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Localization and distribution of antigens related to calcium-rich deposits in the gills of several freshwater bivalves

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In the gills of freshwater unionid mussels, calcium concretions may account for up to 50% of the dry weight of the gill and are composed primarily of calcium phosphate with an associated glycoprotein. The role of these structures in relation to calcium physiology of bivalves is unknown. A study of the origin and distribution of these structures and their possible relationship to specific organ systems or tissue types was undertaken. An antiserum to decalcified concretions was prepared and utilized in indirect immunofluorescence studies on fresh-frozen sections of excised gill. Electron microscopy was employed to examine areas of interest revealed by the immunofluorescence microscopy. Calcium concretions provided the bulk of the fluorescence when the sections were examined. In addition to the concretions, other structures including the chitinous rods of the gill filaments, and nerve tracts intimately associated with the concretions also bound the antiserum, suggesting the possibility of some shared antigens. Some previously undescribed cells, also found to react with the antisera, were shown by electron microscopy to contain what appears to be concretion material in stages of assembly and likely represent the synthesis site of the concretions in the gill. Antisera were produced from isolated concretions of two different genera; each reacted similarly when tested against tissues from several different unionid species. These data suggest a similar composition for calcium phosphate concretions within the Unionidae.

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Dans les branchies des moules d'eau douce (Unionidae), les concrétions de calcium peuvent constituer jusqu'à 50% de la masse sèche d'une branchie et elles sont surtout présentes sous forme de phosphate de calcium associé à une glycoprotéine. Le rôle de ces structures dans la physiologie du calcium chez les bivalves est encore inconnu. L'origine et la répartition de ces concrétions et leurs relations à des systèmes organiques spécifiques ou à des types de tissu ont donc fait l'objet d'une étude. L'antisérum des concrétions décalcifiées a été préparé et il a servi au cours d'expériences d'immunofluorescence indirecte sur des portions fraîchement surgelées de branchies prélevées. Le microscope électronique a servi à examiner les régions intéressantes révélées par la microscopie à immunofluorescence. Les concrétions de calcium sont responsables de la plus grande partie de la fluorescence dans les coupes examinées. D'autres structures sont également mises en évidence, notamment les bâtonnets chitineux des filaments branchiaux; des cordons nerveux intimement liés aux concrétions se sont également reliés à l'antisérum, ce qui laisse supposer qu'il y a partage de certains antigènes. Des cellules d'un type nouveau réagissent aussi à l'antisérum et le microscope électronique a révélé qu'elles contiennent ce qui semble être de la matière à concrétions en voie de formation; ces cellules constituent sans doute le siège de la synthèse des concrétions dans les branchies. Des antisérums ont pu être produits à partir des concrétions isolées de deux genres différents; ils réagissent de la même façon au cours des tests sur des tissus de plusieurs espèces d'Unionidae. Ces résultats indiquent que les concrétions de phosphate de calcium ont probablement des compositions très apparentées chez les diverses espèces de moules d'eau douce.

[Traduit par le journal]

Introduction

Lamellar calcium concretions occur in variable amounts throughout the tissues of many freshwater and terrestrial invertebrates. The gill of the large freshwater mussel, *Anodonta grandis*, for example, is at least 50% calcium concretions on a dry weight basis (Silverman et al. 1985), while another species, *Ligumia subrostrata*, contains only 25% (Silverman et al. 1983*a*). In mollusks, such concretions are found frequently in the connective tissues of mantle (Petit et al. 1980; Davis et al. 1982), gill (Silverman et al. 1983*a*), and within specialized calcium cells of the hepatopancreas (Abolins-Krogis 1961; Simkiss 1976, 1982; Burton 1972) of gastropods and bivalves.

The calcium in some of these concretions has been reported as being complexed with pyrophosphate anion and a poorly understood organic component (Simkiss 1982; Howard et al. 1981). Recently, the structure of these concretions in freshwater mussel gills has been studied (Silverman et al. 1983*b*). Chemical analysis has shown the concretion to contain about 75% nonvolatile inorganics, mostly in the form of calcium and phosphate, with the other 25% being volatile organics. Specific histochemical staining of the concretions before and after decalcification by organic acids suggests a portion of the lamellar structure is composed of oxidizable polysaccharides (Silverman et al. 1983*a*). A dense central core which resists hot 1 *N* NaOH treatment involved in concretion isolation, purification, and later decalcification exhibits staining properties suggestive of glycoproteinaceous compounds. The present knowledge of the concretion structure and composition suggests that it is composed of a dense, glycoprotein core which binds calcium phosphates (Abolins-Krogis 1961, 1963*a*, 1963*b*; Silverman et al. 1983*a*).

The ontogeny of the gill calcium concretions is unknown. Structures similar in appearance and composition have been reported in the basophilic calcium cells of snail hepatopancreas where they are said to be formed (Abolins-Krogis 1958, 1961, 1963*a*, 1963*b*, 1968; Howard et al. 1981; Simkiss 1982; Mason and Simkiss 1982). Prior to this study, few concretions had been observed intracellularly in gill tissue, leading to the supposition that the concretions are transported from the site of their development to other parts of the body.

