Early Pattern of Calcification in the Dorsal Carapace of the Blue Crab, *Callinectes sapidus*

Richard Dillaman,* Stephanie Hequembourg, and Mark Gay

Department of Biological Sciences, University of North Carolina at Wilmington, Wilmington, North Carolina 28403

**ABSTRACT** The pattern of calcium carbonate deposition was observed in the dorsal carapace of premolt (D2–D3) and early postmolt (0–48 h) blue crabs, *Callinectes sapidus*, using scanning (SEM) and transmission (TEM) electron microscopy. Samples of dorsal carapace for SEM were quick-frozen in liquid nitrogen, subsequently lyophilized, and viewed using secondary and backscattered electrons as well as X-ray maps of calcium. Pieces of lyophilized cuticle were also embedded in epoxy resin and subsequently sectioned and viewed with TEM and SEM. Fresh pieces of dorsal carapace for TEM were also fixed in 2.5% glutaraldehyde in phosphate buffer followed by postfixation in 1% OsO4 in cacodylate buffer. Calcium concentrations were determined using atomic absorption spectrophotometry and quantitative X-ray microanalysis. Calcium accumulation began in the cuticle at 3 h postmolt at the epicuticle/exocuticle boundary and at the distal and proximal margins of the interprismatic septa (IPS). The bidirectional calcification of the IPS continued until the two fronts met at 5–8 h postmolt. The roughly hexagonal walls of the IPS formed a honeycomb-like structure that resulted in a rigid cuticle. The walls of the canal containing sensory neurons also calcified at 3 h, thereby imparting rigidity to the structure and additional strength to the cuticle. Examination of thin sections of lyophilized cuticle and fixed cuticle revealed that the first mineral deposited is more soluble than calcite and is probably amorphous calcium carbonate. The amorphous calcium carbonate is transformed to calcite along a front that follows the original deposition and is probably controlled by a specialized matrix within the IPS. Since amorphous calcium carbonate is isotropic, it would also make the mineral in the exocuticle stronger by an equal distribution of mechanical stress. J. Morphol. 263:356–374, 2005.

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Calcification is a physiological process that occurs in almost all phyla, although the morphology and extent of the calcification vary widely (Simkiss, 1975). Despite these variations, phases of the process are similar. First there is organic matrix deposition and then an accumulation of ions (Addadi and Weiner, 1985; Falini et al., 1996). These characteristic events set the stage for the nucleation of calcium salts and subsequent crystal growth. Once nucleated, a calcified structure may grow by either increasing the size of a single crystal by dendritic growth or by fusion of crystals from multiple nucleation sites (Simkiss, 1975).

Crustaceans have been called “the champions of mineral mobilization and deposition in the animal Kingdom” (Lowenstam and Weiner, 1989). Consequently, the blue crab, *Callinectes sapidus*, while it may not represent all of the members of the Crustacea, is an excellent model in which to study many aspects of calcification (Roer and Dillaman, 1993).

Calcification in the blue crab progresses in stages that separate the deposition of an organic matrix, the influx of calcium ions, and the nucleation of crystals, enabling examination of these processes separately. These predictable sequential events occur periodically as the blue crab molts, so crabs in various stages of the molting process can be collected and those separate components of calcification can be examined. Calcification must occur quickly after ecdysis (emergence from the old exoskeleton), for a hardened exoskeleton is tantamount to survival, but in mature *C. sapidus* the calcification process is long enough that the sequence of events may be easily observed.

In response to complex endocrine signals (Quackenbush, 1986; Chang et al., 1993), the molt cycle begins with apolysis, the separation of the old exoskeleton from the hypodermis (Drach, 1939; Jeuniaux, 1959a; Green and Neff, 1972). Growth of a new, noncalcified cuticle occurs under the old carapace, the epicuticle deposited first, followed by the exocuticle (Drach, 1939; Travis, 1963). The crab then sheds the old cuticle and rapidly calcifies the new cuticle. During postmolt, the pre-ecdysial layers (epicuticle and exocuticle) first tan, then mineralize.
The third layer, the endocuticle, is subsequently deposited and calcified (Drach, 1939; Krishnan, 1951; Travis and Friberg, 1963; Vacc and Finger- man, 1975a,b). This continues until the crab deposits (but does not calcify) the fourth and final layer of the cuticle, the membranous layer (Passano, 1960). This signals the final stage of the molt cycle, inter- molt, when there is no further cuticle deposition.

Calcification occurs when Ca\(^{2+}\) and CO\(_3\)\(^{2-}\) accumulate in concentrations that exceed their solubility product within an organic matrix (Simkiss, 1975). Once crystals have nucleated, crystal growth continues throughout the matrix (Akita et al., 1992). In the crustacean cuticle, the organic matrix is composed of chitin-protein fibrils, with the exception of the lipoprotein epicuticle (Green and Neff, 1972; Giraud-Guille, 1984b). Blood calcium concentrations over the molt cycle have been examined in a number of species, including the spiny lobster, Panulirus argus (Travis, 1955), and the green crab, Carcinus maenas (Robertson, 1960), and, as summarized by Greenaway (1985), the blood of premolt crustaceans has a higher concentration of calcium than intermolt crustaceans, with the lowest calcium concentrations in postmolt crustaceans. The elevated premolt blood calcium values were attributed to resorption from the old endocuticle layer, and the low postmolt values to uptake of calcium from seawater (Greenaway, 1983) and subsequent calcium transport from the blood to the new postmolt cuticle.

