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Ultrastructure and Mineral Composition of the Tergite Cuticle of the Iulid Millipede *Ophyiulus pilosus*

(Myriapoda, Diplopoda)

by

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Abstract: The tergite integument of *Ophyiulus pilosus* (NEWPORT) during the resting or "intermoult" phase was investigated ultrastructurally and chemically with a view to characterizing cuticular waterproofing and hardening in diplopods. The epicuticle organization follows the same basic scheme found in insects. From inside to outside it consists of the inner epicuticle, the laminated cuticulin layer, a wax layer and a cement-like layer. Dermal gland ducts and pore canels associated with tubular filaments extend from these superficial layers to the epidermis. Calcium and magnesium salts, representing about 70 % of the cuticular dry weight, impregnate the whole exocuticular and endocuticular layers of the procuticle.

1. Introduction:

The cuticle of arthropods is commonly regarded as a highly specialized cell coat providing a protective exoskeleton against hostile environments. It is also admitted that some improvements of the outer epicuticular layers in waterproofing and sclerotization of procuticular layers in cuticular hardening play an important role for insects and arachnids in achieving their success in a terrestrial mode of life. In this respect, diplopods appear very original since several authors (SHRIVASTAVA 1970 b, CARMIGNANI & ZACCONE 1977, ANSENNE et al. 1990) reported that their cuticle is hardened by impregnation of calcium salts as it is the general rule in crustaceans. However, information concerning either the ultrastructure or the mineral composition of the cuticle of diplopods is still scarce.

The present paper summarizes recent observations concerning the intermoult cuticle of the iulid *Ophyiulus pilosus* (NEWPORT) with special reference to the organization of the epicuticle and the pore canal system and to the extent of mineralization in the procuticle.

2. Material and Methods:

Ophyiulus pilosus individuals, collected in an oak wood near Stams (Tirol, Austria) were obtained from Dr. E. Meyer (Innsbruck). – For transmission electron microscopy, samples were fixed by direct immersion for 3 h at room temperature in a 2.5 % glutaraldehyde solution buffered with 0.1 M Na-cacodylate at pH 7.4 and rinsed in the same buffer. Demineralization was realized for 2 - 3 days at 4° C in 0.2 M EDTA at pH 8.0, fixed again in glutaraldehyde for 1 h and post-fixed for 3 h at 4° C in buffered 1 % OsO_4. After rinsing in distilled water and dehydration in ethanol and propylene oxide series, they were embedded in Epon 812, sectioned with a diamond knife

(ultramicrotome Reichert-Jung), contrasted with uranyl actetate and lead citrate and examined in a JEOL 100 SX electron microscope at 80 kV accelerating voltage.

For scanning electron microscopy, fractured cuticle samples were fixed for 3 h in buffered 2.5 % glutaraldehyde and post-fixed overnight in 1 % OsO_4. After washing, dehydration and embedding in PELDRI II, they were dried by sublimation of the embedding medium, mounted on aluminium stubs and coated with evaporated gold-palladium before examination in a JEOL-JSM 840A electron microscope at 20 kV accelerating voltage.

Cuticle calcium and magnesium concentrations were estimated by atomic absorption from the demineralization solutions (N HCl overnight).

3. Ultrastructural Organization:

The tergite cuticle of *O. pilosus* consists of a thin (1 μm) superficial epicuticle (Figs 1 and 2) covering a fibrous lamellate procuticle about 80 μm thick (Fig. 4). It is vertically crossed by pore canals and dermal gland ducts. Its surface bears numerous microplates (about 30.000 per mm^2) regularly assembled like roof tiles (Figs 1 - 3).

