Ultrastructural study of the embryonic and larval shell of *Anodonta cygnea*

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An ultrastructural study of the embryonic and larval shell of *Anodonta cygnea* was carried out by light, polarization, and scanning electron microscopy, and X-ray diffraction analysis. A thin outer layer (cuticle), composed of β-keratin fibrils organized in parallel rows and enveloped by chitin material, was observed. The hooks, spines, and teeth are constituted by cuticular formations only. A calcareous inner layer presenting convergent prismatic monocrystals mainly of aragonite, which form prismatic structures around the pores and inside of irregular polygonal border lines, was also shown. Two clearly differentiated calcareous valves were already observed on very young embryos. The valvular pores totally covered by the cuticle may be involved in the metabolic cell exchange by penetrating cytoplasmatic extensions of the mantle cells. The irregular polygonal configuration observed on the internal surface of young decalcified cuticles supports the hypothesis of a role for the matrix as a regulator of shell formation, inducing the prismatic organization of the calcareous layer. Shell mineralization from the border lines to the center of each polygonal area may occur.

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La microstructure de la coquille de l'embryon et de la larve d'*Anodonta cygnea* a été étudiée au microscope photonique, au microscope polarisant, au microscope électronique à balayage ainsi que par analyse de diffraction aux rayons-X. La couche externe est mince (cuticule) et se compose de fibrilles de β-kératine en rangs parallèles entourées d'une substance chitineuse. Les crochets, les épines et les dents sont de nature essentiellement cuticulaire. Une couche interne calcaire contenant des monocristaux prismatiques convergents, surtout de l'aragonite, forme des structures prismatiques autour des pores et à l'intérieur de lignes de bordure irrégulières polygonales. Deux valvules calcaires bien différenciées sont déjà présentes chez les embryons très jeunes. Les pores des valvules sont complètement recouverts de cuticule et il se peut qu'ils assurent les échanges métaboliques cellulaires en pénétrant dans les prolongements cytoplasmiques des cellules du manteau. La configuration polygonique irrégulière à la surface interne de jeunes cuticules décalcifiées appuie l'hypothèse selon laquelle la matrice joue un rôle régulateur dans la formation de la coquille en déclenchant l'organisation prismatique de la couche calcaire. Il est possible qu'il y ait minéralisation de la coquille à partir des lignes de bordure vers le centre de chaque région polygonale.
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Introduction

Embryonic shells of molluscs are usually composed of two layers, one cuticular and one calcareous (Dawydoff 1928; Grassé *et al.* 1970; Fretter and Pilkington 1971; Spiess 1972; Haas *et al.* 1979; Kniprath 1981; Eyster 1986). The elemental composition and ultrastructural characteristics of shells prior to metamorphosis are little known (Eyster 1986). Nevertheless, some authors pointed out that the inner calcareous layer of bivalve larval shells is a crystalline-like formation, presenting many pores which extend perpendicularly through it but do not penetrate the cuticle (Giusti *et al.* 1975; Calloway and Turner 1978). The timing of initial mantle attachment to the shell may be relevant to the formation of these pores.

A comparative study was undertaken by Dinamani (1976) in bivalve species but very little was added to our knowledge of the structural and elemental composition of the shell layers. Giusti *et al.* (1975) and Durfort (1984) suggest that the external surface of the cuticular layer is covered with a great number of small villus-like processes organized in rows.

The main purpose of the present work is to study some ultrastructural aspects of the embryonic and larval shells of *Anodonta cygnea*.

Materials and methods

Adult gravid animals of *Anodonta cygnea* were collected, with some water and sediments from Mira lagoon in the north of Portugal, and kept in aerated and dechlorinated water at room temperature for a few days.

"Embryo" refers to all developmental stages occurring inside the capsule wall (vitelline membrane) and "larva" refers to all developmental stages outside the capsule wall.

