calcium sur les branchies des unionidés ne se lient pas indifféremment à tous les cations bivalents, mais font un contrôle spécifique des cations bivalents qui seront incorporés. Ce phénomène est d'ailleurs confirmé par l'absence de relation entre les concentrations de cations du sang ou de l'eau de l'étang et les concentrations des divers cations présents dans les concrétions. Ces concrétions sont mobilisées durant la période de reproduction et elles servent de source de calcium aux glochidies; il serait donc désavantageux que ces concrétions se lient à des cations bivalents, même pour assurer la désintoxication.

[Traduit par la revue]

Introduction

Some organisms are able to tolerate exposure to relatively high concentrations of toxic trace metals. Mechanisms of tolerance include exclusion of the metal entry into the organism, effective excretion of the toxic metal, and binding of the metal into insoluble complexes, which decreases the amount of metal circulating and interacting with sensitive enzyme and organ systems.

Perhaps the most extensively studied binding mechanism is the binding of divalent cations by the metallothionein class of proteins. These proteins contain sulfhydryl-based binding sites which have been shown to bind metals such as cadmium, copper, mercury, silver, and zinc. These proteins are often concentrated in particular organs, and sometimes are concentrated in metal-containing granules thought to be polymerized oxidation products developed from lysosomes (George et al. 1982; George 1983), most notably in molluscan kidneys. Another system that has been implicated in the binding of divalent toxic metals, without sulfhydryl binding sites (Howard et al. 1981), consists of inorganic, amorphous crystalline concretions assembled on organic matrices. Many of the invertebrate phyla are known to produce calcium concretions in one or more of their tissues (George 1982). In some organisms, these concretions have been shown to bind toxic metals, and the suggestion is that this binding is associated with a detoxification function (Simkiss 1981). The best examples of concretions thought to show detoxification binding are those produced by cells of the hepatopancreas of snails and other invertebrates (Simkiss 1981; Becker et al. 1974). These cells produce concretions intracellularly and have been shown, by elegant labelling experiments. to incorporate a number of divalent cations. Many of the concretions produced by these cells eventually appear in the lumen of the digestive tract, thus suggesting both a binding and an elimination of toxic metals through the digestive tract.

The calcium concretions in the gills of freshwater mussels have a similar structural and, to some extent, chemical composition to concretions found elsewhere in other invertebrates, including the hepatopancreas concretions mentioned above. These concretions are known to function as a storage site for calcium, and to mobilize this calcium specifically during reproduction in females (Silverman et al. 1985). The mobilized calcium serves as a source of calcium for the construction of the glochidial shell (H. Silverman, manuscript submitted for publication). Since calcium concretions in the gill are mobilized annually, their use as a detoxification storage site for metal ions would not appear to be particularly adaptive. Release of toxic cations stored throughout the year would increase circulating toxic material during the period of reproductive stress.

In spite of this logic, the similarity in structure and chemical makeup of gill concretions to those of other concretions suggests that they may also have the capacity to bind many different cationic metals. The chemical similarity to hepatopancreas concretions includes a phosphate crystal matrix built on an organic core (Howard et al. 1981). The organic core appears to include some specific calcium-binding proteins (H. Silverman,

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manuscript submitted for publication) which might be expected to show some binding with other divalent cations. The current study documents the divalent cations present in gill concretions in animals collected from unpolluted ponds. Additionally, exposure to high levels of Zn, Cd, and Mn demonstrates that the concretions do bind these cations, but that the specific ionic content of the blood must be prolonged and relatively high, compared with normal blood content, before any significant binding will occur. Further, single-label injection experiments demonstrate that during initial partitioning of a ^{65}Zn label, concretions do not specifically concentrate the Zn, but are loaded to a lesser extent than soft tissues, including gonad and mantle. We suggest that although some binding of these metals occurs *in vivo*, the data are not consistent with a primary detoxification function for the gill concretions.

