# The hydrophilie of the larval test of Ascidiae: functional role played by test cells

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Ascidian swimming larvae are entirely surrounded by a hyaline extracellular coat, called as tunic or test, on which numerous test cells adhere. The functional role played by test cells in larvae of various ascidian species consists in depositing submicroscopic structures known as ornaments and/or substances of proteoglycan nature in the larval test surface. The deposition of ornaments would render the larval test hydrophilic and thus allow the larvae to swim being immersed in sea water. Ultrastructural investigations reported in literature on larvae of Cionidae and Ascididae families have not evidenced the presence of ornaments in the swimming larval test. For these Ascididae families it has been hypothesized that test cells secrete an amorphous substance that would let them to adhere to larval tunic. In order to clarify the functional role played by test cells of swimming larvae of the Ascididae family, ultrastructural and cytochemical investigations have been carried out on test cells of *Ascidia malaca* swimming larvae. The ultrastructural observations have evidenced that these cells are metabolically active and show an amoeboidic behaviour as they mainly adhere to the surface of the test. Their cytoplasm is characterized by the presence of a Golgi and large granules that gradually empty their contents and release the same on the test surface. The cytochemical investigastions carried out at ultrastructural level have evidenced that the substances secreted by test cells and deposited on the larval test consisting of glycosaminoglycans. According to the data reported in literature the results of the present investigations confirm that the deposition of glycosaminoglycans enables the adhesion of test cells on the surface of larval tunic and would render the *Ascidia malaca* larva hydrophilic and able to swim being immersed in sea water.

**Keywords** ascidian larvae; ultrastructure; cytochemical study

## 1. Introduction

After hatching and throughout the entire swimming larva stage, in many Ascidian species test cells keep adhering to the $C_1$ cuticular layer of the test. Various, at times controversial, functions have been attributed to test cells during the swimming larva stage. In some ascidian families (referred to as type I) during larval test formation, test cells release the contents of their granules on the outer layer of the larval test, in the form of granular and/or filamentous submicroscopical structures known as ornaments, together with proteoglycan substances [1-6]. In these Authors' opinion, ornaments and glycosaminoglycans deposition on the larval test renders it hydrophilic and allows the larva to swim being fully immersed in the sea. According to Cloney [3] proteoglycan deposition would also ease the adhesion of ornaments and test cells to the $C_1$ outer layer of the test. However, the ultrastructural investigations carried out on swimming larvae belonging to the Cionidae and Ascidiidae families (referred to as type II) have failed to evidence the presence of ornaments on the $C_1$ outer cuticular layer of the larval test [7-10]. The function hypothesized for the test cells of these swimming larvae is thus that of secreting a substance which would allow them to adhere to the test . In order to shed further light on the functional role played by test cells at the larval stage in Cionidae and Ascididae families, the present ultrastructural and cytochemical investigations have been made on swimming larvae of *Ascidia malaca.*

## 2. Material and Methods

Adult specimens of *A. malaca* were collected from the Gulf of Palermo during the July-November period. The most vital animals were transferred to an aquarium and kept at 16-18°C. Following gamete removal, fertilization occurred in Syracuse dishes containing pasteurized and Millipore-filtered sea water (MFSW). Embryos were left to develop up to the stage of swimming larva (3-6 hrs. after hatching) and then utilized for optical and electron microscopy investigations.

- Light microscopy

*A. malaca* swimming larvae vitally stained for 1-2 min. with Nile Blue sulphate ($1:10^5$ in MFSW), were observed and photographed with a Leitz Orthoplan microscope using an Ilford FP4 plus film.

- Transmission electron microscopy

a) A lot of the *A. malaca* swimming larvae was fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer pH=7.5, and postfixed in 1% osmium tetroxide in the same buffer.

b) The other lot of the larvae was fixed in a mixture containing 2.5% glutaraldehyde and 4% tannic acid in 0.2 M phosphate buffer pH=7.5 and postfixed in 1% osmium tetroxide in the same buffer.

 

The fixed material was dehydrated in a graded ethanol series and embedded in Epon 812. The sections obtained with the Ultracut E (Reichert-Jung) microtome were contrasted with uranyl acetate and lead citrate and photographed with Phillips EM 410 at 80 kV accelerating voltage using Kodak electron microscope film (Estar thick base 4489).

## 3. Results

- *Light microscopy investigations*

Fig. 1a shows the image of an *A. malaca* swimming larva (3-6 hrs after hatching) after *in vivo* staining for 1-2 min with Nile Blue sulphate. The larva is entirely surrounded by an outer coat known as tunic or test on the outer layer of which adhere numerous test cells equally distributed along the whole larval surface. The anterior region of the larva consists of the cephalenteron wich presents three adhesive organs known as palps or papillae and the sensorial organs inside the cerebral vesicle. Attached to the cephalenteron is a long tail, whose motion allows the larva to swim and shift about the marine environment.

