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CYTOCHEMICAL DEMONSTRATION OF ACID MUCOPOLYSACCHARIDES IN THE EPICUTICULAR SURFACE COAT OF THE CRAB *CARCINUS MAENAS* (L.) (CRUSTACEA, DECAPODA)

by

PHILIPPE COMPÈRE and GERHARD GOFFINET
Laboratoire de Biologie générale et de Morphologie ultrastructurale,
Institut de Zoologie, Université de Liège,
quai Ed. Van Beneden 22, B-4020 Liège (Belgium)

SUMMARY

The epicuticle of marine decapod crustaceans is composed of three sublayers : the inner epicuticle, the cuticulin layer, and the surface coat which was previously described as an external layer rich in tannophilic proteins. Positive staining by ruthenium red and alcian blue suggests the presence in this layer of polyanionic sites and acid mucopolysaccharides. The surface coat might be viewed as a hydrophilic glycoproteinaceous layer reducing the surface tension between the hydrophobic cuticulin layer and the aqueous environment.

Keywords : Acid mucopolysaccharides, cuticle, *Carcinus maenas*, cytochemistry.

INTRODUCTION

The epicuticle is defined as a thin, multilayered, outer covering of the arthropod cuticle. Its complex structure and chemical composition vary considerably with the ecological conditions and the physiology of the integument. The presence of surface waxes reducing water loss is one of the most important adaptative features of terrestrial arthropods (insects, arachnids). In marine decapod crustaceans, the epicuticle is composed of three sublayers : the inner epicuticle, the cuticulin layer, and the outermost surface coat (KÜMMEL *et al.*, 1970 ; COMPÈRE, 1988, 1995). The inner epicuticle and the cuticulin layer are common structures of the arthropod cuticle while the surface coat was only described in crustaceans (COMPÈRE, 1995). In *Carcinus maenas (op. cit.)*, the surface coat is deposited during late premoult after the cuticulin layer, the inner epicuticle, and a great part of the pigmented layer. After the moult, it appears as a relatively thick (0.1 µm), electron-dense, fuzzy coat overlying the cuticulin layer. The recent demonstration that this coat is mainly proteinaceous (showing a strong tannophilic reaction, COMPÈRE and GOFFINET,

1992) and hydrophilic raises the question : might this surface coat contain acid mucopolysaccharides and electrostatic surface charges ? The hypothesis that it is negatively charged is supported by the fact that, in the gill cuticle of fresh water acclimatised crabs *Eriocheir sinensis* (MILNE-EDWARDS, 1854) intoxicated with $HgCl_2$, it accumulates Hg^{2+} ions (BARRADAS and PÉQUEUX, 1995).

The purpose of this study is to detect polyanionic sites and acid mucopolysaccharides at the ultrastructural level in the epicuticular surface coat of the Atlantic shore crab *Carcinus maenas,* using cytochemical methods based on staining with ruthenium red and alcian blue.

MATERIAL AND METHODS

Cuticular samples excised from branchiostegites and gills of the crab *Carcinus maenas* were fixed by immersion for 2 h at 20° C in 2.5 % glutaraldehyde, 50 mM $MgCl_2$, 0.05 M Na-acetate buffer (pH 7.4 or 5.8) containing either 0.1 % ruthenium red (LUFT, 1971) or 1 % alcian blue 8GX (SCOTT and DORLING, 1965 ; SCOTT, 1970 ; LEWIS and KNIGHT, 1977). Control experiments were carried out in the presence of 1 M MgCl2 according to Scott's (1970) critical electrolyte method. Branchiostegite samples were decalcified under the same conditions (pH, buffers, $MgCl_2$ and stain concentrations) for 24 h at 4° C in a solution containing 0.1 M EGTA and 1.25 % glutaraldehyde, then fixed again for 90 min in 2.5 % glutaraldehyde solutions as detailed above. To enhance staining, we post-fixed all the samples for 1 h at 4° C in 1 % OsO_4, using the same buffer, pH and $MgCl_2$ concentration. Ruthenium-red- and Alcian-blue-treated samples were washed in buffer and OsO_4-fixed, respectively, with or without the stain. All samples were then processed classically without further staining and examined in a transmission electron microscope (JEOL 100-SX) at 80 kV accelerating voltage.

