The Structure and Calcification of the Crustacean Cuticle[1]

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SYNOPSIS. The integument of decapod crustaceans consists of an outer epicuticle, an exocuticle, an endocuticle and an inner membranous layer underlain by the hypodermis. The outer three layers of the cuticle are calcified. The mineral is in the form of calcite crystals and amorphous calcium carbonate. In the epicuticle, mineral is in the form of spherulitic calcite islands surrounded by the lipid-protein matrix. In the exo- and endo-cuticles the calcite crystal aggregates are interspersed with chitin-protein fibers which are organized in lamellae. In some species, the organization of the mineral mirrors that of the organic fibers, but such is not the case in certain cuticular regions in the xanthid crabs. Thus, control of crystal organization is a complex phenomenon unrelated to the gross morphology of the matrix.

Since the cuticle is periodically molted to allow for growth, this necessitates a bidirectional movement of calcium into the cuticle during postmolt and out during premolt resorption of the cuticle. In two species of crabs studied to date, these movements are accomplished by active transport effected by a Ca-ATPase and Na/Ca exchange mechanism.

The epi- and exocuticular layers of the new cuticle are elaborated during premolt but do not calcify until the old cuticle is shed. This phenomenon also occurs *in vitro* in cuticle devoid of living tissue and implies an alteration of the nucleating sites of the cuticle in the course of the molt.

INTRODUCTION

While the crustacean cuticle has been the subject of study for over 250 years (Réaumur, 1712, in Drach, 1939), the focus of those investigations has generally been concerned with the process of molting. Our approach will be slightly different; we will deal with the exoskeleton of the Crustacea as a mineralized tissue that is made particularly interesting by the fact that its structure is affected by a cyclic molting process.
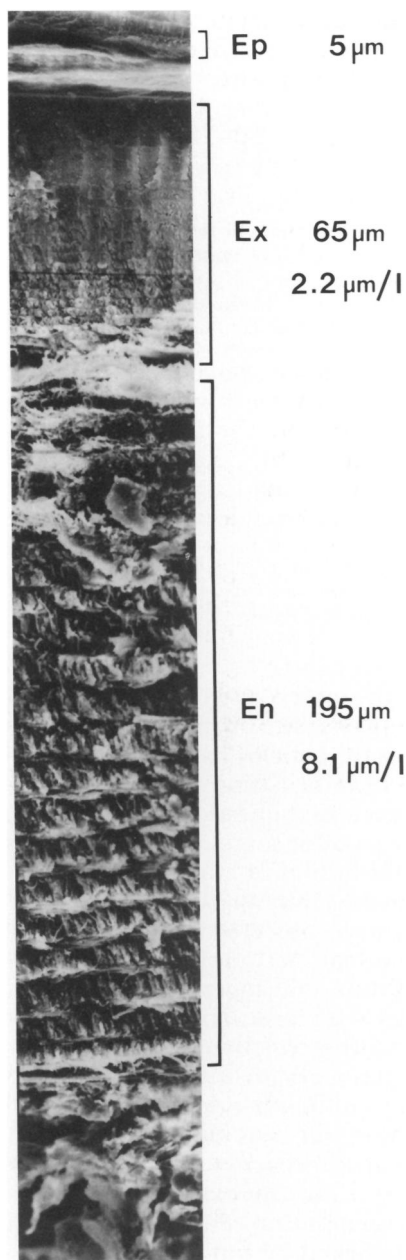
When investigating any mineralized tissue, one must address some basic problems: 1) the chemical nature and crystalline form of the mineral; 2) the nature and form of the organic matrix; 3) the relationship between the organic and inorganic components of the tissue and the influence of the matrix on crystal morphology; 4) the sources of mineral for deposition; 5) the pathways for mineral movement into or out from the mineralized structures; 6) rates of mineral deposition; and 7) the nature and location of nucleation sites

within the matrix and mechanisms for control or cessation of crystal growth. The crustacean cuticle has provided a rich source of information with respect to each of these central questions. In addition, the Crustacea offer some unique problems for those interested in biomineralization.

Since the mineralized exoskeleton of the Crustacea is subjected to periodic molting, bidirectional net movement of mineral during different stages of the molt cycle is necessary. This situation is in sharp contrast to other calcifying systems in which net accretionary growth patterns or slightly shifting equilibria are the rule. Furthermore, crustaceans demonstrate drastic temporal differences within the same tissue with regard to the extent of and capacity for mineralization. Such temporal differences, marked by rapid and discrete transitions, allow one to ask very specific and answerable questions about the control of nucleation and mineralization.

The subject of crustacean cuticles is as diverse as is the taxon and, hence, an encyclopedic overview is impossible. We will restrict our review, in large part, to the decapod crustaceans although examples from other orders will serve to remind us of the hazards of sweeping generalizations.

] Ep   5 μm

Ex   65 μm

2.2 μm/l

En   195 μm

8.1 μm/l

FIG. 1. Montage of the mineralized cuticular layers of *Carcinus maenas* carapace (from Roer 1979). En = endocuticle, Ex = exocuticle, Ep = epicuticle. Numbers represent the total thickness of the individual layers and the thickness of endo- and exocuticular lamellae (l).

STRUCTURE OF THE DECAPOD INTEGUMENT

Basic cuticular structure

The integument of the decapod Crustacea is, in general, comprised of a rigid exoskeleton or cuticle underlain by the cellular hypodermis (Richards, 1951). The cuticle is not homogeneous, but contains four discrete layers. Despite the fact that this observation was made more than a century ago (Williamson, 1860) and has been the subject of numerous subsequent studies, the nomenclature of the four layers of the cuticle varies from author to author. The terminology of Travis (1963), however, has come to be widely accepted and will be used exclusively in the present work. These layers from the most external to the most internal are: the epicuticle, the exocuticle, the endocuticle and the membranous layer (Fig. 1).

The epicuticle is the outermost and thinnest layer of the cuticle. It consists of tanned lipoprotein impregnated with calcium salts (Travis, 1955a). It is bilaminar, with the basal layer pervaded by mineral-filled canals normal to the surface (Hegdahl *et al.*, 1977c).

The exocuticle immediately underlies the epicuticle. It is composed of chitin-protein fibers stacked in layers of continuously changing orientation (Travis, 1955a; Green and Neff, 1972). In *Cancer pagurus* 34% by weight of the organic component is chitin (Welinder, 1975b). The exocuticle is hardened by quinone tanning and calcification (Travis, 1955a), with the mineral crystals situated between the fibers (Bouligand, 1970; Hegdahl *et al.*, 1977b).

The endocuticle is the thickest and the most heavily calcified layer of the cuticle (Travis, 1955a, 1965). The endocuticle, like the exocuticle, is composed of horizontal lamellae of chitin-protein fibers with continuously changing orientation (Green and Neff, 1972; Hegdahl *et al.*, 1977a), the organic material being 73% chitin by weight in *Cancer pagurus* (Welinder, 1975b). The endocuticle is apparently not tanned, but hardened solely by Ca salts (Travis, 1955a).

The membranous layer is the innermost layer of the cuticle and is in contact with the hypodermis. It consists of chitin and protein, the chitin representing 74% of the organic material in *Cancer pagurus* (Welinder, 1975b), but contains no mineral (Travis, 1955a).

The mineral in the outer three layers of the cuticle is $CaCO_3$ in the form of calcite or poorly crystalline, amorphous calcium carbonate (Travis, 1963).