Specifically, within the gills of freshwater unionid mussels, calcium concretions are found associated with selected areas in the gill tissue (Dietz et al. 1985; Silverman et al. 1985). Ex-

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tensive nerve tracts which innervate the gill filaments are paralleled by abundant depositions of calcium concretions. This study was initiated to define the distribution of calcium concretions in freshwater bivalves and to establish ontogenic relationships to specific cell or tissue types. Our current interpretation of the structure and composition of calcium concretions led us to believe that at least a portion of the matrix could serve as an antigen, leading to the development of an antibody probe for cytological study. With such a probe and suitable labels, information could be acquired on the distribution, formation, and ultimate fate of the concretions in the particular animals being studied; and possible similarities with other calcium-binding structures in entirely different systems could be investigated.

Methods

Animals used in the experiments included the following: male *Ligumia subrostrata* (20–70 g) collected locally; *Anodonta grandis* (150–300 g) collected locally; *Margaritifera hembeli* (75–100 g) collected in northern Louisiana; and *Elliptio crassidens* (75–100 g) purchased through Carolina Biological Supply Co. Animals were maintained in the laboratory in artificial pond water before use (Silverman et al. 1983*a*).

Concretion isolation and purification

Calcium concretions (CCS) that were used in preparing the antigens were isolated by alkaline digestion of the soft tissue of the gill. Lateral gills excised from both *Ligumia subrostrata* and *Anodonta grandis* were homogenized in a Tissue-Mizer and the homogenate incubated 1 h at 60°C in 1 *N* NaOH. Following centrifugation at 2600 × *g* (5 min × 3) (Silverman et al. 1983*a*), the remaining concretions were collected (and checked for purity by electron microscopy), rinsed in several changes of distilled water, and dried in an oven prior to use.

Antibody preparation

Dried, purified concretions were partially decalcified by suspending in 0.1 *M* succinic acid at a concentration of 30–60 mg/cm^3 for at least 1 h. The washed concretion organic component was then mixed with an equal amount of Freund's complete adjuvant (Sigma) in a tissue homogenizer. Two groups of mature mice (inbred strain C57B1/6J) were injected intraperitoneally with 0.1 cm^3 of the resultant slurry prepared from concretions isolated from either *Ligumia subrostrata* or *Anodonta grandis*. Each group of animals was reinjected with a similar preparation (but using Freund's incomplete adjuvant) on the 7th, 10th, and 13th days, and the sera were collected on the 15th day. The sera (from each species similarly challenged) were pooled and heated in a waterbath at 56°C to denature complement. Control serum from an uninjected group of mice was handled identically.

The possibility of nonspecific reactions between the mouse serum and mussel tissues was reduced by reacting the sera with mussel tissue not containing the CCS antigen. Tissue containing the fewest concretions (muscle from the foot) was homogenized and CCS extracted by sucrose (2.5 *M*) density centrifugation. The CCS-free material was lyophilized, mixed with the reactive and normal sera, and incubated at 37°C in an agitating waterbath for 1 h. The sera were then centrifuged for 15 min at 11 200 × *g*, and separated from the tissue pellets.

A slide agglutination test was performed on each of the sera using particulate decalcified CCS to check for activity and specificity. Each serum containing a gill concretion antibody caused a visible agglutination of its respective antigen to a dilution of 1:5. No agglutination was evident when either antigen was tested against control serum or water.

Immunohistochemistry

Gills from mussels to be prepared for antibody labelling were removed from the animals by cutting the adductor muscles, opening the shell, and excising the gill pair from the body at the basal surface. The gills were coated in OCT compound (Ames Co., Elkhart, IN), oriented as desired on cryostubs, and frozen in liquid nitrogen. The tissues were warmed to −20°C in a cryostat (American Optical) and sectioned to a thickness of 20 μm. Sections were collected on slides and allowed to air dry.

Sections were examined for preservation and orientation by light microscopy before treatment with antibody. The adsorbed sera were serially diluted in doubling increments with phosphate buffer (0.3 *M*, pH 7.0 + sodium azide) and 100 μL applied to the sections. Cover slips were applied to spread the sera and reduce desiccation. The slides were incubated in moist chambers at 37°C for 1–2 h. The slides were washed in distilled water to remove the cover slips and serum, and stored in buffer. Rabbit antimouse IgG conjugated to fluorescein isothiocyanate (FITC-AB) (U.S. Biochemical) was diluted and applied to the tissue sections. After a period of at least 1 h, but not more than 3 h, the sections were washed in distilled water, rinsed briefly in buffer, and cover slips applied. The preparations were viewed on a Leitz Ortholux II microscope equipped with epifluorescence illumination and a Leitz H2 prism module (excitation 390–490 nm; mirror 510 nm; suppression 510 nm). Photographs were taken using Kodak VR-1000 color print film. Experiments using varying dilutions of concretion antisera and FITC-AB revealed optima to be as follows: concretion antisera, 1:4; FITC-AB, 1:10. These dilutions were used throughout the course of the study.