3.1. The Epicuticle:

Although cytochemical data are still lacking, the epicuticle of *O. pilosus* shows a basic organization comparable to that of insects (FILSHIE 1982), arachnids (HADLEY & FILSHIE 1979) and of *Glomeris marginata* (VILLERS) (ANSENNE et al. 1990). It consists of a cement layer, a wax layer, a cuticulin layer and an inner epicuticle successively (Fig. 5). Any comparison of this organization plan with the epicuticle of other millipedes, such as *Orthoporus ornatus* (GIRARD) (WALKER & CRAWFORD 1980) or *Polyzonium germanicum* BRANDT (WEGENSTEINER 1982) is difficult owing to the confusing terminology used by these authors and also the fact that these superficial layers often undergo dramatic structural modifications when animals enter the resting phase.

In *O. pilosus* the cement layer appears as an electron dense material irregularly overcoating microplates (Fig. 5). As has been established in insects (FILSHIE 1982) and suggested in *Glomeris marginata* (ANSENNE et al. 1990), in all likelihood, this material is discharged at the cuticular surface via the numerous dermal gland ducts passing through the cuticle. The waxy material covering the cuticulin layer constitutes a strongly osmophilic layer of high electron density and of variable thickness (Fig. 5). As a general rule it accumulates in the folds between microplates and locally forms large thickenings exhibiting an irregular stratified pattern (Fig. 7). The cuticulin layer is a continuous sheet of about 20 nm thick (Fig. 5). Its laminated appearence (two electron dense outer leaflets separated by a clear median sheet) is comparable to that described in insects (LOCKE 1966), arachnids (HADLEY & FILSHIE 1979), in decapods (KÜMMEL et al. 1970, COMPERE 1988) and recently in terrestrial isopods (COMPERE 1990). This supports the view it constitutes an universal structure in Arthropods.

The inner epicuticle, of variable thickness, is composed of a fine granular matrix of medium electron density. It is however significantly thicker (0,6 μm) in the prozonite cuticle in comparison with the metazonite one where microplates, particularly their posterior margin, are reinforced with procuticular material (Figs 2 and 10). In both the prozonite and metazonite the lower part of the inner epicuticle has a higher electron density and shows a porous appearance resulting from numerous sectioned tubules limited by a dense wall (Figs 5, 6). The tubules, the inner diameter of which is about 12 nm, form a complex circumvoluted network. Locally, they seem to reach the cuticulin layer and to be in direct relation with the distal ends of the procuticular pore canals (Figs 10, 11).

3.2. The Procuticle:

As in Insects (FILSHIE 1982) and in other diplopods (*Polyxenus lagurus* (L.), SEIFERT 1967; *Cingalobolus bugnioni* CARL, *Aulacobolus excellens* (SILVESTRI), RAJULU and