For morphological studies embryos were observed *in vivo* by light microscopy. Schwejeninoff's technique (Ganter and Jolles 1969) was used to detect the presence and location of the calcareous layer in the very young embryonic shell. Semithin sections for light microscopy, stained with methylene blue Azur II, were used to investigate whether

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the presence of the mantle tissue lining the young embryonic or larval shell occurs. A crystallographic study was carried out, with an Ortoplan Leitz microscope, on embryonic and larval shells previously cleaned. The removal of soft parts, as well as organic tissue from the shell and the vitelline membrane, was carried out by bacterial treatments with tap water for 3 to 6 days in air contact. After organic decomposition the shells were cleaned with distilled water and air dried. For scanning electron microscopic (SEM) studies, the precleaned and air-dried embryonic and larval shells were mounted on SEM specimen stubs with conductive silver paint, coated with gold, and observed with a JEOL JSM-35C scanning microscope operated at 25 kV. The identification of calcium deposition in embryonic shells was also made by energy dispersive microanalysis (EDS). Some embryonic and larval shells were decalcified with very dilute HCl, thus removing the inner calcareous layer to study the internal surface of the cuticle and its protuberances. Over a period of 10 months, other larval shells were exposed to partial dissolution by water, to emphasize the irregular polygonal areas in the calcareous layers, since in normal conditions these areas are not very visible.

X-ray diffraction analysis of larval shells, previously cleaned by bacterial decomposition, was carried out using a Debye-Sherrer camera 11.4 cm in diameter and a Philips power supply PW 1130/00 operated at 40 kV and 20 mA (radiation CuK_α).

Results

Two very small valves with an approximate length of 150 μm having calcareous deposits and apparently without any attachment to visceral mass were observed on very young embryos by light microscopy (Fig. 1). Our observations indicate that the mantle lines the later embryo shells only. The early existence of calcareous material was shown by EDS and also by Schwejeninoff's reaction, which produced typical gypsum crystals on the areas of $CaCO_3$ deposition. It was also shown by EDS that several protuberances present on the edge of the larval shell (Fig. 2) do not contain $CaCO_3$. The valves of larval shell may reach 400 to 500 μm in length.

Two differentiated layers, a thin outer cuticular layer and a thicker inner calcareous layer adhering to it, were shown by SEM in the embryonic shell (Fig. 3). The calcareous layer seems to be composed of prismatic, needle-like monocrystals about 3 μm long (Figs. 3, 5, 12*c*). There are also many pores which extend perpendicularly through the calcareous layer but do not penetrate the cuticle (Figs. 3, 6). These pores, with an approximate diameter of 1.0 μm, are uniformly distributed in the shell (Figs. 6, 8).

A black cross, derived from light beam extinction, and birefringence zones are shown by polarization microscopy on very young embryonic shell, being clearer on larval shells even when shell rotation occurs (Figs. 4*a*, 4*b*). These observations were completed with X-ray diffraction analysis through larval shells (Figs. 11*a*, 11*b*), identifying some reflections such as 1.2, 1.7, 1.8, 1.9, 2.1, 2.3, 2.7, 3.4, and 4.23 Å of aragonite; 3.07 Å of calcite; 4.4–5.4 and 10.5 Å of chitin; and 6.6 Å of β-keratin.

By light and polarization microscopy (focusing in and out the objective lens), it was possible to distinguish polygonal lines in the calcareous surface of the shells precleaned by bacterial decomposition. The pattern is shown in Figs. 12*a* and 12*b*. Irregular polygonal line arrangement with nearly central pores was also observed by SEM (Fig. 6). These irregular polygonal lines are sites prone to fracture (Fig. 5). Polygonal border lines are similarly reproduced on the internal surface of very young embryonic cuticle subjected to decalcification with HCl (Fig. 7). It was proved by EDS analysis that these border lines do not present $CaCO_3$ deposits after decalcification.

Figures 9 and 10 show flexible cuticles, removed from larval shells, without pores but with specialized formations such as hooks, spines, and teeth on the edge. Figures 3, 8, and 12*c* show that this cuticle forms depressions over the pores of the calcareous layer.

Discussion

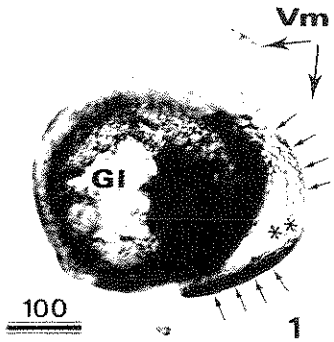
Glochidia were first extensively studied by Lillie (1895) and later by Lefevre and Curtis (1910*a*, 1910*b*, 1912). The ultrastructural aspects of molluscan shells are not yet very well known (Eyster 1986) and are the subject of some controversy.

