THE EXTRACELLULAR MINERAL CONCRETIONS IN *ANODONTA CYGNEA* (L.): DIFFERENT TYPES AND MANGANESE EXPOSURE-CAUSED CHANGES

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ABSTRACT The mantle and gill extracellular mineral concretions of normal unpolluted and manganese-incubated specimens of the freshwater mussel, *Anodonta cygnea* (L.), were studied for their elementary composition and morphology. The mineral concretions of the gills, as well as those analyzed on the haemolymph side of the intrapallial epithelium, revealed a phosphate nature, being related to manganese accumulation. On the contrary, mineral formations located on the haemolymph side of the extrapallial epithelium are mainly built of carbonate salts, probably being more related to the shell biomineralization process than to the toxic metal detoxification. The concretions also differ markedly in their morphological aspects, with the phosphate formations being spherical, and the carbonate formations quite irregularly shaped.

KEY WORDS: biomineralization, detoxification, mussel, gill, mantle, concretion, manganese

INTRODUCTION

Many invertebrate phyla are known to produce calcium concretions in one or more of their tissues (George 1982). The freshwater bivalve, *Anodonta cygnea*, has been reported to use this insoluble store of calcium for shell deposition (Istin and Girard 1970a).

The extracellular concretions of *Anodonta* and other related species have been described in the mantle (Pekkarinen and Valovirta 1997) and gill (Silverman et al. 1983, Silverman et al. 1985, Silverman et al. 1987a). The composition and structure of these concretions has been studied in *Ligumia subrostrata* (Silverman et al. 1983), *Margaritifera margaritifera* (Pekkarinen and Valovirta 1997), and *Anodonta* sp. (Pynnönen et al. 1987), through histochemistry, electron microscopy, and chemical analysis.

We now report the results of an annual monitoring of these extracellular concretions in normal specimens of the freshwater mussel, *Anodonta cygnea*, with respect to their morphology and mineral composition. Both concretions from the mantle and gill epithelia were examined, as well as the alteration of their normal characteristics after manganese exposure, to establish a connection between these microstructures and their possible physiological roles, namely those related to shell production and heavy metal detoxification.

MATERIALS AND METHODS

Lake mussels, belonging to the species *Anodonta cygnea* (L.), were collected through dragging from the clay bottom of the Mira lagoon (Aveiro, Portugal). The length of the animals treated varied from 12.0 to 18.0 cm, with a mean of 15.0 cm.

Six specimens were collected each month, were transported to the Institute laboratory facilities in iceboxes containing natural pond water; kept in the laboratory in aerated pond water without feeding; and processed within 24 h of collection. The animals were considered healthy if they showed active ventilation, powerful valve closing or water ejection upon disturbance, and if their nacre presented a smooth and shiny look.

For the manganese-exposure assay, 12 specimens of about 200-g body weight, collected in the same way during March, were randomly assigned to two treatment categories, control and heavy metal exposure, and kept in plastic tanks with naturally collected water at a temperature similar to natural conditions. The tanks were placed in relative darkness and the water was aerated and used without any pretreatment.

For the heavy metal treatment, manganese, as $MnCl_2$, was added to the tank water to reach a final concentration of around 10^{-6} M (between 1 and 2 ppm) from a stock solution previously prepared with distilled water. Manganese was the heavy metal chosen because of the well-known relationship with the mineral structures (Silverman 1989). The exposure period lasted for 16 h, during which the mussels showed normal ventilation. After exposure, both manganese-exposed and control animals were subjected to the same treatment as the specimens collected each month.

Gill and mantle fragments were extracted by cutting the mussel adductor muscles, opening the shell, and cutting the middle region of the tissue free from the body. After dissection, small (3–4 mm) mantle pieces were separated into their two composing epithelia, i.e., the one facing the pallial cavity (intrapallial epithelium) and the other facing the shell side (extrapallial epithelium). These fragments were then placed in aluminum cylinders with their haemolymph-bathed side up (showing the basal face of both epithelia), and left to dry at room temperature.