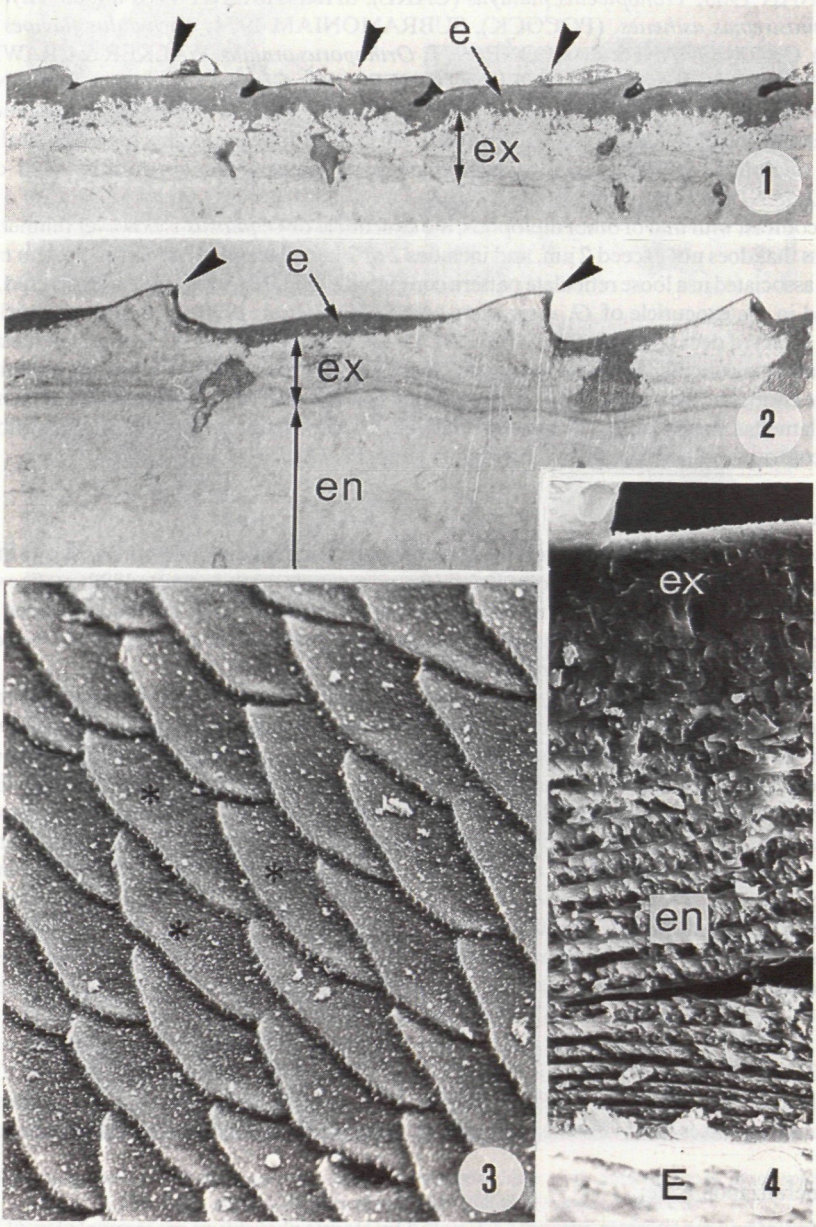


Fig. 1: Thin section through the tergite prozonite of *Ophyiulus pilosus* showing the regularly spaced microplates (arrow heads). e epicuticle; ex, exocuticle. x 7.000.

Fig. 2: Thin section through the metazonite cuticle showing the reinforced margins of microplates (arrow heads). e, epicuticle; en endocuticle; ex, exocuticle. x 8.500.

Fig. 3: S.E.M. micrograph showing the arrangement of tergite microplates (asterisks). x 2.200.

Fig. 4: S.E.M. general view of a vertical fracture through the whole cuticle. E, epidermis; en, endocuticle; ex, exocuticle. x 850.

KRISHNAN 1968; *Gonoplectus malayus* (CARL), SHRIVASTAVA 1970 a (sub *Thyroglutus m.*); *Spirostreptus asthenes* (POCOCK), SUBRAMONIAM 1974; *Pachyiulus flavipes* (C.L. KOCH), CARMIGNANI & ZACCONE 1977; *Orthoporus ornatus*, WALKER & CRAWFORD 1980; *Polyzonium germanicum*, WEGENSTEINER 1982; *Glomeris marginata*, ANSENNE et al. 1990) two distinct layers are recognized in the procuticle of *O. pilosus:* the outer exocuticle and the inner endocuticle (Figs 1, 2, 4, 10). Both show a lamellate appearence due to the classical chitin-protein microfibre arrangement in a twisted plywood system common to arthropod cuticles (BOULIGAND 1972).

In contrast with that of other diplopods, the exocuticle of *O. pilosus* is however thinner, with a thickness that does not exceed 2 μm, and includes 2 or 3 lamellae only. The chitin-protein microfibres are associated in a loose reticulate pattern comparable (Fig. 10), even it is less structured, to that observed in the exocuticle of *G. marginata* (ANSENNE et al. 1990) or in the exocuticle (pigmented layer) of decapod crustaceans (GIRAUD-GUILLE 1984). At the level of the metazonite microplates exocuticular fibres are very scarce and loose their reticulate and lamellate organization.

