Silverman et al 1983

THE JOURNAL OF EXPERIMENTAL ZOOLOGY 227:177–189 (1983)

Calcium Concretions in the Gills of a Freshwater Mussel Serve as a Calcium Reservoir During Periods of Hypoxia

H. SILVERMAN, W.L. STEFFENS, AND T.H. DIETZ
Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803

ABSTRACT The gill of the freshwater mussel, *Ligumia subrostrata*, is composed of 25% calcium concretions on a dry weight basis. These concretions are located extracellularly in the connective tissue of the gill. The concretions have a lamellar structure and occur singly, multiply, and in clumps. The concretions are composed primarily of calcium phosphate and an organic matrix. The concretions are more numerous toward the base of the gill and less frequent in the tip of the gill. The average size of the concretions is 2–3 μm. The concretions are not solubilized in animals exposed to prolonged hypoxic conditions. Instead, as calcium increases in the blood under hypoxic conditions the percentage of the gill dry weight attributed to concretion material increases and the calcium content of the concretion material is elevated. The calcium content of the concretions also is inversely correlated with blood pH. This study indicated that the concretion material in the gills was not mobilized under hypoxic conditions, but served as a calcium reservoir during periods in which the animal was liberating calcium from other sites.

Calcium concretions are found in the body tissues of freshwater bivalves and gastropods. The lamellar concretions are usually found extracellularly within connective tissue in muscle (Kapur and Gibson, '68) and mantle (e.g., Davis et al., '82; Petit et al., '80). Similar appearing concretions also are seen intracellularly in the basophilic calcium cells of the hepatopancreas where they are presumably formed (Abolins-Krogis, '58, '61, '63a,b, '68; Simkiss, '82; Mason and Simkiss, '82; Howard et al., '81). Since the concretions contain calcium and are common in mantle tissue, a role in shell calcification has been suggested for these mineral spherites (Wagge, '51; Abolins-Krogis, '68; Watabe et al., '76; Davis et al., '82). In support of this hypothesis certain tissues (particularly the hepatopancreas) show an apparent increase in the number of spherites present following shell damage and during subsequent repair of the shell (Abolins-Krogis, '73). These studies show a relationship between what appears to be concretion formation in the hepatopancreas and abnormally high shell deposition in freshwater molluscs during periods of shell repair. In further support of the above hypothesis, Abolins-Krogis ('73) suggests that wandering amoebocytes may transport concretions to the mantle after the concretions are formed. However, Marsh et al. ('81) note that no amoebocytes can be found above mantle tissue unless the mantle has been damaged during experimental manipulation of the shell.

Yet another hypothesis (Istin and Girard, '70) for the role of concretions suggests that the calcium in these spherites can be mobilized under hypoxic conditions much as the shell of these organisms is often resorbed under similar conditions. In this report we note the extensive distribution of calcium concretions in gill tissue of the Unionid freshwater mussel *Ligumia subrostrata*, yet the gill is quite removed from the area of shell deposition. We report the results of morphological and histochemical analyses of the calcium concretions found in the gill tissue. We observe that calcium concretions of the gills are not mobilized under the most severe hypoxia; in accord with recent chemical evidence suggesting calcium in the concretions of the hepatopancreas is mainly bound to a pyrophosphate complex and not to either or-

Address correspondence to Dr. H. Silverman.

thophosphate or carbonate (Howard et al., '81). Instead, we report the concretions serve as a calcium phosphate reservoir which is capable of increasing its calcium content under anoxic conditions. The increase in calcium content in the concretions is concomitant with increasing levels of calcium in mussel blood and correlated with the decreased pH of the blood during the anoxic period. It appears that this freshwater mussel has a mechanism for conserving calcium which might otherwise be lost to the environment following its liberation from the shell as a result of hypoxia.

MATERIALS AND METHODS

Male *Ligumia subrostrata* collected locally (Baton Rouge, La) were used in all experiments. Mussels (25 to 70g) were acclimated to artificial pond water (0.5 mM NaCl, 0.4 mM $CaCl_2$, 0.2 mM $NaHCO_3$, 0.05 mM KCl at 22–25°C) in the laboratory for at least a week, and all experiments were initiated within six weeks of collection.

Histochemistry

Gills were prepared for histochemistry by cutting the mussel adductor muscles, opening the shell, and cutting the gills free from the body at their basal surface.The gill pair on each side of the mussel was treated as a unit. Each unit was rapidly coated with O.C.T. Compound (Ames Co., Elkhart, IN), oriented, and frozen in liquid nitrogen. The gills were allowed to warm to −20°C in a cryostat (American Optical) and sectioned at a thickness of 12 μm.

The histochemical method employed to detect calcium in the gill tissue was the alizarin red S reaction following the methods of Dahl ('52) without employing counter stain. Alizarin red S reactions also were performed after pretreatment of gill sections with either 10 mM EGTA (pH 8.5), 0.1 M succinic acid (pH 2), or potassium acetate buffer (pH 4.4) for 1 h in an attempt to remove calcium from the tissue. Following the pretreatments, the sections were washed 5–6 times in distilled water to remove the preincubation fluid prior to the alizarin red S reaction.

Oxidizable polysaccharides in gill tissue were demonstrated using a modification of the silver methenamine method of Martino and Zamboni ('67) without gold substitution, and by the periodic acid-Schiff reaction of McManus ('46) substituting Carnoy for formalin fixation. The PAS reaction was run before and after a 1-h pretreatment in amylase to remove glycogen, and also following EGTA pretreatment. As a tissue control, all the glycogen in the muscle fibers of the mussel foot was removed using the amylase procedure, suggesting that PAS staining cannot be attributed to glycogen.

Some sections were analysed for Ca-ATPase following the methods of Padykula and Herman ('55) with and without ATP as a substrate. For general orientation purposes we also stained some sections with 1% toluidine blue and Paragon.