Materials and methods

Animals

Anodonta grandis (200–400 g) and *Ligumia subrostrata* (30–70 g) were collected from ponds in the Amite and Tickfaw river flood plains near Baton Rouge, Louisiana. The animals were collected from sand or clay substrate at least 1 m from the bank of the pond. Animals were kept in the laboratory, in artificial pond water (0.5 m*M* NaCl, 0.05 m*M* KCl, 0.2 m*M* $NaHCO_3$, 0.4 m*M* $CaCl_2$) for 3–4 days before the initiation of any experiments. Pond water samples were collected from the sites of animal collection for ion analysis. *Anodonta grandis* and *L. subrostrata* both contain concretions which have the same reproductive function (H. Silverman, manuscript submitted for publication), antigenic similarity in the organic matrix (Steffens et al. 1985), and normal divalent cation content (Silverman et al. 1983, 1985). The smaller *L. subrostrata* are the better experimental species for *in vivo* labelling experiments, while the larger *A. grandis* are easier to use for the rest of the experimentation described. The smaller animals were used for *in vivo* radiolabelling experiments to reduce the overall amount of label necessary and to allow handling of smaller amounts of radiolabelled material. The larger *Anodonta grandis* were preferable for the rest of the experimentation, as each animal contains 0.5–1 g of concretion material as opposed to 50–100 mg found in *L. subrostrata*.

Gill concretion isolation

Gills were removed from the animal and homogenized in H_2O. The homogenate was boiled for 1 min to denature proteins. The homogenate was centrifuged (5 min, 4000 × *g*), and the pellet resuspended in 1 *N* NaOH and heated to 80°C for 1–2 h to digest soft tissue. The insoluble concretions were centrifuged and washed in distilled H_2O (3×) and recentrifuged to obtain a pure concretion pellet. Purity was checked using light microscopy, and has previously been checked using electron microscopy (Silverman et al. 1983). These concretions show little structural alteration following NaOH treatment.

To assure that minimal modification of the surface of the concretions was caused by the NaOH isolation procedure, all experiments performed in this study were repeated on concretions isolated on a discontinuous sucrose gradient. Briefly, the gill tissue was homogenized as described above, and the homogenate filtered through cheesecloth to remove tissue debris. The homogenate was then layered over 2.5 *M* sucrose and allowed to sit for 1–2 h. The concretions settle rapidly into the sucrose, while remaining tissue material does not. The concretions were pelleted by centrifugation (5 min, 4000 × *g*), washed free of sucrose (4×), and pelleted by a final centrifugation.

Atomic absorption spectrophotometry

For analysis of Zn, Cd, Hg, Mn, and Fe, 8–10 mg of dry concretion material was dissolved in 5 mL 1% HCl. Blood samples, taken by pericardial puncture before concretion isolation, were centrifuged to sediment particulates and mixed in equal volume with 1% HCl for analysis of divalent metal ions. Dilutions for measurement were made from these stocks where necessary. For Ca and Mg measurement, concretion and blood samples were dissolved in 5% HCl : 1% LaO_3. Water samples from the collection site were handled similarly to blood. All measurements were made on a Perkin–Elmer 5000 atomic absorption spectrophotometer.

Measurement of ionic content of the concretions and blood of individual animals was performed for each of the following experimental groups: (*i*) a control group bathed in artificial pond water; (*ii*) animals placed in 65 ppm Zn as $ZnSO_4$ for 1 week; (*iii*) animals placed in 50 ppm Cd as $CdCl_2$ for 3 days; (*iv*) animals placed in 100 ppm Mn as $MnCl_2$ for 1 week; (*v*) animals placed in 10 ppm Zn as $ZnSO_4$ for 1 month.

Measurement of the ion of interest was made on blood and concretions from at least three animals from each group. Blood values are expressed as millimoles/litre (m*M*) and concretion values as a percent dry weight of concretion material.