Electron microscopy

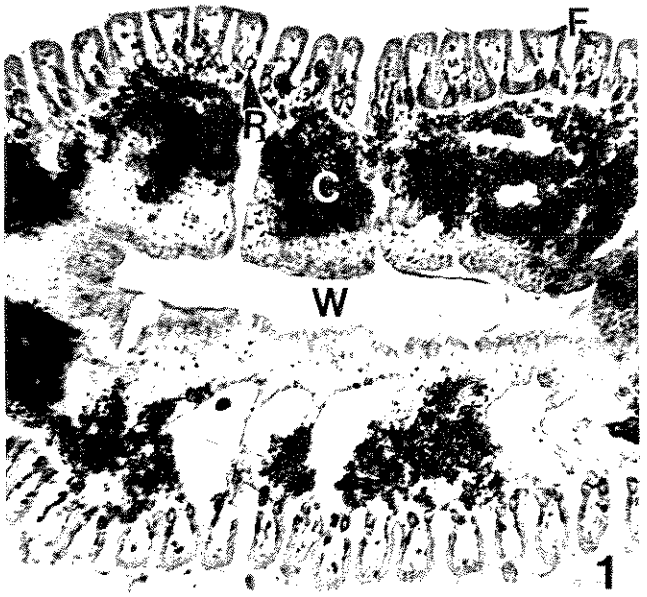
Material to be examined by electron microscopy was prepared by excising the entire demibranch from the animal, transferring it to pond water, removing the desired portion noting orientation, and transferring it to fixative. The fixation schedule consisted of 1.5 h at room temperature in 2% (v/v) glutaraldehyde at pH 7.8 containing 1 m*M* ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA). Postfixation was 1 h in buffered 1% OsO_4 at room temperature followed by ethanol dehydration and embedding in L.R. white resin. Thin sections were observed and photographed in a JEOL 100-CX operating at 80 keV.

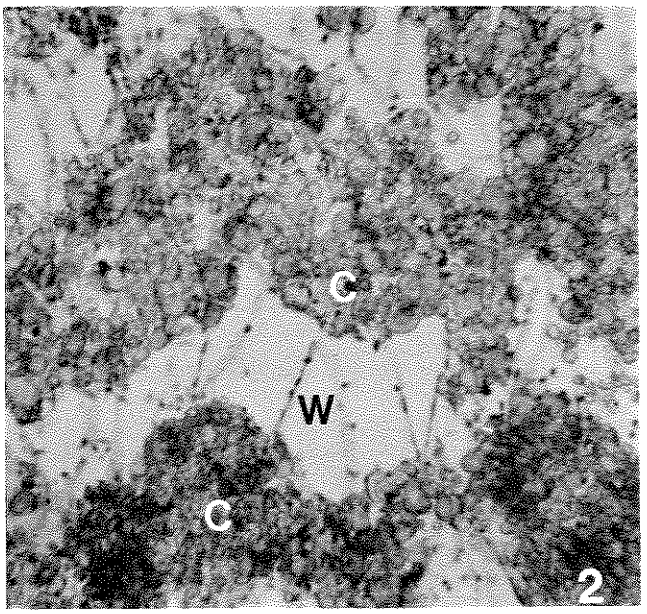
Results

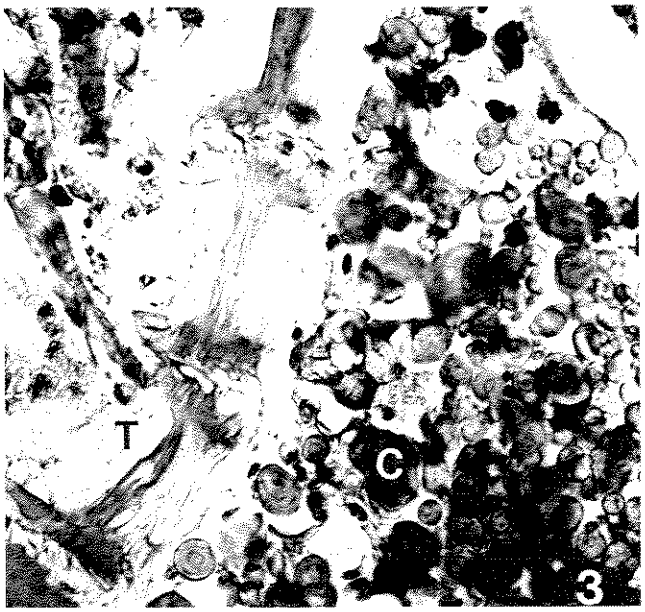
General observations on the distribution of calcium concretions

Observations of histological sections through various planes of the gills reveal a predictable consistency in the pattern of occurrence of the calcium concretions (CCS) with only slight differences among the three unionid genera studied. In the typical anterior–posterior cross section through the gill demibranch, bright-field microscopy reveals dense granular clumps in the loose connective tissue below the inner and outer lamellar gill surfaces abutting the epithelium of the inner water channels (Fig. 1). When the demibranch is sectioned and viewed *en face*, a distinct pattern in the distribution of the bulk of the CCS is observed. The clumps observed in the horizontal section plane are seen as long, regularly spaced parallel rows of depositions of CCS (Fig. 2) arranged anterior–posterior along prominent nerve tracts which innervate the epithelium and muscle of the gill filaments. At a higher magnification (Fig. 3), these dense clumps are revealed as quantities of CCS which differ individually in size (0.5–100 μm), shape (spherical–oblong), and color (light – dark brown).