The composition of concretions from both mantle epithelia, on the haemolymph side, was analyzed on the assembled material by energy dispersive X-ray spectroscopy (EDS), with a spectrum collection time of 60 seconds. The elements chosen for analysis were Ca, P, C, Cl, Na, Mg, and Mn, from pilot determinations. Gill tissue fragments. on their haemolymph-bathed side and equally assembled, were also analyzed with this technique, to establish

eventual differences between the concretion populations from both organs. The morphology of the different mineral concretions, determined by their elemental composition, was then studied by scanning electron microscopy (SEM), also using the dried tissue samples without any other treatment.

RESULTS

Results of the mineral concretion composition reveal no major variations, either between individuals or throughout the year, the only significant differences being those between tissue-type analyzed, and those exposed to manganese. Therefore, comments will focus on these elements, using the EDS records of each group of animals (i.e., normal and metal-exposed) and tissues (i.e., gill, extrapallial and intrapallial epithelia).

Using the March records, which also correspond to the control group for the contamination study, we find high amounts of phosphate-like concretions on both the gill (see Fig. 1) and the intrapallial epithelium, on its haemolymph side (Fig. 2). The major phosphate composition of these mineral formations is demonstrated by their higher content of phosphorus, compared to carbon—which is probably more related to the surrounding tissue present—as shown by the record of the intrapallial epithelium itself (Fig. 4). In these phosphate concretions, and throughout the whole year, there seems to be a good correlation with the manganese level, suggesting a constant relationship between them.

On the other hand, at the haemolymph side of the shell-facing extrapallial epithelium, the mineral concretions reveal a carbonate nature (Fig. 3), although with some phosphate, which is usually rather low (residual). This second group of concretions does not show the same tight relationship with the manganese composition, with a lower level being the general rule.

The two kinds of mineral concretions, now detected also show morphological differences (Fig. 5), with the carbonate concretions (3) being usually larger and more irregular. The phosphate formations (1,2) are present in higher amounts and show a regular spherulitic morphology, although with important size variation. They are also tightly covered by organic material presenting a net-like structure, in which they seem to be formed. Both concretion types are well attached to the respective epithelium, always giving the same distribution results when both tissues are separated from each other (see Fig. 6 for better a understanding of the overall arrangement).

These general features were not significantly changed in the manganese-incubated specimens. These results show that the manganese is preferentially bound by the phosphate-bearing granules, being increased only in those formations in response to the contamination situation, compared to the normal control animals. The association between manganese and phosphate minerals is also supported by our observations on the carbonate-composed concretions, where higher amounts of this metal were associated with the samples with the highest level of residual phosphate.