The interprismatic septa (IPS) have been implicated as initial sites of calcification (Bouligand, 1970; Giraud-Guille, 1984a; Compère et al., 1992, 1993). The IPS are contiguous, roughly hexagonal cylinders in the exocuticle supposedly created by the lateral membranes of the hypodermal cells during pre-exuvial cuticle deposition (Vitzou, 1882; Dennell, 1947; Green and Neff, 1972; Giraud-Guille, 1984a). The IPS have been visualized and characterized by a variety of techniques. Ruthenium red staining of the IPS in Carcinus maenas indicated that they contain glycosaminoglycans with many anionic groups, thereby enabling the IPS to bind calcium ions (Giraud-Guille, 1984a). Fluorescent-labeled lectin staining of Callinectes sapidus, Procambarus clarkii, and Ocypode quadrata was used to further describe the glycoprotein components of the cuticle (Marlowe et al., 1994). Some lectins, such as Jacalin and Concanavalin A, did not bind to the IPS, but only to the prismatic spaces (i.e., the areas bounded by the IPS), indicating qualitative differences in the carbohydrate content of the two regions. Still other studies have used energy-dispersive X-ray microanalysis in combination with pyroanti- monate precipitation to demonstrate an increase in calcium within the IPS from premolt, stage D\(_2\), to postmolt, stage A\(_2\), and a consistently lower amount of calcium in the prismatic spaces (Compère et al., 1992).

The pattern of calcium crystal nucleation and growth in the cuticle during the molt cycle was first described in Carcinus maenas based on observations at intervals no shorter than 24 h (Bouligand, 1970). It was observed that calcification began at the epicuticle interface and spread inwards towards the endocuticle layers. This front was not uniform, but rather the mineral appeared first in the IPS, laterally in-filling the prisms only after the calcified front of the IPS had extended to the endocuticle. Crystal nucleation and growth, therefore, was said to move in two directions, inward towards the endocuticle and laterally, filling in the prismatic spaces. This proposed calcification pattern was consistent with previous (Travis, 1963) and subsequent calcium localization studies (Giraud-Guille and Quintana, 1982; Giraud-Guille, 1984a; Compère et al., 1992, 1993).

Despite numerous studies on calcium localization and the calcification process in crustaceans (Giraud-Guille and Quintana, 1982; Giraud-Guille, 1984a,b; Compère et al., 1992, 1993), until recently the early hours postecdysis (0–5 h) have not been explored. Investigations of these early hours after molt have proven to be extremely important, as many postecdysial changes occur in the cuticle proteins and glycoproteins during that interval (Shafer et al., 1994, 1995; Coblenz et al., 1998). Those changes have been referred to as postecdysial cuticle alterations (PECA) (Shafer et al., 1994), and it has been suggested that they are related to initiation of the calcification process (Pierce et al., 2001; Roer et al., 2001).

To examine calcium distribution during the very early hours of postmolt, a tissue preparation was needed that would not extract calcium from a cut or fractured piece of cuticle. Since previous biochemical assays (Shafer et al., 1994, 1995) used quick-freeze lyophilization, microscopic investigations were performed using the same processing to investigate cuticle morphology and mineral deposition. During preliminary SEM investigations of quick-frozen, lyophilized cuticle, a more complex pattern of calcium deposition was indicated than that previously suggested by Bouligand (1970). Since this new pattern had implications for CaCO\(_3\) crystal nucleation and growth, the present study was undertaken to more fully describe the calcium distribution pattern during the early hours of the calcification process.

**MATERIALS AND METHODS**

**Specimen Collection**

The cuticle and underlying hypodermal tissue were harvested in May 2001 from sexually mature male and female blue crabs, Callinectes sapidus, 92–166 mm carapace width, at Endurance Seaford in Kill Devil Hills, North Carolina. Postmolt crabs were sacrificed at 0, 2, 3, 4, 5, 6, 7, 8, 12, 24, and 48 h postecdysis (n = 3; except at 7 h, n = 1 and 12 h, n = 2), premolt crabs were sacrificed at D\(_3\) (n = 2) and D\(_2\) (n = 1). The postecdysial times were chosen to bracket the initiation of deposition and calcifica-
tion of the endocuticle layer at 5 h postmolt, while allowing for variation in the timing of events among individual animals. One piece of the dorsal-branchial cuticle from the left side was removed to be used in the SEM studies. From that piece, a 1 cm² subsample was removed from approximately the same place in each sample and frozen in liquid nitrogen for total calcium measurements using atomic absorption spectrophotometry (AAS). An additional piece was removed from the right side and used for the TEM studies.

**Atomic Absorption Spectrophotometry**

Tissue pieces were lyophilized, weighed, and extracted overnight in 0.5 ml nitric acid. Extracts and standards (Fisher, Fair Lawn, NJ) were diluted with 1.17% lanthanum oxide (Sigma, St. Louis, MO) in 5.0% HCl (Sidiropoulos, 1969; Dillaman and Roer, 1980). Calcium content was measured using a Perkin-Elmer (Norwalk, CT) 107 atomic absorption spectrophotometer.

**Tissue Preparation for Scanning Electron Microscopy**

Immediately after harvesting, the cuticle pieces were placed in cryotubes and plunged into liquid nitrogen. They were then stored in a –70°C freezer until lyophilization. After lyophilization, the cuticle pieces were fractured, mounted fractured side up on aluminum stubs using colloidal graphite (Ted Pella, Redding, CA), and sputter-coated with 6 nm of platinum/palladium (80/20) on a Perkin-Elmer 208HR Sputter Coater. The mounted specimens were stored in a desiccator until viewed using a Philips XL30S FEG scanning electron microscope.

**Scanning Electron Microscopy**

Secondary electron (SE) images were typically collected at 5 kV accelerating voltage with a spot size (condenser setting) of 3 and back-scattered electron (BSE) imaging was done at 20 kV accelerating voltage with a spot size of 5. A Si(Li)-super ultrathin window detector with a resolution of 135 eV was used to collect the X-ray signal. Energy dispersive X-ray analysis software (EDAX v. 3.32) was used to analyze and display spectra and map elements in the samples. X-ray spectra were collected for 200 live sec at 20 kV accelerating voltage, a working distance of 7.5 mm, a spot size of 5, counts per second of at least 800, and a dead time of 50 μsec. Quantitative data were collected using a Perkin-Elmer (Norwalk, CT, v. 5.5).