Estimates of the size range of the calcium concretions were made using a Wild light microscope and an ocular micrometer.

Isolation of the calcium concretions

Two isolation procedures were used to isolate a pure fraction of calcium concretions from the gill tissue for chemical analysis. The first involved homogenizing gill tissue with an Ultrasonics tissue homogenizer, heating the homogenate (100°C for 2 min) to denature protein, and incubating the mixture in 1 N NaOH at 60–70°C for an hour. After several washes in NaOH followed by distilled water, calcium concretions were centrifuged into a pellet (5 min spin at 4,000g). The second method of purifying the calcium concretion fraction from gill tissue involved a differential centrifugation in a discontinuous sucrose gradient. The tissue was homogenized in distilled water layered over a 2.5 M sucrose layer, and the calcium concretions were collected by centrifugation (20 min at 2500g). The fractions used for X-ray microanalysis were homogenized using a ground glass homogenizer and were not allowed to contact any metal instruments. The purity of the calcium concretion pellets isolated was checked directly using electron microscopy. Some of the concretion material was dehydrated in an ethanol series and embedded in L.R. White resin for ultrathin sectioning. Another portion was similarly dehydrated, mounted on copper troughs, and sputter coated with 10 nm Au-Pd for examination using scanning electron microscopy. Some of the material was also placed on carbon-coated parlodion films for observation of whole concretions using transmission electron microscopy. All electron microscopy was done on concretions immediately after isolation and also after treatment with 10 mM EGTA to remove calcium from the concretions.

In some mussels the mantle was isolated and a calcium concretion fraction was pre-

pared using the same techniques applied to the gill pairs.

Chemical analysis of the concretion fraction

The concretions were analyzed for calcium by atomic absorption spectrophotometry (Perkin-Elmer) using methods previously described (Murphy and Dietz, '76). Concretion bicarbonate was estimated using manometry to measure the liberation of CO_2 from the calcium concretions by dilute sulfuric acid (Dietz and Branton, '75; Potts, '54). Concretion phosphate was extracted by 10% TCA and quantitated by molybdate colorimetry (Huxtable and Bressler, '73). Blood from mussels in which the gill concretions were isolated was also analyzed for osmolarity, Ca content, pH, phosphate, and bicarbonate. Concretions also were ashed at 450°C to determine the percentage of the concretion material attributed to volatile organic matter.

Production of an hypoxic environment

Conditions of hypoxia were produced in two independent ways. One method consisted of placing mussels in a bell jar continuously gassed with water saturated N_2 creating a slight positive pressure. The mussels were placed on a support above the water in the bell jar. Under these conditions the mussels were not permitted to excrete metabolic byproducts into water. This method of hypoxia produced considerable stress as demonstrated by the change in blood variables measured and by the attendent mortality of mussels between days 3 and 6 of the study.

Hypoxic conditions were also established with the mussels in N_2 saturated artificial pond water. Single mussels were placed in quart jars sealed with rubber stoppers. The artificial pond water in each jar was changed once each 24-h period to prevent the accumulation of metabolic waste products. The replacement pond water was gassed with N_2 for 20 min before it was used to replace pond water in the jars. Winkler O_2 titration indicated that this treatment of pond water leaves less than 1% saturation with oxygen. Mussels remained under these hypoxic conditions between 2–4 weeks.

Electron microscopy of gill sections

Gills from both control and hypoxic (in water) treatment groups were prepared for electron microscopy by excising the gill pair and fixing the whole outer gill filament in 0.02 M cacodylate buffered 2.5% glutaraldehyde for 20 min. The gills were then cut into 5-mm wedges which identified the orientation of the tissue and fixed an additional 1–2 h. The tissue soaked overnight in 0.02M cacodylate buffer and was postfixed for 1 h in 1% OsO_4. The tissue was dehydrated in either an acetone or ethanol series and embedded in either Spurr or L.R. White resin. Ultrathin sections were taken and contrasted with lead citrate and with saturated uranyl acetate (50% ethanolic). The sections were viewed with a JEOL 100CX operating at 80 keV.

X-ray microanalysis

Concretions isolated on sucrose gradients were transferred to distilled H_2O, then air-dried. The resultant pellet of purified concretions, as determined previously, was attached (using drawn glass probes for handling) to a spectroscopically pure carbon SEM stub with Aqua Dag (Fullam). After verification of sample purity by SEM, elemental composition was determined subsequently on the same sample using energy dispersive X-ray spectroscopy (EDS). A Hitachi HS-450 equipped with an Edax unit was used for all work. Spectrum collection time was 25 sec.

RESULTS

Morphology of the calcium concretions in the gill

Calcium concretions are located throughout the connective tissue of the gill. They appear singly, as multiples, and often clumped together as clusters. In the loose connective tissue forming the core of the gill bars (Fig. 1), the concretions exist as isolated single or doublet concretions. In the denser connective tissue toward the base of the gills the concretions are numerous and exist as clusters (Figs. 1,2), which can virtually fill several hundred micrometers of basal connective tissue. They predominate particularly in the region of the gill connective tissue lying adjacent to the kidney tubule, and surrounding the branchial nerves.

The concretions range in size from tenths of microns to over 100 μm, but the average size of the concretions in gill material is between 1–3 μm with less than 1% over 20 μm as determined by light microscopy of isolated gill concretions. The concretions react histochemically with both alizarin red S (Figs. 1,2) and PAS following amylase reaction (Fig. 3). The alizarin red S reaction can be eliminated by pretreatment with either EGTA (Fig. 4) or succinic acid. Although the calcium can be removed, structures are still present which


1
Cr
C


2
Cr
C
C


4
C


3


5

correspond to concretion material. Reaction with PAS following amylase treatment indicates that the material which is not removed by either acid or EGTA (Fig. 5) is either polysaccharide or has a polysaccharide moiety associated with it. The concretions also react specifically with silver methenamine (Fig. 6) supporting the PAS results.