The hypodermis is heterogeneous, having numerous cell types arranged in three principal layers (Travis, 1955a, b, 1957, 1965). The layer in contact with the cuticle and apparently responsible for its formation is the outer epithelial layer or the cuticle secreting cells of Green and Neff (1972). The outer epithelial layer is one cell thick and may range from squamous to columnar as will be discussed below. Beneath the outer epithelium is the sub-epithelial connective tissue layer (Travis, 1955a, b) which consists of oval reserve cells, blood sinuses with hemocytes, lipoprotein cells (Sewell, 1955) and pigment cells (Green and Neff, 1972). Proximal to the connective tissue layer is the inner epithelium which is similar to the outer epithelium but reduced in size. The inner epithelium is bounded on its inner margin by a basement membrane with the exception of those regions covering the branchial chamber, where the inner epithelium elaborates a thin, non-calcified cuticle resembling the outer epicuticle in other respects (Skinner, 1962).

The cuticle is pervaded by vertically running pore canals first described by Valentin (1837, in Richards, 1951). Further examination has revealed these to be cytoplasmic extensions of the outer epithelial cells which emanate from the apical cell borders, extend through the membranous layer, endocuticle and exocuticle, and terminate at (Travis, 1963; Green and Neff, 1972) or in (Hegdahl *et al.*, 1977c) the epicuticle. The pore canals may have a typical helical or twisted ribbon morphology (Drach, 1939; Neville *et al.*, 1969) with the pitch of the helix being equal to the lamellar period (Drach, 1939). These pore canals are extremely numerous. In *Cancer pagurus*

there are an estimated 150,000–220,000 pore canals per mm^2 (Hegdahl *et al.*, 1977a); in *Carcinus maenas* there are about 950,000/mm^2 (Roer, 1980); and *Orconectes virilis* there are 50–90 pore canals emanating from each epithelial cell or about 4,000,000/mm^2 (Travis, 1963). Thus, the cuticle is in close contact with the hypodermis at all levels and should be considered living tissue.

The molt cycle

Rather detailed accounts of the phenomena related to the molt cycle of the Crustacea come from the last century (Vitzou, 1882), but the first method for describing the molt cycle that could be applied to the Crustacea in general did not appear until much later (Drach, 1939). According to this method, the molt cycle is divided into five stages (A–E), with further subdivisions within each stage. Thus the postmolt period corresponds to stages A_1, A_2, B_1, B_2, C_1, C_2, and C_3; intermolt is stage C_4; premolt is comprised of stages D_0, D_1', D_1'', D_1''', D_2, D_3, and D_4; and the act of ecdysis or molting is stage E (Drach and Tchernigovtzeff, 1967).

The intermolt integument

Most adult decapods spend the majority of the molt cycle in the intermolt condition (stage C_4), molting only once or twice yearly (Passano, 1960). The cuticle has its four layers complete and is fully calcified (Fig. 2). The hypodermis is in its most reduced state at this time. The epithelial cells in contact with the cuticle are extremely squamous (Green and Neff, 1972) having a height of only 9 μm in *Orconectes virilis* (Travis, 1965). The secretory activity of the Golgi of the epithelial cells is at a minimum (Chassard-Bouchaud and Hubert, 1973; Hubert and Chassard-Bouchaud, 1978) and the endoplasmic reticulum is reduced (Green and Neff, 1972). The pore canals are still structurally intact and contain cytoplasm (Green and Neff, 1972) or may be partially filled with calcite crystals (Travis, 1963; Travis and Friberg, 1963; Hegdahl *et al.*, 1977a, b, c).

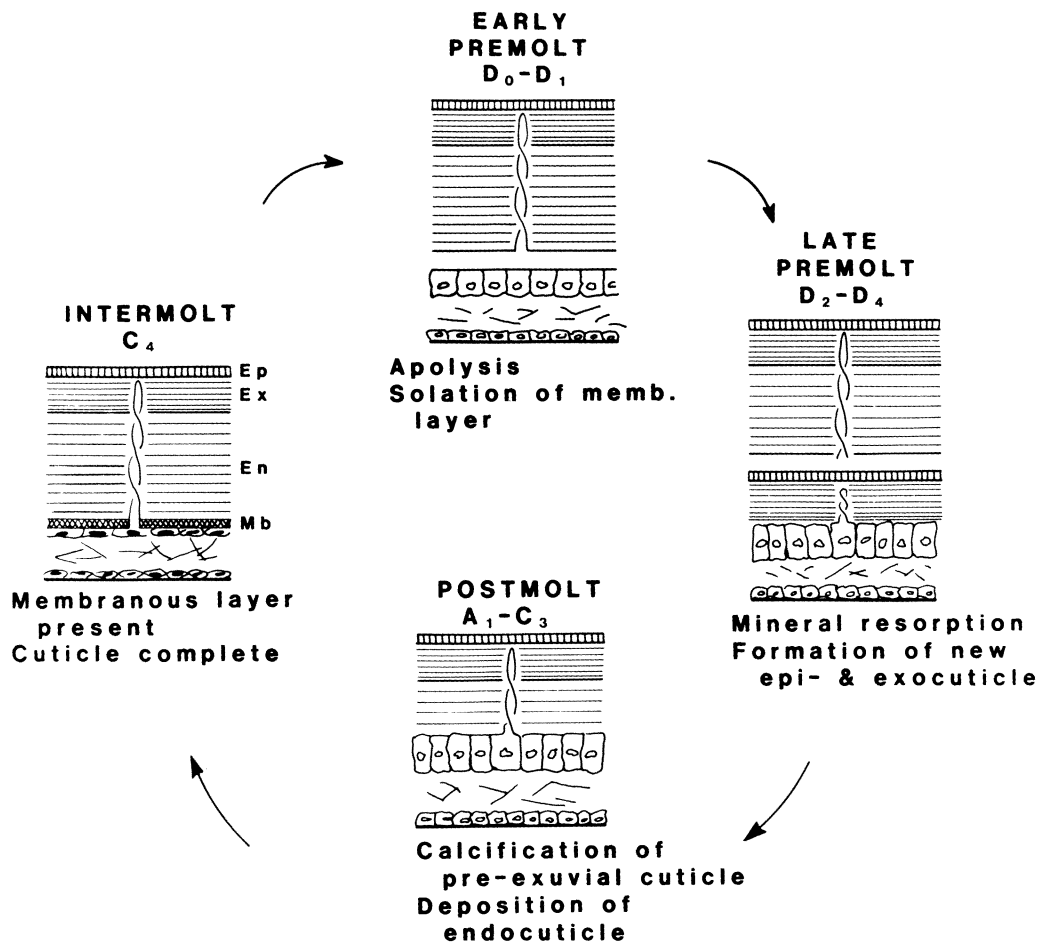The hypodermis is not completely inac-

FIG. 2. Schematic representation of the cuticular events associated with progression through the molt cycle. Ep = epicuticle, Ex = exocuticle, En = endocuticle, Mb = membranous layer.

tive, however, for a peak in RNA synthesis is seen during C_4 (Skinner, 1966) and the reserve cells of the highly vacuolated and reduced connective tissue are engaged in the storage of materials for the ensuing premolt period (Travis, 1957).

