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Calcification initiation sites in the crab cuticle: The interprismatic septa

An ultrastructural cytochemical study

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Summary. In the crab cuticle the interprismatic septa (IS), which correspond to imprints left in the cuticle by the margins of the epidermal cells, penetrate the twisted structure of the chitin-protein matrix. The ultrastructure and geometric relationship between the fibrous architecture and the pattern of the prisms is described.

The cytochemical characterization of the IS, by pronase treatment and ruthenium red staining, supports the hypothesis that this material corresponds to cell-coat glycoproteins released in the cuticle during secretion of the organic matrix.

Calcification begins after ecdysis in the external laminae of the pigmented layer and along the IS. The presence of cation-binding glycoproteins in the sites where calcification is initiated could induce the nucleation of the mineral phase by concentrating calcium. The extracellular distribution of carbonic anhydrase, which favours carbonate deposition, is observed on ultrathin sections over the IS.

Key words: Calcification – Crab cuticle – Cell coat glycoprotein – Carbonic anhydrase

The structure of the exoskeleton of decapod crustaceans consists of four distinct layers designated in relation to their sequence of formation and their chemical composition (Drach 1939) (Table 1).

The organic matrix of the crab cuticle is mainly composed of fibrous chitin-protein units (Rudall 1965) spatially organized according to a twisted polywood model, i.e. all fibrils are horizontal and parallel in a given plane, and rotate continuously along a vertical line from one plane to the next (Bouligand 1972). The organization is made more complex by the presence of pore canals, which are fine cytoplasmic extensions of the epidermis cells, and by the presence of polygonal divisions in the pigmented layer (Pi), the interprismatic sepate (IS) (which are prints left in the cuticle by the margins of the epidermal cells) (Vitzou 1882; Drach 1939; Travis (1963) (Fig. 1).

The crabs periodically shed their rigid calcified exoskeleton during ecdysis. Immediately after ecdysis the cuticle is soft; thereafter the calcite mineral confers progressively hardness to the cuticle. Ultrastructural observations of undecalcified cuticle a few hours after ecdysis exhibit opaque mineral deposits over the outermost laminae of the Pi layer, and along the interprismatic septae (IS) (Fig. 1) (Bouligand 1970). These regions of the organic matrix therefore correspond to the sites where calcification is initiated.

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Table 1. Designation of layers in the crab cuticle

Sequence of formation		Chemical composition
Preecdysial layers	1 Epicuticle	Lipoprotein
	2 Pigmented layer	Chitin-protein, $CaCO_3$
Ecdysis		
Postecdysial layers	3 Principal layer	Chitin-protein, $CaCO_3$
	4 Membranous layer	Chitin-protein

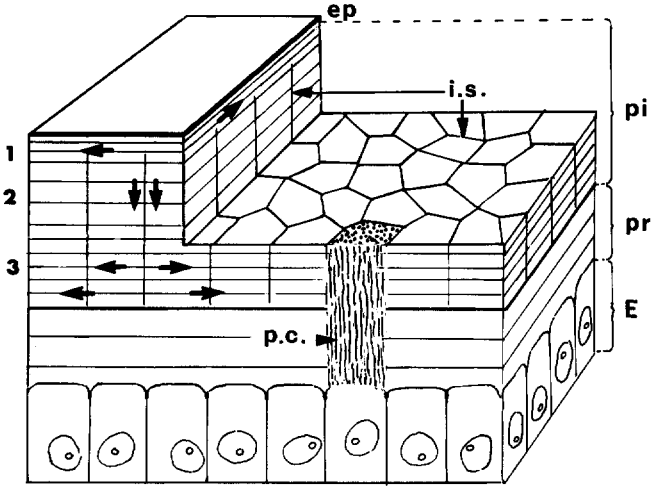


Fig. 1. (Modified from Giraud, 1981). Diagram of the integument of a crustacean cuticle at stage A_2. The pre-exuvial layers, epicuticle (*ep*) and pigmented layer (*pi*) are completely secreted. The postexuvial, principal layer (*pr*) is just beginning to be secreted and will be followed by the membranous layer. The interprismatic septa (*i.s.*) correspond to imprints left in the cuticle by the margins of the epidermis cells (*E*); pore canals (*p.c.*). Fine extensions of the epidermal cells are represented for one cell. Mineralization begins a few hours after ecdysis. The $CaCO_3$ deposits first appear all along the outer laminae of the Pi layer (*1*), then progresses along the IS (*2*), and radially fills the free space left by the organic matrix (*3*) (Drach 1939; Bouligand 1970)

The purpose of our investigation was to study the ultrastructure and the chemical nature of the IS and to observe the manner in which these specific regions of the cuticle may play a role in initiating calcite deposition. The geometric relationship between the epidermal cell margins and the IS imprints in the cuticle directed our cytochemical approach towards characterization of cell-surface glycoprotein.