This pattern of distribution of CCS in gills of the unionids was common to all species studied with any observed differences being primarily quantitative. *Anodonta grandis* had the most extensive depositions of CCS. This was followed by *Elliptio* and *Ligumia*. *Margaritifera* (a margaritiferid) had the least accumulation of CCS of all species studied. This present investigation was carried out on all animals at the same time of year (October–November), and the amount of CCS observed in each species does not necessarily remain constant throughout its yearly cycle (Silverman et al. 1985). Our references made







to precise quantitative content of CCS for any given species are therefore valid for these animals only during this specific period of the year.

Immunohistochemistry

When either a slide agglutination test or the indirect immunofluorescence technique is performed using antiserum on isolated decalcified CCS (prepared similarly to those antigens used to produce the antisera), each antiserum reacts with its specific antigen. Normal (control) mouse serum has virtually no affinity for isolated CCS from either *Ligumia* or *Anodonta*.

When antisera produced from concretions derived from *Anodonta* and *Ligumia* CCS are reacted with fresh-frozen sections of gills from the respective animals followed by FITC-labelled antimouse serum, the CCS acquire the label (Fig. 4). The pattern of antibody fluorescence is identical with the pattern described above (compare Fig. 4 with Fig. 2). The greenish-yellow fluorescence produced by the FITC on the CCS is variable *in situ*, probably because of differing degrees of calcification among individual CCS and a resultant inhibition of Ab penetration with increasing degree of calcification. Smaller CCS tend to have the label uniformly distributed over the surface and, judging from their observed levels of fluorescence, throughout at least some of the interior. Large CCS are labelled variably, with a very intense fluorescence around the periphery and a darker interior is commonly observed (Fig. 4). A light green autofluorescence was observed in the epithelial tissue.

When antiserum to CCS from one species was exposed to fresh-frozen sections of gill demibranch from another species, cross-reactivity was demonstrated. Figure 5 shows reaction between anti-*Ligumia* CCS serum to *Anodonta* gill. These cross-reactions were judged to be of comparable intensity to the species-specific antiserum reactions.

In addition to the CCS, other structures are observed to acquire the fluorescent label. Foremost among these are the chitinous rods which occur in interrupted pairs running the length of the gill bar from the dorsal gill base to the ventral tip (Fig. 6). In anterior–posterior cross section, the edges of the chitinous rod often acquires as much label as concretions, while the interior of the rod remains unreacted. More specifically, a crescent-shaped region on the inner edge of the chitinous rod is highly reactive (Fig. 7). The corresponding area can be observed using transmission electron microscopy (Fig. 8). Note the similar appearance of this edge of the rod with the outer edge of the larger CCS (Fig. 9). Also labelled immunohistochemically are elongated fibrous elements which are observed in *en face* view as running in the same posiiton as the nerve

FIG. 1. A bright-field (toluidene blue freeze-dried preparation) anterior–posterior cross section through a lateral demibranch of *Anodonta grandis*. Deposits of calcium concretions (C) are demonstrated occurring in the connective tissue between the gill filaments (F) of both epithelial lamella and the water channel (W). R, paired chitinous rods in each filament. ×75. FIG. 2. A bright-field (toluidene blue freeze-dried preparation) proximal to distal section across the face of a gill demibranch of *Anodonta grandis* demonstrating the regular arrangement of large depositions of calcium concretions (C). These concretions surround nerve tracts (not visible in this section). Water channels (W) lie between concretion rows. ×160. FIG. 3. A bright-field (toluidene blue freeze-dried preparation) cut in the same orientation as Fig. 2. In this section the plane passes directly through the nerve tract (T) indicating the concretion (C) distribution paralleling the tract. ×650.