The premolt integument

The onset of premolt (stage D_0) is marked by apolysis, or the separation of the hypodermis from the cuticle through the action of secreted chitinase, chitobiase and protease (Jeuniaux, 1959a, b; Bade and Stinson, 1978) which cause the solation of the membranous layer. Apolysis results in the severing of the pore canals (Green and Neff, 1972) (Fig. 2). The outer epithelial cells begin to increase in height and complexity.

Through the premolt period they change from squamous to columnar. In *Orconectes* they double their height between D_0 and D_2, reaching a maximum of 54 μm (six times their height in C_4) by D_3 (Travis, 1965); the maximal height is about 56 μm in *Carcinus* (Roer, 1980). The Golgi begin to secrete a proteinaceous paracrystalline substance and an increase in smooth endoplasmic reticulum associated with mitochondria is observed (Hubert and Chassard-Bouchaud 1978). Mitotic activity is apparent in the epithelial cells during stages D_0, D_1' and D_1''; and there are peaks in O_2 consumption, protein synthesis and chitin synthesis during stage D_2 (Skinner, 1962, 1966; Stevenson, 1972).

Premolt is also the period during which

the matrix of the new epicuticle and exocuticle is laid down beneath the old cuticle. Epicuticular deposition takes place during stage D_1, and exocuticular deposition begins at stage D_2. Because these matrices are deposited before the molt, they are referred to as the pre-exuvial layers (Drach, 1939). Although the organic matrix of the epi- and exocuticle is laid down pre-exuvially, these layers do not calcify until after the molt (Paul and Sharpe, 1916; Travis, 1963; Travis and Friberg, 1963). The epicuticle is, however, tanned before the molt (Krishnan, 1951).

Concomitant with pre-exuvial deposition is the partial resorption of both the mineral and organic portions of the old cuticle (Drach, 1939; Travis, 1965; Roer, 1980). In *Gecarcinus lateralis*, more than 75% of the cuticle is resorbed (Skinner, 1962); while in *Panuliris argus* only about 20% of the carapace is resorbed, the endocuticle being completely resorbed in some areas and the exocuticle being partially resorbed in certain regions (Travis, 1955a). In *Carcinus*, 15–20% of the mineral is resorbed during premolt (Graf, 1978). Resorptive activity reaches a maximum during stage D_2 (Drach, 1939; Green and Neff, 1972), and results in a rise in hemolymph Ca^{++} concentration (Robertson, 1960) and HCO_3^- concentration. This causes a slight alkalosis of the hemolymph, the pH rising from 7.9 to 8.1 in *Astacus leptodactylus* (Dejours and Beekenkamp, 1978).

The postmolt integument

The initial events following the molt are the tanning of the exocuticle and the calcification of the pre-exuvial layers. The exocuticle is tanned by quinone formation from dihydroxyphenols under the action of a polyphenol oxidase transported to the cuticle from the epithelial cells (Krishnan, 1951; Travis, 1957; Vacca and Fingerman, 1975a, b). The pre-exuvial layers begin to calcify during stage A_1. The first crystals of $CaCO_3$ are evident in *Carcinus* 10 hr after the molt (Drach, 1937); and in *Astacus fluviatilis* the rate of Ca deposition reaches a peak two days postmolt (Welinder, 1975a). Calcification of the epi- and exocuticles begins in the most external regions

and proceeds proximally (Travis and Friberg, 1963; Bouligand, 1970). Mineral apparently reaches the outer portions of the cuticle via the pore canals. Calcium is concentrated in the distal portions of the epithelial cells and appears to be extruded in vertical rows corresponding in position to the pore canals of the new cuticle (Travis, 1957, 1963, 1965; Travis and Friberg, 1963; Chockalingham, 1971). Stage B is marked by the onset of endocuticle deposition; here calcification is concomitant with matrix formation, each organic lamella being mineralized as it is laid down (Drach, 1939; Travis, 1957, 1963, 1965; Travis and Friberg, 1963).

Mineral deposition continues through postmolt. Calcification spreads throughout the exocuticle and eventually is found to pervade the walls of the pore canals (Travis, 1963; Travis and Friberg, 1963). Calcite crystals may finally be seen within the lumina of the pore canals as the cell processes apparently recede to be replaced with mineral (Travis, 1963; Travis and Friberg, 1963; Hegdahl *et al.*, 1977a, b, c). The end of postmolt is marked by the deposition of the membranous layer during stage C_3 and the cessation of net calcium deposition (Passano, 1960).

The postmolt changes in the hypodermal cells are also marked. By one day postmolt the dedifferentiation of these epithelial cells has already begun (Green and Neff, 1972). In *Orconectes*, the epithelial cells decrease from their maximal height of 54 μm to 21 μm within two days of ecdysis, the decrease continuing until a minimum is reached at intermolt (Travis, 1965). The secretory activity of the Golgi and abundance of endoplasmic reticulum also decreases through postmolt to a virtual absence in C_4 (Hubert and Chassard-Bouchaud, 1978).

THE RELATIONSHIP BETWEEN MINERAL AND ORGANIC COMPONENTS

The epicuticle

The epicuticle, as mentioned above, differs from the exo- and endocuticles in its lack of chitin and lamellar organization. This organizational difference is manifested in the orientation of the mineral. A transmission electron microscopic and

radiographic study by Hegdahl and co-workers (1977c) on the epicuticle of Cancer pagurus revealed that mineral was restricted to vertical canals, 100–250 nm in diameter, in the proximal layer. These canals are presumably the distal terminations of the pore canals. The mineral was in the form of $CaCO_3$ crystals or crystal aggregates and were distributed unevenly and surrounded by epicuticular tissue.

We have also observed a non-homogeneous and discontinuous distribution of mineral in the epicuticle of Carcinus maenas. While a 24 hr treatment of Carcinus cuticle with 5.25% sodium hypochlorite results in a complete removal of the epicuticle, brief treatment renders the tissue only partially anorganic and reveals mineral clumps which are spherulitic aggregates (Figs. 3 and 4). The loss of these aggregates following the complete hypochlorite removal of the organic material suggests that these are both discrete from one another and from the mineral components of the underlying exocuticle.

The exocuticle and endocuticle

The exo- and endocuticles have in common a regular array of chitin-protein fibers arranged in lamellae defined by a rotation in the orientation of parallel sheets of these fibers (Mutvei, 1974 and Figs. 6 and 16). The two layers differ in the lamellar spacing with the interlamellar distance being less in the exocuticle than the endocuticle (approximately 2 μm and 8 μm respectively in Carcinus; Dillaman and Roer, 1980).

Mineral first appears as small crystals of calcite observed around the perimeters of the pore canals and then throughout the chitin-protein fibrillar network (Travis, 1963; Yano, 1975). Calcification of the exocuticle appears to be concentrated initially in the periphery of and interstices between prisms defined by the margins of

the hypodermal cells underlying the cuticle (Drach, 1939; Travis, 1963; Hegdahl et al., 1977b; Giraud-Guille and Quintana, 1982). When calcification is complete, however, the distribution of mineral within these layers is relatively homogeneous (Hegdahl et al., 1977a, b; Giraud-Guille and Quintana, 1982).