In vivo *labelling experiments*

Male *Ligumia subrostrata* were injected with 1.5×10^6 cpm (10 μL) ^{65}Zn or ^{45}Ca into the foot muscle. Eight hours after injection, blood was collected by cardiac puncture and analyzed for calcium and zinc content using atomic absorption spectrophotometry, and for radiolabelled zinc or calcium by scintillation spectrometry. The specific activity of the ^{65}Zn and ^{45}Ca circulating in the blood was calculated from the blood data; the specific activity of ^{65}Zn was 1000-fold higher than ^{45}Ca because of the relative content of unlabelled Ca (4 m*M*) and Zn (0.004 m*M*) in the blood. Small pieces of gonad, gill, kidney, and mantle were excised, washed, blotted, and dried to constant weight. Each sample (5 mg dry weight) was digested in concentrated HNO_3 (200 μL), and ^{45}Ca or ^{65}Zn in the sample was determined by scintillation counting. A large portion of one demibranch was excised, washed, blotted, and placed into hot (80°C) 1 *N* NaOH for several hours until the soft tissue of the gill had been digested. Purification and washing of concretion material was as described above. Concretions were dried to constant weight, and digested in HNO_3, as described above for tissue analysis. Label bound to concretion material was determined by scintillation counting. Quench curves were constructed for both Zn and Ca, and counts per minute were corrected for both background and quenching. Data are expressed as cpm/milligram dry weight of tissue or as cpm/millilitre of blood.

In vitro *binding studies*

Isolated concretion material (5 mg/mL) was incubated with $0.5–1.0 \times 10^5$ cpm/mL of ^{65}Zn or ^{45}Ca to determine the rate and amount of label incorporation with time. The experiments were done in solutions containing 0–5 m*M* $CaCl_2$, or 0–5 m*M* $ZnCl_2$, in distilled water or in clam blood (4 m*M* Ca). Analysis of label incorporation was by scintillation counting. Some of the samples containing millimolar quantities of Ca or Zn were analyzed by atomic absorption spectrophotometry to determine Ca or Zn ion loss from the incubation solution, and concomitant ion gain by the concretion material.

Energy dispersive X-ray microanalysis

The methods used for our X-ray microanalysis studies have been previously described (Silverman et al. 1983).

Results

Normal divalent cation content in concretions

The normal divalent cation content of concretions from the gills of *A. grandis* is shown in Table 1, and illustrated by energy dispersive X-ray microanalysis (Fig. 1). The major ions appearing by the X-ray microanalysis are Ca, Mn, and Fe. These metals make up most of the cationic content found using atomic absorption spectrophotometry, and are the only divalent cations contributing individually more than 0.5% of the total concretion weight. Mg and Zn are present naturally, but occur only as minor components of normal concretions. The ionic content of the pond water at the collection site (Table 1) indicates that Mg, Mn, and Ca are all present in the water in the range of 0.04–0.057 m*M*. Therefore, the concretions do not strictly mimic the divalent content of the freshwater environ-

TABLE 1. *Anodonta grandis* blood, pond water, and gill concretion divalent cation content (means ± SEM (*n*))

	Blood (m*M*)	Pond water (m*M*)	Concretion (% dry wt.)
Ca	4.4±0.3(3)	0.041±0.015(4)	21.3±2(6)
Mn	0.12±0.01(4)	0.057±0.020(4)	8.6±0.3(4)
Fe	0.03±0.01(3)	0.009±0.007(5)	0.5±0.0(4)
Mg	0.19±0.02(3)	0.047±0.008(5)	0.19±0.01(8)
Zn	0.005±0.000(11)	0.0009[a](3)	0.004±0.000(6)

[a]Value is at the limit of detection.

ment. The concretion's divalent ion content does not mimic blood ionic content either. Calcium content is highest in both blood and concretions, but blood magnesium is higher than blood manganese, and yet magnesium is only a minor component of normal concretions. Further, the manganese:calcium ratio is almost 10-fold greater in the concretions than the ratio of these metals in the blood. Zinc is a trace component of normal concretions; atomic absorption spectrophotometry was unable to demonstrate any Cd, Pb, or Hg.

