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ULTRASTRUCTURAL SURVEY OF TUNIC MORPHOGENESIS IN THE LARVAL AND YOUNG ADULT ASCIDIAN *ASCIDIELLA ASPERSA* (TUNICATA, ASCIDIACEA)

by

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SUMMARY

In *Ascidiella aspersa,* tunic development begins during the tail-bud stage. In the trunk, the larval cuticle and three tunic layers arise consecutively. In the tail, just the larval cuticle and one tunic layer are formed. Shortly before metamorphosis, a new very thin layer arises limiting the tail tunic interiorly. The tunic is composed of granular and fibrillar material embedded in an electron-transparent ground substance. In the course of metamorphosis, the tail is retracted. The larval cuticle and the first tunic layer are discharged. The young ascidian is just surrounded by a cuticle and one tunic layer.

Key words : Ascidian, *Ascidiella,* tunic.

INTRODUCTION

For a long while the ascidian tunic, that covers the whole body of the larvae and the adult as a protective layer, has been the subject of many investigations since it was the first animal structure where a cellulose-like polysaccharide, called tunicin, could be demonstrated (for review see : SAINT-HILAIRE, 1931 ; PRUVOT-FOL, 1951).

Although the consistency of the tunic varies remarkably among different species, some structural and biochemical features seem to be characteristic of the ascidian tunic. The adult tunic is a fibrous structure consisting of a thin cuticular layer supported by a large fundamental layer of hydrated ground substance (VAN DAELE, 1989), comprising fibrils and various types of intratunical cells (ENDEAN, 1961 ; STIÉVENART, 1970, 1971 ; DE LEO *et al.,* 1976, 1977 ; DE LEO and PATRICOLO, 1980 ; VAN DAELE and GOFFINET, 1987). In contrast to other invertebrates, eg. the arthropods where the cuticle comprises the whole non-cellular part of the integument, in tunicates the terms cuticle or cuticular layer are reserved only for the external layer of the tunic.

Chemical and histochemical studies of the tunic revealed the presence of cellulose and other polysaccharides (containing galactose, glucose, mannose, xylose and fucose), proteins, glycoproteins, sulfated glycans, as well as acid and neutral mucopolysaccharides (ENDEAN, 1961 ; DECK *et al.*, 1966 ; STIÉVENART, 1970, 1971 ; PATRICOLO and FERRARELLA, 1973 ; D'ANCONA LUNETTA and NUARA, 1975 ; PATRICOLO and DE LEO, 1979 ; ALBANO and MOURAO, 1983, 1986).

The main fraction of fibres consists of tunicin, a cellulose-like polysaccharide with associated proteins and acid mucopolysaccharides (SMITH and DEHNEL, 1970 ; DE LEO *et al.*, 1977, 1981 ; PATRICOLO and DE LEO, 1979 ; VAN DAELE *et al.*, 1988).

Recent investigations paid attention to the origin of the larval tunic, regarding the following species : *Ciona intestinalis* (LINNAEUS, 1767) (DILLY, 1969 ; MANCUSO, 1973, 1974 ; GIANGUZZA and DOLCEMASCOLO, 1980), *Perophora orientalis* ÄRNBÄCK, 1936 (TERAKADO, 1970), *Corella inflata* HUNTSMAN, 1912 and *Ascidia paratropa* (HUNTSMAN, 1912) (CLONEY and CAVEY, 1982) and *Distaplia occidentalis* BANCROFT, 1899 (CAVEY and CLONEY, 1984).

To our knowledge, no study has been done following morphogenesis of the tunic from the very beginning up to the young ascidian.

This paper presents ultrastructural investigations on the whole course of tunic development in *Ascidiella aspersa* (MÜLLER, 1776). Cytochemical studies at the ultrastructural level are in progress and will be presented in a subsequent paper.

MATERIAL AND METHODS

Embryos : breeding

Adult specimens of *Ascidiella aspersa* were collected during their breeding season by divers of the Biologische Anstalt Helgoland (BAH) in the vicinity of Heligoland (North Sea).

Sperm and oocytes of this self-fertile species were obtained separately by puncturing their gonoducts. After artificial insemination, the embryos were raised in millipore filtered seawater at room temperature (22° C). The determinate bilateral cleavage is followed by the blastula, gastrula, neurula and tail-bud stage. About 14 hours later, they hatch as tadpole larvae. The free swimming period, lasting just a few hours, is terminated by their settling on a substrate and the beginning of a radical metamorphosis (for description of the normal development in *Ascidiella aspersa* see : NIERMANN-KERKENBERG and HOFMANN, 1989).