Although Bouligand (1970) claims that there is no orientation of the calcite crystals with respect to the chitin-protein fibers in Carcinus, other evidence points to the contrary. The crystal aggregates clearly are aligned with the organic fibers as determined by transmission electron microscopy in Gaetice depressus (Yano, 1975) and in Cancer pagurus (Hegdahl et al., 1977a, b), and by secondary ion mass spectroscopy and X radiodography in Carcinus (Giraud-Guille and Quintana, 1982). Indeed, Hegdahl and co-workers (1977a) have clearly demonstrated rod-shaped crystal aggregates interspersed with the chitin-protein fibers.

We have further investigated, by scanning electron microscopy, the orientation of the mineral components and the relation of this orientation to the organic fibers. The organization of the cuticle is made more apparent by the comparative observations of untreated cuticles, EDTA-decalcified cuticles and those rendered anorganic by sodium hypochlorite. In Carcinus (Figs. 5–8) the composite nature of the exoskeleton is clearly seen as the interspersion of the organic fibers with similarly oriented crystal aggregates. The orientation of the chitin-protein fibers demonstrates a continuous spiral rotation with each lamella corresponding to a 180° deviation (Fig. 6). Throughout the exo- and endocuticles of Carcinus, the mineral is found as long rod-shaped elements displaying the same continuously changing orientation as seen in the chitin-protein

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PLATE 1.   Scanning electron micrographs of Carcinus maenas cuticle. FIG. 3. Partially anorganic epicuticle. ×700. FIG. 4. Partially anorganic epicuticle. ×5,500. FIG. 5. Untreated endocuticle. Note pore canals (arrow). ×10,000. FIG. 6. Decalcified endocuticle. Note pore canals (arrow). ×5,000. FIG. 7. Anorganic endocuticle. Note the pore canal space (ps). ×10,000. FIG. 8. Anorganic endocuticle. Note the individual spherulitic elements comprising rod-shaped crystal aggregates (arrow) ×40,000.
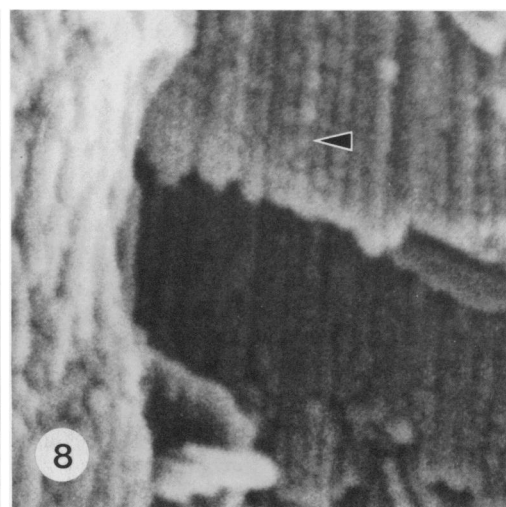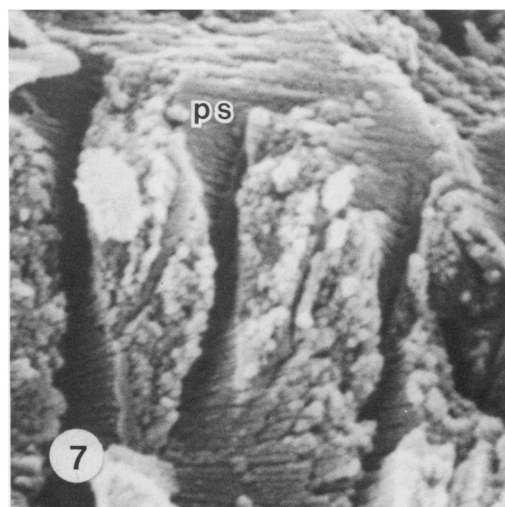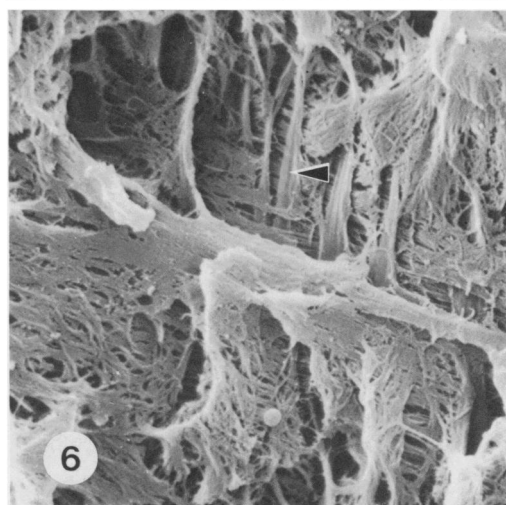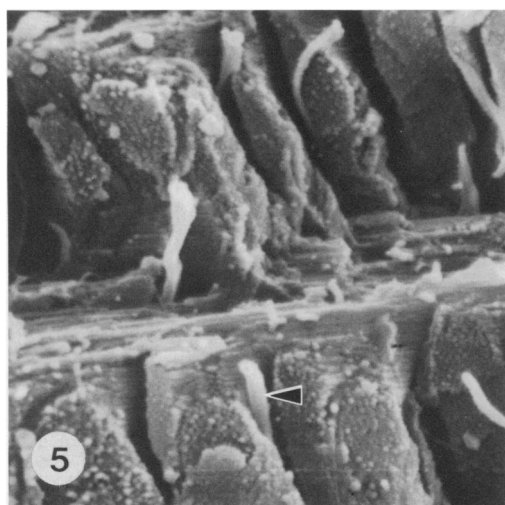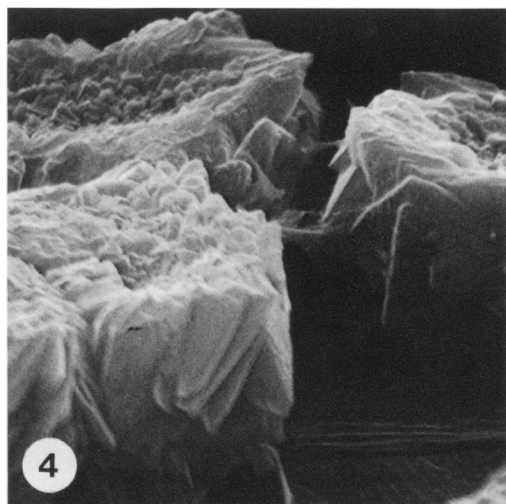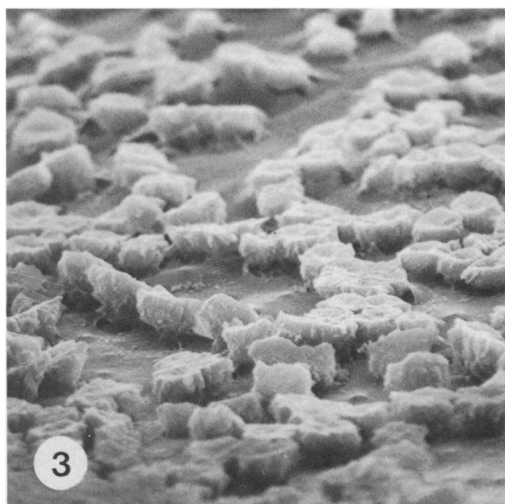
fiber network (Figs. 7 and 8). Inspection of the mineral rods at higher magnification reveals that these are aggregates of calcite spherulites strung together in the orientation of the organic fibers (Fig. 8).