In vivo versus in vitro loading of Cd, Zn, and Mn

Our attempt to load concretions with Cd, Zn, or Mn by elevating the concentration of these metals in artificial pond water had limited success. Since a major question is whether these concretions can bind metals in a detoxification role, mussels were placed in concentrations of metals which were found to more than double the blood concentrations of the metals after about a week of exposure (values were extrapolated from Eisler 1977). Cadmium was, as expected, quickly lethal at a concentration of 50 ppm. Of the nine animals in the treatment group, only four survived for 3 days. In the Zn group at 65 ppm, six of the nine animals survived for 1 week. The surviving animals were analyzed for blood and concretion content of Cd, Zn, and Mn. None of the Mn-treated animals were lost during the week of exposure. The results appear in Table 2 and, in all cases, there was a significant increase in ionic content in the blood. The concretions appeared to increase in trace ionic content, but in the case of Zn and Mn the increase is not significantly different from Zn or Mn content in concretions isolated from control animals. Further, the apparent increase in the Zn content of the concretions is accounted for by concretions isolated from only two of the six animals tested. The other four animals had concretion Zn content at control values. Although there is about 8% by weight Mn in the concretions as collected from normal animals, a significant increase in the amount of Mn in the blood did not result in a significant increase in the amount of Mn in the concretions on a dry weight basis (Table 2). Following a slightly different tack, administration of only 10 ppm Zn but over a 1-month period in artificial pond water caused a significant increase in the amount of Zn accumulated by concretions; 0.044 ± 0.03% dry wt. in controls versus 0.19 ± 0.02% dry wt. in the treated group.

These experiments lead to the question of whether the concretions bind Zn or Ca extracellularly by nonspecific adsorption, or by specific binding, or whether these ions must be added intracellularly. Although this question is not easy to answer without extensive experimentation (see George 1983), an attempt was made to show that Zn and Ca can be bound to isolated concretions *in vitro*. Concretions isolated by discontinuous sucrose gradient and by NaOH digestion of soft tissue were compared. Neither of these isolation methods degrades concretions morphologically (Silverman et al. 1983), and the results were compared to assess whether NaOH isolation caused a change in the concretion surface attraction to divalent cations. Both Zn and Ca bind to concretions rapidly as indicated in Fig. 2. However, the binding of Zn was not affected by external concentrations of either Zn or Ca. Calcium concentrations tested in the bathing medium included 0, 0.1, 0.4, 1, 2, and 5

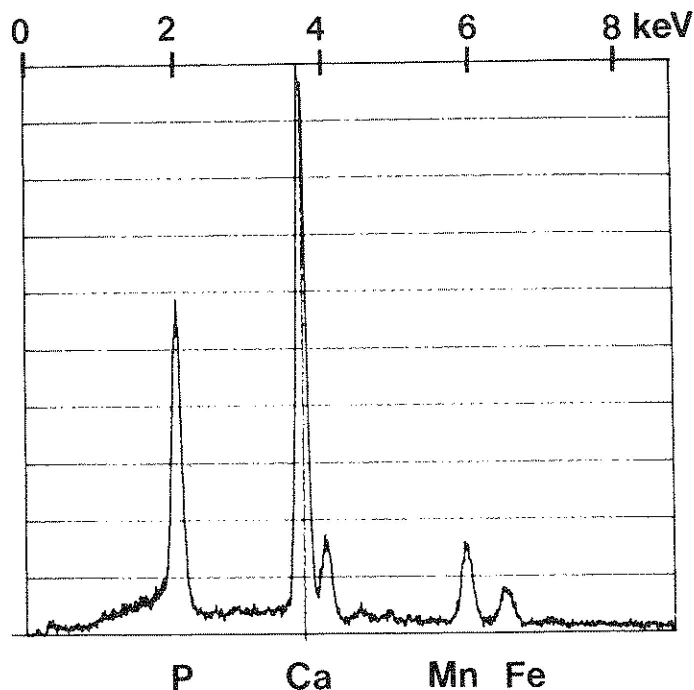

FIG. 1. Energy dispersive X-ray spectrum of a single gill calcium concretion from *Ligumia subrostrata*. Note that the major cation is calcium with phosphate as the anion. Manganese and iron are also detected. Only these components are found above 0.5% by weight in chemical determination of the divalent cations.