RESULTS

The ruthenium red and alcian blue staining methods give quite similar qualitative results, staining being only slightly more intense with ruthenium red. In the samples fixed at pH 7.4 (Figs 1, 2, 5 and 6), the surface coat appears very electron-dense, but less so than the outer leaflets of the cuticulin layer (COMPÈRE, 1988, 1995). The chitin-protein fibres of the pigmented layer are also contrasted while the inner epicuticle remains electron-lucent. In all layers but the cuticulin layer, positive

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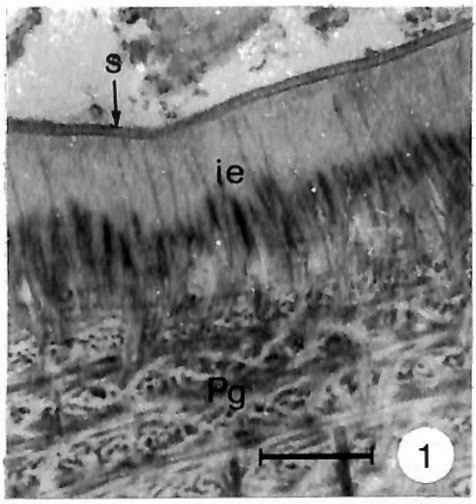
Figs 1-8. Branchiostegite epicuticle of the crab *C. maenas* — 1-4. Treated with alcian blue — 5-8. Treated with ruthenium red — 1, 2, 6. at pH 7.4 and 50 mM $MgCl_2$ — 3, 5, 7 at pH 5.8 and 50 mM $MgCl_2$ — 4, 8. at pH 7.4 and 1 M $MgCl_2$. c, cuticulin layer ; ie, inner epicuticle ; Pg, pigmented layer ; s, surface coat ; ie, inner epicuticle ; Pg, pigmented layer ; s, surface coat. Scale bars in 1, 5 : 1 μm ; in 2-4, 6-8 : 100 nm.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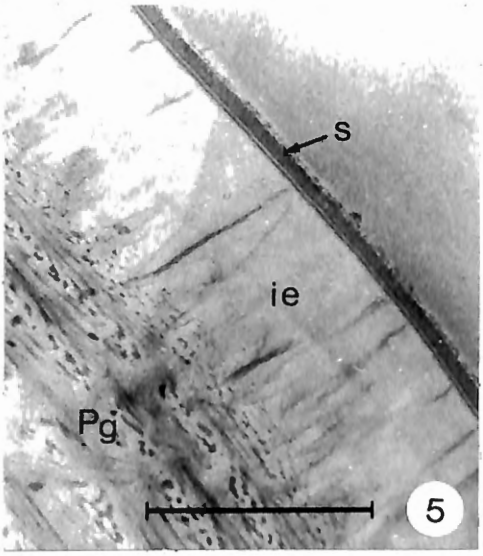
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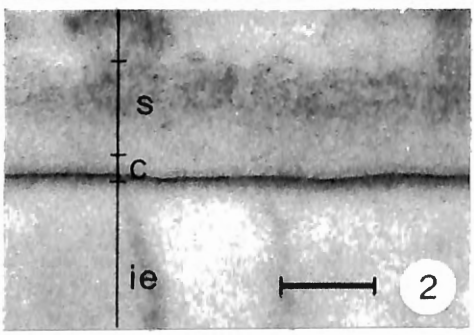
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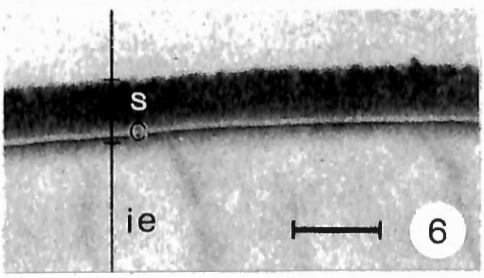
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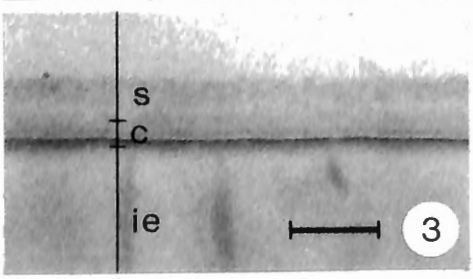
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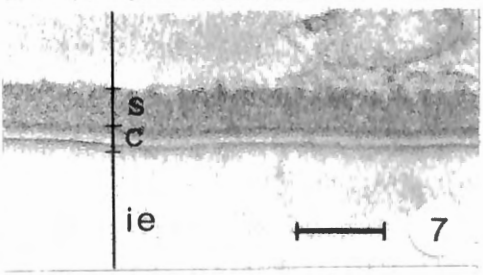
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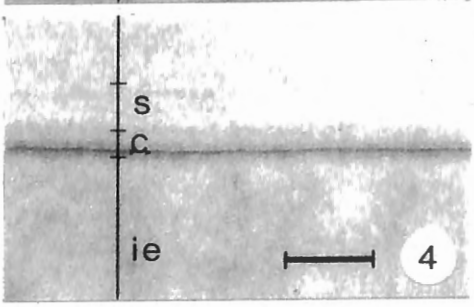
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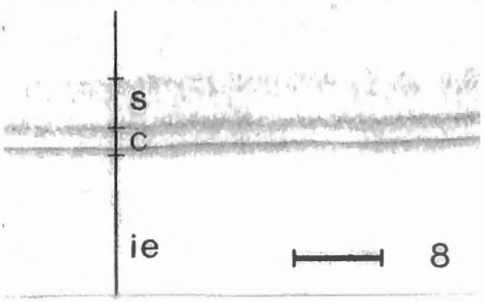
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staining appears slightly less intense in the samples fixed at pH 5.8 (Figs 3 and 7) and completely absent in control samples treated in the presence of 1 M $MgCl_2$ (Figs 4 and 8).