Furthermore, carbonic anhydrase, which catalyzes the hydration of carbon dioxide and favours $CaCO_3$ deposition, is commonly associated with the calcification process. The extracellular presence of this enzyme has been demonstrated in the cuticle by a manometric method (Giraud 1981). Assays were made to reveal the distribution of carbonic anhydrase on ultrathin sections of cuticle.

Material and methods

The material studied, the exoskeleton of the Atlantic shore crab *Carcinus maenas* L., was obtained from Roscoff, France. Fragments of cuticle are removed from the cephalothorax of small animals (1–2 cm in width) at initial stages of the intermolt cycle (A_2 and B_1, according to Drach 1939) when mineralization begins and the pre-exuvial layers are still in close relationship with the epidermis.

Classical transmission electron microscopy (TEM)

Cuticle fragments were fixed 1 h with 3% glutaraldehyde in cacodylate buffer 0.4 M pH 7.4 at 4° C, and rinsed in the same buffer. Samples were then postfixed for 50 min in buffered 1% OsO_4 at 4° C. The osmolarity of all samples was adjusted to 1100 mOsm by addition of sucrose. The cuticle was decalcified with 10% ammonium citrate solution in a vacuum chamber, then dehydrated in a graded series of ethanol dilutions and propylene oxyde. The samples were embedded in Araldite. Thin sections were cut with a diamond knife and contrasted with uranyl acetate and lead citrate (Reynolds 1963). The grids were examined in a Philips EM 201 electron microscope at 80 Kv.

Treatment with pronase

The cuticle was fixed with 3% glutaraldehyde and decalcified as above indicated. Samples are embedded in a hydrosoluble resin glycol-methacrylate (GMA).

Enzymatic extractions were effected on floating sections transferred in successive fluids with a ring. Pronase extracted from *Streptomyces griseus* is an exo- and endopeptidase with a strong proteolytic action at neutral pH and optimum activity at 40° C (Monneron 1966). Semi-thin sections (1 μm) were placed on a 0.5% pronase solution (45000 proteolytic units per dry weight) buffer at pH 7.4 for 60 min at 40° C. Control sections were placed on buffered distilled water under the same conditions. Dehydrated sections were stained with methyl blue 1% (in sodium borate 1%) and azur B 1%, vol/vol at 60° C, and observed by light microscopy.

Ruthenium red staining

The inorganic dye ruthenium red (RR) stains extracellular glycosaminoglycanes. The reagent was added to the fixatives at 20° C (Luft 1971). Cuticle specimens were fixed 60 min with 3% glutaraldehyde in cacodylate buffer 0.2 M pH 7.3 with 0.2% RR rinsed in a 0.15 M cacodylate buffer. Specimens were postfixed 3 h in OsO_4 2% added with 0.2% RR; briefly rinsed, dehydrated and embedded according to classical TEM procedures. Thin sections were observed without further staining at 60 Kv. Controls were treated in parallel with fixatives without RR.

Ultrastructural localization of carbonic anhydrase

The reaction of Hansson (1967) which localizes carbonic anhydrase activity on cryostat sections, was performed on ultrathin sections embedded in GMA, and transferred through successive solutions with a ring. Cuticle fragments were fixed with the FGP (formaldehyde 4%, glutaraldehyde 3%, picric acid 0.05%) of Ito and Karnovsky (1968) in cacodylate buffer 0.1 M pH 7.3. This fixative retains only 5% of the original carbonic anhydrase activity (Sugai and Ito 1980). Samples rinsed in the buffer were embedded in GMA. Thin sections were incubated for 4 min at 20° C on a staining medium containing 1.86 mM $CoSO_4$, 55.9 mM H_2SO_4, 3.73 mM KH_2PO_4, 158 mM $NaHCO_3$, as described in Sugai and Ito (1980). Thin sections were examined without further staining. Control sections were treated with incubation medium to which 10^{-5} M acetazolamide, a specific inhibitor of carbonic anhydrase, was added.