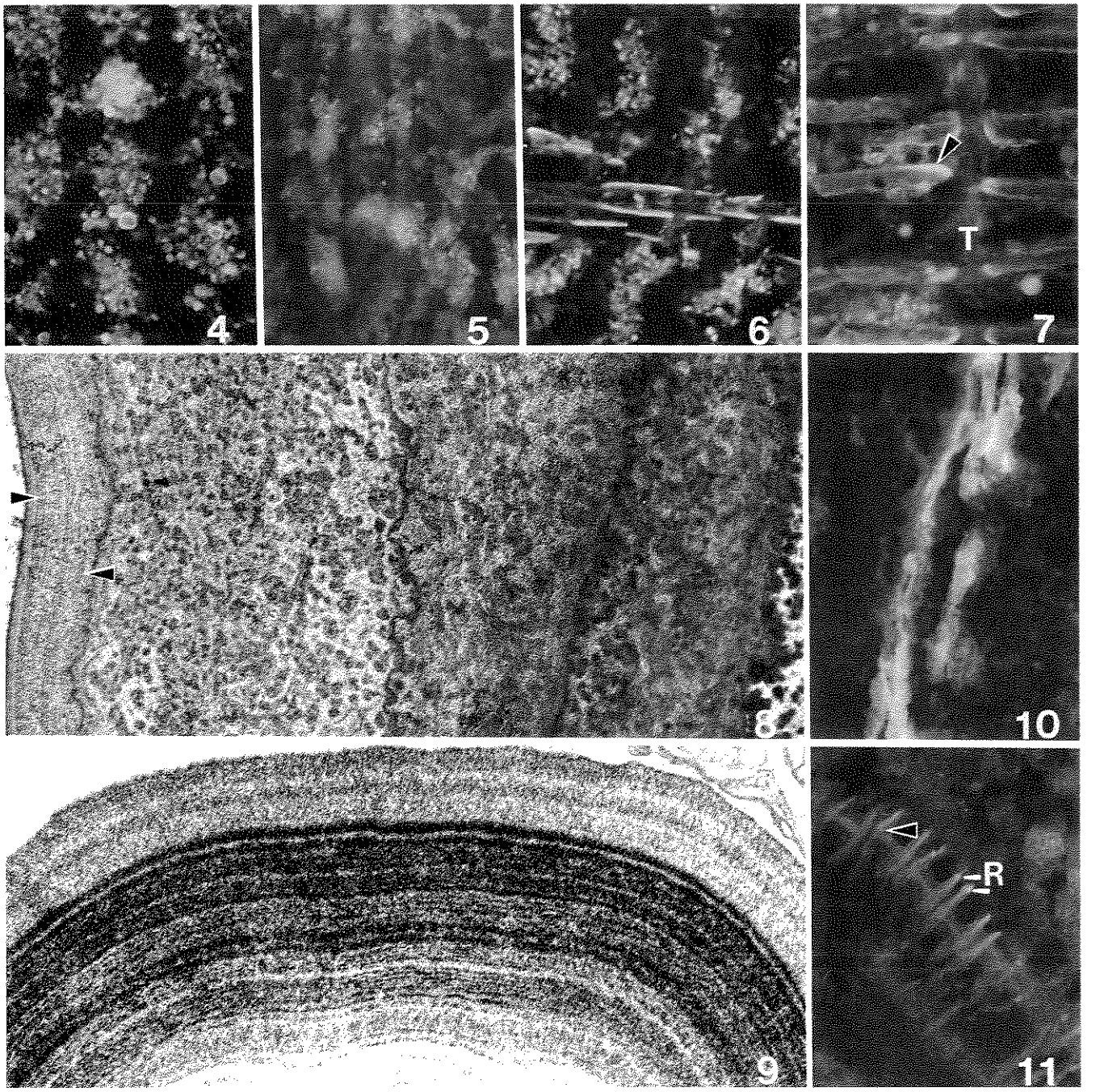
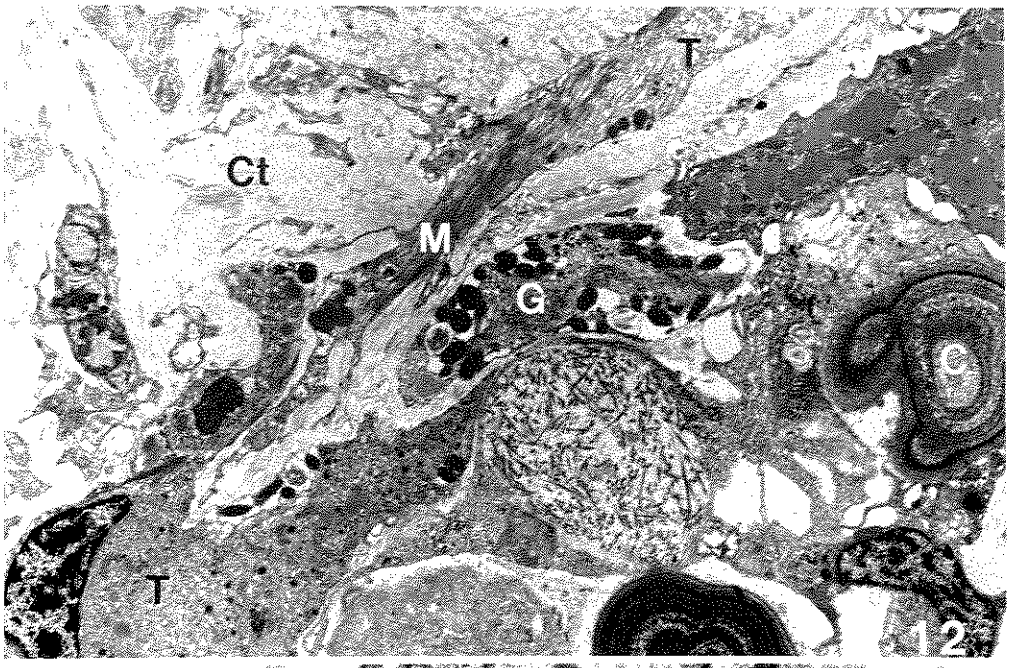