**Transmission Electron Microscopy**

Immediately after harvesting, the right-hand pieces of cuticle were fixed in 2.5% glutaraldehyde in Sörensen’s phosphate buffer (pH 7.4, 890 mOsm) or frozen in liquid nitrogen for lyophilization. Glutaraldehyde-fixed tissue was postfixed in 1% OsO₄ in 0.2 M (pH 7.4, 890 mOsm) or frozen in liquid nitrogen for lyophilization. Mid segments containing atomic absorption spectrophotometry (AAS). An additional piece was removed from the right side and used for the TEM studies.

**RESULTS**

Secondary electron imaging of a 24 h postmolt cuticle clearly illustrates three layers: epicuticle, exocuticle, and endocuticle (Fig. 1). The epicuticle is the thin, dense outermost layer ~1–2 μm thick. Its surface is covered with setae, projections of cuticle present over much of the exoskeleton. The outermost component of the epicuticle is a thin homogeneous layer that often will separate from the remainder of the epicuticle when the cuticle is fractured (Fig. 2a). Below this thin outer layer the remaining subunits of the epicuticle are tightly packed vertical elements perpendicular to the surface (Figs. 2a, 5a,b). The outer exocuticle appears very dense parallel to the epicuticle-exocuticle interface and the same morphology extends into the IPS, where a solid, granular matrix characterizes the IPS, with fine fibers periodically projecting from the fractured surface (Fig. 2a,b). The remainder of the exocuticle, the prismatic spaces between the IPS, and successive horizontal layers of the fibers are shifted relative to each other to a uniform degree so that fracture surfaces through a single plane of the exocuticle appear mulitlamellate (Figs. 1, 2a).

At lower magnifications the endocuticle is distinguished from the exocuticle by its thicker lamellae (Fig 1). The horizontal fibers of the endocuticle are more regularly arranged than those in the exocuticle and form small cylindrical channels that are surrounded by a homogeneous matrix (Fig. 2d). Vertical fibers are often in the shape of a twisted ribbon. The hypodermis borders the endocuticle, but cellular features are not retained in this lyophilized preparation (Fig. 1).

Figures 3 and 4 illustrate the changes in the cuticle over a 48 h period postmolt as revealed by three different imaging modes: secondary electron (SE) imaging, backscattered electron (BSE) imaging, and X-ray mapping. Premolt cuticles (stages D₂ and D₃) were omitted from this series because they were indistinguishable from 0 h cuticle.

The SE and BSE images are very similar in the 0 h and 2 h postmolt cuticles (Fig. 3a1-2, b1-2). They both appear very homogeneous, with the exception of a slightly stronger BSE signal on the distal margin of the 2 h cuticle. In the 0 h calcium map the cuticle cannot be distinguished from the background (Fig. 3a3), whereas in the 2 h calcium map low levels of calcium, evenly distributed over the entire cuticle,
allow it to be differentiated from the background (Fig. 3b3). With SE the 3 h cuticle lamellae (Fig. 3c1) appear more distinct than the earlier cuticles, and the BSE image indicates an increased signal at the boundaries of the exocuticle, both at the epi-exocuticle (distal) margin and the exo-endocuticle (proximal) margin (Fig. 3c2). The intensity of the calcium X-ray pattern matches the BSE pattern, with a slightly higher calcium signal on the distal and proximal margins of the exocuticle (Fig. 3c3). As a result, the cuticle can be easily differentiated from the background and the underlying hypodermis.

A comparison of the morphology of the epicuticle-exocuticle boundary before and after the accumulation of calcium reveals an interesting feature. Prior to calcium accumulation this zone is characterized by smooth fibrous elements (Fig. 5a), whereas by 3 h there is an accumulation of fused granular elements on and among the fibrous elements (Fig. 5b). In addition, both the BSE image and calcium X-ray map of 3 h cuticle reveal short vertical elements perpendicular to the exocuticle margins protruding from the exo-endocuticle border (Fig. 3c2-3). These partial vertical elements, found only in the exocuticle, are the interprismatic septa (IPS). Higher magnification reveals that calcium deposition within the IPS begins at both the distal (Fig. 5c) and proximal (Fig. 5d) margins of the exocuticle. At 3 h after ecdysis the IPS are contiguous with the continuous calcified layer at the epicuticle-exocuticle boundary (Fig. 5c), whereas they are not connected to any such continuous calcified layer on the proximal surface of the exocuticle (Fig. 5d). Neither is there any evidence of any calcified endocuticle layers at this stage of cuticle deposition.

A chance fracture through a 3 h lyophilized cuticle provided additional insight into the pattern of mineral deposition in the blue crab cuticle. The fracture reveals a longitudinal section of a canal containing a sensory hair that penetrates the cuticle as well as its associated hair sensilla (Fig. 6a). In this BSE image one can also see that, in addition to the more intense signal at the inner and outer margin of the cuticle
due to calcium deposition, there is also an intense signal along the walls of the sensory canal that is contiguous with the layers at the inner and outer margins of the exocuticle. This was verified with a calcium map (not shown). When the hypodermis is removed and the proximal surface of a 3 h cuticle is viewed in the proximity of a sensory canal, one can see a revealing aspect of the mineralization pattern (Fig. 6b). The BSE image indicates a relatively dense mineralized ring surrounding the canal and a reticulated pattern on the adjacent inner surface of the cuticle. A higher-magnification BSE image of the flat surface of the cuticle reveals that the reticulated pattern consists of a connected series of polygons ~5–10 μm in diameter (Fig. 6c). Also evident are numerous small pores that cover the surface of the cuticle.
At 4 h postmolt, the exocuticle margins are more prominent in both the BSE and X-ray images, whereas the center of the exocuticle has less signal (Fig. 3d2-3). In addition, the IPS are more prominent than in the 3 h BSE image (Fig. 3d2), extending farther from both the exo-endocuticle interface and the epi-exocuticle interface. These same margins are also more distinct in the calcium map, although the IPS are somewhat less distinct than in the BSE image (Fig. 3d3).