TABLE 2. Loading of heavy metals into the blood and gill concretions of *Anodonta grandis* exposed to elevated metals in artificial pond water (blood values are expressed as m*M* ± SEM (*n*), concretion values are expressed as % dry weight)

	Control	Metal-treated
Blood		
Zn[a]	0.004±0.0004(5)	0.013±0.002(5)*
Cd[b]	Undetected[c]	0.012±0.002(3)
Mn[d]	0.12±0.01(4)	0.28±0.05*
Concretion		
Zn	0.044±0.03(4)	0.085±0.02(6)
Cd	Undetected[c]	0.05±0.025(3)
Mn	8.65±0.25(4)	8.98±0.5(4)

[a]Zn present in pond water at 65 ppm Zn as $ZnSO_4$ for 1 week.
[b]Cd present in pond water at 50 ppm Cd as $CdCl_2$ for 1 week.
[c]Limits of Cd detection: blood, <0.001 m*M*; concretions, <0.004% Cd by weight.
[d]Mn present in pond water at 100 ppm Mn as $MnCl_2$ for 1 week.
*Significantly higher than control values, $p < 0.05$.

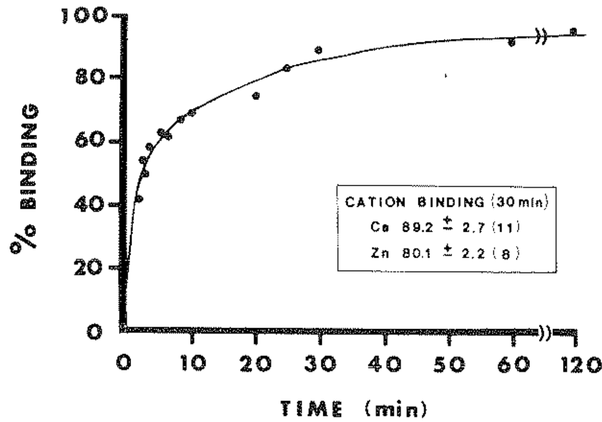

FIG. 2. Binding of ^{45}Ca by isolated concretions. One millilitre of 2 mM Ca containing 5×10^4 cpm ^{45}Ca was used as the incubation medium. The curve remains unchanged if the label is added to incubation media containing 0–5 mM Ca or 0–5 mM Zn. Half of the label is bound in 2 min. Zn binding is similar in kinetics although slightly less Zn is bound maximally, shown by comparison of Zn and Ca binding at 30 min (inset). Maximum binding of calcium, expressed as % bound, equals 485 ± 40 μmol/mg concretion (n = 10).

mM and Zn was tested at 0, 2, and 5 mM in the external bath. For each of the concentrations tested, ^{65}Zn and ^{45}Ca binding was equally rapid, and the curve of Ca binding shown in Fig. 2 for 2 mM Ca in the external bath is the same as the curve for ^{45}Ca binding in 5 mM Ca. No difference in divalent cation binding was seen between the two isolation groups. The same is also true for ^{65}Zn binding at the various external calcium concentrations tested. Zn attraction, therefore, is relatively nonspecific. Further, taking Ca binding as the example, binding in differing external calcium concentrations was similar regardless of ^{45}Ca specific activity. Both Zn and Ca binding capacity was reduced when the assay was done in complete mussel blood. Although exact binding constants for these cations have not been determined, these data show that the concretions can bind Ca and Zn *in vitro*, and that the binding capacity for both cations is about 500 μmol/mg. These results suggest nonspecific binding that is too rapid for us to determine exact binding constants under the experimental protocol described here. The concentrations of Ca tested were in the physiological range for *A. grandis*, and the decline at 10 mM suggests that a binding limit is reached at a concentration well above the normal physiological range seen in the blood of these animals. The data from the trace ion exposure *in vivo* suggest that divalent trace ion binding is limited and strictly regulated, since high blood levels of zinc do not cause rapid binding to the concretions. Further, little correlation was found among the Zn-treated animals relating blood load to Zn accumulation by concretions.