FIG. 4. A fluorescence micrograph of a frozen section across the face (distal to proximal) of a demibranch of *Anodonta grandis* which has been reacted with anti-*Anodonta* concretion mouse serum. The FITC fluorescence from the labelled antimouse serum is confined to concretions in this micrograph. Note the vertical symmetry in the fluorescence. The gill filaments are running horizontally and the concretions are organized along the nerve tracts. ×75. FIG. 5. A fluorescence micrograph of a frozen section across the face of a demibranch from *Anodonta* reacted with anti-*Ligumia* mouse serum. The orientation is similar to Fig. 4. ×65. FIG. 6. A fluorescence micrograph of *Anodonta* gill which has been reacted with anti-*Anodonta* concretion mouse serum demonstrating the similarity in labelling of concretion deposits (running vertically) and paired chitinous rods (running along the filaments or horizontal in this micrograph). ×75. FIG. 7. A similar preparation to that described in Fig. 6. At this higher magnification note the labelling of a crescent-shaped area (arrowhead) on the chitinous rods which occur along the regularly spaced interruptions marking the site of nerve tract (T) passage between filaments. ×135. FIG. 8. An electron micrograph of a cross section through a chitinous rod. The section is cut at the level of the fluorescent crescent in Fig. 7. Note the similarity of lamellar organization and electron density between the edge of the rod in this region (between the arrowheads) and the outer region of a concretion as shown in Fig. 9. × 11 500. FIG. 9. An electron micrograph of a section through a large calcium concretion. Note the similarity in structure to the chitinous rod shown in Fig. 8. × 19 900. FIG. 10. Immunofluorescent labelling of a nerve tract in a face section of *Ligumia* gill which has been reacted with anti-*Anodonta* concretion mouse serum. ×350. FIG. 11. An immunofluorescently labelled nerve tract passing through disruptions (arrowhead) in the more intensely fluorescing tips of the chitinous rods (R) in an *en face* sectioned demibranch of *Anodonta*. ×75.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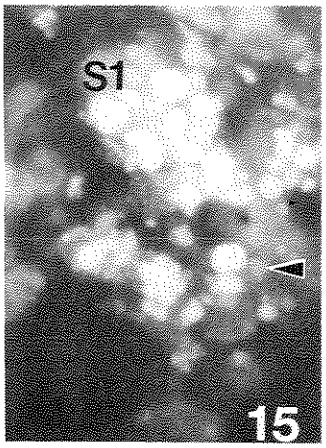
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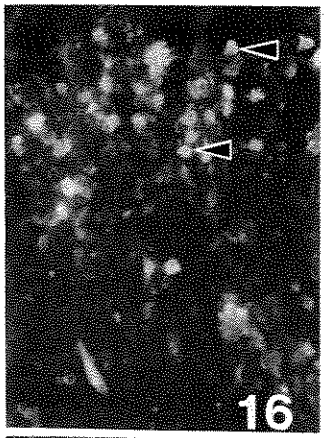
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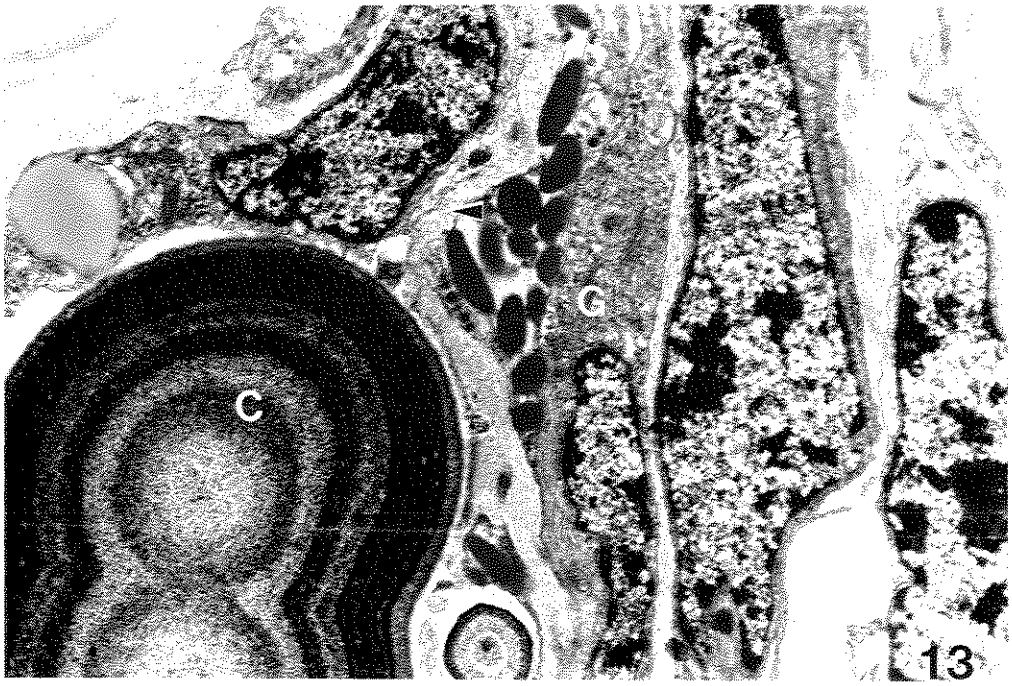
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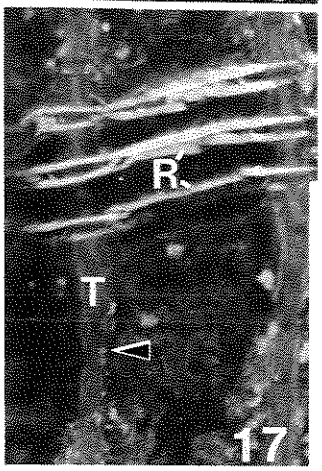
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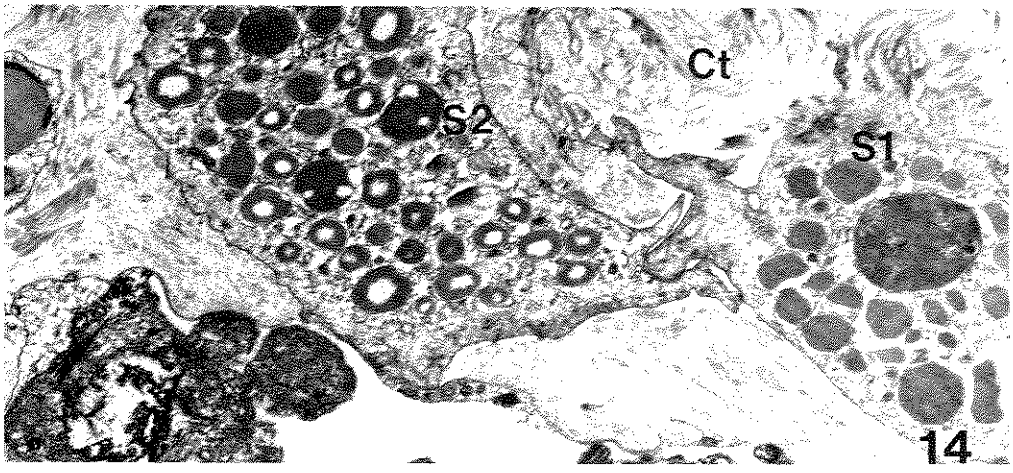
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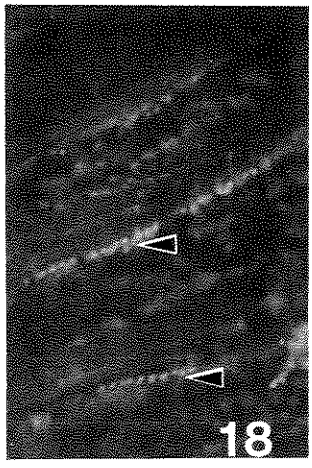
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tracts observed in bright field (Figs. 10 and 11). Associated with these nerve tracts are several characteristic cell types: muscle and interstitial glial cells (after Vitellaro-Zuccarello et al. 1983) (Figs. 12 and 13) and stellate-shaped cells containing what appears to be intracellular concretion material (Fig. 14). Similar appearing cells are fluorescent following immunohistochemistry (compare Fig. 15 with Fig. 14). Other as yet unidentified cells that appear to be associated with the elongated neural elements also display FITC labelling (Fig. 16).