DISCUSSION

Anodonta cygnea uses calcium concretions as calcium stores for shell growth (Istin and Girard 1970b), associated with anhydrase activity as a regulatory feature (Istin and Girard 1970a). Concretions are reported both in the gills (Silverman et al. 1983, Silverman et al. 1985, Silverman et al., 1987a) and in the mantle (Pekkarinen and Valovirta 1997) connective tissues of this and other related (i.e., freshwater) species.
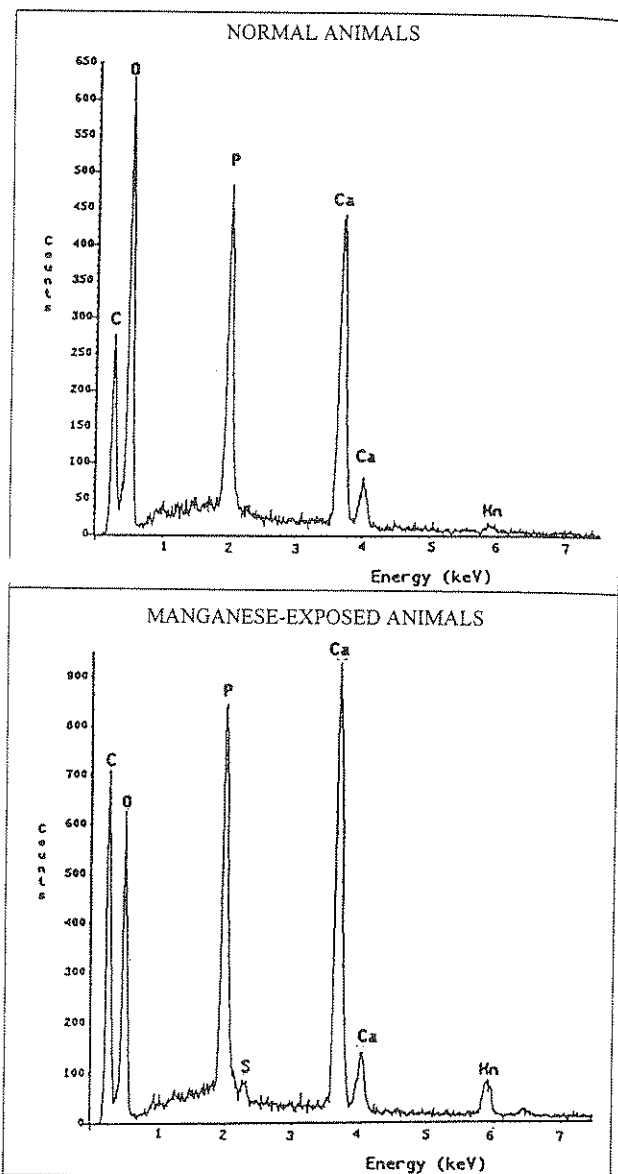

Figure 1. EDS records from analyses of mineral concretions in the gills of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).

In the unionid, the calcium of gill concretions is bound to inorganic or organic phosphate (Silverman et al. 1983, Silverman et al. 1987b; Pynnönen et al. 1987, Lautié et al. 1988) and is associated with an organic matrix (Silverman et al. 1983; Silverman et al. 1987b). According to Silverman et al. (1983), 25% of the concretion weight is organic, calcium makes up 25%, and phosphate represents 36%–39%. These extracellular concretions are composed of phosphorus, calcium, manganese, and iron, with smaller amounts of Mg, Al, S, Cl, Zn, and Ba (Lautié et al. 1988). No carbonate can be detected in these concretions by Raman analysis (Lautié et al. 1988).