DISCUSSION

Our results point to the presence of polyanionic sites and acid mucopolysaccharides in the epicuticular surface coat of the branchiostegite and gill cuticle of the shore crab *C. maenas*. The slightly higher contrast in samples treated at pH 7.4 than in ones treated at pH 5.8 is presumably attributable to weakly negative sites, *i.e.* the carboxyl groups of proteins, which are not stained at pH 5.8 in the presence of a low concentration (50 mM) of $MgCl_2$ (SCOTT and DORLING, 1965 ; LEWIS and KNIGHT, 1977). At pH 5.8, the appearance of contrast strongly suggests the presence of negatively charged acid mucopolysaccharides. Furthermore, the absence of contrast in control samples treated in the presence of a high concentration of electrolyte (1 M $MgCl_2$) confirms the electrostatic nature and the specificity of the reaction (*op. cit.*). The persistence of the high electron-density of the cuticulin layer leaflets in these controls is probably due to a nonspecific reaction.

In combination with the previous demonstration of tannophilic proteins (COMPÈRE, 1990a ; COMPÈRE and GOFFINET, 1992), the presence of polyanionic sites and acid mucopolysaccharides suggests that the epicuticular surface coat of the crab *C. maenas* forms a hydrophilic, glycoproteinaceous, external coat. This composition explains the affinity of this sublayer for Hg^{2+} ions in *E. sinensis* gills after intoxication by $HgCl_2$ (BARRADAS and PÉQUEUX, 1995). Accumulation of Hg^{2+}, moreover, is greater in fresh-water-acclimatised animals than in seawater-acclimatised ones, being inversely proportional to the electrolyte concentration.