The concretions also stain with toluidine blue giving a refractile appearance. The concretions also give an apparently positive reaction for the presence of Ca-ATPase using the methods of Padykula and Herman ('55) (Fig. 7), but incubation of the sections without the ATP substrate indicates the reaction is not an enzymatic one but is indicative of a calcium phosphate complex already present, and is histochemical evidence for a calcium phosphate component in the concretions.

Also of interest in the gill sections studied with light microscopy is the similarity in staining pattern of the paired chitinous rods supporting individual gill bars and the concretions (see Figs. 1,2,4,7). The chitinous rods of Unionids have been reported to have a calciferous nature (Ridewood, '03), and this study indicates their composition to be similar to the calcium concretions of the gill. This also is consistent with the electron microscopic appearance of the two structures (Fig. 8).

The histochemical results suggest the presence of a high concentration of calcium and phosphate (confirming the report of Janssens, 1893) in both the concretions and chitinous rods of the gill, and also suggest that the concretions occupy much of the volume of the deep connective tissue of the gills. Isolation of the concretion material from the soft tissue of the gill was readily accomplished in a discontinuous sucrose gradient indicating the difference in density between the concretions and the other components of

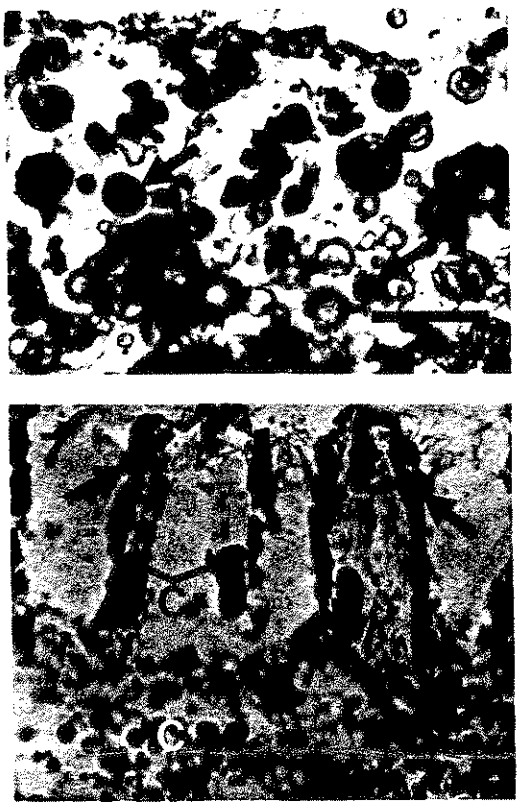


Fig. 6. Calcium concretions in the deep connective tissue of the mussel gill stained with silver methenamine. Several of the concretions exhibit a lamellar appearance. Some of the large concretions demonstrate a dense staining core (arrows). Bar = 5 μm.

Fig. 7. A section through two mussel gill bars reacted for histochemical Ca-ATPase activity. The epithelium of the gill bars show dark staining ATPase activity (arrows), but the calcareous chitinous rods (Cr) and the calcium concretions (C) show a more intense staining than the epithelium. The calcium phosphate nature of these two structures is responsible for the staining. Bar = 20 μm.

Fig. 1. An anterior to posterior section across the ventral edge of a mussel gill lamella stained with alizarin red S to demonstrate calcium deposits. Calcium concretions stain and are found singly (C) and in large clumps (arrows) within the connective tissue of the gill. Also stained are the calcareous chitinous rods (Cr) which support individual gill bars. Note the area occupied by particulate calcium within this section of gill. The section is typical of the midregion of the lamellae. Bar = 40 μm.

Fig. 2. A dorsal-ventral section across the plane of a mussel gill lamella stained with alizarin red S. The area shown is near the base of the lamella. Note the calcareous chitinous rods (Cr) running the length of the gill bars. Also note the apparent organized clusters of calcium concretions (C) along the length of the rods. The calcium concretions increase in number and area of connective tissue occupied toward the base of the gill (compare this figure with Fig. 1). Bar = 100 μm.

Fig. 3. An anterior to posterior section across the ventral edge of a mussel gill lamella stained with PAS. The calcium concretions stain positive with PAS (C) and exhibit a typical concentric lamellar structure. The calcareous rods (arrows) as well as the connective tissue in the immediate area of the rods also react positively with PAS. Bar = 40 μm.

Fig. 4. A section with similar orientation to that seen in Figure 1. This section has been preincubated in 10 mM EGTA before subsequent staining with alizarin red S. After EGTA or acid treatment neither the calcium concretions nor the calcareous chitinous rods (Cr) stain with alizarin red S. Bar = 40 μm.

Fig. 5. A section of gill lamella adjacent to a water channel preincubated with EGTA before PAS staining. The concretion material loses shape and organization but still stains positive with PAS (arrows). Bar = 20 μm.

the gill tissue. Electron microscopy of the isolated gill concretions shows the purity of the isolated concretion fraction.

In situ, the calcium concretions have a lamellar appearance in cross-section viewed by electron microscopy (Figs. 8,9). The concentric lamellae also can be seen in the light microscope using either phase contrast or Nomarski interference optics or after toluidine blue staining. The number of lamellae is variable, depending in part, on the size of the concretion. The lamellae surround an inner electron dense material making up the core of the concretions. Sections of isolated concretions have the same appearance (Fig. 10), and when pretreated with EGTA for 1 min the lamellae of the concretions disappear from the sections (Fig. 11). A few of the dense cores from the concretions remain. Although the size of the concretions varies widely, the core of these concretions are generally the same size (0.1 μm).