TEM-microscopy

Specimens of the appropriate stages were fixed in 5 % glutaraldehyde buffered in 0.1M s-collidin (pH 7.4) containing 0.5 % alcian blue, and postfixed in osmium tetroxide buffered in 0.1M s-collidin compounded with 0.8 % $K_3(FeCN)_6$. They

were dehydrated in a graded series of ethanol solutions and embedded in Epon 812 (LUFT, 1961).

Ultrathin sections were cut with a Porter-Blum MT2-B ultramicrotome, collected on copper grids, stained with uranyl acetate and lead citrate (REYNOLDS, 1963) and observed with a Jeol JEM-1OO SX electron microscope at 80 kV accelerating voltage.

RESULTS

The tunic of the hatched tadpole larva of *Ascidiella aspersa,* that is occupied by several hundreds of test cells, covers the entire epidermis and solely builds up the larval fins. Hence, it is very important in larval locomotion. The tunic of the trunk is pulled out to form six or seven fins that can reach a height of up to 30 µm. The tail possesses a ventral and a dorsal fin, each of about 80 µm in height, and a caudal fin with a length of approximately 170 µm.

The first signs of the larval tunic are seen during the tail-bud stage. A small discontinous ribbon of fibrous material lies upon the epidermis (Pl. I, 1).

Only a few minutes later, the entire surface of the embryo is covered by a thin tunic layer. It consists of a network of interwoven fibrils and patches of granular material embedded in an electron-transparent ground substance. The outer limit is characterized by condensed granular and fibrillar material. The tunic of the trunk forms many thin protrusions that are perpendicularly oriented to its surface and can reach a height of up to 1,6 µm (Pl. I, 2) ; in the tail, the tunic has a rather wave-like appearance (Pl. I, 3).

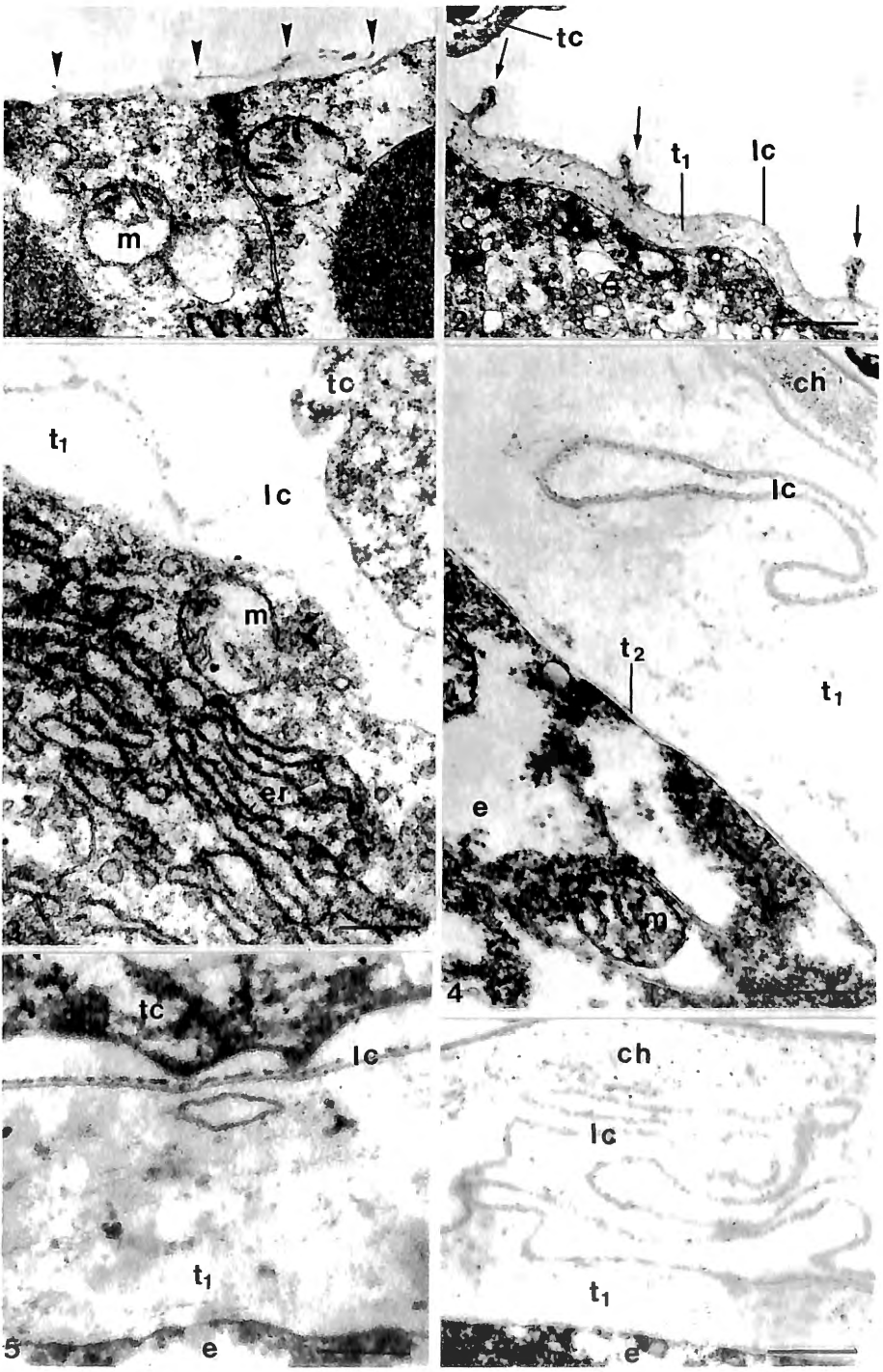
During further progress, an additional fine line of fibrous material appears upon the trunk epidermis (Pl. I, 4). The tunic of the tail enlarges, but there is no further layer visible (Pl I, 5). At the places of the future larval fins, long folded extensions develop. They are made up of the outer dense border and the first tunic layer (Pl. I, 6).