These features resemble those reported here for the gill and intrapallial epithelium, except for the trace element composition, which were not studied in the present work. The phosphate concretions we studied are probably identical to the ones reported by Lautié et al. (1988) and, therefore, the carbon amount de-
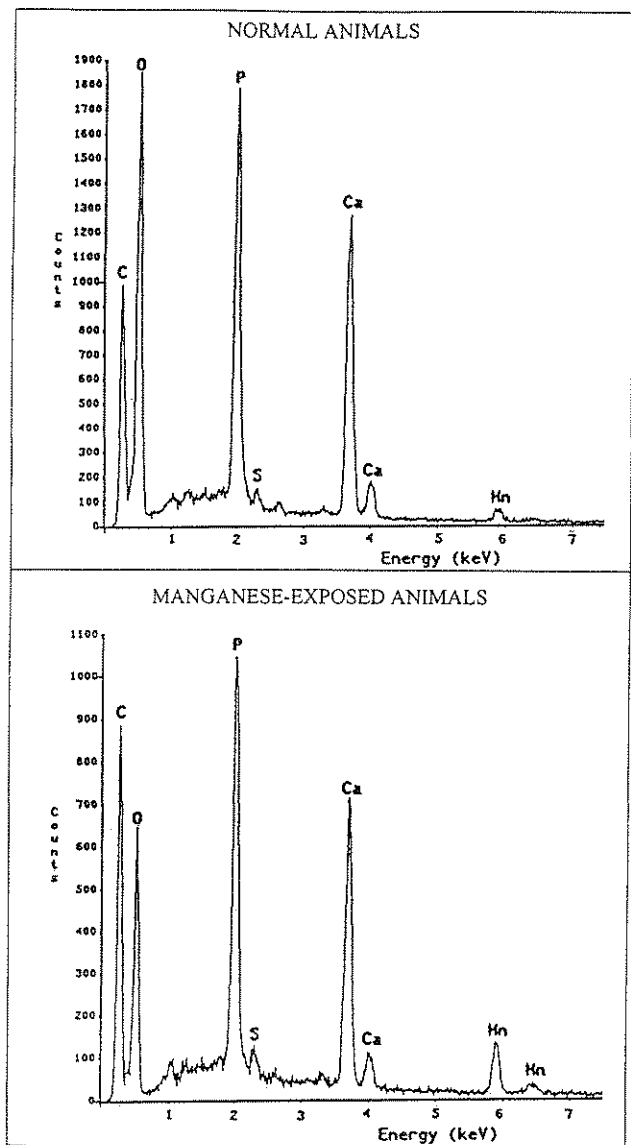
Figure 2. EDS records from analyses of mineral concretions on the intrapallial epithelium of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).
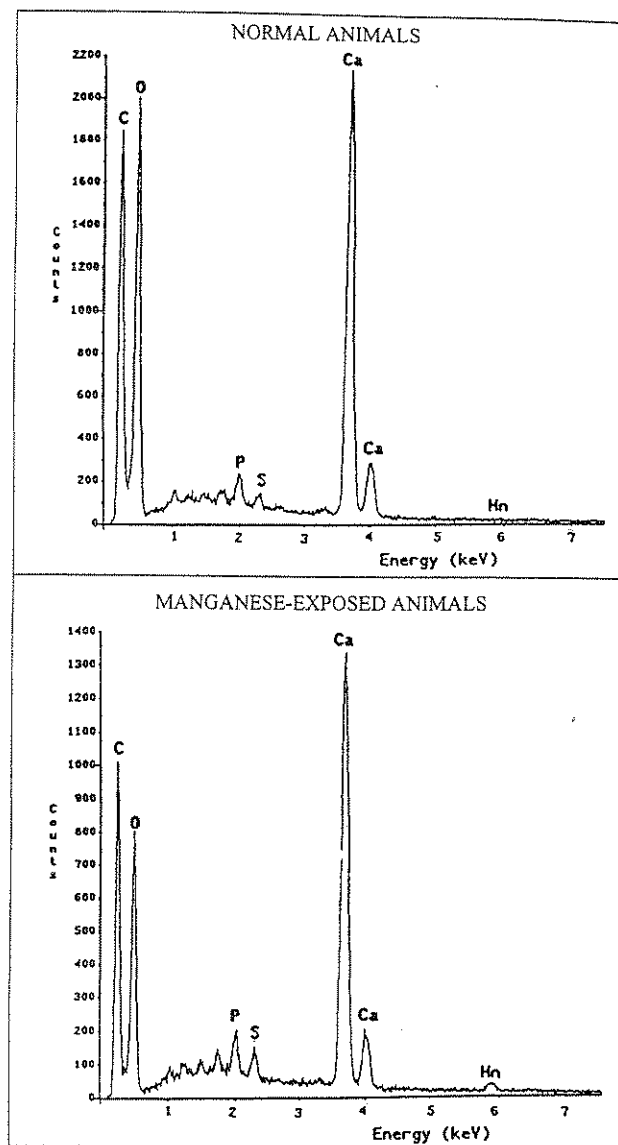

Figure 3. EDS records from the analyses of mineral concretions on the extrapallial epithelium of *Anodonta cygnea* specimens collected in March, from the natural environment (above) or after manganese-contamination (below).

tected could be related to the organic material present. The calcium/phosphate proportions are, in fact, parallel to the data now presented.

The phosphate spherules react positively to PAS following amylase reaction, suggesting the presence of polysaccharides (Silverman et al. 1983). However, although being partially composed of polysaccharides, the organic core of the concretions described by these authors does not appear to have sulfate groups, in contrast to the results of Davis et al. (1982), who found sulfur with X-ray microanalysis.