Further support for a selective *in vivo* regulatory mechanism preventing Zn binding comes from single-injection radiolabel studies. Injection of 1.5×10^6 cpm ^{65}Zn into animals was monitored to determine if ^{65}Zn would be accumulated by concretions, thereby removing it from circulating blood. These experiments were paralleled by similar injections of ^{45}Ca in separate animals. We have previously established that a Ca label will be concentrated into concretions within hours of injection (H. Silverman, manuscript submitted for publication). The label found in mantle, gonad, gill, and isolated concretions 8 h after injection is shown in Table 3. The data are presented as cpm/milligram dry tissue weight. The amount of ^{65}Zn label found in 1 mg of soft tissue for all of the tissues assayed is

TABLE 3. Uptake of ^{65}Zn and ^{45}Ca by *Ligumia subrostrata* tissues and concretions following a single injection of 1.5×10^6 cpm (values are expressed as cpm/mg dry weight ± SEM, n = 4)

	^{65}Zn	^{45}Ca
Gonad	5046±1020	1518±219
Mantle	3460±1146	1156±303
Kidney	2999±983	1942±619
Gill	4270±1331	1642±424
Gill concretions	1941±393	6938±2445
Ratio of cpm/mg isolated concretion: cpm/mg gill tissue	0.525±0.075	4.63±1.11*

*The ^{45}Ca ratio is significantly higher than the ^{65}Zn ratio, $p < 0.02$ (t-test, following arc sine transformation).

greater than that found per milligram dry weight of concretion. Note that the opposite result is seen for ^{45}Ca. In this case, concretions contain about 4 times the amount of ^{45}Ca label seen in the gill tissue. Since *L. subrostrata* gill contains 25% concretions by weight (Silverman et al. 1983), most of the calcium present in the gill is bound to the concretions. These results suggest that the ^{65}Zn label is not rapidly deposited into the concretions in a detoxification mode, but is nonspecifically added to the concretions in a distribution that is similar to or even less than its distribution in the soft tissues. This result is demonstrated by the ratio of cpm/milligram isolated gill concretion : cpm/milligram isolated gill tissue. For calcium the ratio is about 4, while for zinc this same ratio is 0.5. In contrast to ^{65}Zn, ^{45}Ca is rapidly accumulated into the concretions, and remains there for months (H. Silverman, manuscript submitted for publication).

Discussion

While it is postulated that some calcium concretions serve as detoxification systems, particularly those associated with the hepatopancreas in crustaceans and molluscs (Simkiss 1981; Howard et al. 1981), it would appear that such a mechanism would be disadvantageous in an organism that mobilizes the divalent cation pool of the concretions during the reproductive period. Some similar concretions from other molluscs have been evaluated biochemically to determine their ability to specifically bind metals such as cadmium (George 1983). Although these granules are not strictly concretions, but kidney tertiary lysosomes, the results of these studies suggest that any binding, as studied *in vitro*, appears to be by nonspecific adsorption. This would appear to differ from the high affinity binding sites for such metals found in the metallothionein proteins (see George et al. 1979).