In the trunk, the development of a third tunic layer follows. Pl. II, 7 shows the trunk tunic of a 13h 05min old embryo. The outer dense border, in the following called larval cuticle (according to DILLY, 1969), consists of three sheets. The outer electron-dense one has a dotted appearance in some sections (Pl. I, 4 and 5). The middle sheet is electron-transparent, the inner one an electron-dense continous line.

In the first tunic layer, no special organization of the relatively dense material is recognizable. In contrast, the fibrils of the third tunic layer are arranged almost parallel to the epidermal surface. The second layer, that separates the first and the third from each other, is very thin and resembles the innermost cuticular sheet.

All layers are involved in larval fin formation (Pl. II, 8). At high magnification, it is possible to detect granular material attached to the fibrils of the first tunic layer (Pl. II, 8a).

PLATE I



When the larva hatches, 13 h 40 min after artificial insemination, the larval cuticle is thicker and has lost its stratified arrangement (Pl. II, 9).

The tail tunic still consists of just the larval cuticle and the first tunic layer, that both form the larval fins of the tail (Pl. II, 10).

Some hours later, shortly before the larva attaches to a substrate in order to begin metamorphosis, the tunic of the trunk and the tail looks very different (for comparison see Pl. III, 11 and 12). Now, in the trunk, the larval cuticle and the first tunic layer are thinned out and almost all material is lost. In contrast, the third tunic layer is enlarged. Near the epidermis, the density of fibrils is higher than further outwards. There, they are oriented parallel to the epidermis. In the middle part of the layer, they are arranged reticularly. Near the second tunic layer, they have the propensity to run parallel to it. (Pl. III, 11).

The thickness of the tail tunic hasn't changed, but the arrangement of fibrils is less dense than before. A new, very thin layer limits the tunic interiorly. A small cleft is visible between this layer and the epidermis (Pl. III, 12).

In the course of metamorphosis, the whole tail is resorbed and the entire body is rotated 180° (for description of ascidian metamorphosis see : BARNES, 1980 : p. 1041). The tunic of the tail is shed. The trunk is just surrounded by one tunic layer and a cuticle as soon as metamorphosis is completed. In the tunic, fibrils of random directions are embedded in an electron-lucent ground substance. Near the epidermis, they tend to lie parallel to it. The cuticle is composed of dense granular and fibrillar material which is outwards disaggregated (Pl. III, 13). It is the first time that intratunical cells are seen (Pl. III, 14).

PLATE I

Fig. 1 : First signs of the trunk tunic (arrow heads) on the surface of the epidermis. Tail-bud stage ; age of the embryo : 11 h 10 min. Scale bar : 0.5 µm.

Fig. 2 : The trunk tunic consists of the larval cuticle (lc) and the first tunic layer (t_1). Many protusions (arrows) are formed perpendicularly to the surface of the tunic. Tail-bud stage ; age of the embryo : 11 h 15 min. Scale bar : 1 µm.

Fig. 3 : The tail tunic has a wave-like appearance. The epidermis exhibits a conspicuous rough endoplasmic reticulum (er). Tail-bud stage ; age of the embryo : 11 h 15 min. Scale bar : 0.5 µm.