The negative reaction for the carboxyl and sulfhydryl radicals of the organic matter from concretions has been explained by the tightly bound metal amounts, which can keep the active groups from reacting (Lautié et al. 1988). Alternatively, these contradictory results can be explained by the presence of two kinds of

mineral concretion differently located, and separated from each other, one having sulfated organic material and the other being poorer in that respect. This is, in fact, what we found in both epithelia of *Anodonta cygnea* mantle.

The extracellular spherocrystals of the mantle are usually shown to be composed of calcium carbonate (Istin and Girard 1970b), a conclusion taken after studying the action of the pH, CO_2 partial pressure, and carbonic anhydrase activity in the calcium movements, and the location of Ca and this enzyme in the concretions. Alternatively, Pekkarinen and Valovirta (1997) and others reported the presence, in this same tissue, of calcium phosphate concretions in both *Anodonta* and *Margaritifera*, similar to those found in the gills of the same animals. The present work demonstrates, for the first time to our knowledge, that both types of concretion composition are present simultaneously in the mantle
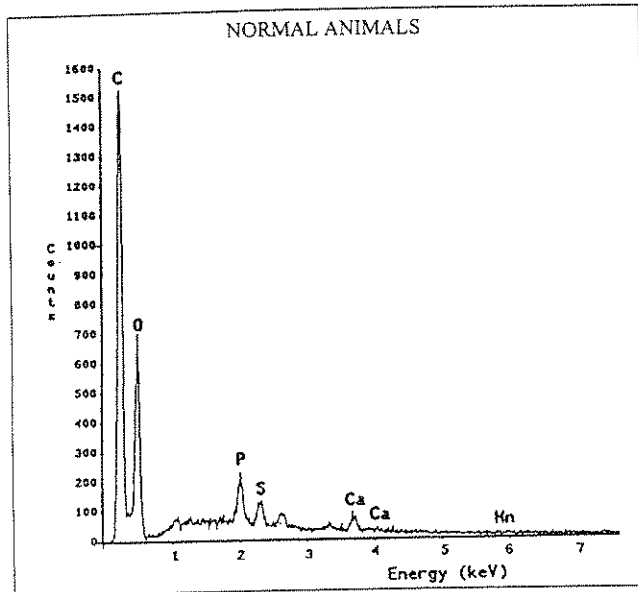
Figure 4. EDS records from the analyses of the intrapallial epithelium from normal *Anodonta cygnea* specimens collected in March.

Figure 6. Schematic representation showing the localization of the different pallial concretion types of *Anodonta cygnea*. (1) external medium; (2) intrapallial epithelium; (3) haemolymph compartment; (4) extrapallial epithelium; (5) extrapallial compartment; (6) shell; CF = spherical calcium phosphate concretions; CC = irregularly shaped calcium carbonate concretions.

of *Anodonta cygnea,* which explains these contradicting results, and confirms the hypothesis proposed by Pekkarinen and Valovirta (1997).

On the other hand, lamellar concretions have been described singly or clustered (Silverman et al. 1983), both in muscle (Kapur and Gibson, 1968) and mantle (Davis et al. 1982) tissues; they are related to the biomineralization process of the shell, because they disappear during shell regeneration (Watabe et al. 1976).