The endocuticle constitutes by far the bulk of the procuticle (Fig. 4). It includes a great number of thick lamellae that progressively decrease in thickness towards the epidermis. The microfibrils are of low contrast and homogenously distributed (Figs 12, 13).

3.3. The Pore Canal System:

Except for some distinct features, the cuticular pore canal system of *O. pilosus* shows the same basic organization as other Arthropods. Briefly the pore canals exhibit a twisted ribbon-like vertical pathway extending from the epidermis (Fig. 13) up to the epicuticle (Fig. 10). They are lined by vertical fibres forming a twisted sheath around an axial pore canal lumen as it appears in vertical sections (Fig. 12) or in oblique fractures (Fig. 9).

The distinct features of the *O. pilosus* pore canals concern their luminal content and the morphological relationships they exhibit with the epicuticular layers. S.E.M. observations of vertical fractures (Fig. 8) show the open pore canal lumens running through the mineralized endocuticle. With the T.E.M. they appear densely walled and filled with a fine granular cytoplasmic-like material (Figs 12, 13). At the level of the epidermis-cuticle junction, similar material seems to be spread in a cleft separating the first lamella of the procuticle from the apical cell plasma membrane (Fig. 13). In the exocuticle the pore canal ends are dilated and form bulb-like structures joining the epicuticle (Figs 10, 11). These distal ends contain clusters of circumvoluted tubular filaments (inner diameter: 10 nm; outer diameter: 20 nm) that seem to extend into the inner epicuticular matrix up to the cuticulin layer forming a complicated meshwork. Although they do not typically arise from axial dense filaments, the tubular filaments seen in *O. pilosus* could correspond to the filaments described in *G. marginata* (ANSENNE et al., 1990). These were compared to the "multiple filaments" (DELACHAMBRE 1971), also called "wax canal filaments" (LOCKE 1961), of insect (FILSHIE 1982) and arachnid (FILSHIE & HADLEY 1979) cuticles. In insects, LOCKE & KRISHNAN (1971) and WIGGLESWORTH (1985) consider these filaments as probable transport routes for waxes and reducing components (tanning precursors) to the cuticle surface.

3.4. Mineralization Rate:

Calcium and magnesium amounts estimated by atomic absorption spectrometry in *O. pilosus* tergites, account for 27.6 (± 4.2) % and 0.4 (± 0.1) % of the total dry weight respectively. Considering that Ca and Mg are freed mainly from carbonates, minerals in *O. pilosus* tergite cuticle should thus constitute about 70 % (70.4 ± 10.8) of the total cuticle dry weight. This rate is comparable to that recorded in *G. marginata* tergites (ANSENNE et al. 1990) but is lower than of the hardest cuticular regions of some marine decapod crustaceans (about 85 %) (WELINDER 1974, JEUNIAUX et al. 1986). This relatively high mineralization rate can be closely connected with the large