The antisera against CCS from *Anodonta* and *Ligumia* also cross-reacted with fresh-frozen sections of gill from the other species. *Elliptio crassidens* and *Margaritifera hembeli* were checked for cross-reactivity with the two antisera. Both displayed fluorescent labelling. Again, the most prominently fluorescing tissue components were the CCS, chitinous rods, and neural components (Fig. 17). *Elliptio*, however, showed one additional instance of cross-reactivity. A high percentage of the fluorescence not associated with CCS or chitinous rods was localized within small cells which were in turn associated with areas rich in neural components (Fig. 18). These cells lined the elongated neural elements. This cellular element has not been studied carefully at the ultrastructural level, but may be a precursor to the stellate cells mentioned previously. *Ligumia* and *Anodonta* showed more fluorescence associated with similar cells associatesd with neural components.

Interestingly, *Margaritifera* showed the only nonspecific FITC binding observed. In one of the controls, where FITC-labelled rabbit-antimouse serum was applied without first exposing the tissue to antiserum to CCS, the chitinous rods acquired a fluorescence differing from the autofluorescence when no antiserum was used. This nonspecific binding of the rabbit serum was seen only in the chitinous rods of *Margaritifera*, and was not observed in any other tissue components of this or any other species. Sections of *Margaritifera* which were exposed to either of the anticoncretion antisera typically showed bright fluorescence in the neural components and CCS.