While this pattern of mineralization appears to obtain in all of the Cancridae and Portunidae thus far investigated, the situation in the Xanthidae appears to vary somewhat. The overall appearance of the untreated cuticles of the xanthids is quite similar to that of the other families (Fig. 9) but, macroscopically, has a porcelainous character and, in general, is considerably thicker, particularly in the stone crab, *Menippe mercenaria*. Likewise, decalcified preparations of *Menippe* or *Neopanope texana* cuticle show no major differences from those of other crabs studied (Fig. 10). Upon bleach treatment, however, the anorganic cuticles demonstrate regions of mineral in which the orientation and structure differ markedly from one another. These different regions of mineral in the anorganic *Menippe* cuticle are not merely surface features nor artefacts resulting from the hypochlorite treatment. The atypical regions are cone-shaped and evident at the inner surface, at the exocuticular/endocuticular boundary and throughout the intervening endocuticle (Figs. 11 and 12). This pattern is not evident in *Carcinus* (Fig. 13), but is likewise apparent in *Neopanope*, the other xanthid we have observed to date (Fig. 14).

The ultrastructure of the untreated and the decalcified cuticle of *Menippe* shows patterns of organization very similar to that of *Carcinus* even as far as the interlamellar distances of the exo- and endocuticles (Figs. 15 and 16). In the regions of the anorganic cuticle not contained within the cone-shaped structures, the mineral morphology and orientation is also very similar to

Carcinus: minute spherulites are organized in strands interspersed between the organic fibers (Fig. 17). In the cone-shaped regions, however, the organization of the mineral is not as well defined by the chitin-protein fiber orientation (Figs. 18 and 19). The crystal aggregates form spherules of much larger diameter (0.25 μm as compared to 0.05 μm) and demonstrate a far less organized orientation (Fig. 20). The fusion of these aggregates with one another is also apparently not as robust as the fiber-oriented region, as the large spherules are often displaced by hypochlorite treatment.

The lack of correlation in the xanthids between the chitin-protein fiber orientation and, in certain regions, the orientation of the crystal aggregates precludes a simple hypothesis for the control of mineral morphology and organization. Thus it does not appear that calcitic spherules merely nucleate randomly upon the organic fibers and simply fill the spaces between them. It is clearly not solely the orientation of the chitin-protein lamellae which governs the orientation of the crystal aggregates. While the cone-shaped regions of mineral in *Menippe* and *Neopanope* do not appear to correspond to any morphological or anatomical features of the integument, clearly the nature of the matrix or of the cells underlying the matrix differs in these regions. It may be that while the organic fibers appear uniform, they may possess nucleation sites whose characteristics vary from place to place.
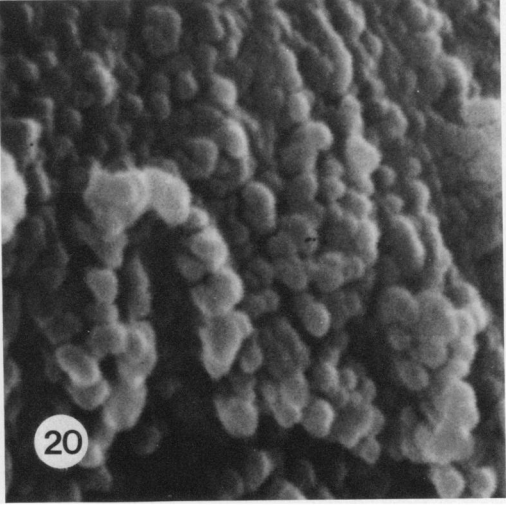
SOURCES OF MINERAL FOR DEPOSITION
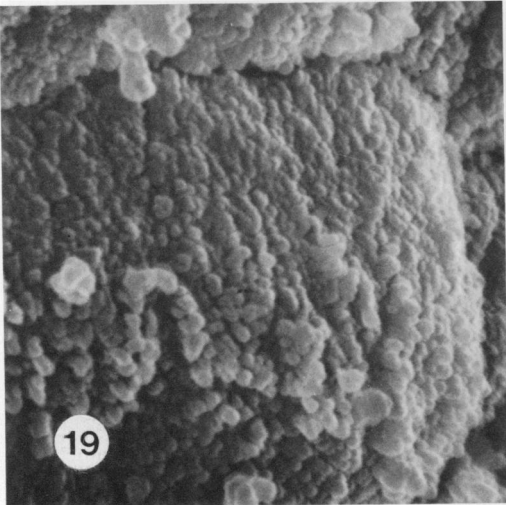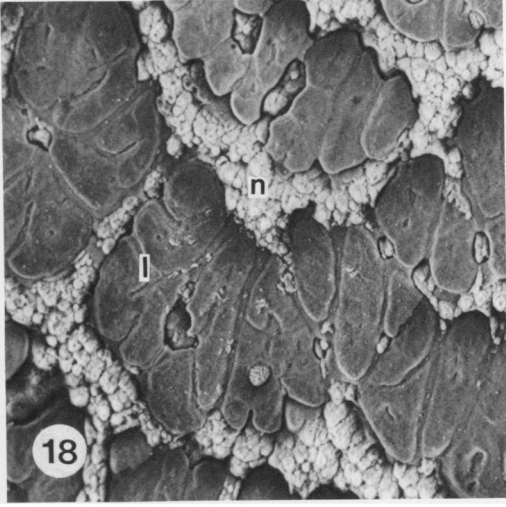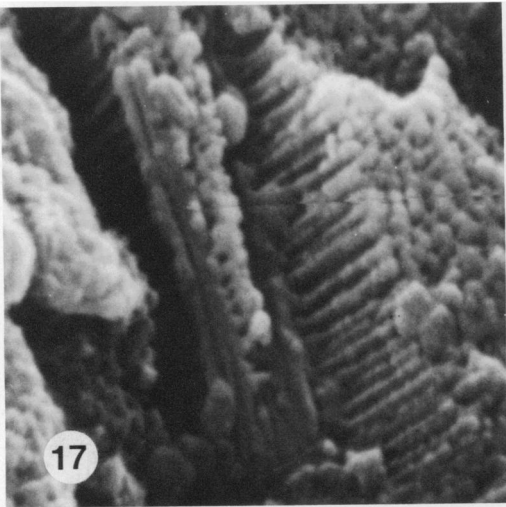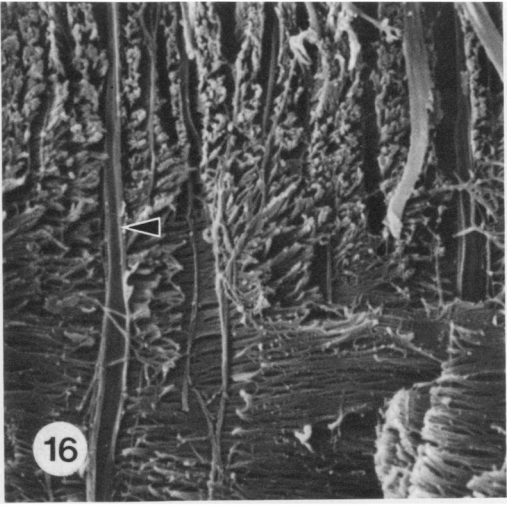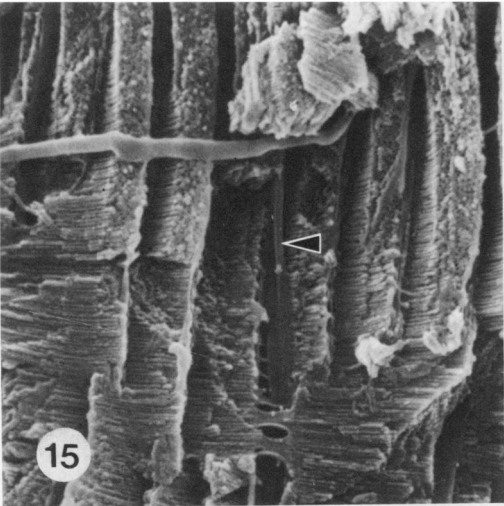
Most of the Crustacea possess some means for the retention and storage of part of the calcium resorbed from the cuticle during premolt. The postmolt mobilization of this calcium provides an endogenous source of calcium for the calcification