Results

Geometry and ultrastructure of the prisms

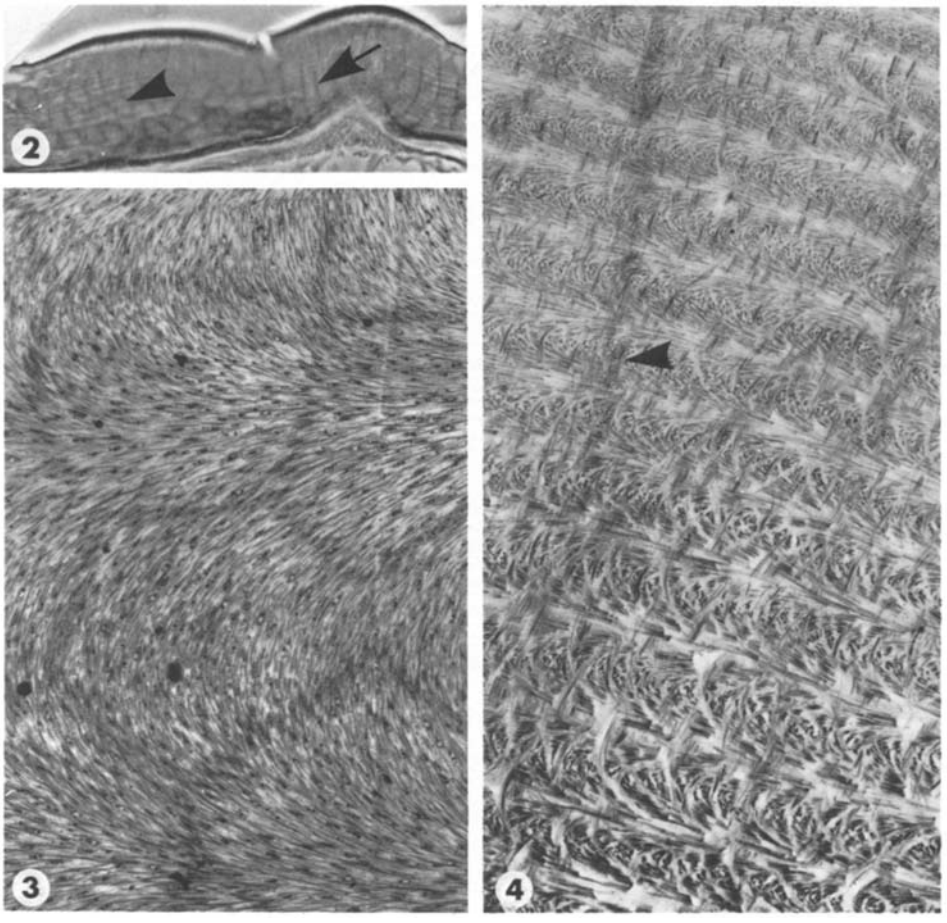
The polygonal pattern of prisms, considered as the vertical projection of the margins of epidermal cells into the cuticule, is superimposed on the twisted polywood architecture of the fibrous chitin-protein macromolecules. The interprismatic septae penetrate the mass of the twisted fibrous system in the pigmented layer.

The spatial arrangement of the chitin-protein fibrils is similar to the geometry of cholesteric[1] liquid crystals (Bouligand 1972). All fibrils are horizontal; their direction rotates by successive angles that are constant from one plane to the next; a rotation of 180° of the fibrils corresponds to one lamina. Over each lamina, this twisted structure in oblique thin sections, exhibits series of nested arcs. At first these bow-shaped arrangements were interpreted as curved filaments. In fact this appearance is an illusion caused by overlapping of horizontal layers of microfibrils. In a twisted system the arcs are visible in oblique section, but absent in horizontal and longitudinal ones (Livolant et al. 1978).

The patterns of the prisms vary in form and size as a function of the obliqueness of the plane of section (Figs. 2–4); parallelly the width of the arcs over each lamina appears wide or narrow. The geometric relationship between the two independent organic networks (fibrous architecture and prisms) observed by electron microscopy is shown in Fig. 5, in which four different section planes are represented with the corresponding cuticle section as seen in the microscope.

In a vertical section (1), perpendicular to the laminae, the organic fibrils are alternatively seen in longitudinal, oblique, and cross sections; the pattern of the IS forms vertical septae. In an oblique section (2), close to vertical, the or-

[1] The term cholesteric was given to this precise geometric organization because it was first described in benzoate of cholesterol



Figs. 2–4. Morphology of the interprismatic septa (IS). Polygonal divisions visible in the Pi layer are interpreted as imprints left in the cuticle by the margins of the epidermal cells

Fig. 2. Photomicrograph of a semi-thin section of the pre-ecdysial layers realized in a region where the cuticle is bent inside the araldite block. This allows visualization of two different section planes in the same section. In a oblique section (left side of the photograph, ▶) the IS appear polygonal. In a plane almost vertical to the cuticle surface (right side of the photograph, →) the IS appear as divisions perpendicular to the laminae. Methyl blue and azur B staining, ×420

Fig. 3. TEM of a close to horizontal section (parallel to the cuticle surface) in the Pi layer. The nested arcs, characteristic of the cholesteric organization of the chitin-protein microfibrils, appear very large and run through several prisms. The polygonal IS are electron-dense and correspond to material deposited at this level. Uranyl acetate and lead citrate. ×5100

Fig. 4. TEM of a oblique section in the Pi layer (almost transverse to the cuticle surface), arcs are very narrow, the margin of each prism covers 10 or more laminae. The stain by uranyl acetate and lead citrate reveals a fine fibrillar structure over the IS, ×5400

ganic fibrils appear as narrow arcs, and the IS pattern forms asymetric polygons elongated perpendicularly to the laminae. Each surface of a prism covers several laminae (about 10 in our micrographs). In oblique sections (3), close to horizontal, the organic fibrils form wide arcs, and the polygonal pattern of the IS cover a portion of arc only; several prisms are superimposed over the lamina. In horizontal sections (4), the organic fibrils are parallel, so that the pattern of the prisms corresponds to the exact projection of the margins of epidermal cells.