At 5 h postecdysis (Fig. 3e1-3), the SE image appears more homogenous than the cuticle at ear-
lier sample times (Fig. 3e1). The BSE signal from the outer part of the epicuticle is not as bright as the exocuticle just underneath it (Fig. 3e2). It is also evident that the entire expanse of the exocuticle is crossed with calcium-filled IPS. The IPS are also distinguishable in the calcium map, although not all of them are solid lines that are connected to each other at the distal and proximal ends (Fig. 3e3). In both the BSE and X-ray images, the exo-endocuticle margin has an intense band that is thicker than in the 4 h cuticle, indicating more deposition of endocuticle (Fig. 3e2-3). At 6 h

Fig. 4. Composite images of representative samples of cuticle from *Callinectes sapidus* viewed at 6, 8, 12, 24, and 48 h postecdysis using secondary electron (SE), back-scattered electron (BSE), and calcium mapping in the X-ray mode (Ca map). Each row is the same piece of cuticle.
after ecdysis (Fig. 4a1-3), the epicuticle is less apparent than the underlying bright exocuticle and the IPS are more defined in both the BSE image and X-ray map (Fig. 4a2-3). In the BSE image, the endocuticle appears as a homogeneous band along the proximal margin (Fig. 4a2). The calcium map likewise shows a thicker band of calcium present in the endocuticle area (Fig. 4a3) as well as an increased, but diffuse, calcium signal between the IPS. More calcium is also present in the areas near the outer and inner margins of the exocuticle (Fig. 4a3).

The 8 h cuticle SE image looks similar to the 6 h (Fig. 4b1). The IPS continue to be distinct using BSE (Fig. 4b2), and the calcium map indicates there is even more calcium present in the prismatic spaces (regions between the IPS) than in the 6 h cuticle (Fig. 4b3). The BSE image (Fig. 4b2) also reveals a
Fig. 6. Back-scattered electron images of a 3 h postecdysial cuticle from *Callinectes sapidus*. **a:** Fracture through a canal (c) containing processes from a sensory hair (H) and demonstrating mineralization along the canal as well as along the inner and outer margins of the exocuticle (arrows). Also visible are the beginnings of mineralization along the interprismatic septa on the proximal and distal margins of the exocuticle (arrowheads). **b:** Proximal surface of the exocuticle of *Callinectes sapidus* showing the canal of a sensory hair (C) surrounded by a reticulated mineralized ring. Note that the ring is contiguous with a less dense reticular pattern on the remainder of the surface. **c:** Higher magnification of the reticular pattern formed by interprismatic septa on the inner surface of the cuticle. Note the pores in the cuticle that form the paths for the cytoplasmic extensions of the pore canals (arrowheads).
thicker endocuticle layer and higher magnification (not shown) showed it to consist of three or four distinct lamellae.

The 12 h cuticle sample has a very uneven fracture surface (Fig. 4c1) and the IPS, although present, are not seen as solid lines as in the 6 h and 8 h cuticle samples (Fig. 4c2-3). Although there is a strong X-ray signal for calcium at the margins of the exocuticle (Fig. 4c3), less calcium is indicated between the IPS than in the 8 h cuticle. With both BSE and X-ray imaging, the endocuticle appears to consist of at least as many lamellae as the 8 h cuticle (Fig. 4c2-3). The epicuticle continues to be less noticeable with BSE when compared to the strong signals exhibited by the exocuticle below it (Fig. 4c2). The exocuticle and endocuticle can be clearly delineated in the SE image of the 24 h cuticle (Figs. 1, 4d1), as the endocuticle lamellae are noticeably wider than those of the exocuticle. The BSE signals reveal a thicker, higher atomic number layer in the outer exocuticle than in the previous hours and many more layers of endocuticle (Fig. 4d2). The calcium map is very homogeneous in the endocuticle, and a thicker homogeneous region also lies under the epicuticle. The calcium signal is also higher in the prismatic spaces of the exocuticle (Fig. 4d3), but the IPS are still clearly evident.

Secondary electrons again reveal striking morphological differences between the exocuticle and endocuticle at 48 h (Fig. 4e1). The BSE image reveals a thick layer of higher atomic number material in the outer exocuticle margin and an endocuticle with 15–20 dense lamellae (Fig. 4e2). The calcium X-ray map illustrates that calcium has preferentially filled the prismatic spaces between the vertical IPS elements in the distal two-thirds to three-quarters of the exocuticle, whereas the endocuticle is uniformly filled with calcium (Fig. 4e3).

Quantitative X-ray data were compiled from the same analyses as those used to generate the calcium maps (Figs. 3, 4), but also included pieces of premolt (D2 and D3) cuticle. Elements in the analysis included both intrinsic (carbon, oxygen, phosphate, calcium) and exogenous (aluminum, platinum, palladium), as well as some elements that could potentially be found either within or external to the cuticle (sodium, chlorine, silicon). The weight percent of representative elements is displayed in Figure 7a. Although variations occur within each sampling time, general trends are apparent. During premolt (D2 and D3) and early postmolt (0–2 h), the weight percent of calcium is very low. However, at 3 h postmolt there is an increase in the weight percent of calcium in the cuticle, and this value increases in subsequent samples. Carbon is relatively stable at a high weight percent from D2 to 12 h postmolt and is present in lower amounts 24 and 48 h postmolt. Oxygen weight percent is maintained at ~20, rising to 30 at 24 and 48 h. Magnesium, palladium, and phosphate have a consistently low weight percent throughout the times sampled, although there are slight variations within sampling times.