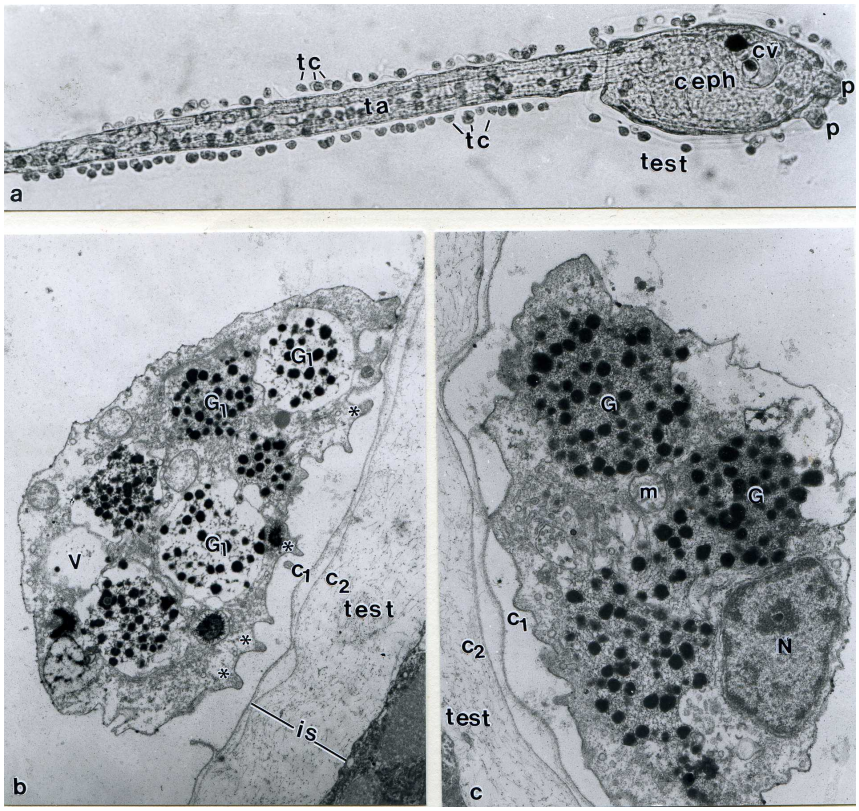


**Fig. 1** (**a**) *A. malaca* swimming larva (3-6 hrs after hatching), vitally stained with Nile Blue sulphate. The larva consists of a cephalic region or cephalenteron (ceph) and a tail (ta) for swimming in seawater. The cephalenteron presents, in its anterior region, three adhesive organs as palpi (p) and contains the cerebral vesicle (cv). The larva is completely covered by a coat called test (test) adhering to which lie numerous test cells (tc) equally distributed along the entire larva surface. (**b, c**) Test cells adhere to the larval surface both through finger evaginations of their membrane similar to pseudopodes and through numerous small membrane protrusions resembling micropoints (*). The cytoplasm of test cells is almost entirely filled with large granules (G) containing electron-dense spherical particles scattered in a highly compacted finely granular matrix. Some granules progressively empty their contents thus changing their ultrastructure ($G_1$) and eventually turning into vacuoles (v) containing scarce remnants of electron-dense material. $C_1$ = outer cuticular layer of the test; $C_2$ = inner cuticular layer of the test; is = hypocuticular compartment; N = nucleus; m = mitochondria. (a) 200x; (b) 10.000x; (c) 16.000x.

- Ultrastructural investigations

The ultrastructural investigations were carried out on swimming larvae of *A. malaca* (3-6 hrs after hatching). Observations of the test surrounding the larval cephalenteron have evidenced that, at this stage, it consists of two cuticular layers, $C_1$ and $C_2$, delimiting a hypocuticular compartment made of an amorphous matrix with abundant fibrillar structures scattered inside (figs. 1a, 1c). Numerous test cells adhere to the outer cuticular layer $C_1$ of the test and no deposition of ornaments by test cells on the outer cuticular layer $C_1$ has been observed in the present research. Test cells present the ultrastructure typical of cells with an amoeboidic behaviour due to the presence of numerous extrusion shaped as micropoints (figs. 1b, 1c) and finger shaped evaginations of their membrane similar to pseudopodes through which they adhere to the test surface (fig. 2a). At this stage the test cell cytoplasm is characterized by the presence of

large granules. Typically, these granules contain electron-dense spherical particles scattered in a finely granular, strongly compacted matrix (figs. 2a, 2b).