The interpretation that the IS are imprints of the margins of epidermal cells left in the cuticle is corroborated by ultrastructural observations. Indeed, in transverse sections of the integument immediately after ecdysis, a good correlation exists between the disposition of the IS vertical limits, and the cell nuclei. Moreover the spacing of the tight junctions, visualized on the apical plasma membrane of the epidermal cells, is similar to that of the IS.

Morphological observations show that the IS begin two or three laminae under the ep-Pi junction, and continue up to the last laminae of the Pi layer (Figs. 2–4).

The organic material forming the IS is electron dense; in a close to vertical section this material appears to be made of short fine fibrils after uranyl acetate and lead-citrate staining (Fig. 4). A fine granular appearance has been observed after staining with phosphotungstic acid (Y. Bouligand, personal communication). The uniform dispositions of the pore canals (fine cellular expansions of the epidermal cells) and their localisation over the polygonal pattern (Fig. 3), argues in favour of the penetration of pore canals into the IS.

Treatment with pronase

The polygonal pattern of IS can be observed on a transverse semi-thin control section stained by azur B and methyl blue (Fig. 6). The necessity of placing the control section on

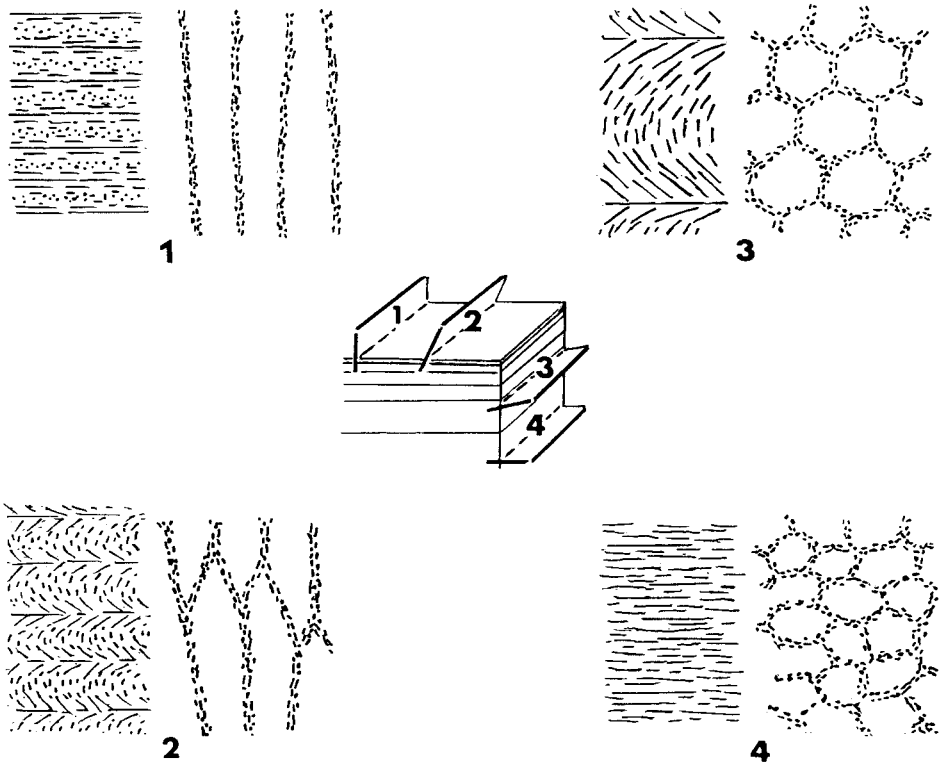
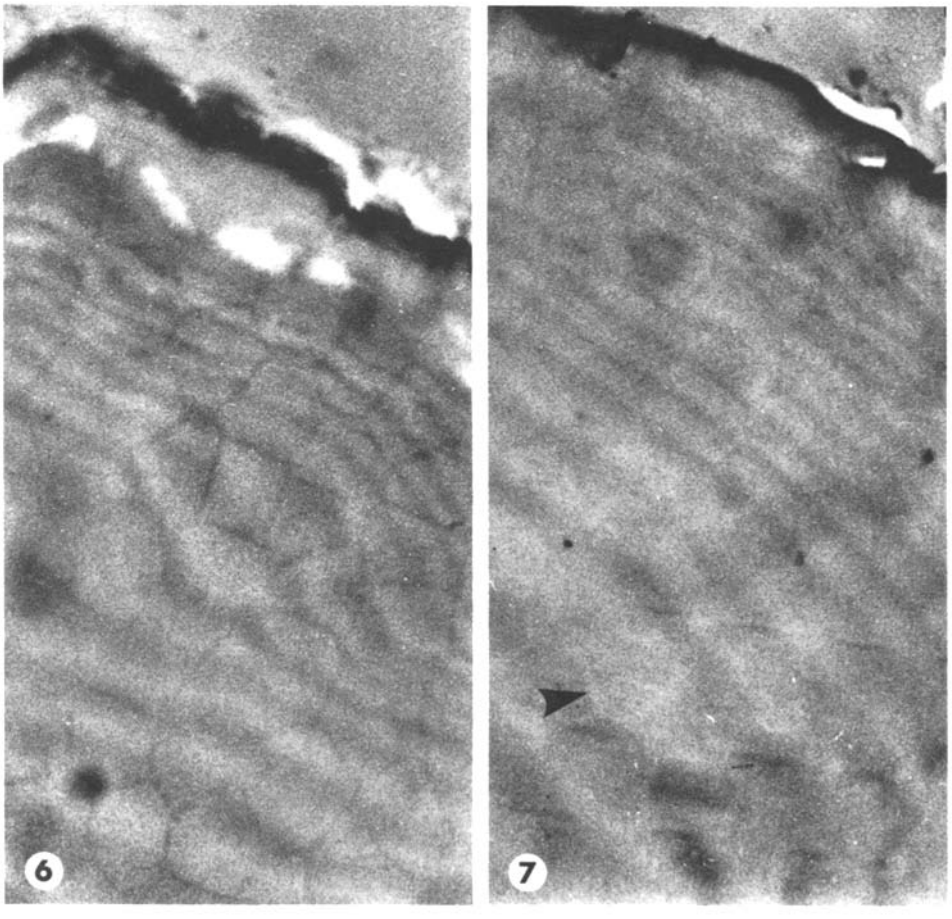