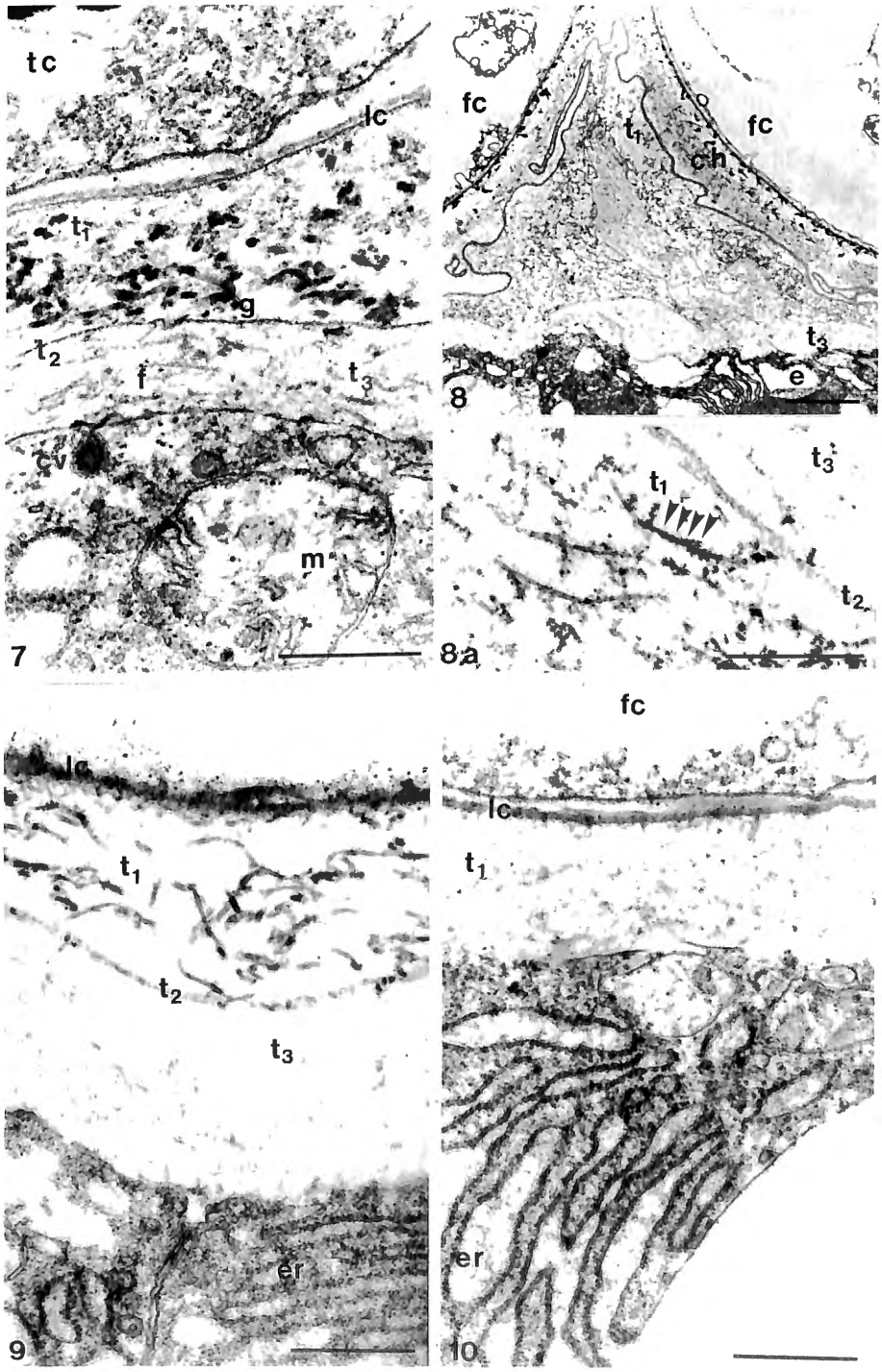
Fig. 4 : In the trunk a second tunic layer (t_2) develops. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.5 µm.

Fig. 5 : No further layer differentiates in the tail. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.2 µm.

Fig. 6 : Long folded extensions mark the places of the future larval fins of the tail. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.5 µm.

ch : chorion ; e : epidermis ; er : endoplasmic reticulum ; lc : larval cuticle ; m : mitochondria ; t_1 : first tunic layer ; t_2 : second tunic layer ; tc : test cell ; v : vesicle ; y : yolk granule.

PLATE II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During the studied period of the embryonic and larval life, the epidermis of the trunk is a columnar (Pl. IV, 15), that of the tail a squamous epithelium (Pl. IV, 16), both resting on a basal lamina. The epidermal cells possess a large nucleus, an extensive rough endoplasmic reticulum (Pl. II, 9 and 10; Pl. III, 11), a Golgi apparatus and many vesicles, ranging in diameter from 0.08 μm to 0.5 μm (Pl. I, 2). These vesicles were never seen fused with the apical plasma membrane. In contrast, coated vesicles are found in the apical region of the epidermal cells, as well as coated pits and coated grooves in the apical plasma membrane (Pl. II, 7).