The present study has demonstrated that the embryonic and larval shells are formed by a thin outer organic layer (cuticle) and a thicker inner calcareous layer with many pores not penetrating the cuticle. The presence of calcareous structures in very young embryonic stages suggests that in *A. cygnea* shell mineralization may begin shortly after the first organic shell material is secreted. This resembles the timing of initial shell mineralization of some gastropod species described by Eyster (1986). Any comparison with bivalve species is limited, since, according to Eyster (1986), the number of studies on the subject is insufficient. Nevertheless, LaBarbera (1974) pointed out that calcification begins before the organic component of the shell is completed in *Tridacna squamosa*. The timing of initial shell mineralization in *A. cygnea* will be determined by cytochemical methods and the results will be reported in another paper (F. Castilho and J. Machado, in preparation).

Inner layer

The extinction black cross and birefringence zones shown by polarization microscopy on young shells, even when shell rotation occurs, suggest that the calcareous layer is a crystalline structure with convergent crystals. X-ray diffraction analysis indicates that $CaCO_3$ deposition forms calcite, probably vaterite, but mainly aragonite crystals. The typical occurrence

FIG. 1. Light microscopy of very young embryo mounted *in vivo*. Gastrula (Gl), calcified valves (small arrows), cuticle (**), vitelline membrane (Vm). Bar, 100 μm. FIG. 2. Ventral extremity of the larval shell by scanning electron microscopy (SEM). Cuticular hook (*), cuticular spines (arrows). Bar, 10 μm. FIG. 3. Fractured edge of the embryonic valve observed by SEM. Cuticular layer (Cl), prismatic calcareous layer (*), pore (Po), cuticular niche (arrows). Bar, 1.0 μm. FIG. 4. Observation of the internal