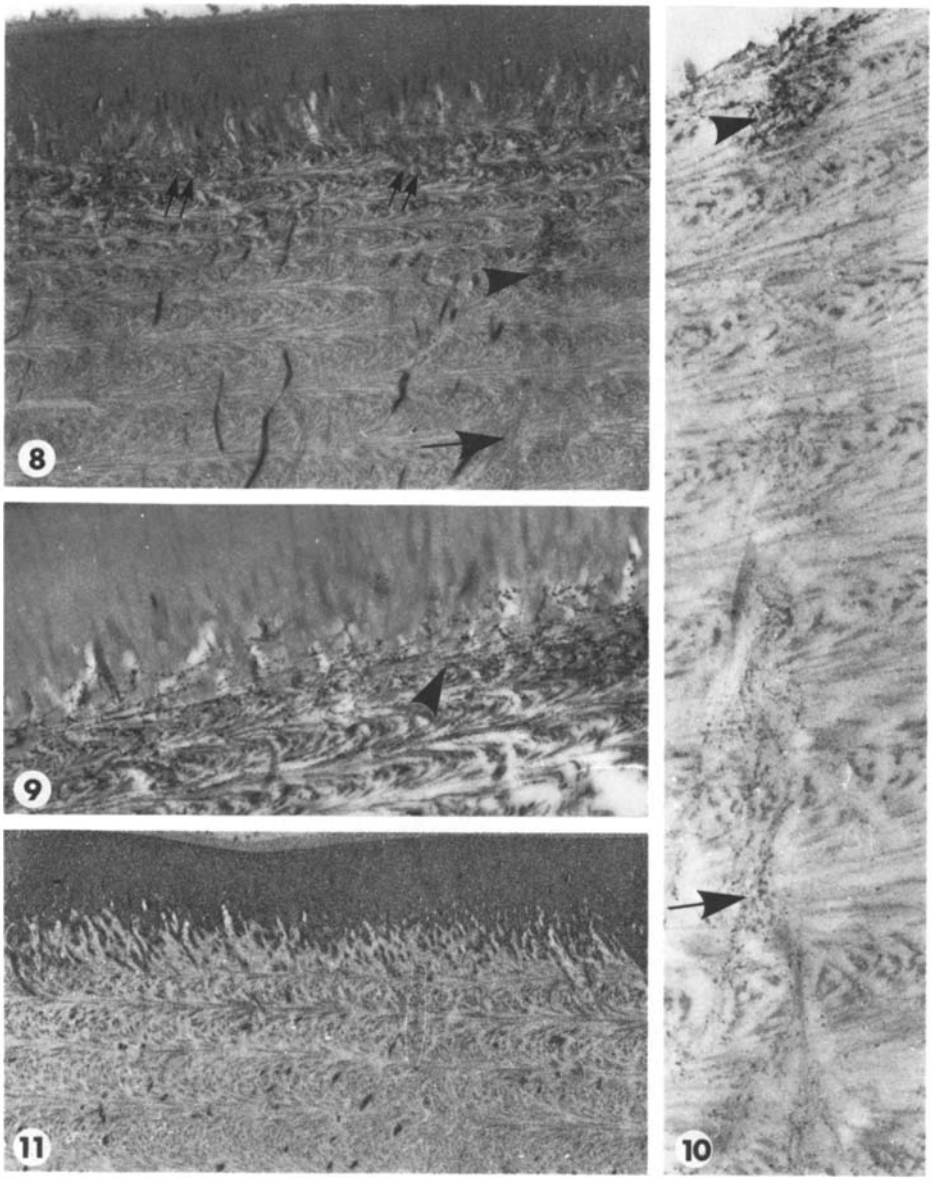
Fig. 5. IS pattern in function of the fibrous cholesteric organization: In a vertical section (*1*) perpendicular to the cuticle surface, the chitin-protein fibrils are seen alternatively in cross, intermediate and longitudinal sections, the IS appear as vertical limits. In an oblique section (*2*) (close to vertical) organic fibrils appear as narrow arcs. The IS pattern consists of polygons elongated perpendicularly to the laminae. In an oblique section (*3*) (close to horizontal) the organic fibrils appear over each lamina as large arcs covering several IS polygonal patterns. In a horizontal section (*4*) the organic fibrils are parallel, the IS patterns correspond to the exact straight projection of the cell margins