During the process of metamorphosis, the epidermis of the trunk changes considerably. It becomes a squamous epithelium with a flat, but still prominent nucleus and many mitochondria (Pl. III, 13 and 14). Yolk granules, very common during embryonic and larval life (Pl. I, 1 ; Pl. II, 8 ; Pl. IV, 15 and 16), are now consumed. Cell organelles which characterize a secreting cell are no longer visible. The endoplasmic reticulum has almost disappeared (Pl. III, 13 and 14).

DISCUSSION

Tunic development in the larvae of *Ascidiella aspersa* is executed in several successive phases that are summarized in Fig. 1

Tunic deposition starts during the tail-bud stage. In the trunk, the larval cuticle and three tunic layers arise consecutively. In the tail, the tunic is just built up by the larval cuticle and one tunic layer (Fig. 1, A, B, C, D, E). All layers participate in larval fin formation, in the trunk and the tail respectively.

PLATE II

Fig. 7 : The trunk tunic consists of the larval cuticle (lc) and three tunic layers (t_1, t_2, t_3). The larval cuticle has a tripartite arrangement (see also Figs 4-6). Note the large amount of granular material (g) in the first and the fibrils (f) in the third tunic layer. Tail-bud stage ; age of the embryo : 13h 05min. Scale bar : 0.5 μm.

Fig. 8 : All tunic layers participate in the larval fin formation of the trunk. Tail-bud stage ; age of the embryo : 13 h 05 min. Scale bar : 2 μm.

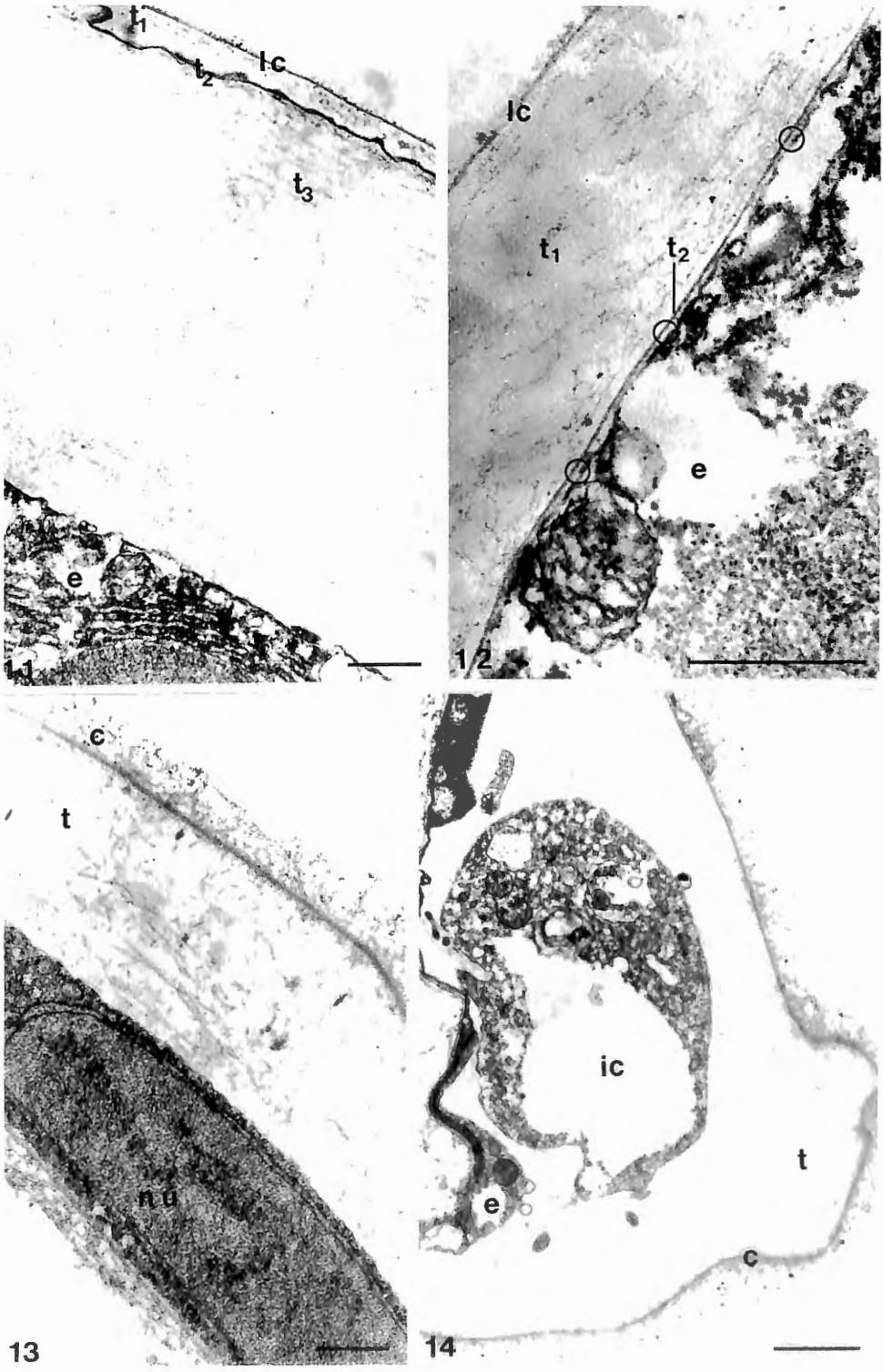
Fig. 8a : Enlargement of Fig. 8. Granular material (arrow heads) is attached to the fibrils (f) of the first tunic layer (t_1). Scale bar : 0.5 μm.