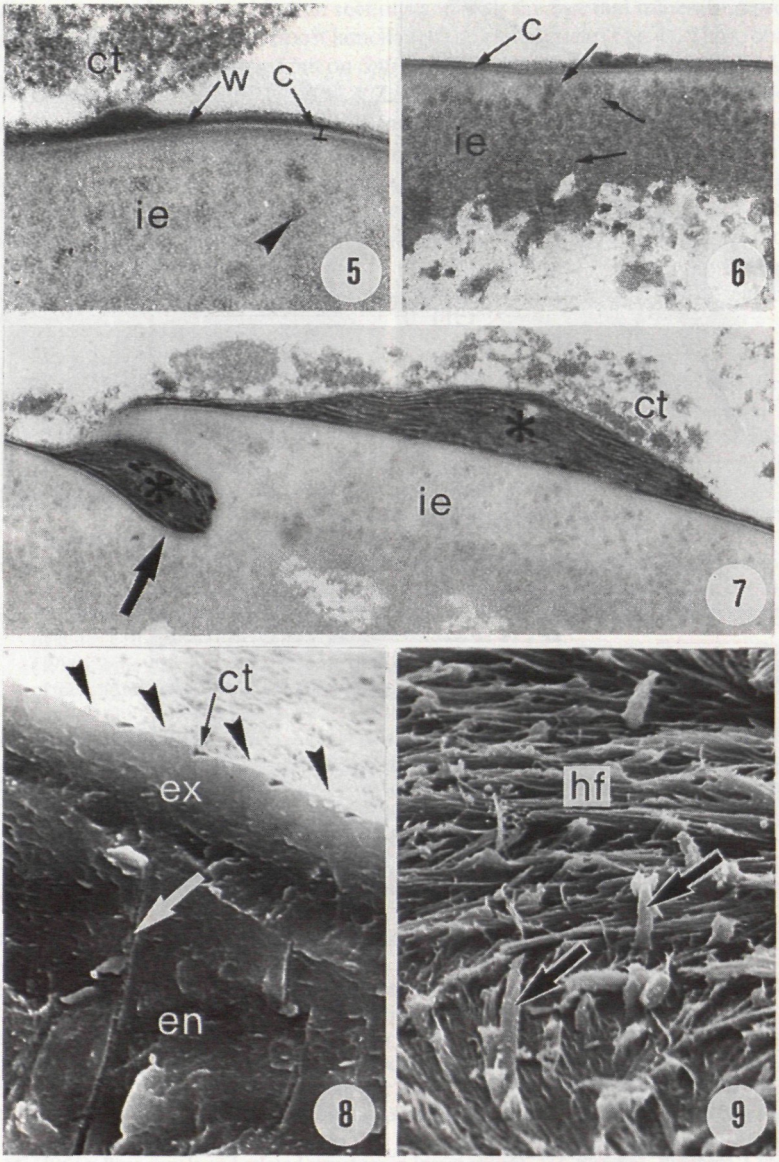


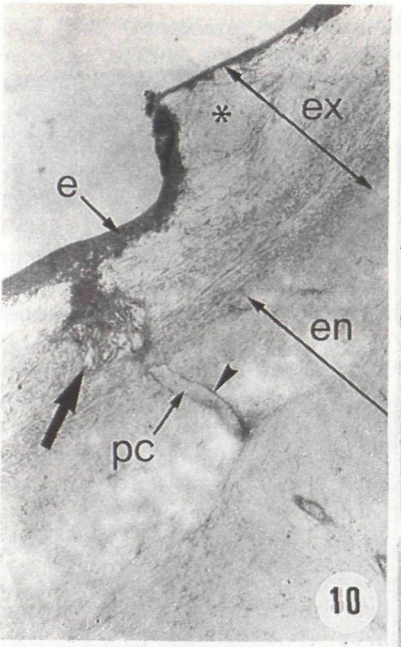
Fig. 5: The successive epicuticular layers: ct, cement layer; w, wax layer; c, cuticulin layer; ie, inner epicuticle; arrow head, section of intraepicuticular tubular filaments. x 100.000.

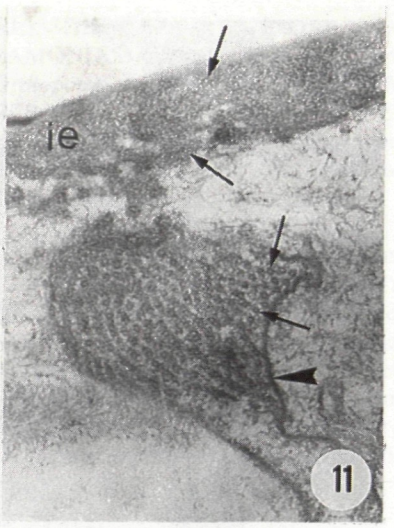
Fig. 6: Vertical section of the epicuticle showing intraepicuticular tubular filaments (arrow) in the lower part of the inner epicuticle (ie). These filaments run up to the cuticulin layer (c). x 38.000.