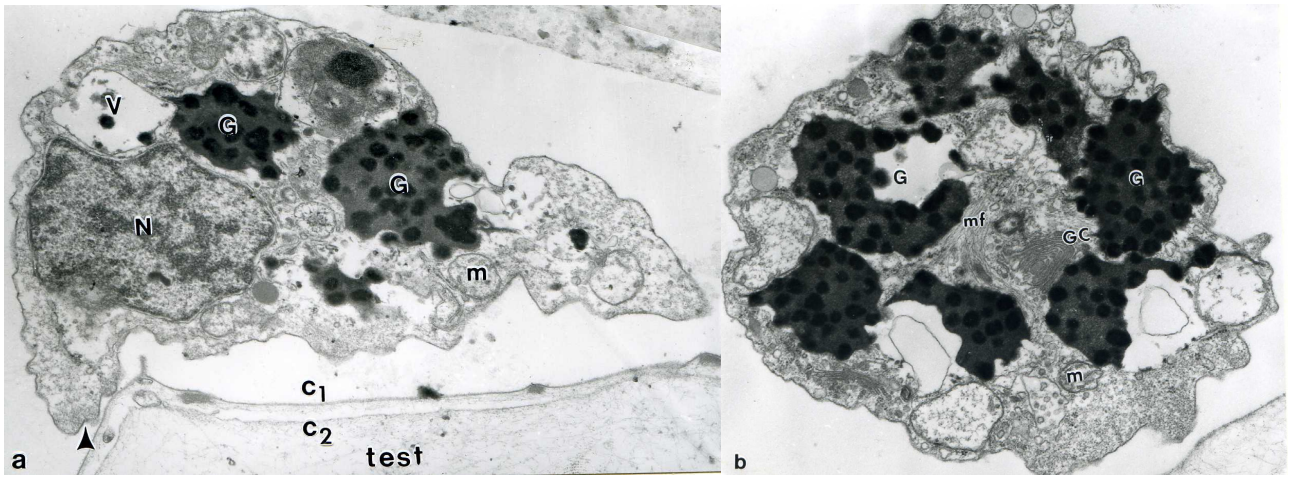


**Fig. 2** (**a, b**) The test cells cytoplasm is characterized by the presence of large granules (G). Some of the granules progressively empty their contents and they significantly change their structure. In the final modification phase, the granules empty their contents almost entirely and turn into vacuoles containing scarce residues of electron-dense spherical particles. (**b**) The Golgi complex (GC), actively involved in synthesis, is made of cisternae stacked in piles whose dilated ends by gemmation give rise to numerous vesicles. Besides the presence of regularly shaped mitochondria, the observations have evidenced microfilaments (mf) sometimes arranged in bundles. (a) 14.000x; (b) 18.000x.