Fig. 9 : The larval cuticle (lc) has lost its tripartite arrangement (see also Fig. 10). Age of the larva : 13 h 40 min, just before hatching. Scale bar : 0.5 μm.

Fig. 10 : The tunic of the tail is still just made up of the larval cuticle (lc) and the first tunic layer (t_1). Age of the larva : 13 h 40 min, just before hatching. Scale bar : 0.5 μm.

ch : chorion ; cv : coated vesicles ; er : endoplasmic reticulum ; f : fibrils ; fc : follicle cell ; g : granular material ; lc : larval cuticle ; m : mitochondria ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer ; tc : test cell.

PLATE III



The larval tunic of *Ascidiella aspersa* consists of a network of interwoven fibrils and patches of granular material embedded in an amorphous ground substance. The larval cuticle and the second tunic layer are distinguished by compact fibrils and a small amount of ground substance.

The onset of tunic formation with respect to the developmental stage of the embryo is comparable in all studied species (*Halocynthia roretzi* (v. DRASCHE, 1884) : NISHIKATA *et al.*, 1987 ; *Ciona intestinalis* : MANCUSO, 1973 ; GIANGUZZA and DOLCEMASCOLO, 1980 ; *Distaplia occidentalis* : CAVEY and CLONEY, 1984 ; *Corella inflata* and *Ascidia paratropa* : CLONEY and CAVEY, 1982).

In contrast, the organization of the larval tunic is distinct among different species. Like in *Ascidiella aspersa* the second and third tunic layers are restricted to the trunk in *Corella inflata, Ascidia callosa* STIMPSON, 1852, *Ascidia paratropa, Clavelina huntsmani* VAN NAME, 1931 and *Molgula occidentalis* TRAUSTEDT, 1883 whereas in *Ciona intestinalis, Distaplia occidentalis, Styela partita* STIMPSON, 1852 and *Boltenia villosa* STIMPSON, 1864 both the trunk and the tail are surrounded by all tunic layers (CLONEY and CAVEY, 1982 ; CAVEY and CLONEY, 1984 ; CLONEY, 1990).

It is also known that in the course of metamorphosis, the tail is retracted by contractile fibres of the epidermis (CLONEY, 1978). The tunic of the tail is left behind.

The tunic of the young metamorphosed *Ascidiella aspersa* is composed of one tunic layer and the cuticle (Fig. 1, F). Ultrastructurally it appears that in addition to the larval tunic of the tail, the larval cuticle and the first tunic layer of the trunk are also lost during metamorphosis. In this respect, probably the second tunic layer of the larva has become the cuticle of the young ascidian.

PLATE III

Fig. 11 : The trunk tunic still consists of the larval cuticle (lc) and three tunic layers (t_1-t_3), but their thickness has changed considerably. The dense granular material is lost. Age of the swimming larva : 20 h 40 min. Scale bar : 0.5 µm.

Fig. 12 : A further layer (t_2) limits the tail tunic interiorly. A small cleft is visible between the tunic and the epidermis (circles). Age of the swimming larva : 20 h 40 min. Scale bar : 0.5 µm.

Fig. 13 : The young ascidian is just surrounded by the cuticle (c) and one tunic layer (t). Age of the ascidian : 4 days after metamorphosis. Scale bar : 0.5 µm.