surface of larval shell by polarization light microscopy with crossed Nicol filters. A black cross derived from the light beam extinction is clearly visible and presents parallelism with polarization directions (crossed arrows) even if shell rotation occurs. (*a*) Shell in horizontal position, (*b*) shell in diagonal position. Bars, 100 μm. FIG. 5. Observations of the internal surface of fractured embryonic valve. Polygonal border lines of prismatic structures (arrows), prismatic aragonite monocrystals (*). Bar, 1.0 μm. FIG. 6. Internal surface of larval valve partially dissolved by water (SEM). Calcareous layer arranged in polygonal areas, with central pores (Po) and polygonal border lines (arrows). Bar, 5.0 μm. FIG. 7. The internal surface of embryonic cuticle where the calcareous layer was removed by very dilute HCl. Irregular polygonal areas (*), darkened border lines (arrows). Bar, 10 μm. FIG. 8. SEM external observations of the outer layer of glochidium. β-Keratin fibrils (arrows), cuticular niches (*). Bar, 1.0 μm.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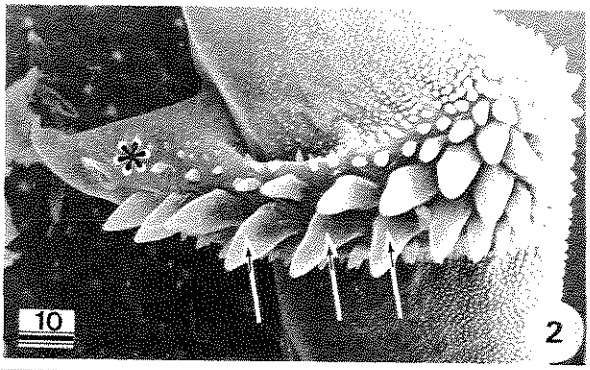
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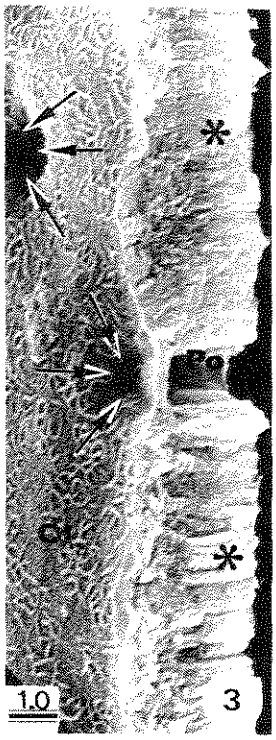
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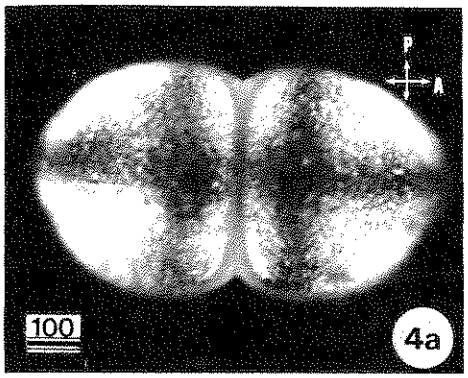
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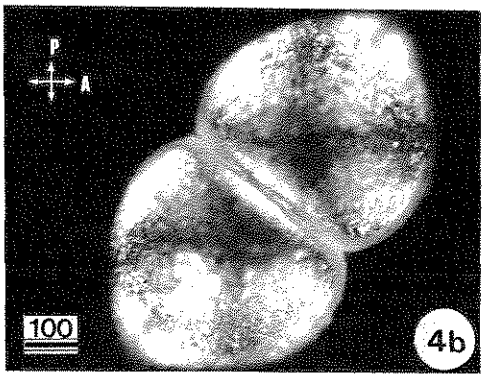
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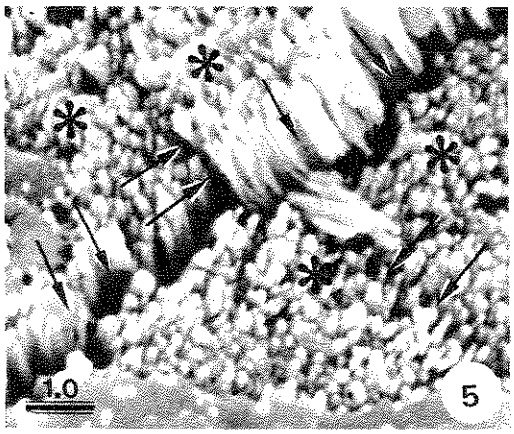
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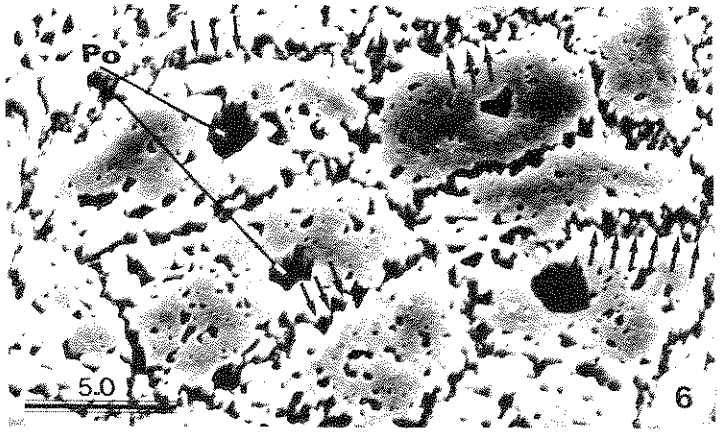
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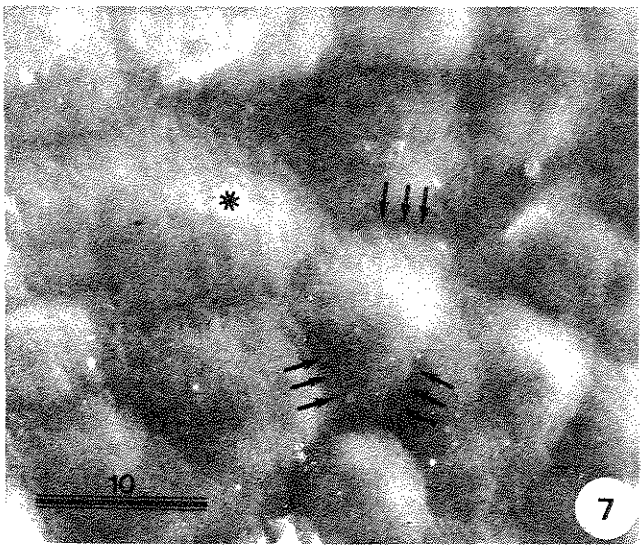
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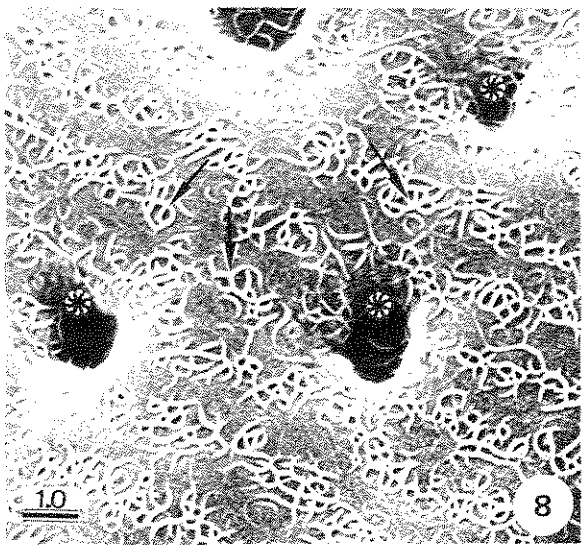
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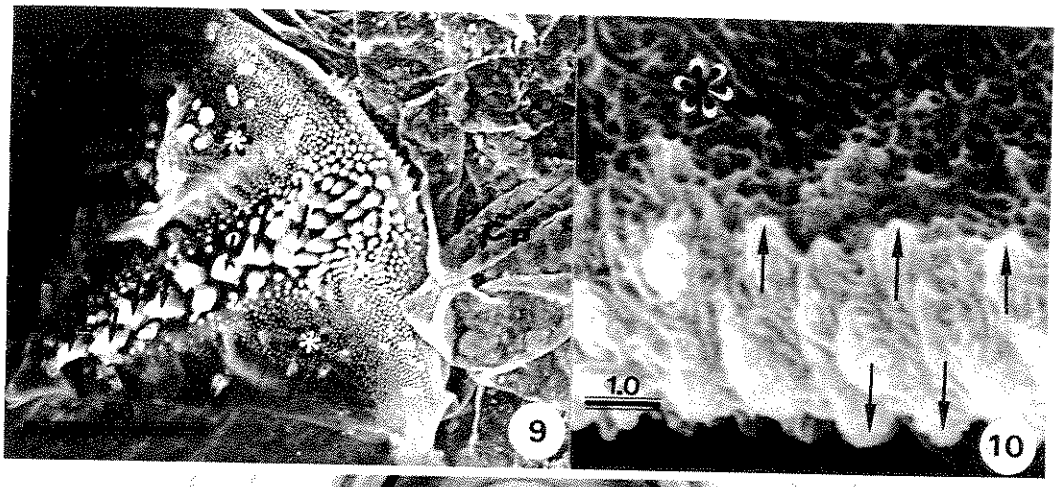