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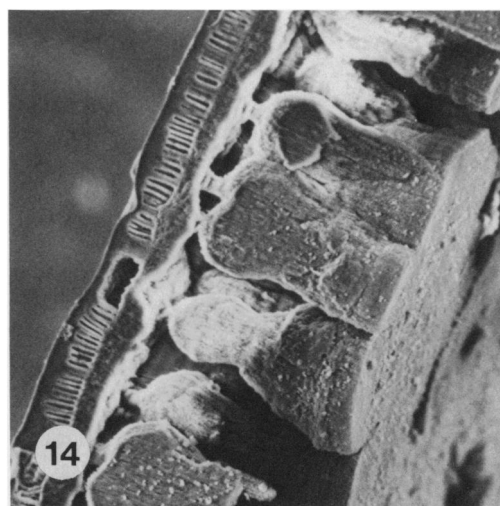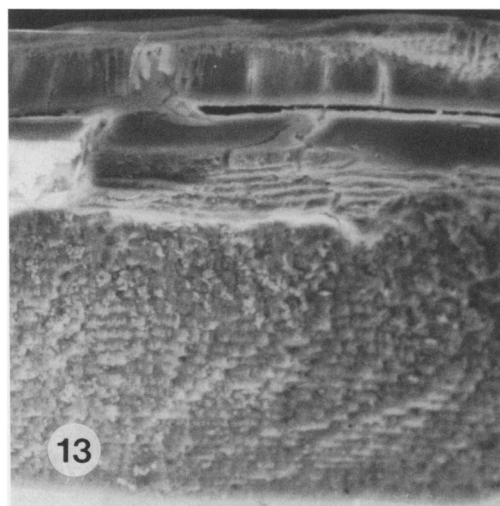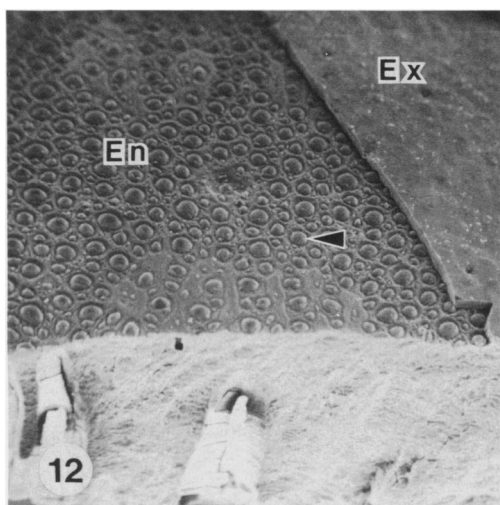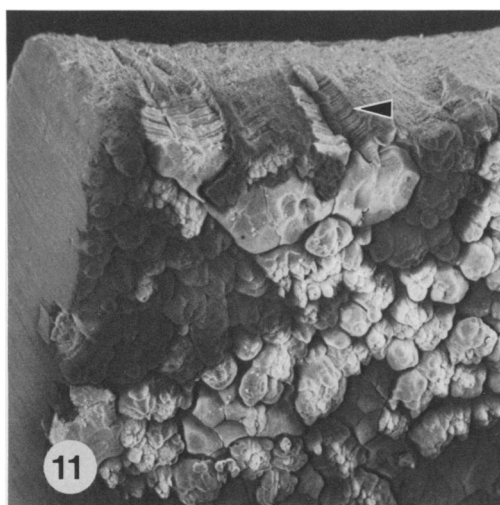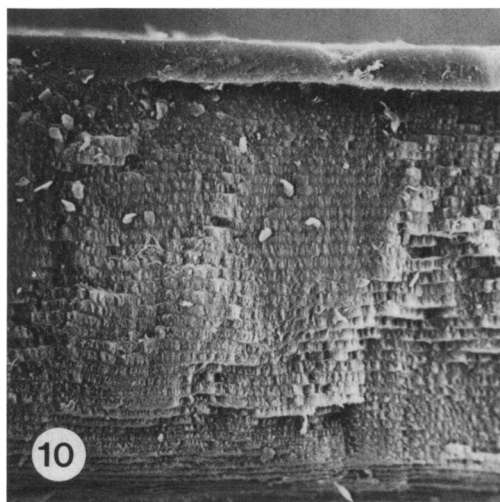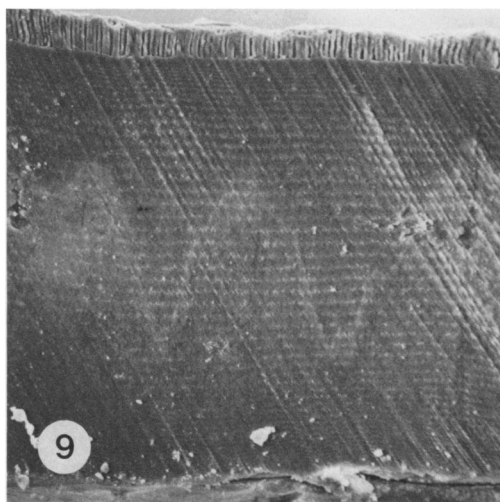
of the new cuticle. The storage sites may be in the form of gastroliths, the calcareous concretions beneath the cuticle lining the cardiac stomach in some freshwater macrurans and terrestrial brachyurans; sternal concretions within the anterior cuticle during the biphasic molt in isopods; concretions within the posterior caeca of the midgut in amphipods; and the general deposition of calcareous spherules in the hepatopancreas and increase in protein-bound calcium within the hemolymph (see Graf, 1978 for review). The importance of endogenous calcium for postmolt mineralization is dependent upon the habitat of the animal; Crustacea in sea water depend very little upon stores, as calcium is readily available from the medium while reserves are more important for freshwater and terrestrial forms. Thus over 92% of body calcium is lost upon exuviation in *Carcinus* while only 25% and 40% are lost in the supralittoral and terrestrial isopods *Ligia* and *Oniscus* respectively (Graf, 1978).

Uptake of calcium from the food and water is the source of exogenous calcium, but the relative importance of these two sources varies. In general, the food accounts for little of the total calcium of the new cuticle in the marine brachyura despite the eating of the exuvia in many species (Drach, 1939; Graf, 1978). Such is not the case of the mole crab *Emerita asiatica* in which there is no storage and the food accounts for approximately 82% of the calcium for mineralization (Sitaramaiah, 1967).