Discussion

Reports describing the extent and distributions of CCS in mussel gills are very recent (Silverman et al. 1983*a*, 1983*b*, 1985) and likely reflect the past tendency to relate any type of molluscan calcium granule with a role in shell calcification, thus limiting studies on their ontogeny and physiology mainly to those found in the mantle and hepatopancreas. Studies concerning the pattern of distributiion of CCS in the gill tissues of freshwater mussels have now revealed an anatomical relationship between CCS and other gill structures. Silverman et al. (1983*b*) have demonstrated a correspondence between the pattern of deposition of CCS and neural elements in the gills of several unionid mussels. In some species at certain times of the year, CCS are abundant and may be observed macroscopically, imparting to the gill a brown coloration. The CCS appear as dense lines running anterior–posterior along the length of demibranchs of each gill pair (Silverman et al. 1985). The heavy depositions of CCS are adjacent to a well-defined system of nerve fibers which have branched off the major branchial nerve trunks and innervate the gill filaments at regular intervals through discontinuations in the chitinous rods (Silverman et al. 1983*b*; Dietz et al. 1985).

It was hoped that an antibody against a part of the CCS could be utilized to identify specific areas within the animal where assembly of the CCS was occurring, the logic being that cells labelled by the probe and containing no visible CCS or CCS in various stages of development could represent a site of assembly of CCS. Since these CCS can range up to 100 μm in size, it is unlikely they are produced by hepatopancreas cells and transported to the gills. We have demonstrated at least two cells showing FITC labelling after binding to the CCS antibody. One of these cells suggests concretion assembly activity (Fig. 14). Prior to this investigation, nervous tissue was not one of the areas suspected as being associated with CCS ontogeny (the nerves themselves may not be directly related). However, the similar antigenic reactions seen between CCS and nerve tract elements suggest that they share at least one common antigen. Evidence presented suggests that at least one of the organic components of the CCS may be common to nerve tract elements present in the gill. This information, combined with the appearance of the putative CCS-producing cells (calcium stellate cells), suggest the CCS are produced *in situ* and not at some other site, transported throughout the body, and deposited in the discrete pattern where they are found. The numerous "companion cells" (Fig. 18) that acquire the label and are closely associated with the nerves in *Elliptio* require further study to elaborate their relationship to the process of concretion production in mussel gills.

It was suggested in the earlier part of the century (see Ridewood 1903) that the so-called chitinous rods were composed in part of calcium phosphate. Silverman et al. (1983*a*) present histochemical and cytochemical evidence supporting this contention. When viewed in section with the electron microscope, some of the structures of the CCS and chitinous rods become virtually indistinguishable (compare Figs. 8 and 9). Both exhibit very similar reactions with specific histochemical labelling techniques for calcium and polysaccharide. Both appear to have the complex lamellar structure.

This investigation and a previous one (Silverman et al. 1983*b*) demonstrate considerable similarities between the gill anatomies and pattern of CCS distribution in several unionid species. More importantly, it demonstrates shared antigenic similarity between an important gill component and major anat-

FIG. 12. An electron micrograph of a demibranch of *Ligumia* seen in anterior–posterior cross section revealing a prominent nerve tract (T) with associated glial interstitial cell (G), muscle (M), and small concretions (C) within loose connective tissue (Ct). ×5160. FIG. 13. An electron micrograph of a *Ligumia* gill in the vicinity of a large nerve tract, revealing a neuron (arrowheads), glial interstitial cell (G), and a very large double concretion (C). × 11 000. FIG. 14. An electron micrograph of two stellate cells (S1 and S2) containing what appears to be concretion material in various stages of assembly. These cells are found in loose connective tissue (Ct) often adjacent to the water channel epithelium. ×5500. FIG. 15. Immunofluorescent labelling of two stellate cells (one of which is labelled S1) with anticoncretion mouse serum. The concretionlike material (arrowhead) within the cells is fluorescent. ×75. FIG. 16. Immunofluorescent labelling (arrowheads) of a cell type possibly representing neuronal cell bodies in the gill of *Anodonta* following treatment with anti-*Anodonta* concretion mouse serum. ×75. FIG. 17. Immunofluorescent labelling of chitinous rods (R), nerve tracts (T), and concretions (arrowheads) in the demibranch of *Margaritifera* following treatment with anti-*Anodonta* concretion mouse serum. ×75. FIG. 18. Unidentified regularly arranged small cells (arrowheads) displaying immunofluorescence in the demibranch of *Elliptio* following treatment with anti-*Anodonta* concretion mouse serum. ×70.

omical features associated with this component, even in the gills of the dissimilar *M. hembeli*. One factor which makes the gill concretion system rather interesting is that the substance in question, the organic component of gill CCS, is capable of binding calcium, and this component appears similar in all the species examined. In fact, there is some evidence indicating that the CCS may bind several divalent cations (Simkiss 1982; Silverman et al. 1983*a*), suggesting that it may be a general accumulator of divalent cations. It will be of interest to determine whether all pyrophosphate concretions found, from helminth calcium corpuscles to vertebrate arthritic crystallization, have any antigenic similarity. If this is the case, the ability of the mussel to mobilize the concretions (Silverman et al. 1985) may be an important model in the attempt to modify abnormal calcium phosphate deposition in vertebrates.

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