Figs. 6–7. Treatment with pronase. Photomicrographs of semi-thin sections (1 μm) of crab cuticle embedded in a hydrosoluble resin (GMA). Methyl-blue and azur B staining. × 2340

Fig. 6. Control section floated 1 h at 40° C on buffered distilled water. The IS pattern can be observed all over the section

Fig. 7. Section floated 1 h at 40° C on pronase 0.5%. The IS are much attenuated and even appear negatively at the bottom of the photograph (▶)



Figs. 8–11. Ruthenium red staining. Electron micrographs of vertical thin sections in the upper laminae of the Pi layer

Fig. 8. The staining by RR is visible all along the two or three first laminae of the Pi layer (⇉) and over the IS (a maximum at first (▶), very lightly downwards (→). ×8100

Fig. 9. At a higher magnification the RR-OsO_4 deposit is seen on the organic fibrils forming the first laminae of the Pi layer (▶), and absent in the epicuticle. ×16150

Fig. 10. The RR staining of the IS first appears as a dense deposit (over a few μm (▶) and continues as a light deposit visible all along the IS pattern in the thickness of the Pi layer (→). ×25000

Fig. 11. Control section from cuticle prepared by aldehyde/OsO_4 fixation without RR. The epicuticle (*ep*) presents roots in its lower part penetrating in the first laminae of the Pi layer. No staining is visible. ×7800

the buffer 1 h at 40° C reduces the staining of IS compared with that of a non-floated section. Enzymatic extraction of protein by 0.5% pronase (Fig. 7) reduces unequally the IS pattern in relation to the level in the cuticle.

A very light pattern remains visible over the IS located in the outer Pi layer close to the epicuticle. On the contrary, the polygonal design appears in negative contrast in the lower laminae. All observations were made on serial sections.

The presence of protein constituents forming part of the IS material can be inferred from these observations. However, the deposition of protein seems to be unequal; a rather low protein fraction is present in the outer cuticle laminae of the IS and a richer one underneath.

Ruthenium red staining (RR)

The fine-structural localization of 0.2% ruthenium red reveals a dense deposit over the two or three first laminae of the Pi layer (the staining is continuous all along the laminae), and on the outermost part of the interprismatic septae over approximately 3 μm (Fig. 8). At a higher magnification (Fig. 9) the RR-OsO_4 deposit is observed specifically on the fibrous organic matrix of the first Pi layer laminae, the roots of the epicuticle being unstained. Expanding downwards a light staining continues over the IS down to the lowest laminae of the Pi layer (Fig. 10). The control section (Fig. 11) is unstained.

Since the staining by RR is considered (Luft 1971) as due to the presence of anionic groups of glycosaminoglycans, our results demonstrate their presence in the cuticle. The distribution corresponds to specific regions of the cuticle involved in the initiation of calcification. Over the IS an unequal distribution of glycans can be inferred as a function of staining density, with a higher proportion in the outermost IS, and less in the lower IS.