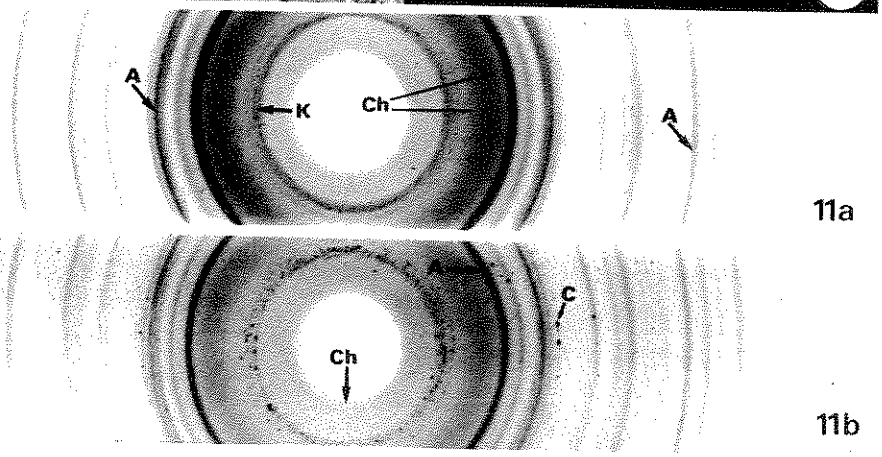

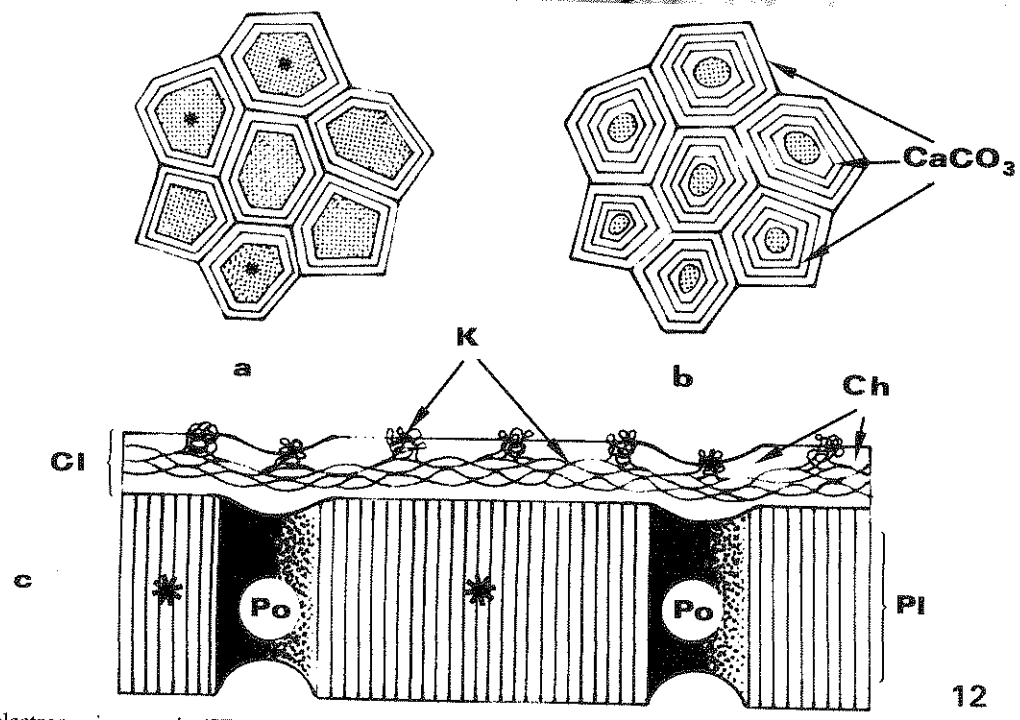

FIG. 9. Scanning electron microscopic (SEM) observations of the ventral cuticular extremity from a larval shell decalcified with very dilute HCl. Cuticular hook (*), cuticular spines (arrows), cuticular pellicle of the valve (Cp). Bar, 50 μm. FIG. 10. The cuticular edge from a larval shell exposed to long bacterial decomposition and to decalcification with very dilute HCl. Sclerotized β-keratin fibrils (*), cuticular teeth (arrows). Bar, 1.0 μm. FIG. 11. (*a*) X-ray diffraction analysis of a larval shell with rotation; (*b*) X-ray diffraction analysis without rotation. Aragonite, A (1.2, 1.7, 1.8, 1.9, 2.1, 2.3, 2.7, 3.4, and 4.23 Å); calcite, C (3.07 Å); chitin band, Ch (4.4–5.4 and 10.5 Å); protein-like β-keratin, K (6.6 Å). FIG. 12. Diagram of the development of the calcareous layer (Pl) by prismatic monocrystal formation over the internal surface of the cuticular layer (Cl). (*a*) Small polygonal crystalline areas with pores (*) in young valves; (*b*) enlargement of the crystalline areas ($CaCO_3$) towards the center of the polygon reducing the pore diameter; (*c*) chitin material (Ch), β-keratin fibrils (K), prismatic monocrystals (*), pores (Po).

of aragonite in *A. cygnea* is in accordance with observations of Eyster and Morse (1984), indicating that most early shell material is aragonitic in premetamorphic molluscan shells; for example *Crassostrea virginica* (Stenzel 1964), *Ilyanassa obsoleta* veliger (Ivester 1972), and *Aeolidia papillosa* (Eyster 1982). According to Stenzel (1964), aragonite crystals have the possible advantage of both greater structural strength and a lesser tendency to cleave. Thus, the present observations suggest that the calcareous layer in embryonic shell of *A. cygnea* is mainly formed of prismatic, needle-like monocrystals of aragonite in a convergent position.