The concretions often have associated with them an amorphous material at their outside surface similar to that reported for the intracellular concretions of the hepatopancreas (Simkiss, '82). This amorphous material often has embedded within it crystalline structures which are removed by EGTA treatment. Rarely the lamellar components on the outside surface of the concretion appear to have crystals embedded in them as well (Fig. 12). Following EGTA treatment, fragments of these lamellae can often be found displaying a negative image of the solubilized crystals (Fig. 13). Although the concretions themselves rarely gave an electron diffraction pattern the crystals embedded in the lamellae and in the amorphous coat did produce diffraction patterns indicative of a true crystalline structure. Scanning electron microscopy reveals these crystals extending from the surface of the otherwise smooth concretion surface (Fig. 14). Other concretions demonstrate no crystal protrusions and are smooth (Fig. 15).

Chemical analysis of the gill concretions

Chemical analysis of the gill concretions confirms the histochemical results reported above. The isolation procedures (both sucrose gradient and NaOH extraction) were reasonably free of contamination and gave an average concretion fraction yield of 25% of gill dry weight (see Fig. 16). It is of interest that the concretion material appears resistant to 60°C 1 N NaOH exposure for as long as an hour and can withstand over a week in 1 N NaOH without showing any apparent change in morphology at the electron microscopic level. Ashing of the isolated concretions at 450°C indicates 25% of the concretion material to be of an organic volatile nature with the remaining 75% being nonvolatile inorganic matter.

The major inorganic components of the concretion material are phosphate and calcium (Table 1) as determined both by X-ray microanalysis and chemical assay. Calcium makes up 25% of the normal concretion material (atomic absorption spectrophotometry), and phosphate accounts for another 36–39% of the concretion material's weight (molybdate colorimetry). The concretion material also contains notable amounts of other cations. Manganese and iron are detected in the characteristic X-ray spectrum, and barium appears to be present in trace amounts (Fig. 17). The major anionic component associated with the concretions is phosphate. Bicarbonate (respirometry) accounts for less than 4% of the concretion weight. X-ray microanalysis of the material reveals no detectable sulfur; thus sulfate does not appear to be present as a component of either the inorganic or organic portion of the concretions.

Fig. 8. An electron micrograph through portions of the calcareous chitinous rods of a single mussel gill bar. Note the concentric lamellae of the isolated concretion in the upper right corner of the micrograph. The similarity betwen outer layers of the gill bar and the lamellae of the concretion is apparent (arrows). Bar = 2 μm.

Fig. 9. An electron micrograph of several calcium concretions in the connective tissue of a mussel gill. The concretions are often found asociated with amoebocytes (A) but are rarely seen intracellularly in the gill tissue. Note the electron dense cores found in several of the concretions (arrow). The concretions vary in size but all have a similar concentric lamellar appearance. Bar = 4 μm.

Fig. 10. An electron micrograph of a concretion isolated from mussel gill by sucrose density centrifugation. The concretion has been sectioned and demonstrates the same lamellar appearance as in situ concretions. Note the typical electron dense core of the concretion. Bar = 1 μm.

Fig. 11. An electron micrograph of isolated calcium concretions from mussel gill sectioned and treated for 1 min with EGTA. The portion of the concretion relatively resistant to this treatment is the electron dense core (Co) and, in some cases, the very outer lamellae of the concretion (arrows). Bar = 0.5 μm.

10

11

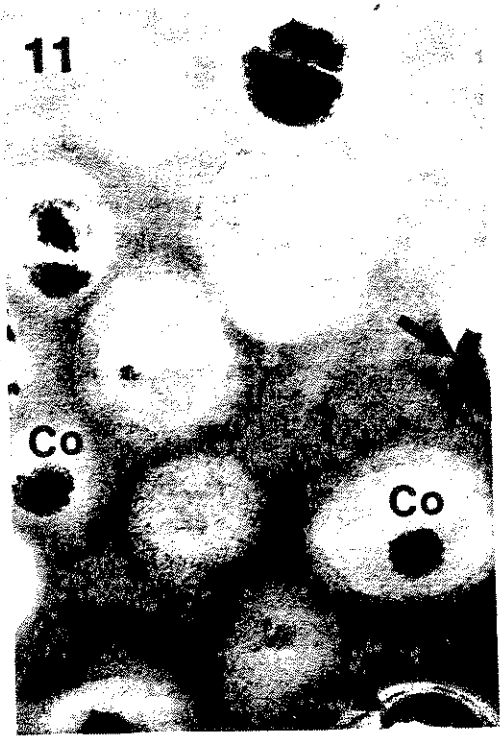


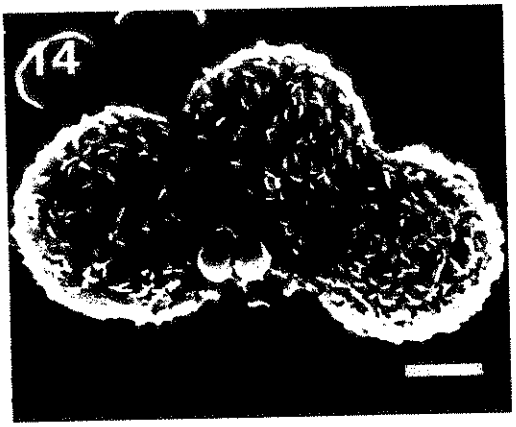
Fig. 12. A high magnification electron micrograph of the outer lamellae of a concretion. Crystalline material is embedded in an organic matrix (in some concretions). Bar = 1 μm.

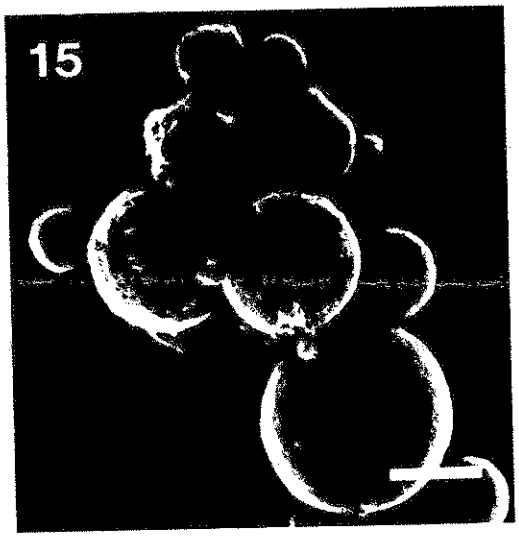
Fig. 13. A micrograph of an outer lamellae from a concretion treated with EGTA. Note the negative images (arrows) where crystalline material was apparently embedded before the EGTA treatment. Bar = 1 μm.