The presence of this hydrophilic external sublayer is probably a general feature of the epicuticle of aquatic arthropods, since a surface coat or corresponding layers have been reported by several authors or can be structurally identified on published micrographies in many crustaceans (decapods : KÜMMEL *et al.*, 1970 ; FOSTER and HOWSE, 1978 ; GREEN and NEFF, 1972, COMPÈRE, 1990a, 1995 ; COMPÈRE and GOFFINET, 1992 ; copepods GHARAGOZLOU-VAN GINNEKEN and BOULIGAND, 1973, 1975 ; cladoceran branchiopods : HALCROW, 1976) and different pycnogonid species (COMPÈRE *et al.*, 1993, FAHRENBACH, 1994). In the terrestrial oniscoid isopods, the surface coat is also present but appears much thinner than in marine decapods (COMPÈRE, 1990b). On the other hand, POQUET *et al.* (1994) have shown that in a parasitic copepod in mussel gills, the external coat of the epicuticle (« el », according to the terminology of GHARAGOZLOU-VAN GINNEKEN and BOULIGAND, 1973, 1975) gives a positive reaction with phosphotungstic acid (PTA) at pH 0.3, indicating the presence of glycoproteins (RAMBOURG, 1971). No homology can be proposed, however, between this external coat and the epicuticular surface coat of decapods. Apart from the observation of the surface coat deposition in the sclerites of *C. maenas* (COMPÈRE, 1988, 1995) and *Oniscus asellus* (L., 1758) (COMPÈRE, 1990b)

during late premoult, there is no information in the litterature on the morphogenesis of these external epicuticular layers.

Concerning the role of these outermost layers, many different highly specialised functions have been proposed but all are hypothetical. In some pycnogonids, the filamentous coat is believed to act as a protective layer against nematocysts (FAHRENBACH, 1994). POQUET *et al.* (1994) suggest that the glycoproteinaceous coat of parasitic copepods plays a role in the attachment of bacteria or protects the copepod against host reactions. According to ARNAUD *et al.* (1988), sublayer « e1 » plays a key role in the agglutination of food particles in the labral glands of calanoid copepods, but the presence of similar or at least analogous external epicuticle sublayers in a variety of aquatic arthropods suggests, rather, that these layers have a common basic function. In all cases, the surface coat directly overlies the cuticulin layer, which is known to be hydrophobic and membrane-like structure (NEVILLE, 1975 ; FILSHIE, 1982 ; WIGGLESWORTH, 1985). Similarly, *C. maenas* (COMPÈRE and GOFFINET, 1992), the cuticulin layer appears to form the main permeability barrier of the cuticle. The surface coat might thus be viewed as a hydrophilic layer protecting the cuticulin layer and/or reducing the surface tension between the hydrophobic cuticulin layer and the aqueous environment. In this respect, the surface coat would resemble the cell coat or the polar heads of plasma membrane phospholipids.

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REFERENCES

ARNAUD, J., M. BRUNET and J. MAZZA (1988) — The labral glands in *Centropages typicus* (Copepoda, Calanoïda) : I. Site of synthesis. *J. Morphol.*, **197** : 21-32.

BARRADAS, C. and A. PÉQUEUX (1995) — Influx and accumulation of mercury in the gills of euryhaline chinese crab *Eriocheir sinensis* (Milne-Edwards). In *Proc. 1st Iberian Cong. of Contamination and Environmental Toxicology. Proscientia,* Coimbra, Portugal : 67.

COMPÈRE, PH. (1988) — Mise en place de l'épicuticule chez le crabe *Carcinus maenas*. In P. LE GAL and A. VAN WORMHOUDT : *Aspects récents de la Biologie des Crustacés*. IFREMER, Brest, A.C. **8** : 4754.

COMPÈRE, PH. (1990a) — Structure, mise en place et cytochimie de l'épicuticule et des systèmes de canaux intracuticulaires du crabe *Carcinus maenas* au cours d'un cycle de mue et en relation avec la specialisation physiologique de diverses regions tégumentaires. Doctorat thesis, Université de Liège : 167 pp.

COMPÈRE, PH. (1990b) — Fine structure and elaboration of the epicuticule and pore canal system in tergite cuticle of the land isopod *Oniscus asellus* during a molting cycle. In

P. JUCHAULT and J.P. MOCQUARD : *Biology of terrestrial Isopods III.* Université de Poitiers, Poitiers : 169-175.

COMPÈRE, PH. (1995) — Fine structure and morphogenesis of the sclerite epicuticle in the Atlantic shore crab *Carcinus maenas. Tissue Cell* (in press).