The presence of irregular polygonal lines on the internal surface of this layer indicates that the monocrystals of aragonite are arranged into polygonal areas, forming irregular prismatic structures at the three-dimensional level. These prismatic structures are very similar to the ones described by Taylor *et al.* (1969) and Wise (1971) on adult animals of other species, by Waller (1980) on *Arca imbricata*, and by Roer and Dillaman (1984) on crustacean cuticles. The polygonal border lines also reproduced on the internal surface of very young cuticle leads us to think that the organic material may act as an active surface to control the crystal organization by epitaxy, according to Wilbur (1976, 1984) and Greenfield *et al.* (1984).

From the present observations it was also shown that the pores are placed for the most polygonal areas in a nearly central position. According to the structural reports of calcified cuticles in crustaceans by Roer and Dillaman (1984), it is very probable that these pores were completely filled by cytoplasmic extensions which emanate from the apical membrane of the mantle cells and terminate at the cuticle. Thus, it seems obvious that the appearance of the pores implicates a crystallization process from the polygonal border lines towards the center of each polygonal area. This process may be stopped subsequently by cytoplasmic extensions of mantle cells, which attach to the shell at the last embryonic stages, according to Wood (1974), Durfort (1984), and present observations. As to the probable function of the valvular pores, judging by the cytoplasmic extensions into the valves, we think that it is possible that metabolic exchange with the environment takes place.