Ultrastructural localization of carbonic anhydrase

Ultrathin cuticle sections, embedded in GMA and treated according to the procedure of Sugai and Ito (1980), showed a strong reaction over the IS, and a light staining over

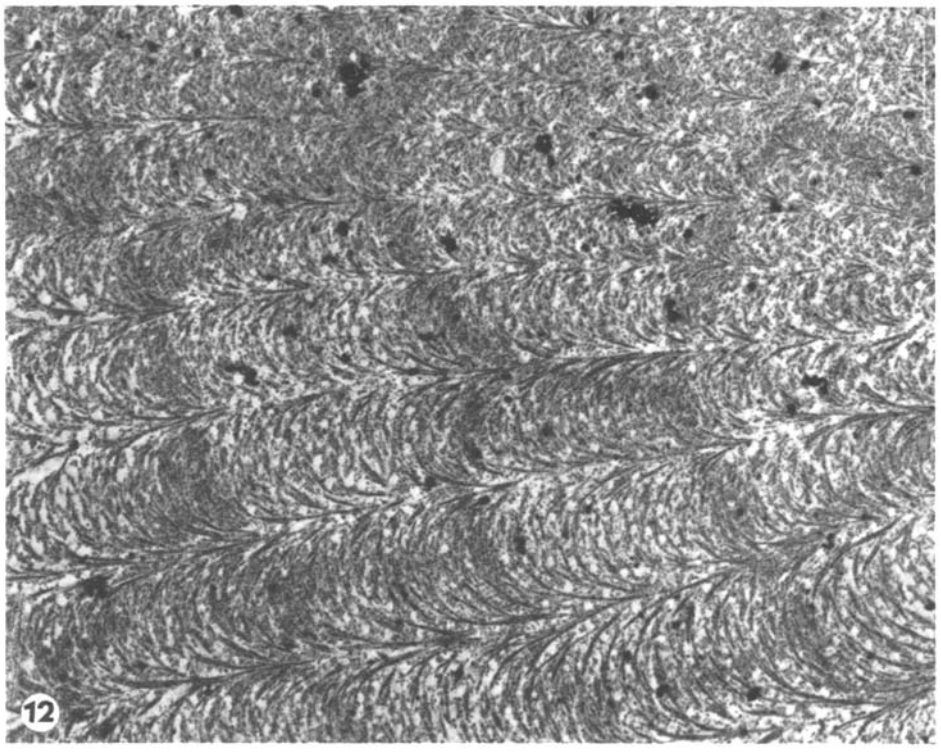


Fig. 12. Ultrastructural localization of carbonic anhydrase. The Hansson reaction applied on thin floating sections of cuticle embedded in GMA reveals a maximum cobalt reaction product over the IS polygonal pattern in an oblique cuticle section. A light staining apparent all over the section could be attributed to the uncatalysed reaction (see discussion). × 14000

the whole section (Fig. 12). Incubation time was limited to 4 min. Indeed, the spontaneous deposition of the cobalt complex corresponding to the progressive accumulation of the uncatalyzed reaction product appeared rapidly, i.e., within 10 min the section was greyish. The reaction control on semi-thin sections incubated in presence of 10^{-5} M acetazolamide showed only a partial inhibition of the reaction. The validity of the cuticular staining cannot be firmly asserted in view of the controversy about the specificity of the Hansson reaction and the validity of the method (Maren 1980; Lönnerholm 1980; Sugai and Ito 1980) or the unreliability of the acetazolamide inhibition (Muther 1977; Anderson et al. 1982).

Nevertheless, the presence of the enzyme in the cuticle is doubtless. Indeed the extracellular presence of carbonic anhydrase in the crab cuticle was demonstrated by measuring the enzyme activity in cuticle homogenates with a manometric method (Giraud 1981). So the precise localization of the cobalt reaction product by our cytochemical study argues in favour of the presence of carbonic anhydrase at the IS level.

Discussion

Nature and deposit of the IS

The cytochemical characterization of the IS imprints visible in the cuticle supports the hypothesis that this material corresponds to cell-coat glycoproteins of the epidermal cells released in the cuticle.

Pronase treatment resulted in the hydrolysis of the material forming the IS, the action is unequal with respect to the cuticle level. This observation allows us to conclude that pronase has a clear action in the cuticle, and that the IS have a varying composition. Indeed there exists a progressive increase in density of the chitin-protein complexes deposited throughout the Pi layer (Giraud-Guille 1983), and the enzyme action is at its maximum (negative contrast) where the organic matrix density is also at its maximum.

No effect of pronase was observed at the ultrastructural level on the fibrous chitin-protein matrix, the macromolecular links of this fibrous chitin-protein twisted structure being particularly resistant.

The inorganic dye ruthenium red is a hexavalent cation that reacts by ionic interactions with extracellular polyanions (Luft 1971). The RR staining is uneven over the IS. This staining localizes anionic charges, in the exact sites of the crab cuticle, were beginning of calcification occurs.