Changes in the mussel after hypoxia

After establishing the normal distribution and structure of the gill calcium concretions, experiments to determine whether their form and composition would change under hypoxic conditions were initiated. To establish the stress on individual animals placed in the hypoxic environment, and to correlate this stress with changes seen in concretion form and content, analyses of blood were performed. Table 2 shows the average values recorded for blood composition in normal mussels, in mussels placed in a water saturated N_2 atmosphere, and in mussels placed in pond water saturated with N_2.

Blood pH drops significantly in the animals under hypoxia. Both groups of hypoxic animals also show significantly increased calcium content and, interestingly, only the atmosphere hypoxic group show a significant increase in total blood osmolality. These results have been reported earlier (Dugal, '39; Dietz, '74; Wieser, '81) and led to the assumption that the increased calcium found in the blood possibly could be coming from the two large calcium reserves; the shell and the calcium concretions. Mussels placed in an hypoxic environment do not show any decrease in the content of concretions within the gill on a dry weight basis. In fact, there is a significant increase in the dry weight of the concretions per mg dry weight of gill under these conditions. Further, when the calcium content of concretions is plotted against the blood pH of individual mussels there is a significant ($P < 0.002$) inverse relationship between blood pH and calcium content in the concretions (Fig. 18). A relationship also exists between the calcium concentration in the blood and the calcium content of the concretions (Fig. 19). The results demonstrate that concretions in the gill of these mussels are not losing calcium to the blood during a time of increased blood calcium, but are instead gaining it.





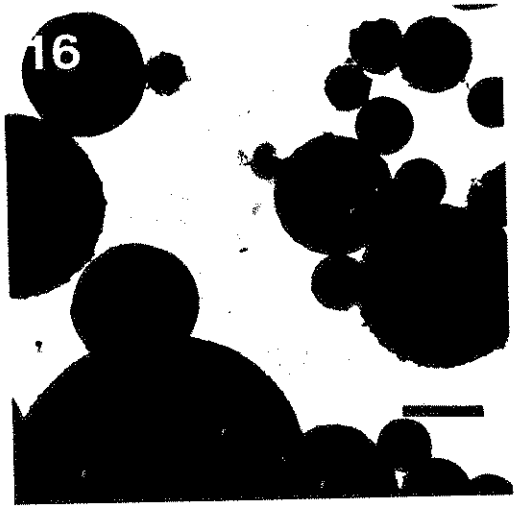


TABLE 1. Composition of gill concretions in pondwater acclimated Ligumia subrostrata

CCs/dry weight gill (%)	24.6 ± 1.2(29)
Ca/dry wt CCs (%)	25.3 ± 1.5(13)
HCO_3/dry wt CCs (%)	3.4 ± 0.4 (3)
P/dry wt CCs (%)	13.1 ± 0.4 (6)
Calculated PO_4/dry wt gill (%)	39.9
Calculated P_2O_7/dry wt gill (%)	36.5
Volatile material (%) (at 450°C for 4 h)	25 ± 1.8 (4)[1]

[1]Calcium plus phosphate account for 60–64% of the calcium concretions (CCs). Organic material accounts for another 25%, and X-ray microanalysis indicates quantities of iron, manganese, barium, and sodium can account for 10% of the CCs on a dry weight basis.

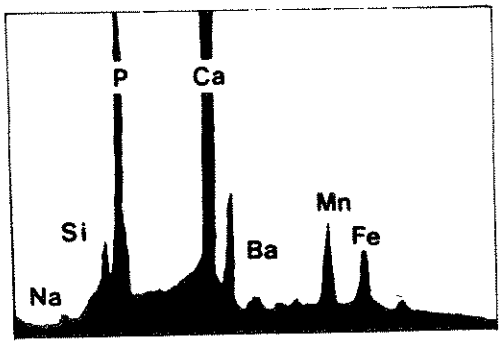


Fig. 17. A characteristic X-ray spectrum. Spectrum collection time was 25 sec. The predominant elements are calcium and phosphorous, with barium, manganese, and iron also present. Sodium was detectable and silcon may have been a glass contaminant.

Of further interest is the observation that bicarbonate in the blood does not increase with calcium but significantly declines (Table 2). If calcium does not come from the concretions it could come from the shell where it is partially complexed as calcium carbonate (Dugal, '39). However, blood bicarbonate is reduced under hypoxic conditions (Table 2).

Fig. 14. A scanning electron micrograph of a group of isolated concretions showing crystalline structure in the outer surface lamellae. Bar = 1 μm.

Fig. 15. A scanning electron micrograph of a group of concretions with a smooth outer lamellar surface. These are as common as those appearing in Figure 14. Bar = 1 μm.

Fig. 16. A transmission electron micrograph of concretions isolated by discontinuous sucrose density gradient. This preparation was used for X-ray microanalylsis. The micrograph demonstrates the typical purity achieved with our isolation methods. Bar = 2 μm.