In freshwater and estuarine organisms specific uptake mechanisms exist for the accumulation of calcium from the dilute media. In fact, *Gammarus pulex* can attain complete calcification in media containing 0.1 mM calcium (Wright, 1980). The freshwater crayfish, *Austropotamobius pallipes*, takes up calcium against an electrochemical gradient by a system displaying saturation kinetics with a Km = 0.13 mM Ca (Greenaway, 1974). Active uptake of calcium may also be induced in euryhaline crabs in dilute media. *Callinectes* is able to fully calcify its integument in 10 ppt seawater, although at a rate slower than that in 30 ppt seawater (Price Sheets and Dendinger, 1983). Both *Callinectes* and *Carcinus* have calcium transport mechanisms with a low affinity, Km = 10 mM Ca (Greenaway, 1983).

The uptake mechanism for Ca in *Austropotamobius* is in part dependent upon the presence of HCO_3^- in the external medium (Greenaway, 1974), however very little is known regarding the sources nor uptake pathways for the carbonate for mineralization. While a certain amount of carbon is likely to be absorbed from consumption of the exuvium and from food, other possible sources include HCO_3^- from the water and metabolic CO_2.

In preliminary experiments in which we injected postmolt (B_1) *Carcinus* with 50 μCi each of $^{45}CaCl$ and $NaH^{14}CO_3$ and etched the cuticle (according to Dillaman and Ford, 1982) following a 6 hr incubation, we found an unequal distribution of label in the mineral throughout the cuticle (Fig. 21). The specific activity of ^{14}C was uniform from the inner surface out to the epicuticle while the specific activity of ^{45}Ca was highest at the inner surface and decreased monotonically toward the epicuticle. Nowhere in the cuticle was the specific activity of ^{14}C as high as that of ^{45}Ca despite the fact that the specific activity of $H^{14}CO_3$ was higher in the hemolymph than was that of ^{45}Ca.

These data imply that there is on the one hand a much larger tissue pool for carbonate than for calcium and, on the other hand, that equilibrium of ^{14}C in the tissue pools and within the cuticle is much faster than that of ^{45}Ca. The location and extent of

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PLATE 3.   Scanning electron micrographs of *Menippe mercenaria* cuticle. FIG. 15. Untreated endocuticle. Note pore canals (arrow). ×5,100. FIG. 16. Decalcified endocuticle. Note pore canals (arrow). ×5,000. FIG. 17. Anorganic endocuticle from lamellar region. ×21,000. FIG. 18. Inner surface of anorganic endocuticle. Note regions of lamellate (l) and non-lamellate (n) organization. ×25. FIG. 19. Crystal organization in non-lamellate region of endocuticle. ×10,000. FIG. 20. Spherulites of non-lamellate region of endocuticle. ×20,000.
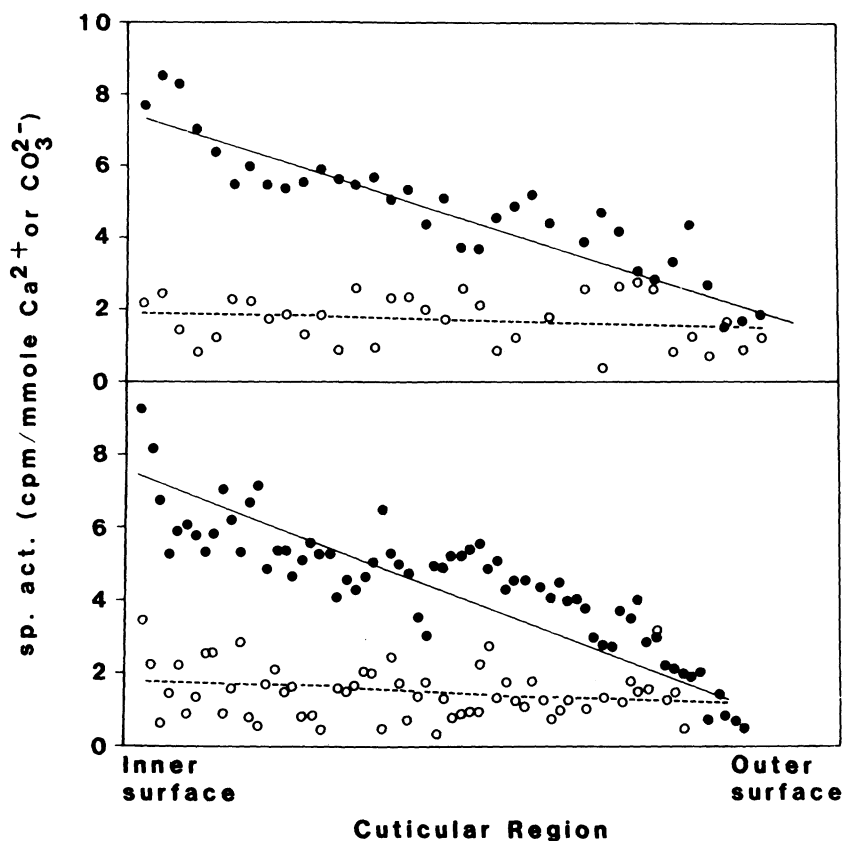
FIG. 21. Distribution of $^{45}Ca^{2+}$ (●) and $^{14}CO_3^{2-}$ (O) in two pieces of cuticle from a postmolt *Carcinus maenas* 6 hr after injection as determined by controlled EDTA-etching analysis.

the tissue pools of calcium and carbonate are as yet unknown, however.

PATHWAYS AND MECHANISMS OF MINERAL TRANSLOCATION

Few studies have been performed in an effort to elucidate the mechanisms by which calcium and carbonate are translocated into and out from mineralized structures within the Crustacea. Digby (1964, 1965) has postulated that mineral deposition of crustacean cuticle occurs through electrode action mediated by the semi-conductor nature of the quinone moieties of the cuticle itself in response to the differing saline concentrations within and without the animal. The lack of such divergent concentrations in most Crustacea and the relatively large potentials which must be induced appear to preclude such an expla-

nation for normal calcification. Indeed, evidence points to a transepithelial transport of mineral to effect both deposition and resorption of minerals from calcareous tissues.

Ultrastructural studies of the posterior caecal epithelium of *Orchestia* (Graf and Meyran, 1983) and of the gastrolith epithelium of *Procambarus clarkii* (Ueno, 1980) have demonstrated cells displaying the properties of a typical transport epithelium with elaborate apical microvilli, basal infoldings and numerous mitochondria. Mizuhira and Ueno (1983) have hypothesized an active role of the mitochondria in calcium translocation in the gastrolith epithelium. They detected large amounts of calcium within the mitochondrial matrices by energy dispersive X-ray analysis and electron energy-loss spectroscopy. Their

proposal is that the mitochondria effect the transepithelial translocation of calcium during the elaboration of the gastroliths.

In the hypodermis of the shrimp, *Crangon crangon*, electron-probe X-ray microanalysis has also demonstrated calcium peaks within the epithelial cells during the periods of maximal resorption of the carapace mineral (stage D) and while maximal rates of deposition are occurring (stage B) (Hubert and Chassard-Bouchaud, 1978). Epithelial involvement in the processes of calcium translocation during premolt resorption and postmolt deposition of carapace mineral was further elucidated by Roer (1979, 1980) in *Carcinus. In vitro* unidirectional flux studies were performed on premolt hypodermis removed from beneath the dorso-branchial carapace and net calcium uptake experiments were conducted on pieces of postmolt integument from the same region. These data demonstrated that both resorption and deposition of calcium were effected by active transport across the hypodermis. Maximal *in vitro* resorption rates were 22.6×10^{-8} mole/cm$^2 \cdot$hr in stage D$_2$, and deposition rates were $8-9 \times 10^{-8}$ mole/cm$^2 \cdot$hr in stages A$_1$ and A$_2$. These stages correspond to those in which maximal resorption and deposition have been found to occur *in vivo* (Drach, 1939; Green and Neff, 1972; Welinder, 1975a; Vigh and Dendinger, 1982).