Outer layer

The outer organic layer (cuticle) is a flexible pellicle without pores but with specialized formations such as hooks, spines, and teeth on the shell edge. These cuticular protuberances do not contain $CaCO_3$ in normal shells.

The micrographs show that the cuticle forms depressions over the pores, limited only to the calcareous layer. Our observations are in accordance with Giusti (1973), Giusti *et al.* (1975), and Calloway and Turner (1978), who described a cuticle without pores, but with small niches in *A. cygnea*, *A. cataracta*, and *Lampsilis radiata*. However, Rand and Wiles (1982) pointed out that these pores completely penetrate the mantle and the valves in *A. cataracta* and *A. implicata*. It is possible that Rand and Wiles (1982) observed an artifact as a result of dissolution of the cuticle with some chemical compound, since the cuticle is not visible in the respective micrograph.

So far as the elemental composition of organic material is concerned, the reticular distance of 6.6 Å obtained by X-ray diffraction analysis provides evidence for the presence of β-keratin with a fibrous texture, according to Lehnninger (1982). It also detected the existence of chitin material, since the chitin reflections of 4.7 and 10.3 Å referred to by Rudall (1963) correspond very approximately to the mean of the reflection bands, 4.4–5.4 and 10.5 Å, obtained in the present study. According to Rudall (1963) these bands are not well defined when an association of chitin with a protein occurs. Some preliminary cytochemical results emphasizing the presence of keratin and chitin material were also obtained (F. Castilho and J. Machado, in preparation). A chitinoid material was also noted by Ghose (1962) on the embryonic shell of *Achatina fulica*, but we do not believe that keratinoid material has been reported.

According to Giusti *et al.* (1975) and Durfort (1984), the external surface of the shell is covered with a great number of small villus-like processes. We think that the presence of microvilli on the shell surface is unlikely for at least four reasons. First, even after exposing the larvae to a long bacterial decomposition to remove all organic material, these fibrillar structures remain intact, suggesting sclerotized fibrillar structures. Secondly, since mantle formation occurs later than shell formation, most probably the "microvilli" do not penetrate the calcareous layer. Thirdly, these processes resemble anastomosed fibrils which are not typical of microvilli. Finally, our results suggest the presence of β-keratin with a fibrous texture enveloped by chitin material.

Thus, all these observations confirm that the villus-like processes may correspond to the β-keratin fibrils forming visible parallel rows in an homogeneous chitin material.

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CALLOWAY, C. B., and TURNER, R. D. 1978. New techniques for preparing shells of bivalve larvae for examination with the scanning electron microscope. Bull. Am. Malacol. Union Inc. pp. 17–24.

DAWYDOFF, C. 1928. Traité d'embryologie comparée des invertebrés. Masson, Paris.

DINAMANI, P. 1976. The morphology of the larval shell of *Saccostrea glomerata* (Gould, 1850) and a comparative study of the larval shell in the genus *Crassostrea sacco*, 1897 (Ostreidae). J. Molluscan Stud. **42**: 95–107.

DURFORT, M. 1984. Ultrasestrutura de les valves i del mantel larvari d'*Anodonta cygnea* L. Biologia del Desenvolupament (Societat Catalana de Biologia), **2**: 85–93.

EYSTER, L. S. 1982. Embryonic shell formation in the nudibranch *Aeolidia papillosa*. Am. Zool. **22**: 981.

———— 1986. Shell inorganic composition and onset of shell mineralization during bivalve and gastropod embryogenesis. Biol. Bull. (Woods Hole, Mass.), **170**: 211–231.

EYSTER, L. S., and MORSE, M. P. 1984. Early shell formation during molluscan embryogenesis, with new studies on the surf clam, *Spisula solidissima*. Am. Zool. **24**: 871–882.

FRETTER, V., and PILKINGTON, M. C. 1971. The larval shell of some prosobranch Gastropods. J. Mar. Biol. Assoc. U.K. **51**: 49–62.

GANTER, P., and JOLLES, G. 1969. Histochimie normale et pathologique. Gauthier-Villars, Paris.

GHOSE, K. C. 1962. Morphogenesis of the shell gland, lung, mantle and mantle cavity of the giant land snail, *Achatina fulica*. Proc. Malacol. Soc. Lond. **35**: 119–126.