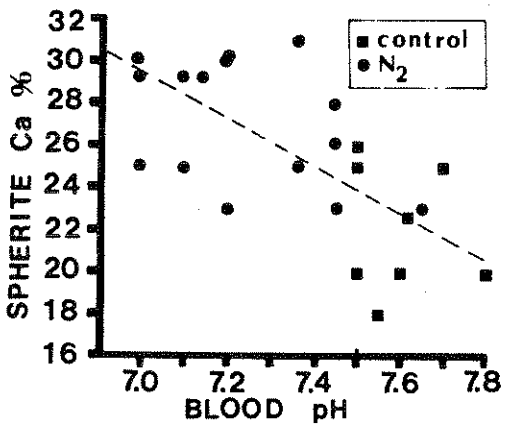


Fig. 18. The dry weight percentage of calcium in isolated concretion material (spherite) plotted against the pH of the blood in the animal from which the concretions were extracted. The regression of calcium content in isolated concretions on mussel blood pH (plotted line) is significant ($P < 0.002$; $r = 0.38$).

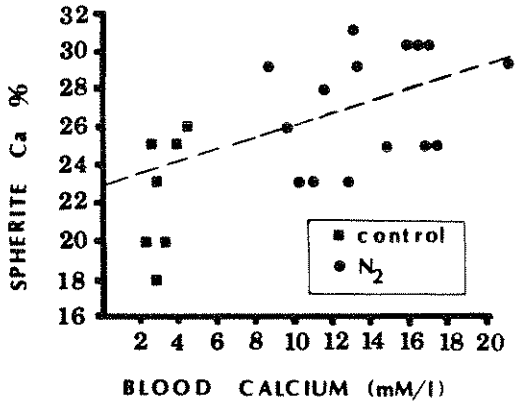


Fig. 19. The dry weight percentage of calcium in isolated concretion (spherite) material plotted against calcium content in the blood of the animal from which the concretions were extracted. A regression of calcium content in isolated mussel gill concretions on mussel blood calcium content (plotted line) is significant ($P < 0.0007$; $r = 0.43$).

TABLE 2. Ligumia subrostrata *blood solutes*

	Normal	N_2 (Gas)	N_2 (Water)
pH	7.60 ± 0.06	7.27 ± 0.07**	7.26 ± 0.07**
mM Ca	3.9 ± 0.5	14.0 ± 1.1*	12.7 ± 1.3*
mM Na	21.0 ± 0.6	20.0 ± 0.6	12.4 ± 0.4**
mM HCO_3	13.6 ± 0.6	7.1 ± 1.4*	—
mM PO_4	0.13 ± 0.01	0.18 ± 0.01	—
Total solute mOsm	45.0 ± 0.9	69.6 ± 3.4*	46.6 ± 2.5

Sodium values from Dietz ('74).
*Higher than normal ($P < 0.05$).
**Lower than normal ($P < 0.05$).

DISCUSSION

In *Ligumia subrostrata*, calcium concretions (CCs) have been observed in the connective tissue of the mantle and foot and extensively examined in the gill. In all three locations CCs are extracellular. In other freshwater and land molluscs CCs located intracellularly have been studied, particularly, in the "calcium cells" of the hepatopancreas (Abolins-Krogis, '58, '61, '63b, '68; Simkiss, '82; Mason and Simkiss, '82; Howard et al., '81). The CCs in these cells are composed mainly of an inorganic amorphous crystalline calcium phosphate (Howard et al., '81) associated with an organic matrix. Our results indicate that the extracellular CCs of *L. subrostrata* have a similar composition of inorganic calcium phosphate associated with an organic matrix.

The presence of CCs in mantle tissue led previous investigators to search for a putative role in shell calcification. Evidence supporting this theory include observations of an apparent increase in CCs in hepatopancreas cells of molluscs with experimentally damaged shells (Abolins-Krogis, '61, '63b, '73). Wagge ('51) suggested that calcium material for shell regeneration was produced in the digestive diverticula. Petit et al. ('80) and Abolins-Krogis ('73) have observed CCs in wandering amoebocytes and suggest these cells may transport CCs to the mantle during times of shell formation and repair. Although there is enough calcium in the concretions to make them a possible source of calcium during shell formation, the calcium in the CCs is in the form of calcium phosphate which is relatively difficult to solubilize (Simkiss, '82), even under extreme

conditions of hypoxia as shown by results of our study. Howard et al. ('81) have analyzed the CCs calcium phosphate composition and find it to be composed of pyrophosphate which would be resistant to breakdown. Since the aragonite crystals of the shell of these molluscs are calcium carbonate, one would have to envision a liberation of calcium in the connective tissue and its transport to the mantle for the CCs to play a major role in shell calcification; possibly by the amoebocytes. However, Timmermans ('73) notes the relationship between amoebocyte number and position in the mantle may be related to mantle damage rather than shell repair during shell injury experiments. Marsh et al. ('81) suggest that it would be difficult to determine if amoebocytes are actually transporting shell building material or are phagocytizing debris in an area of injured cells. These questions raise doubt as to whether the CCs actually contribute to shell regeneration, and perhaps whether they contribute to shell calcification processes under normal conditions. Bierbauer ('57) has presented evidence that the CCs do not play a role in shell calcification.

We suggest it is appropriate to view the CCs as playing an overall role in calcium homeostasis within the organism. This hypothesis is supported, in part, by the distribution of the CCs in the connective tissue of the gill. On a dry weight basis gill tissue contains twice as much CCs material as mantle tissue, and this distribution is difficult to reconcile with a role in mantle-directed shell formation. Further, cursory examination of other mussel tissues (unpublished) reveals the CCs are widely distributed in the connective tissue throughout the organism. Simkiss' ('76) approach has suggested that original CCs formation within hepatopancreas cells is a possible cellular mechanism for maintaining proper intracellular calcium activities in the cytosol.