Fig. 14 : Cells of probably mesenchymal origin have invaded the tunic. Age of the ascidian : 4 days after metamorphosis. Scale bar : 1 µm.

c : cuticle ; e : epidermis ; ic : intratunical cell ; lc : larval cuticle ; nu : nucleus ; t : tunic (after metamorphosis) ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer.

PLATE IV

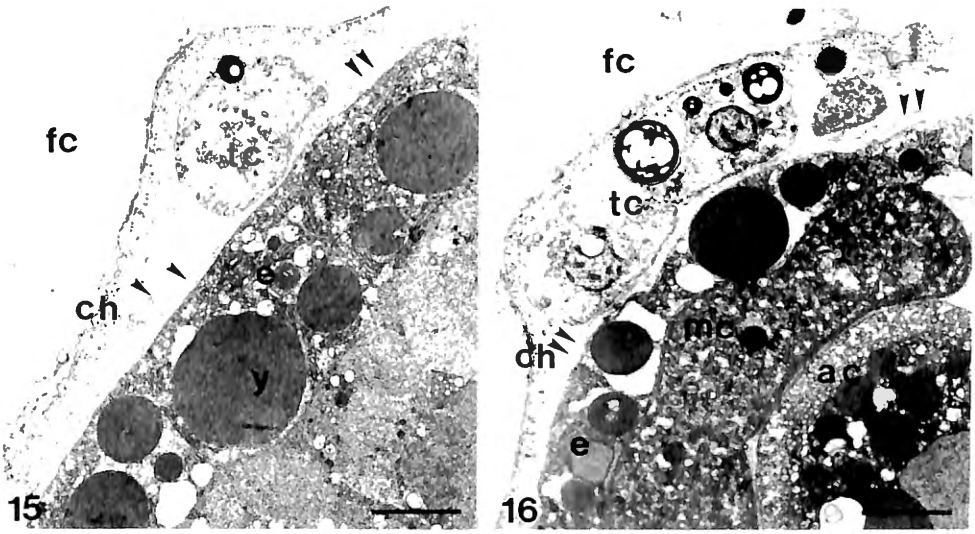


PLATE IV

Fig. 15 : The epidermis (e) of the trunk is a columnar epithelium. Age of the embryo : 11 h 15 min. Scale bar : 5 µm.

Fig. 16 : The epidermis (e) of the tail is formed by a squamous epithelium. Age of the embryo : 11 h 15 min. Scale bar : 5 µm.

ac : axial complex ; arrow heads : larval tunic ; ch : chorion ; e : epidermis ; fc : follicle cell ; mc : muscle cell ; tc : test cell ; y : yolk granule.

The ultrastructure of the epidermis signals secretory activity, both during embryonic and larval stages. The cells possess a vast rough endoplasmic reticulum, a Golgi apparatus, many vesicles and several coated vesicles, especially in the apical cell region, indicating that the epidermis is concerned with the release of components into the larval tunic. This supports the investigations of GIANGUZZA and DOLCEMASCOLO (1980) who studied the ultrastructural changes of the epidermal cells of *Ciona intestinalis* during tunic deposition. They concluded that the larval

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FIGURE 1

A : Tail-bud stage ; age : 11 h 10 min ; B : Tail-bud stage ; age : 11 h 15 min ; C : Tail-bud stage ; age : 11 h 20 min ; D : Larva just before hatching ; age 13 h 40 min ; E : Larva before metamorphosis ; age : 20 h 40 min ; F : Young ascidian ; 4 days after metamorphosis.

e : epidermis ; t : tunic (after metamorphosis) ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer.