The morphological characteristics reported for mantle concretions can also be explained by the presence of both kinds of mineral formations. We found spherulitic concretions together with more lamellar-shaped formations, both described in an isolated fashion by these authors. Perhaps each different kind show, in fact, some variation in their amounts inside the mantle (as those re-
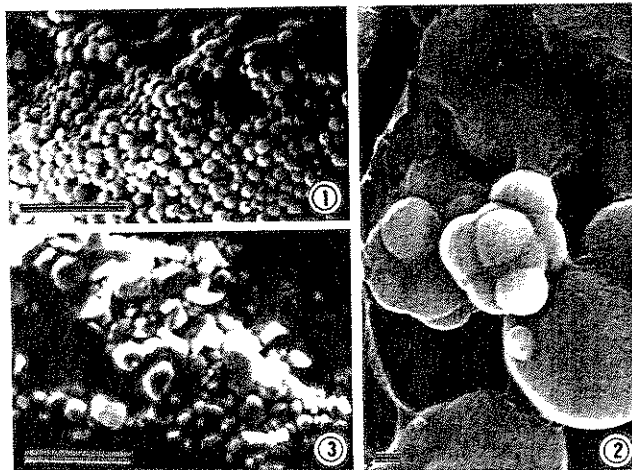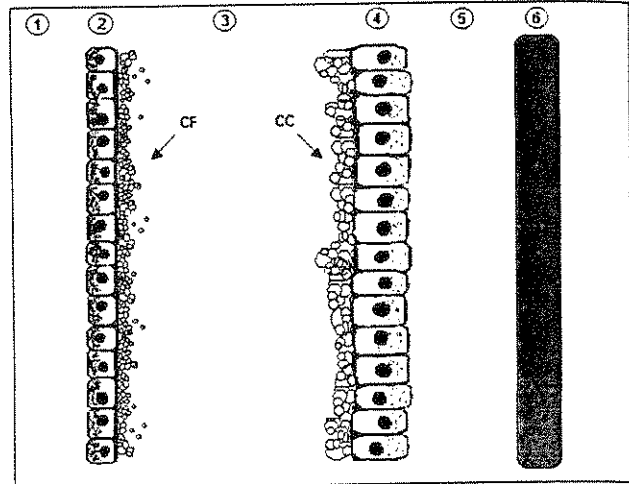

Figure 5. SEM photographs showing the morphology of *Anodonta cygnea* mineral concretions. (1) Spherical concretions, located on the surface of the intrapallial epithelium, on its haemolymph side. Bar 10 μm. (2) Higher magnification of the structures seen in (1). Bar 1 μm. (3) Irregular-shaped concretions, located on the surface of the extrapallial epithelium, on its haemolymph side. Bar 10 μm.

ported for the shell regenerating specimens, with respect to the lamellar formations), which can explain why both types were not found by each worker mentioned. Such a fluctuation is not detected in our results, where, although with some variations, both types coexist throughout the year.

The hypothetical functions of these mineral concretions have been studied by several authors (e.g., Istin and Girard 1970a). Concretions may be involved with calcium storage for shell regeneration or normal growth (Vaidya and Nagabhushanam 1980), pH buffering during metabolic and/or respiratory acidosis (Istin and Girard 1970b), and detoxification of heavy metals (Mason and Simkiss 1982).

During acidosis, bivalves dissolve their calcium reserves to buffer the pH (as reviewed by Burton 1983), causing a significant increase of the circulating levels of calcium (e.g., Pynnönen, 1990a, Byrne and McMahon 1991, Pekkarinen and Suoranta, 1995), carbonates and P_{CO_2} (Burton 1983, Pynnönen 1994), while the internal pH stays only slightly decreased (Collip 1921).

The excessive calcium arising from this situation, on the other hand, can partially leak to the external mantle cavity (Pekkarinen and Suoranta 1995) and be lost to the environment, or be again absorbed after the acidic stress period. The calcium remaining in the biological fluids can be incorporated into the shell (Pekkarinen and Suoranta 1995), in the same way as the one seasonally liberated by the succinic acid formed in the summer (Machado et al. 1990).

The reported high permeability of the mantle to calcium ions (Coimbra et al. 1988) will allow this excess calcium to be rapidly transported to the mineralizing front, causing the fast formation of a calcified pellicle on the inner side of the shell (Machado et al. 1988).

A different model is proposed by other authors, in which the pH is buffered with the dissolution of calcium carbonate from the shell itself (Sorokina and Zelenskaya 1967). However, their studies dealt mainly with marine species that present much smaller amounts of calcium microspherules (Machado et al. 1988). In addition, freshwater organisms do not have an abundant supply of

calcium and, therefore, try to avoid any loss. During a metabolic hypoxia, for example, the animal needs a mechanism to retain the calcium liberated from dissolved minerals. Accordingly, Machado et al. (1988) propose significant differences between marine and freshwater mollusks with respect to the origin of calcium to be dissolved for pH buffering purposes.