COMPÈRE, PH. and G. GOFFINET (1992) — Organisation tridimensionnelle et cytochimie de l'épicuticule et des systèmes canaliculaires des sclérites du crabe *Carcinus maenas* (Crustacé décapode). *Mém. Soc. r. belge Ent.*, **35** : 715-720.

COMPÈRE, PH., THIRY PH., J.-C. BUSSERS and G. GOFFINET (1993) — Ultrastructure of the integument of the sea spider *Pycnogonum littorale* (Ström) (Pycnogonida). *Belgian J. Zool.*, **123** (suppl. 1) : 11-12.

FAHRENBACH, W.H. (1994) — Microscopic anatomy of Pycnogonida : 1. cuticle, epidermis and muscle. *J. Morphol.*, **222** : 33-48.

FILSHIE, B.K. (1982) — Fine structure of the cuticle of insects and others Arthropods. In R.C. KING and H. AKAI : *Insect Ultrastructure,* vol. 1. Plenum Press : 281-312.

FOSTER, C.A. and H.D. HOWSE (1978) — A morphological study on gills of the brown shrimp, *Penaeus aztecus. Tissue Cell.*, **10** : 77-92.

GHARAGOZLOU VAN GINNEKEN, I.O. and Y. BOULIGAND (1973) — Ultrastructures tégumentaires chez un Crustacé Copépode, *Cleptocampus retrogressus. Tissue Cell.*, **5** : 413-439.

GHARAGOZLOU VAN GINNEKEN, I.O. and Y. BOULIGAND (1975) — Studies on the fine structure of the cuticle of *Porcellidium,* Crustacea Copepoda. *Cell Tissue Res.*, **159** : 399-412.

GREEN, J.P. and M.R. NEFF (1972) — A survey of the fine structure of the integument of the fiddler crab *Uca pugilator. Tissue Cell,* **4** :137-171.

HALCROW, K. (1976) — The fine structure of the carapace integument of *Daphnia magna* Strauss (Crustacea, Branchiopoda). *Cell Tissue Res.*, **169** : 267-276.

KÜMMEL, F., H. CLAASSEN and R. KELLER (1970) — Zur Feinstruktur von Cuticula und Epidermis beim Flusskrebs *Orconectes limosus* während eines Haütungszyklus. *Z. Zellforsch. mikrosk. Anat.*, **109** : 517-551.

Lewis, P.R. and D.P. KNIGHT (1977) — Staining methods for sectioned material. A.M. GLAUERT : *Practical Methods in electron Microscopy, vol. 6.* North Holland Publ. Company, Amsterdam, N.-Y., Oxford : 311 pp.

LUFT, J.H. (1971) — Ruthenium red and violet. II. Fine structural localization in animal tissues. *Anat. Rec.*, **171** : 369-392.

NEVILLE, A.C. (1975) — Biology of the arthropod cuticle. W.S. HOAR, J. JACOBS, H. LANGER and M. LINDAUER : *Zoophysiology and Ecology vol. 4/5.* Springer Verlag, Berlin Heidelberg, New-York : 448 pp.

POQUET, M., E. RIBES, G.M. BOZZO and M. DRUFORT (1994) — Ultrastructure and cytochemistry of the integument of *Modiolicola gracilis,* parasitic copepod in mussel gills (*Mytilus galloprovincialis* and *Mytilus edulis*). *J. Morphol.*, **221 : 87-99.**

RAMBOURG, A. (1971) — Morphological and histochemical aspects of glycoproteins at the surface of animals. *Int Rev. Cytol.*, **31,** 57-114.

Scott, J.E. (1970) — Histochemistry of alcian blue. I. Metachromasia of alcian blue, astrablau and other cationic phthalocyanin dyes. *Histochemie,* **21** : 277-285.

SCOTT, J.E. and J. DORLING (1965) — Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. *Histochemie,* **5** : 221-233.

WIGGLESWORTH, V.B. (1985) — Sclerotin and lipid in the waterproofing of the insect cuticle. *Tissue Cell,* **17** : 227-248.