Calcium content of the cuticle was also measured by AAS (Fig. 7b). Although the absolute values cannot be directly compared with the weight percent values of the X-ray analysis (Fig. 7a), both methods indicate the same trend in calcium concentrations. Calcium concentrations in the cuticle are low until 3 h postmolt, at which time there is a significant ($P < 0.05$) increase. In spite of high variability observed in the AAS data, the general trend is a continued increase in calcium content over the postmolt period measured.

Lyophilized pieces of cuticle infiltrated with epoxy resin, thin-sectioned onto water and examined with the transmission electron microscope (TEM), reveal some interesting features (Fig. 8a–c). Despite being unstained, one can easily distinguish the epicuticle from the fibrous layers of the exocuticle (Fig. 8a,b). Also apparent are holes or voids in the cuticle. In 2 h
cuticle (Fig. 8a) they appear as small, roughly circular holes scattered along the epi-exocuticle border. Some circular and slightly elongate voids are also present in the inner half of the epicuticle. At 5 h postmolt the density of the voids is such that they form a much thicker, reticulated layer along the epi-exocuticle border and are contiguous with vertical elements extending into the exocuticle (Fig. 8b). It is also apparent in Figure 8b that the epoxy resin has been excluded from the voids because the electron scattering in the voids is much less than in the epoxy resin outside the cuticle. In the 12 h cuticle the pattern at the epi-exocuticle border is more complicated. The inner portion of the epicuticle and the

Fig. 8. Lyophilized cuticle of Callinectes sapidus embedded in epoxy resin and viewed by transmission electron microscopy (a–c) or fractured and viewed by back-scattered electrons in the SEM (d). a: Two-hour cuticle showing the clear distinction between epicuticle (epi) and exocuticle (exo) as well as small spherical voids (arrowheads) along the boundary between the two layers. b: Five-hour cuticle showing a more extensive pattern of voids along the epicuticle/exocuticle boundary as well as along vertical paths corresponding to interprismatic septa (ips). c: Twelve-hour cuticle showing both a dense mineral layer along the epicuticle/exocuticle boundary (*) as well as a parallel void (v) region that is contiguous with voids corresponding to the interprismatic septa (ips). d: Twelve-hour cuticle showing mineralized regions present (arrows) when tissue is not exposed to water.
distal portion of the exocuticle are filled with electron-dense material that appears to be mineral (Fig. 8c). That portion of the mineral extending into the epicuticle appears to consist of parallel acicular elements extending into the proximal region of the epicuticle. Proximal and parallel to the mineral is a large reticulated void that is contiguous with a void perpendicular to the surface of the cuticle. This perpendicular void is similar in morphology to that seen in the 5 h specimen, but is 4–5 times wider. A BSE image of the fractured edge of a 12 h cuticle at approximately the same magnification and orientation as the TEM sections illustrates that a region with a strong backscattered electron signal corresponds to that region of the TEM section that includes both the mineral and the reticulated void (Fig. 8d). Also apparent in this BSE image is the extension of the calcium into the inner half of the epicuticle.

Fractures normal or oblique to the surface of an 8 h lyophilized cuticle viewed with BSE reveal the true structure of the IPS (Fig. 9a,b). The IPS are actually a honeycomb type of structure consisting of roughly hexagonal cylinders that share walls with one another. Consequently, in an 8 h cuticle, where the IPS are calcified near the epicuticle (Fig. 9a) and near the endocuticle (Fig. 9b), they appear as hexagonal arrays in cross section, both in the distal and proximal portions of the exocuticle.

When lyophilized cuticle is embedded in epoxy resin, cut with a dry knife and then viewed with the SEM, one can see the same hexagonal arrays that are apparent in fractures (Fig. 9c). At higher magnification (Fig. 9d) the region of the hexagonal figures appears solid, although somewhat irregular. While there are holes in the region of the epoxy-embedded matrix, they appear to be an artifact of dry sectioning combined with the fibrous nature of the matrix of the cuticle. When companion sections are treated with a drop of water prior to viewing on the SEM one can see that much of the material within the hexagonal arrays is selectively removed (Fig. 9e). Closer inspection (Fig. 9f) reveals that some fibrous components remain in the hexagonal arrays, but that a significant portion of the material has been removed. In this section one can also see that the lamellar nature of the cuticle is recognizable in the region within the hexagonal arrays (the prismatic space) even when infiltrated with resin (Fig. 9e).

An interesting pattern of crystal deposition appears when cuticle is fixed in a phosphate buffer and then stained with lead citrate and uranyl acetate after thin sectioning. In a piece of 24 h cuticle one can see that there is a less dense region in the cuticle that corresponds to the inner portion of the epicuticle and the outermost portion of the exocuticle (Fig. 10a,b). Within this region one can see fibrous elements of both the epicuticle and the exocuticle, but the region is characterized by “empty” spaces between the fibers. Higher magnification of this region (Fig. 10b) indicates that the region immediately proximal to the empty region is filled with small acicular crystals. These needle-like crystals are not found throughout the cuticle, but rather seem to be restricted to two locations. The first location is adjacent to the empty regions (Fig. 10b), and the second is within the pore canals that extend from the hypodermis into the cuticle (Fig. 10c,d). In sections that contain both cuticle and hypodermis (Fig. 10d), one can see that the pore canals are contiguous with the apical surface of the hypodermal cells. Since the pore canals are extensions of the underlying hypodermis, not surprisingly the cytoplasm of the underlying hypodermal cells is also filled with the acicular needles.

**DISCUSSION**

This investigation of CaCO₃ deposition in Callinectes sapidus cuticle during the early hours after ecdysis adds significant features to Bouligand’s (1970) description of the initial calcification process in Carcinus maenas. Detection of these new features in C. sapidus was the result of both the methods of tissue processing used in this study as well as the time course of sampling. As a result, in addition to revealing the temporal and spatial aspects of the calcium distribution, insights were gained on the mechanism of CaCO₃ deposition in the dorsal carapace of C. sapidus.