The calcium carbonate of the shell would be more susceptible to leaching by acidic metabolites than the calcium phosphate of the concretions (Howard et al. 1981), because it is more soluble. This agrees with the fact that these concretions do not seem to be implicated with pH buffering during anoxia in these animals (Silverman et al. 1983), thus different bivalves may have different calcium reserves to be used in this mechanism (Pynnönen et al. 1987).

Keeping *Anodonta cygnea* in acidic water leads to further shell growth, demonstrated by the formation of a calcified new pellicle on the inner surface of the shell (Machado et al. 1988). Therefore, it was suggested that calcium may come from the phosphate concretions (Pekkarinen and Suoranta, 1995), at least in this species.

In other works, however, calcium concretions were not mobilized under the most severe hypoxia or acidic conditions (Silverman et al. 1983, Pynnönen, 1990a, 1990b). On the contrary, the increase of calcium in the blood that follows hypoxia was accompanied by an increase of the concretions level, showing that the concretions in the gill of these mussels were gaining calcium from the circulating fluid during a time of increased blood calcium, which would serve to avoid calcium loss to the environment following its liberation from the shell, as a result of hypoxia (Silverman et al. 1983).

Radiolabeled studies also showed that the source of calcium for the elevated haemolymph levels seems to be the shell (Crenshaw and Neff, 1969). In the freshwater bivalve, *Ligumia subrostrata*, the calcium released from the shell during hypoxia (McMahon, 1979; Silverman et al. 1983) is deposited into calcium phosphate concretions in the gill tissue (Silverman et al. 1983).

Finally, these contradicting results lead to the hypothesis that a fraction of the calcium stored in the concretions is associated with carbonate, being more easily solubilized than calcium phosphate (Pekkarinen and Valovirta, 1997).

All previous results, together with the present observations of gill and mantle mineral concretions in *Anodonta cygnea*, can be explained if we consider this dual nature of the mantle mineral formations. In fact, the phosphate concretions are most probably related to the other possible functions usually assigned to these structures, namely the metal sequestering and detoxification processes, as suggested by the higher incorporation of manganese reported here and in other works (Silverman et al. 1987b). This detoxification mechanism seems to be more important in freshwater species, because this environment is subjected to higher heavy metal fluctuations, according to Mersch et al. (1996). As explained by this author, this type of concretion probably evolved as a form of retaining the calcium that would otherwise be lost during acidosis. Its lower solubility and higher calcium binding ability (Silverman et al. 1987b) allowed for its later use as a detoxifying agent, in view of the higher binding of metal, as calcium analogues (Silverman et al. 1987b). The phosphate granules are also closely related to the reproduction cycle, being rapidly mobilized for the glochidia shell formation (Silverman et al. 1985), in what seems to constitute its primer function (Silverman et al. 1985; Silverman et al. 1987b), an idea also confirmed by the fact that these are the only concretions found in the gills of unionid bivalves, their marsupial organs.

On the other hand, there are the carbonate lamellar granules, formed only by the extrapallial mantle epithelium, directly related to the inner shell growth and bearing some resemblance in structure and composition. These formations can, in turn, be related to the deposition cycling of the nacre formations, a situation suggested by Mason and Simkiss (1982). As calcium carbonate entities, these formations are more readily dissolved in response to metabolic or respiratory acidosis than the phosphate ones. Under the normal seasonal internal pH decrease postulated by Machado et al. (1988), these concretions would dissolve before the shell itself, in view of their particular spatial localization, partially sparing the nacre from this dissolution event. The increased calcium, following this dissolution, would then cross the extrapallial fluid, fulfilling the mechanism proposed by Machado et al. (1988).

This, however, in view of the small amounts of carbonate concretions, would be a limited event, particularly if the acidosis situations, like those experimentally caused, were strong and prolonged. Under these conditions, the shell would be the next candidate for the acid action, being dissolved as commonly reported.

ACKNOWLEDGMENTS

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