A more precise characterization of glycoconjugates at the IS level was attemped with the use of concanavaline A. This lectin binds to specific α-D glucosidic or α-D mannosidic residues (So and Goldstein 1967) on the outer side of the cell membrane. Staining with the fluorescent lectin, wheat germ agglutinine (WGA), binding to N-acetyl-glucosamine (characteristic of chitin), was previously shown to be possible in the complex arthropod cuticle (Mauchamp and Schrével 1977). Fluorescent concavaline A (Con A-FiTC) incubated in GMA gave fluorescent localization of the IS. However controls remained unclear. Absence of autofluorescence was controlled over the IS, but incubation in presence of the inhibiting sugar (0.2 M α-D methyl mannoside) resulted in the crab cuticle in a persisting fluorescence.

The Con A-FiTC binding sites over the IS can therefore only be mentioned.

The correlation established between the material forming the IS and the cell-coat glycoproteins raises several questions on the release and replacement of components of the cell surface.

The presence of fused membranes in the tight junctions of the apical limit of the epidermal plasma membrane forms a barrier to diffusion of cell-coat material from the plasma

membrane located on the epidermal cell sides. RR staining implies a first glycoprotein release on the entire apical cell surface, as RR deposit occurs all along the first Pi layer laminae. Staining is then limited to the IS imprints corresponding to the cell margins. Therefore, the deposit of cell coat-like material is either restricted to limited regions of the cell membrane, or released over the entire apical cell surface and pushed further laterally.

The release of glycoproteins in the cuticle implies the action of proteases or glucosidases. This dynamic process also requires a turnover of individual components of the surface membrane.

Several hours are needed in small crabs (2 cm in width) for the secretion of one lamina of the Pi layer (of an average height of 1 μm), the entire pre-exuvial layers being secreted in several days (Drach 1939). These delays seem sufficient, since biochemical data indicate a rapid turnover (less than an hour) in the replacement of small areas of membrane (Scanlin and Glick 1977).

Role of the IS in calcification

A common feature of biomineralized structures is the presence of an organic matrix which is assumed to have important ion-binding properties. Calcification of the crustacean cuticle is an extracellular phenomenon. The first mineral deposition in the ultrastructures is observed in the outermost laminae of the Pi layer (Bouligand 1970).

Calcification begins after ecdysis and, therefore, after complete deposition of the Pi layer. This withdraws the sites of calcification initiation from the epidermal cells by about 100 μm in small crabs. The mineral deposit is $CaCO_3$ in the form of calcite. The transport of calcium ions in the cuticle is though to be made via the pore canals since electron micrographs show mineral deposits in these regions (Travis 1963).

In the carapace a bidirectional active transport of calcium through the epidermis has been proposed to be effected by a quercitin-sensitive Ca-ATPase and a Na/Ca exchange mechanism. The active outward transport of calcium at stages A_1 and A_2 is associated with the contribution of pore canals to the cell surface area (Roer 1980).

Carbonic anhydrase in the extracellular organic matrix has been directly associated with the calcification process, indeed acetazolamide inhibits the growth of calcite spherulites "in vivo" (Giraud 1981). The ultrastructural localization of this enzyme at the calcification initiation sites is therefore of significance. Calcification in the extracellular organic matrix also depends on a nucleation process, the minimum grouping of ions, with the structure of the crystalline material (presently a calcite germ) that can persist and grow in solution.

Many articles provide experimental evidence for the role of organic molecules, i.e., glycoproteins, in nucleation of the mineral phase in calcified matrices (for review, see Simkiss 1976; Vittur et al. 1979).

The presence of calcium in the IS has been confirmed on ion images, at mass 40, of crab cuticle sections at stage A_2, by secondary ion microanalysis (Giraud-Guille and Quintana 1982). The occurrence in the IS of polyanions with affinity for Ca^{2+}, and therefore concentrating calcium, could induce the initial precipitation phase. These polyanions associated with cell-coat glycoproteins would be released in the cuticle as the Pi layer is secreted.

Inhibitory roles of calcium-binding proteins and polysaccharides have also been proposed. Ca^{2+}-binding soluble protein extracted form oyster shell suppresses carbonate nucleation (Wheeler et al. 1981). The inhibition of $CaCO_3$ crystallization by an acid Ca^{2+}-binding polysaccharide associated with coccoliths has been demonstrated in vitro (Borman et al. 1982).

In the crab cuticle the spatial localization of the cation-binding glycoproteins at the IS level lends support to a role in nucleating the mineral phase. The rapid start of calcification after ecdysis in the outer laminae of the Pi layer can be related, at stage A_2, to a maximum calcium uptake (Roer 1980), a maximum carbonic anhydrase activity (Giraud 1981), and the presence of calcium-binding glycoproteins.

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