In this study, we have demonstrated that *in vivo* gill concretions selectively bind calcium. Our results support a nonspecific adsorption phenomenon for Zn binding to freshwater mussel gill concretions. Zn binding *in vitro* was not strongly influenced by competitor cations nor by limited changes in Zn concentrations. Further, *in vivo* labelling studies indicate that Zn is not incorporated into concretions above circulating blood levels during a time period when calcium is being rapidly accumulated. The same result occurs even though there is a 1000-fold higher specific activity in ^{65}Zn than ^{45}Ca which should favor ^{65}Zn binding to the concretions. All of the

trace divalents added to the bathing medium caused a significant increase in blood levels, but only minor changes in concretion content.

Recent data clearly implicate the calcium concretions found in the gills of unionids as a selective calcium store to be released and used for glochidia shell formation during the reproductive period (Silverman et al. 1985, and manuscript submitted for publication). The evidence for this hypothesis is that the concretions are mobilized concomitant with reproduction; blood levels of calcium do not rise during this mobilization; ^{45}Ca is passed from maternal sources to glochidia in long-term labelling studies; and at least 93% of the calcium forming glochidial shells is of maternal origin.

Arguments suggesting the specific evolution of metal detoxification concretions having adaptive value cannot be related to the binding of the toxic metals themselves, since presumably the majority of organisms studied evolved in an environment where exposure to any significant concentration of toxic metal was minimal or nonexistent. In the case of the mussel gill concretions, obvious advantage accrues from the storage and passage of calcium to larvae developing in the gill water channel. The additional use of the concretions as a detoxification system would result in the liberation of all sequestered divalents at a critical time and would be disadvantageous to both the maternal animal and the developing larvae.

However, this system might be viewed as a model for the evolution of a detoxification function for other organisms. Those organisms not using concretions for the function which first conferred their advantage could use the available concretions as divalent cation "sinks" during exposure to toxic metals, as occurs in anthropogenically disturbed environments. Such logic is necessary to confer an adaptive detoxification function to calcified concretions in general.

However, generalization of function for calcium concretions may be an oversimplification based on the similarity in the morphology of the structures. Chemically, the concretions found in the invertebrates are remarkably diverse, some composed of calcium carbonate and others of calcium phosphate. Documented functions for such concretions include calcium storage during arthropod molting and metamorphosis (Grodowitz and Broce 1983; Sparkes and Greenaway 1984), heavy metal binding as a detoxification strategy (Simkiss 1981), calcium storage for reproductive purposes (Silverman et al. 1985; Fournie and Chetail 1984), and even the binding of excess calcium intracellularly (Becker et al. 1974). All of these functions appear to be well documented and can be attributed to concretion presence in diverse tissues. However, until detailed chemical analyses of both the inorganic and organic fractions of the concretions are presented, the superficial similarity of morphology should not be generalized to similarity of function. Indeed, Mason and Simkiss (1982) have suggested that the phosphate-based concretions show incorporation of metals and are perhaps primary detoxification systems as opposed to carbonate-based concretions.

In the case of the calcium concretions from the gills of freshwater mussels as studied here, the organic matrix of the concretions includes a calcium-binding protein much like calmodulin in molecular weight, antigenicity, and calcium-binding ability (H. Silverman, manscript submitted for publication). These concretions are based on a phosphate anion, and yet specificity for Ca is strong and appears to be important in the construction and function of gill calcium concretions. The relative invariance of manganese content in the concretions, even when blood levels of the ion are doubled, also demonstrates the regulation of divalent ion binding *in vivo*. The critical role of the concretions in female mussels is not consistent with a generalized divalent cation binding related solely to the blood content of the various divalents. Calcium always is the dominant cation, with manganese and iron also present. No experimental manipulation so far attempted that increases trace divalent concentrations in the blood alters this relationship over short time periods. On the other hand, if calcium levels in the blood are raised, the concretions will increase their calcium content; this has been demonstrated directly by monitoring calcium content during increases in blood calcium caused by experimental hypoxia (Silverman et al. 1983). As blood calcium rises, the amount of calcium bound to the concretions also rises. All of these points suggest the strong regulation of concretion ionic content *in vivo* in freshwater mussels.

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