By employing ion substitutions and various inhibitors, this calcium transport mechanism was found to involve both a Ca-ATPase and Na/Ca exchange (Roer, 1980). These results have recently been verified using another brachyuran, *Callinectes sapidus* (C. Mangum, personal communication).

These physiological studies in combination with morphological investigations (Travis, 1963; Bouligand, 1970; Yano, 1975) point to the importance of the pore canals to the calcification of the pre-exuvial cuticle. The epi- and exocuticles are elaborated before the molt, but only calcify during postmolt. Mineral must reach distances 70 μm or more from the apical surfaces of the epithelial cells for the rapid hardening of these layers following exuviation. This transport of calcium, and pre-sumably carbonate, is apparently effected by the cytoplasmic extensions of the hypodermal cells extending up into these regions within the pore canals.

While we now possess some knowledge of the mechanisms of calcium transport associated with mineral structures in select Crustacea, virtually nothing is known regarding the supply of carbonate to these tissues. It has been established by histological and biochemical means, however, that carbonic anhydrase is localized within the epithelial cells of the hypodermis and in the cuticle (pore canals?) of the anomuran *Clibanarius olivaceous* (Chockalingham, 1971) and of *Carcinus* (Giraud, 1977a, b, 1981). The amount of enzyme is highest during postmolt calcification (stages A$_1$ to C$_3$). The role of carbonic anhydrase in the calcification process is unclear, but inhibition of the enzyme by acetazolamide results in decreased rates of mineralization *in vitro* (Giraud, 1977a) and *in vivo* (Giraud, 1981) as judged by polarized light microscopy. Whether the enzyme acts in a transport capacity or simply in the supply of bicarbonate for mineralization has not been established. Acetazolamide has no effect on *in vitro* calcium transport in *Carcinus* hypodermis (Roer, unpublished data), so if a role in carbonate transport is established it would be unlinked to the movement of calcium.

NUCLEATION AND THE CONTROL OF CRYSTAL GROWTH

As mentioned previously, preparation for the molt in the calcified crustaceans involves the resorption of the mineral and organic components of the old cuticle and the simultaneous deposition of elements of the new cuticle. During premolt, the entire organic matrix of the two outermost layers of the new cuticle (the epi- and exocuticle) are elaborated beneath the old cuticle. The pre-exuvial layers do not calcify until after the molt (Drach, 1939; Travis, 1963, 1965; Travis and Friberg, 1963). In *Carcinus maenas*, for example, the first crystals of CaCO$_3$ are not observed in the epi- and exocuticle until 10 hr after ecdysis (Drach, 1937). Moreover, if a premolt crab is prematurely removed from its old exoskele-

ton, the new cuticle becomes leathery but does not calcify (Paul and Sharpe, 1916). Some internal conditions must therefore be met in order that deposition of mineral may occur.

Since the mineral from the old cuticle is resorbed through the pre-exuvial layers of the new cuticle, Travis (1963, 1965) and Travis and Friberg (1963) have suggested that Ca^{++} and $CO_3^=$ are present in metastable solution in these layers before the molt. As calcification does not commence until after the molt, however, they proposed that the epithelial cells underlying the cuticle exert some control over its environment. They add that this control might be realized via the cytoplasmic extensions of the epithelial cells in the pore canals of the pre-exuvial cuticle through the regulation of such factors as Ca^{++}, $CO_3^=$, phosphate or pH.

An alternative hypothesis has been put forth by Yano (1972), who favors the unmasking of nucleation sites in the pre-exuvial cuticle as the means of controlling calcification. He suggested that an acid mucopolysaccharide complexed to the protein fraction of the pre-exuvial matrix inhibits premolt calcification. Postmolt enzymatic degradation of the polysaccharide-protein complex would then allow calcification to proceed. While there is histochemical evidence for acid mucopolysaccharides in the cuticle (Yano, 1972; Giraud, 1977b), no such degradative enzyme has been described.

A significant peak in phosphorylase and alkaline phosphatase activities is noted in the epicuticle and exocuticle of *Clibanarius* in stages A_1 and A_2 when calcification is commencing (Chockalingham, 1971). It is possible that this activity could be related to the removal of nucleation inhibitors or crystal poisons within the matrix.

The histological location of carbonic anhydrase in the interprismatic regions of the exocuticle (Giraud, 1981) also corresponds to the initial sites of mineral deposition (Giraud-Guille and Quintana, 1982). Thus, a possible role of carbonic anhydrase in mineral nucleation must be considered.

We are currently investigating the role of nucleating sites in the carapace of *Uca*

pugilator in the control of pre-exuvial cuticle calcification. In these experiments, pieces of pre-exuvial cuticle were removed from crabs before the molt (late stage D) and cuticle was removed from newly molted crabs (stage A_1 and A_2). Both types of cuticle were stripped of hypodermal tissue and fixed in 4% paraformaldehyde, decalcified overnight in 0.1 M EDTA in 4% paraformaldehyde, and the EDTA was removed by further rinsing in the fixative alone. The cuticles were then subjected to *in vitro* calcification by successive immersions in 0.5 M solutions of $CaCl_2$ and $NaHCO_3$. Subsequent observation of the tissues by polarized light microscopy showed mineralization within the cuticles from postmolt crabs, but no crystal deposition within those of premolt crabs (Roer and Dail, in preparation). It is apparent that some factor within the matrix of the cuticle is altered in the course of the molt and the ensuing few hours; either a nucleating agent is secreted, or an inhibitor of nucleation and/or crystal growth is removed.

The control by the matrix of crystal morphology and the cessation of crystal growth is evident in studies on carapace repair in crabs (Dillaman and Roer, 1980). Mineral deposited within the wound scab in crabs was not formed in the context of the chitin-protein network and appeared as aragonitic granules rather than the normal calcitic morph. Further deposition, which occurred among histologically atypical lamellae was amorphous $CaCO_3$. Repair cuticle growth showed no evidence of cessation, and hence no membranous layer which normally marks the end of deposition. In this context, it is possible that the secretion of the non-calcifying membranous layer serves to curtail further crystal growth in normal cuticle deposition.

CONCLUSIONS AND FUTURE DIRECTIONS

Readdressing the basic questions of mineralization in the Crustacea it seems we have a large body of data concerning the chemical nature and distribution of mineral and organic components within the cuticle and we know, in certain species, the sources of the calcium for mineralization and the rates at which deposition occurs.

We know far less about the relationship between the mineral and the organic components of the cuticle, both in regard to the determination of crystal morphology and in regard to nucleation. Finally, while in the Portunidae we have some knowledge of the mechanisms and pathways for calcium movement, we know nothing with respect to the transport of carbonate.

These latter areas of investigation will prove fertile ground for future work; work which will provide information not only on the physiology of Crustacea but also on the basic principles of mineralization. The bidirectional nature of mineral transport and the sharp temporal transitions in nucleating ability of the cuticular matrix provide ideal systems in which to study these aspects of calcification.

ACKNOWLEDGMENTS

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