The sampling times in this study were chosen so as to encompass the PECA events in the early hours after ecdysis as described by Shafer et al. (1994, 1995). The major biochemical changes documented by those studies occurred between 1–3 h after ecdysis and include changes in the mobility of several electrophoretic bands as well as changes in Concanavalin A and periodic acid-Schiff staining of those bands. The PECA changes in the EDTA-soluble proteins were also coincident with the ability of the cuticle to mineralize, as measured by an in vitro assay. In those studies tissue samples were collected in the field, quick-frozen in liquid nitrogen, and then transported to the laboratory for lyophilization and subsequent chemical and biochemical analysis. Consequently, in order to be consistent with those studies, most tissues examined in this study were never fixed, or even hydrated after removal from the crabs. These freeze-dried tissues were particularly useful in the present SEM study because it could be presumed that ions had not been lost nor had there been transformations of the cuticle due to fixatives or buffers. Fortunately, the infiltration of the same freeze-dried tissue with epoxy resin and subsequent thin sectioning yielded TEM images indistinguishable from those reported by Compère et al. (1993), who used a freeze-substitution protocol to examine calcification in Carcinus maenas.
Figure 9
The SEM has been widely used to describe cuticle morphology, and the preparatory methods used in this study produced cuticle ultrastructure very consistent with previous observations (Green and Neff, 1972; Hegdahl et al., 1977a–c; Giraud-Guille and Quintana, 1982; Roer and Dillaman, 1984). The epicuticle of Callinectes sapidus could be seen to have two distinct layers, a thin outer layer and a thicker inner layer, similar to the epicuticle of Carcinus maenas (Hegdahl et al., 1977c; Compère and Goffinet, 1987; Compère, 1995) and Uca pugilator (Green and Neff, 1972). Fibrous pore canal elements were also visible in the premolt inner epicuticle of C. sapidus, as described for U. pugilator (Green and Neff, 1972) and C. maenas (Compère and Goffinet, 1987). However, in the postmolt cuticle, instead of just fibrous elements, there were spherulitic structures in the innermost portion of the epicuticle. These structures have been interpreted as mineralized regions of the cuticle (Hegdahl et al., 1977c; Roer and Dillaman, 1984) and, indeed, BSE imaging of the region in this study supports that assertion.

The use of secondary electrons, back-scattered electrons, and X-ray imaging provided a clear temporal and spatial pattern of the calcium distribution in the cuticle of Callinectes sapidus. Since calcium and carbonate must exist at saturation levels in order for nucleation to occur, increased calcium concentration is a valid indicator of future calcification (Simkiss, 1975). A calcium concentration in the cuticle that was distinguishable from background was first seen in the 2 h calcium X-ray maps, but in those maps calcium was evenly distributed and not concentrated in any particular region of the cuticle. However, quantitative analyses of 2 h cuticle by both nondispersive X-ray microanalysis and atomic absorption spectrophotometry indicated that cuticle calcium concentrations were so low as to be indistinguishable from values for premolt or 0 h cuticle. This diffuse distribution and slight elevation in calcium is probably due to small increases in free or bound calcium ions in the cuticle as a result of the active transport of calcium by the hypodermis (Roer, 1980; Greenaway et al., 1995; Zannotto and Wheatly, 2003) coupled with a dramatic decrease in the permeability of the cuticle within the first hour after ecdysis (Williams, 2000). In fact, the newly deposited cuticle changes from being a pathway for the movement of ions from the old cuticle to the hemolymph to being a repository for the calcium moved outward across the hypodermis (Roer, 1980; Wheatly et al., 2002; Zannotto and Wheatly, 2003). This investigation reveals that as early as 3 h postmolt, the weight percent of calcium in C. sapidus cuticle began to increase. This coincides with the 3 h biochemical changes during PECA (Shafer et al., 1994, 1995; Coblelitz et al., 1998). Despite the intrasample time variation, it is clear that calcium weight percent continued to increase with time postmolt, corresponding to the increase in calcium seen using X-ray mapping. In previous studies investigating percent calcium in Carcinus maenas, Cancer pagurus, and Maia squinado cuticle, it was found that at 6 h postmolt calcium was 20.4% of the cuticle (Drach and Lafaon, 1942; Welinder, 1974), but calcium weight percent may vary among species and with calcium content of the medium. In fact, in this study calcium weight percent ranged from 5–13% in 6 h postmolt C. sapidus cuticles.

Calcium in high concentration is first visible at the distal (epi-exocuticular) and proximal (endo-cuticular) margins of the exocuticle and in short segments of the IPS at both margins. Calcified distal and proximal margins of the exocuticle have been previously described in Carcinus maenas, where they are preferentially mineralized within the first 24 h postmolt along with the IPS (Giraud-Guille and Quintana, 1982; Compère et al., 1992, 1993). The high concentration of calcium in the inner exocuticle margin at 24 h is also coincident with the appearance of the multiple, calcified layers of endocuticle. The columns of calcified IPS have also been described from time-matched cuticle as being “sandwiched” between the two margins perpendicular to the IPS (Compère et al., 1993). However, the previous study in C. maenas (Bouligand, 1970) only examined cuticle samples 24 h or more after ecdysis, so the proposed early pattern of deposition was an extrapolation. It was presumed that calcification of the IPS proceeded from the distal layer to the proximal layer, but early calcification was not directly observed until the present investigation.