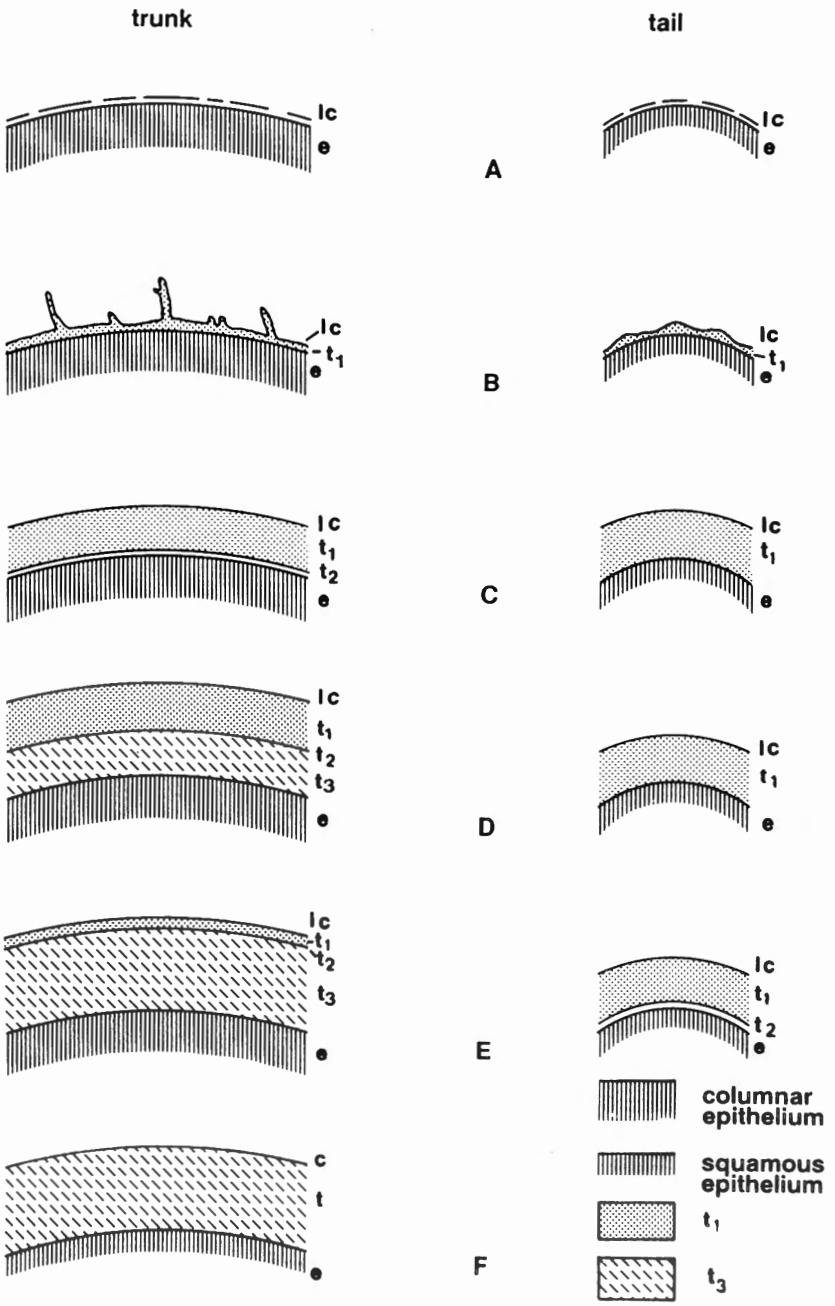

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Fig. 1 : Scheme summarizing tunic morphogenesis in *Ascidiella aspersa*.

tunic is secreted by the epidermis. Furthermore, they have shown cytochemically that coated vesicles are probably involved in this process. These vesicles might play a role as a mediator between the Golgi apparatus and the apical plasma membrane (FRIEND and FARQUHAR, 1967).

But ultrastructural observations alone cannot proove whether the epidermis is the only structure responsible for tunic formation. MANCUSO (1974) demonstrated that the epidermis of *Ciona intestinalis* is able to produce the principal elements of the larval tunic by isolating and cultivating the four animal blastomeres of the eight-cell stage, thus the presumptive ectoderm. These developing partial embryos were covered by a cuticular layer and a hydrated matrix with embedded filaments at the instant when the controls had reached the tail-bud stage.

On the other hand CLONEY and CAVEY (1982) claimed that extraembryonic structures modify the larval tunic. In *Corella inflata* and *Ascidia paratropa,* larval fin formation is inhibited when the embryos are dechorionated before they reach the tail-bud stage. Dechorionation at this stage removes the test cells and the chorion simultaneously. Once tunic secretion and larval fin formation has started, dechorionation has no further influence.

Recent investigations demonstrate that test cells are not necessary for larval fin formation but the chorion serves to contain products of the embryo responsible for the morphogenesis of the larval fins (CLONEY, 1990). The author proposed also that the test cells impart a hydrophilic characteristic to the larval tunic.

ROBINSON *et al.* (1986) described an enhancement of fin formation when dechorionated embryos of *Ascidia callosa* were treated with strongly reducing substances.

In the case of *Distaplia occidentalis,* the test cells are responsible for the ornamentation of the larval tunic (CAVEY, 1976), but it is yet not clarified whether the ornaments change any of its properties.

Less is known on the secretion of the adult tunic and the differences among the species seem to be much greater. Intratunical cells might take part in this process. In *Ascidiella aspersa* they invade the tunic during metamorphosis. It is believed that these cells are either blood cells or other mesenchymal cells passing the epidermis during the first minutes after the onset of metamorphosis (BERRILL, 1950 ; CLONEY and GRIMM, 1970 ; CLONEY, 1978, 1982).

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