Istin and Girard ('70) have suggested that during hypoxic stress the CCs might be a source of the free calcium. Our results indicate that the CCs may contribute to the homeostasis of free calcium in the blood in the opposite way. In the gill tissue the amount of CCs is increased during hypoxic conditions instead of decreased. At the same time the calcium content within the concretions is increased in a manner correlated with the calcium content in the blood (Table 2). The fact that calcium increases at the same time total concretion weight is increasing suggests that the weight gain is largely due to the addition of calcium to the concretions with elevation of calcium in the blood. These results suggest that the calcium liberated by the mussel under hypoxic conditions does not come from the extensive calcium concretion deposits in the gill connective tissue. This also appears to be true of connective tissue CCs found in *Helix* (Burton, '76). Instead, the calcium is probably liberated from the shell (Dugal, '39; Sorokina and Zelenskaya, '67). Freshwater organisms do not have an abundant supply of environmental calcium, and it would be selectively unsound for an animal forced to liberate calcium carbonate buffer derived from the shell in response to metabolic changes (resulting in lower blood pH) due to hypoxia to not have a mechanism to retain the liberated calcium. Clearly, calcium carbonate of the shell (or elsewhere) would be more susceptible to leaching by acidic metabolites than the calcium phosphate (pyrophosphate) of the concretions (Howard et al., '81) due to the solubility characteristics of the two materials under such conditions. It is also not surprising that during the hypoxic conditions, when calcium increased in the blood, there was a decrease in blood bicarbonate. The limit of $CaCO_3$ solubility is about 15 mM, and Dugal ('39) has suggested an unknown organic acid as the balancing anion for the increased calcium in the blood. Wieser ('81) has recently shown that succinate and lactate are produced during hypoxia concomitantly with an increase in blood calcium in *Helix*. In these studies with *L. subrostrata* such anions would not only have to balance the increased calcium in the blood but also make up for an apparent loss of bicarbonate from the normal blood concentrations. The acidosis observed in the present study is the result of hypoxic conditions as maintained by nitrogen gas and not by maintenance of an increased CO_2 environment. In the latter case bicarbonate apparently rises in *Helix* (Burton, '76); it appears that in our hypoxic treatment group, if bicarbonate is being liberated from the shell it is being released from the animal as CO_2 leaving high blood calcium and low bicarbonate levels. These results also can be obtained by infusing HCl into *Helix* in an attempt to mimic metabolic acidosis (Burton, '76).

The unexpected low bicarbonate levels raise the interesting question, if calcium liberation is not tied to the balancing of higher

bicarbonate anion in the blood, why is the calcium concentration in the blood raised so high and in good agreement to decreased blood pH? It has been suggested by Christoffersen ('73) that neurons in the snail *Helix* are depolarized by high external H^+ and hyperpolarized by high Ca^{++}. The increase in blood calcium with acidosis may offset the increase in H^+ and allow nerve function under such conditions (Christoffersen, '73). This explanation of calcium and its regulation of nerve potentials is of interest with reference to the organization of CCs within the gill. The main branchial nerves which lie at the base of the gill are surrounded by extensive accumulations of CCs. The association of CCs with nerves may serve to buffer the changes in the ionic content of the blood. This arrangement of CCs may also partially explain why the mussel can be more tolerant of changes in the ionic composition in its blood (Dietz, '74; Dietz and Branton, '79; Murphy and Dietz, '76; Scheide and Dietz, '82) than other freshwater animals.

Another apparent function of the CCs which has been clearly elucidated by Simkiss and his colleagues (Simkiss, '82; Mason and Simkiss, '82; Howard et al., '81) is the ability of the CCs to bind potentially toxic metals. Such binding to the pyrophosphate core of these granules may serve the organism as a detoxification site during exposure to toxic metal compounds in the environment. The Simkiss reports deal extensively with the CCs found intracellularly in the hepatopancreas. Our study expands on this previous work by demonstrating an extensive accumulation of extracellular CCs in the gills of a freshwater mussel. X-ray microanalysis reveals that the extracellular CCs contain metals, particularly manganese and iron, as also shown by Mason and Simkiss ('82).

The CCs do not contain appreciable amounts of carbonate nor do they appear to have much sulfate because sulfur is not detected by X-ray microanalysis. This is in agreement with the report of Howard et al. ('81). There is an organic core to the CCs as demonstrated by histochemistry. It is composed partially of polysaccharides but does not appear to show the sulfate groups typical of connective tissue matrices. This differs from a recent report by Davis et al. ('82) where sulfur was reported from X-ray microanalysis data. Our spherites were unsectioned and not embedded during analysis and did not exhibit sulfur. Either the region of sulfur content was outside the zone of primary excitation in our preparation (not likely) or Spurr embedding medium may have contributed to the sulfur peak previously reported. The second possibility is supported by the lack of sulfur in the characteristic X-ray spectrum of Petit et al. ('80).

The organic matrix of the CCs is reactive with Schiff and silver methenamine after periodic acid incubation and is likely to be nonsulfated glycoprotein in nature based on the histochemistry of Abolins-Krogis ('61, '63a,b). The structure of the CCs is resistant to 1 N NaOH even at 60°. The organic material of the CCs is also resistant to acids (pH 2) and 10 mM EGTA as indicated by PAS staining after preincubation in such solutions. It appears that a resistant glycoprotein is the likely organic material associated with the CCs.

The CCs in freshwater and land molluscs are a likely calcium reserve which serve many functions including detoxification (by chelation) of heavy metals, a storage site of calcium concomitant with its forced liberation from the shell during hypoxic conditions, and perhaps as a source of shell calcium under the appropriate conditions, although such conditions have not as yet been documented.

ACKNOWLEDGMENTS

The authors wish to thank John I. Scheide for his help and discussion during the course of this study. We thank the faculty of the electron microscopy laboratory at Southwestern Louisiana State University for allowing us to do our X-ray probe experiments in their laboratory. We also thank E.J. Silverman for preparation of our graphs. The work was supported by a grant from the LSU Research Council to H. Silverman and by NSF grant PCM 79-21089-02 awarded to T. Dietz.

LITERATURE CITED

Abolins-Krogis, A. (1958) The morphological and chemical characteristics of organic crystals in regenerating shell of *Helix pomatia* (L.). Acta Zool. (Stockholm) *39*:19–38.