Since this study included examination of earlier times in the calcification process, it revealed that the IPS in-fill with calcium from both the distal and proximal margins. Furthermore, while the distal calcification of the IPS could possibly extend from the calcified layer at the epicuticle-exocuticle boundary, it appears that calcification is also initiated in the IPS in the proximal portion of the exocuticle, as evidenced by the roughly hexagonal arrays with clear centers seen on the inner surface of the cuticle at 3 h. While it had been previously indicated that the interprismatic septa were the sites of initial calcification (Travis, 1960, 1963; Bouligand, 1970; Giraud-Guille, 1984a), it had not been observed that

Fig. 9. Backscattered (a–d) and secondary (e,f) images of 8 h postecdysial cuticle of Callinectes sapidus. a,b: Images of fractures normal to the distal (a) or proximal (b) margin of the exocuticle showing the reticulate array formed by the mineralized interprismatic septa (arrows). c,d: Low (e) and higher (d) magnification images of dry-cut sections of epoxy-embedded 8 h cuticle showing the same reticulate pattern of the mineralized interprismatic septa (arrows in e and between the arrowheads in d). e,f: Low (e) and higher (f) magnification of dry-cut sections treated with water prior to viewing. Note the voids that correspond to the reticular pattern of the interprismatic septa (arrows in e and between the arrowheads in f). Also note the fibers remaining in the voids (arrows).
the IPS accumulates calcium from two directions. This pattern mandates that somehow calcification is triggered at the extremes of the IPS. The mechanism for initiating this pattern may involve the diffusion of an inhibitory component from both the distal and proximal surfaces of the exocuticle soon after ecdysis, as suggested by Coblentz et al. (1998). As previously recognized by Compe`re et al. (1993), initial mineralization of the IPS would allow the cuticle to quickly become rigid, with the minimum amount of calcification. Obviously, it would even further accelerate the process for calcification to begin at both margins and move inward along the IPS and form the solid walls of the hexagonal prisms.
perpendicular to the cuticle layers, thereby resulting in a strong, corrugated structure, or honeycomb, that would resist distortion. This pattern of calcification initiating along margins, and thereby imparting rigidity, is also exemplified by the sensory canal whose vertical walls are also calcified by 3 h postmolt.

A further insight into the calcification process in *Callinectes sapidus* was detected when lyophilized cuticle was infiltrated with epoxy resin and then sectioned. The pattern of voids seen in the early (2–5 h) resin followed the pattern of calcium deposition revealed by the BSE and X-ray maps and initially suggested that all the mineral was dissolved from the sections. However, examination of 12 h cuticle indicated that not all the mineral deposited by that time dissolved, with only the mineralizing front on the proximal half of the deposition at the epicuticle/exocuticle border and along the IPS being dissolved. Due to the higher apparent solubility of those regions we suggest that this mineralizing front in the exocuticle of *C. sapidus* is not calcium carbonate in the form of calcite but rather amorphous calcium carbonate. The differential solubility of the mineral deposited by 12 h was also detected when the cuticle was fixed in the phosphate-buffered fixative and subsequently stained in uranyl acetate. Acellular crystals of calcium phosphate were seen in the regions that corresponded to the soluble amorphous calcium carbonate, suggesting that with phosphate-buffered aqueous fixatives at neutral pH the amorphous phase of the calcium carbonate deposition would dissolve during processing, allowing the free calcium ions in the solution to join with the phosphate ions of the buffer to form calcium phosphate, a process demonstrated by Watabe (1990). In contrast, the calcitic portion of the mineral would remain mostly intact and only be removed by the subsequent acid staining solution (uranyl acetate in ethanol and dilute acetic acid). In some respects those observations were similar to the observations on calcium distributions in the cuticle of *Carcinus maenas* with the K-pyroantimonate method (Compère et al., 1992, 1993). However, the solubilities of magnesium and calcium pyroantimonate are so low as to substitute for both crystalline and amorphous calcium carbonate, whereas only the core of the dog bone spicules of the tunic is amorphous calcium carbonate and, therefore, they were not able to identify the two morphs.

Also revealed by the phosphate fixative was the high level of calcium in the cytoplasmic extensions within the pore canals. This supports the contentions of previous studies in *Carcinus maenas* that suggest calcium and magnesium are transported through these extensions into the cuticle and allow the outer portions of the exocuticle to continue to mineralize after the epicuticle becomes impermeable (Roer, 1980; Compère and Goffinet, 1987; Compère et al., 1993).

The presence of amorphous calcium carbonate in crustacean cuticles has been previously reported by Prennant (1928) and Vinogradov (1953), but the best evidence for its presence in crustaceans is in the calcium storage granules of the terrestrial crustacean, *Orchestia cavimana* (Raz et al., 2002). When investigating *O. cavimana* the authors used X-ray diffraction, infrared, and Raman spectroscopy as well as thermal analysis and elemental analysis to determine that the majority of the calcium carbonate in the storage granules was amorphous rather than calcite. The difficulty in establishing the presence of amorphous calcium carbonate is that it is more defined by what it is not rather than what it is (Levi-Kalisman et al., 2000). Amorphous calcium carbonate does not have any long-range crystal order so it is isotropic in polarized light and, with rare exception (Kingsley et al., 2003), does not display a distinct X-ray or electron diffraction pattern. It is the short-range order that leads to the high solubility of amorphous calcium carbonate as well as providing sites for the substitution of other ions such as Mg$^{2+}$ and PO$_4$$^{-}$\textsuperscript{3-}. Since high magnesium favors the deposition of aragonite rather than calcite, the presence of high magnesium calcites is attributed to amorphous calcium carbonate serving as an intermediate (Raz et al., 2000; Mann, 2001). This could be particularly important in the very early postmolt stages when the epicuticle is more permeable (Williams, 2000) and, as a consequence, the medium for calcium carbonate deposition is similar to surrounding seawater. In fact, during carapace repair in *Carcinus maenas* (Dillaman and Roer, 1980), when an epicuticle is absent but matrix is present, aragonite is deposited on the outside of the repair rather than calcite and amorphous calcium carbonate is deposited in the more proximal portions of the repair cuticle.