GIUSTI, F. 1973. The minute shell structure of the glochidium of some species of the genera *Unio*, *Potomida* and *Anodonta* (Bivalvia, Unionacea). Malacologia, **14**: 291–301.

GIUSTI, F., CASTAGNOLO, L., MORETTI, F. L., and RENZONI, A. 1975. The reproductive cycle and the glochidium of *Anodonta cygnea* L. from Lago Tasimeno (Central Italy). Monit. Zool. Ital. **9**: 99–118.

GRASSÉ, P. P., POISSON, A. R., and TUZET, O. (*Editors*). 1970. Classe des bivalves ou Lamellibranches. *In* Zoologie. I. Invertebrés. Masson et Cie, Paris. pp. 410–452.

GREENFIELD, E. M., WILSON, D. C., and CRENSHAW, M. A. 1984. Ionotropic nucleation of calcium carbonate by molluscan matrix. Am. Zool. **24**: 925–932.

HAAS, W., KRIESTEN, K., and WATABE, N. 1979. Notes on the shell formation in the larvae of the Placophora (Mollusca). Biomineralisation, **10**: 1–8.

IVESTER, M. S. 1972. Ultrastructural study of larval shell formation in *Nassarius obsoletus*. Am. Zool. **12**: 717.

KNIPRATH, E. 1981. Ontogeny of the molluscan shell field: a review. Zool. Scr. **10**: 61–79.

LABARBERA, M. 1974. Calcification of the first larval shell of *Tridacna squamosa* (Tridacnidae: Bivalvia). Mar. Biol. (N.Y.), **25**: 233–238.

LEFEVRE, G., and CURTIS, W. C. 1910*a*. Experiments in the artificial propagation of freshwater mussels. Bull. U.S. Bur. Fish. **28**: 615–626.

——— 1910*b*. Reproduction and parasitism in the Unionidae. J. Exp. Zool. **9**: 79–115.

——— 1912. Studies on the reproduction and propagation of freshwater mussels. Bull. U.S. Bur. Fish. **30**: 109–201.

LEHNINGER, A. L. 1982. Principles of biochemistry. Worth Publishers, New York.

LILLIE, F. R. 1895. The embryology of the Unionidae. J. Morphol. **10**: 1–100.

RAND, T. G., and WILES, M. 1982. Species differentiation of the glochidia of *Anodonta cataracta* Say, 1817 and *Anodonta implicata* Say, 1829 (Mollusca: Unionidae) by scanning electron microscopy. Can. J. Zool. **60**: 1722–1727.

ROER, R., and DILLAMAN, R. 1984. The structure and calcification of the Crustacean cuticle. Am. Zool. **24**: 893–909.

RUDALL, K. M. 1963. The chitin/protein complexes of insect cuticles. *In* Advances in insect physiology. Vol. 1. *Edited by* J. W. L. Beament, J. E. Treherne, and V. B. Wigglesworth. Academic Press, London and New York. pp. 257–311.

SPIESS, P. E. 1972. Organogenese des Schalendrüsenkomplexes bei einigen coleciden Cephalopoden des Mittelmeeres. Rev. Suisse Zool. **79**: 167–226.

STENZEL, H. B. 1964. Oysters. Composition of the larval shell. Science (Washington, D.C.), **145**: 155–156.

TAYLOR, J. D., KENNEDY, W. J., and HALL, A. 1969. The shell structure and mineralogy of the Bivalvia, introduction: Nuculacea-Trigonacea. Bull. Br. Mus. (Nat. Hist.) Zool. No. 3. pp. 1–125.

WALLER, T. R. 1980. Scanning electron microscopy of shell and mantle in the order Arcoidea (Mollusca: Bivalvia). Smithson. Year. **313**: 1–58.

WILBUR, K. M. 1976. Recent studies of invertebrate mineralization. *In* The mechanisms of mineralization in the invertebrates and plants. *Edited by* N. Watabe and K. M. Wilbur. University of South Carolina Press, Columbia, SC. pp. 79–108.

——— 1984. Many minerals, several phyla, and a few considerations. Am. Zool. **24**: 839–845.

WISE, S. W., JR. 1971. Shell ultrastructure of the Taxodont Pelecypod *Anadara notabilis* (Röding), Eclogae Geol. Helv. **64**(1): 1–12.

WOOD, E. M. 1974. Development and morphology of the glochidium larva of *Anodonta cygnea* (Mollusca: Bivalvia). J. Zool. (Lond.), **173**: 1–13.