Abolins-Krogis, A. (1961) The histochemistry of the hepatopancreas of *Helix pomatia* (L.) in relation to the regeneration of the shell. Ark. Zool. *13*:159–202.

Abolins-Krogis, A. (1963a) Some features of the chemical composition of isolated cytoplasmic inclusions from the cells of the hepatopancreas of *Helix pomatia* (L.). Ark. Zool. *15*:393–429.

Abolins-Krogis, A. (1963b) The histochemistry of the mantle of *Helix pomatia* (L.) in relation to the repair of the damaged shell. Ark. Zool. *15*:461–473.

Abolins-Krogis, A. (1968) Shell regeneration in *Helix pomatia* with special reference to the elementary calcifying particles. In: Studies on Structure, Physiology and Ecology of Mollusks. V. Fretter, ed. Academic Press, New York. pp. 75–92.

Abolins-Krogis, A. (1973) In vitro recalcifications of the demineralized shell-repair membrane of the snail, *Helix pomatia* L. Cell Tissue Res. *200*:487–494.

Bierbauer, J. (1957) Untersuchungen uber die Regeneration und Histologie von *Helix pomatia*. Acta Biol. Acad. Sci. (Hung.) 7:419–431.

Burton, R.F. (1976) Calcium metabolism and acid-base balance in *Helix pomatia*. In: Perspectives in Experimental Biology. P.S. Davies, ed. Pergamon Press, New York, vol. 1, pp. 7–16.

Christoffersen, G.R.J. (1973) Chloride conductance and the effect of extracellular calcium concentration on resting neurons in the snail, *Helix pomatia*. Comp. Biochem. Physiol. *46A*:371–389.

Dahl, L.K. (1952) A simple and sensitive histochemical method for calcium. Proc. Soc. Exp. Biol. Med. *80*:474–479.

Davis, W.L., R.G. Jones, J.P. Knight, and H.K. Hagler (1982) An electron microscopic histochemical and X-ray microprobe study of spherites in a mussel. Tissue Cell *14*:61–67.

Dietz, T.H. (1974) Body fluid composition and aerial oxygen consumption in the freshwater mussel, *Ligumia subrostrata* (Say): Effects of dehydration and anoxic stress. Biol. Bull. *147*:560–572.

Dietz, T.H., and W.D. Branton (1975) Ionic regulation in the freshwater mussel, *Ligumia subrostrata* (Say). J. Comp. Physiol. *104*:19–26.

Dietz, T.H., and W.D. Branton (1979) Chloride transport in freshwater mussels. Physiol. Zool. *52*:520–528.

Dugal, L-P. (1939) The use of calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. J. Cell Comp. Physiol. *13*:235–251.

Howard, B., P.C.H. Mitchell, A. Ritchie, K. Simkiss, and M. Taylor (1981) The composition of intracellular granules from the metal-accumulating cells of the common garden snail (*Helix aspersa*). Biochem. J. *194*:507–511.

Huxtable, R., and R. Bressler (1973) Determination of orthophosphate. Anal. Biochem. *54*:604–608.

Istin, M., and J.P. Girard (1970) Dynamic state of calcium reserves in freshwater clam mantle. Calcif. Tissue Res. *5*:196–205.

Jassens, F. (1893) Les branches des acephales. La Cellule *9*:7–91.

Kapur, S.P., and M.A. Gibson (1968) A histochemical study of calcium storage in the foot of the freshwater gastropod, *Heliosoma duryi eudiscus* (Pilsbry). Can. J. Zool. *46*:987–992.

Marsh, M.E., R.D. Summerall, and R.L. Sass (1981) Lysosomes and the modulation of cell morphology in the isthmus region of the outer mantle epithelium in the estuarine clam, *Rangia cuneata*. J. Ultrastruct. Res. *77*:146–159.

Martino, C. de, and L. Zamboni (1967) Silver methenamine stain for electron microscopy. J. Ultrastruct. Res. *19*:273–282.

Mason, A.Z., and K. Simkiss (1982) Sites of mineral deposition in metal-accumulating cells. Exp. Cell Res. *139*:383–391.

McManus, J.F.A. (1946) Histological demonstration of mucin after periodic acid. Nature *158*:202.

Murphy, W.A., and T.H. Dietz (1976) The effects of salt depletion on blood and tissue ion concentrations in the freshwater mussel, *Ligumia subrostrata* (Say). J. Comp. Physiol. *108*:233–242.

Padykula, H.A., and E. Herman (1955) Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. J. Histochem. Cytochem. *3*:161–169.

Petit, H., W.L. Davis, R.G. Jones, and H.K. Hagler (1980) Morphological studies on the calcification process in the fresh-water mussel *Amblema*. Tissue Cell *12*:13–28.

Potts, W.T.W. (1954) The inorganic composition of the blood of *Mytilus edulis* and *Anodonta cygnea*. J. Exp. Biol. *31*:376–385.

Ridewood, W.G. (1903) On the structure of the gills of lamellibranchia. Phil. Trans. Roy. Soc. B *211*:147–284.

Scheide, J.I., and T.H. Dietz (1982) The effects of independent sodium and chloride depletion on ion balance in freshwater mussels. Can. J. Zool. *60*:1676–1682.

Simkiss, K. (1976) Intracellular and extracellular routes in biomineralization. Symp. Soc. Exp. Biol. *30*:423–444.

Simkiss, K. (1982) Cellular discrimination processes in metal-accumulating cells. J. Exp. Biol. *94*:317–327.

Sorokina, Z.A., and V.S. Zelenskaya (1967) Peculiarities of electrolyte composition of molluscan haemolymph. J. Evol. Biochem. Physiol. *3*:25–30.

Timmermans, L.P.M. (1973) Mantle activity following shell injury in the pond snail *Lymnaea stagnalis* L. Malacologia *14*:53–61.