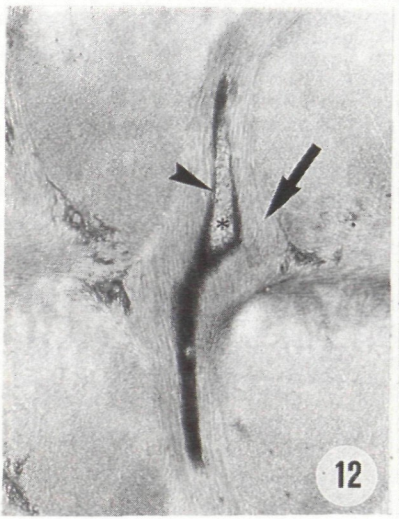
Fig. 7: Local thickenings of the wax layer (asterisks) which appears stratified. ct, cement layer; ie, inner epicuticle; large arrow, fold between two neighboring microplates. x 32.000.

Fig. 8: S.E.M. view of a vertical fracture in the mineralized tergite cuticle. Pore canal lumens (white arrow) appear as vertical open tubes. ct, cement layer; en, endocuticle; ex, exocuticle; arrow heads, microplates. x 4.200.

Fig. 9: Oblique fracture through the endocuticle. Vertical fibre sheath are emerging from pore canals (arrows). hf, horizontal chitin protein fibres. x 2.700.







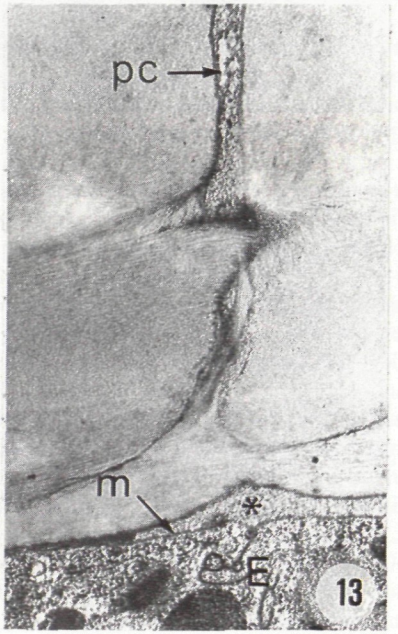


Fig. 10: Longitudinal section of a pore canal (pc) exhibiting a bulb-like dilated end (large arrow) in contact with the epicuticle (e). en, endocuticle; ex, exocuticle, showing a reticulate pattern; arrow head, dense wall of the pore canal lumen; asterisk, reinforced microplate margin. x 17 000.

Fig. 11: Detail of a pore canal bulb-like dilated end containing numerous cross-sectioned tubular filaments (arrows). Tubular filaments also appear in the inner epicuticle (ie). arrow head, dense pore canal wall. x 40.000.

Fig. 12: Longitudinal section of a pore canal in the endocuticle. Arrow, vertical fibre sheath; arrow head, dense wall; asterisk, pore canal lumen filled with cytoplasmic material. x 14.000.

Fig. 13: Longitudinal section of a pore canal (pc) near the epidermis (E). A. granular cytoplasmic-like material appears filling both the pore canal lumen and the large cleft (asterisk) between the cuticle and the epidermal cell apical plasma membrane (m). x 17.000.

interfibrillar spaces we have noted in thin sections and with the fact that minerals impregnate the whole procuticle, including the innermost lamellae of the endocuticle (Fig. 4). These observations disagree with previous studies carried out on *Spirostreptus asthenes* by SUBRAMONIAM (1974) and *Pachyiulus flavipes* by CARMIGNANI & ZACCONE (1977) who consider the endocuticle as an unmineralized procuticular layer. However, in agreement with these authors, the heaviest mineral deposits seem to occur upwards in the exocuticle as well as in the reinforced microplate borders (Figs 2, 10). The fact that the pore canal lumens persist as hollow unmineralized tubes, at least in the endocuticle (Fig. 8), as they do in decapod crustaceans (COMPERE & GOFFINET 1987 b), suggests they originate from epidermal cell process and regress after the mineral deposition has occured.

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