The role of amorphous calcium carbonate as a first step in the formation of calcite was first described for the larval spicules of sea urchins (Beniash et al., 1997) and subsequently for the spicules of the solitary ascidian *Pyura pachydermatina* (Aizenberg et al., 2002). In the latter case the antler spicules of the body consist entirely of stable amorphous calcium carbonate, whereas only the core of the dog bone spicules of the tunic is amorphous calcium carbonate and presumably precedes the formation of the calcitic envelope. Interestingly, Simkiss (1994) pointed out that the amorphous calcium carbonate can dissolve and act as ion sources for more crystalline structures. He used the example of the amorphous calcium carbonate in the crayfish gastroplith as a source of ions to form the calcite of the cuticle. The situation in the blue crab exocuticle differs in that calcium and carbonate from the amorphous calcium carbonate is not transported from one region of the body to another, but amorphous calcium carbonate is first deposited in the exocuticle and then is replaced, in situ, by calcite. The phase transformation of amorphous calcium carbonate into calcite occurs along an expanding front with the calcium and car-
bonate ions from the amorphous form being transferred to adjacent calcite crystals. Since the first solids formed are the most soluble (Simkiss, 1994), one might expect amorphous calcium carbonate to form and quickly be transformed into calcite. However, the presence of a prescribed pattern of amorphous calcium carbonate deposited followed by a significant period of time before transformation to calcite suggests that there is a barrier to that transformation. Studies on stable amorphous calcium carbonate crystals in the plant *Ficus retusa* (Taylor et al., 1993) have concluded that controls imposed on the amorphous phase in biology must be considerable and are most likely to involve the inhibition of the nucleation of the more crystalline phases by the organic matrix. Furthermore, when matrix extracted from either the completely amorphous calcium carbonate antler spicules of the ascidian *P. pachydermatina*, or the amorphous core of the dog bone spicules was compared to matrix from the calcitic shell of the dog bone spicules, using in vitro assays, matrix from the former inhibited crystallization for weeks, while the latter did so for 20 min to 1 h (Aizenberg et al., 2002). In the same study synthetic amorphous calcium carbonate was stabilized by macromolecules isolated from the antler spicules consisting of small spherical particles. It is interesting to note that those spherical particles are very reminiscent, in both shape and size, of the high magnesian calcite reported by Raz et al. (2000) and the spherical particles observed on the cuticular fibers of the early postmolt cuticle in *Callinectes sapidus*.

Since the entire exocuticle eventually calcifies, in order to superimpose a predetermined pattern of mineralization within the exocuticle the blue crab must either direct this pattern by depositing one type of mineralizing matrix in IPS and another in the prism spaces, or by very carefully directing calcium or carbonate ions to the IPS. The calcium maps of 2 h and older cuticles suggest that calcium is first evenly distributed and then precipitates in the IPS, suggesting that the IPS probably has a mineralizing matrix that has a very different affinity for calcium and carbonate than matrix found in the prisms.

In addition to serving as a means of directing the early deposition of mineral in the cuticle so as to form the honeycombed structure, the amorphous calcium carbonate may also endow the exocuticle with better mechanical properties. The isotropic nature of amorphous minerals not only allows them to be formed into more complex and delicate shapes than would be possible using crystalline material, but also makes the delicate structures stronger because there is an equal distribution of mechanical stress, unlike crystals that have weaknesses along certain planes (Levi-Kalisman et al., 2000). By the time the amorphous calcium carbonate in *Callinectes sapidus* exocuticle changes to calcite the prisms are being in-filled with mineral so as to make the exocuticle more solid, and the cuticle is reinforced by the calcified endocuticle.

In summary, the early postmolt cuticle of *Callinectes sapidus* has evolved several strategies for quickly forming a rigid exoskeleton. In late premolt, stages D3 to D4, blue crabs increase their blood hydrostatic pressure to crack the old carapace (Mangum et al., 1985) and after ecdysis they continue to imbibe water so as to both expand the new cuticle and form a hydrostatic skeleton (Taylor and Kier, 2003). The blue crab then preferentially calcifies from both the distal and proximal surfaces of the exocuticle along the interprismatic septa forming a rigid exoskeleton. The initial mineral deposited appears to be amorphous calcium carbonate, whose deposition is probably controlled by a specialized matrix within the IPS, and whose mechanical properties may make the cuticle even stronger. The amorphous calcium carbonate is transformed to calcite along a front that follows the original deposition, which raises the question of whether or not another matrix within the IPS is nucleating the calcite or if the original matrix is somehow altered by the deposition of amorphous calcium carbonate. Since the matrix of the IPS is inseparable from the matrix of the prisms, identification of candidate matrix proteins responsible for the deposition of the two mineral phases must be accompanied by immunocytochemical localization. To date, an immunocytochemical approach to identifying crustacean matrix proteins has been applied in *C. sapidus* to a protein motif in the epicuticle and exocuticle by Hequembourg (2002), based on proteins isolated from *Cancer pagurus* and *Homarus americanus* (Nousianinen et al., 1998; Andersen, 1998, 1999), to a glycoprotein isolated from the exocuticle of *C. sapidus* by Tweedie et al. (2004), and to the localization of orchestin, an acidic phosphoprotein from the terrestrial crustacean *Orchestia carinata* (Hecker et al., 2004). Of these three only orchestin has, as yet, been purified and tested in situ for its capacity to nucleate the different morphs of calcium carbonate. However, while orchestin was originally isolated from concretions that contain amorphous calcium carbonate (Testeniere et al., 2002), in vitro assays only produced calcite, albeit of an increasingly complex morphology as the concentration of orchestin increased. Increasingly, our knowledge of protein composition in the various types of crustacean cuticle is changing from analysis of tissue extracts to identification of proteins based on genetic transcripts isolated from the adjacent epithelia (Hecker et al., 2004). If the identity, location, and sequence of deposition of cuticular proteins and glycoproteins are coupled with a thorough understanding of the pattern and timing of mineral deposition in a particular region of the cuticle, it should be possible to understand the
mechanisms whereby calcification is both induced and controlled (Mann, 2001) in *C. sapidus*.

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