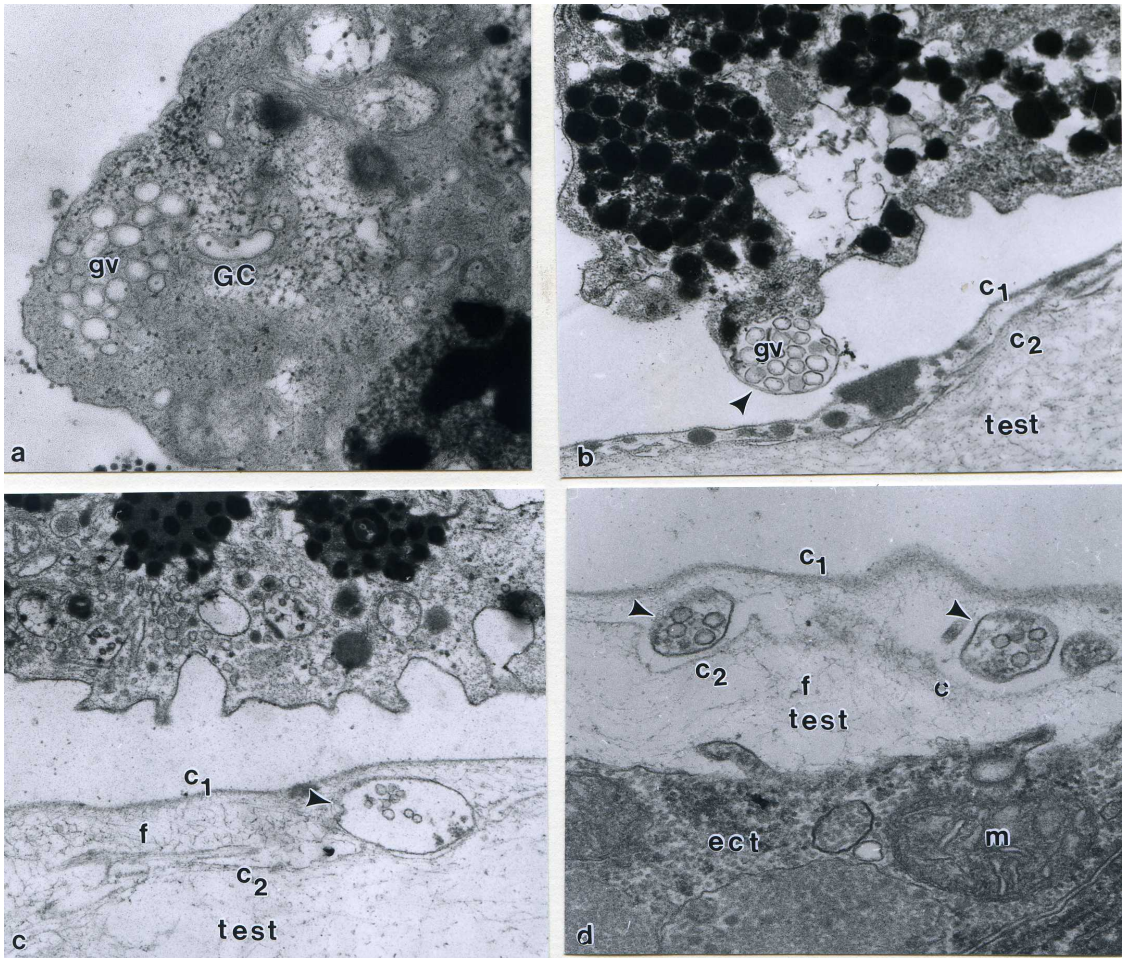


**Fig. 3** (**a, b**) Vesicles of golgian origin (gv), containing some medium electron-dense material, can be noted in the close vicinity of the inner cytoplasm membrane and inside evaginations of the cytoplasm membrane of test cells. (**c, d**) Complexes made of the same vesicle type, surrounded by a delimiting membrane, (arrows) are present inside the larval test in the space between the $C_1$ and $C_2$ cuticular layers. f = fibril. (a) 22.000x; (b) 22.000x; (c) 30.000x; (d) 35.000x.

It has been evidenced that some of the granules progressively empty their contents and during this process they significantly change their structure. The initial phase of ultrastructural modification of the granules ($G_1$) is characterized by reduction in compactness of the finely granular matrix where the electron-dense spherical particles are dispersed. In the final modification phase, the granules empty their contents almost entirely and turn into vacuoles containing scarce residues of electron-dense spherical particles (figs. 1c, 2a). The observations have also evidenced that test cells at this stage still present the characteristics of cells metabolically active. The nucleus shows euchromatine regions and clusters of eterochromatine scattered in the nucleoplasm (figs. 1c, 2a) partially set against the inner membrane of the nuclear envelope. The ultrastructural observations have evidenced the presence of long microfilaments sometimes arranged in bundles (fig. 2b). The Golgi apparatus (fig. 2b), actively involved in synthesis, is made of cisternae stacked in piles whose dilated ends, by gemmation, give rise to numerous vesicles (fig. 3a). In the lumen of the lamellae and in the golgian vesicles some medium electrondense material can be evidenced (figs. 2a, 3b). Golgian vesicles are present in the close vicinity of the cytoplasm membrane of test cells (fig. 3a) and inside protrusions of the membrane itself (fig. 3b). Complexes made of abundant vesicles of the same type, surrounded by a delimiting membrane, can be noted inside the larval test in the interstice between the $C_1$ and $C_2$ layers of the test itself (figs. 3c, 3d).

- Cytochemical ultrastructural investigations (Tannic acid reaction)

The investigations were carried out on swimming larvae of *A. malaca* (3-6 hrs after hatching) fixed in glutaraldehyde-tannic acid mixture and postfixed in osmium tetroxide. The utilization of the glutaraldehyde-tannic acid mixture before osmication can be an advantageous method to allow substances such as proteoglycans to be ultrastructurally visualized. By means of this staining technique the cytochemical investigations made at the ultrastructural level have given positive results visible as highly electron-dense granular particles inside the test cell granules undergoing emptying (figs. 4a, 4b), on the test cells outer membrane surface (figs. 4a, 4b) and above the outer cuticular layer $C_1$ of the larval test where the reaction product settles as a continuous layer (fig. 4c).

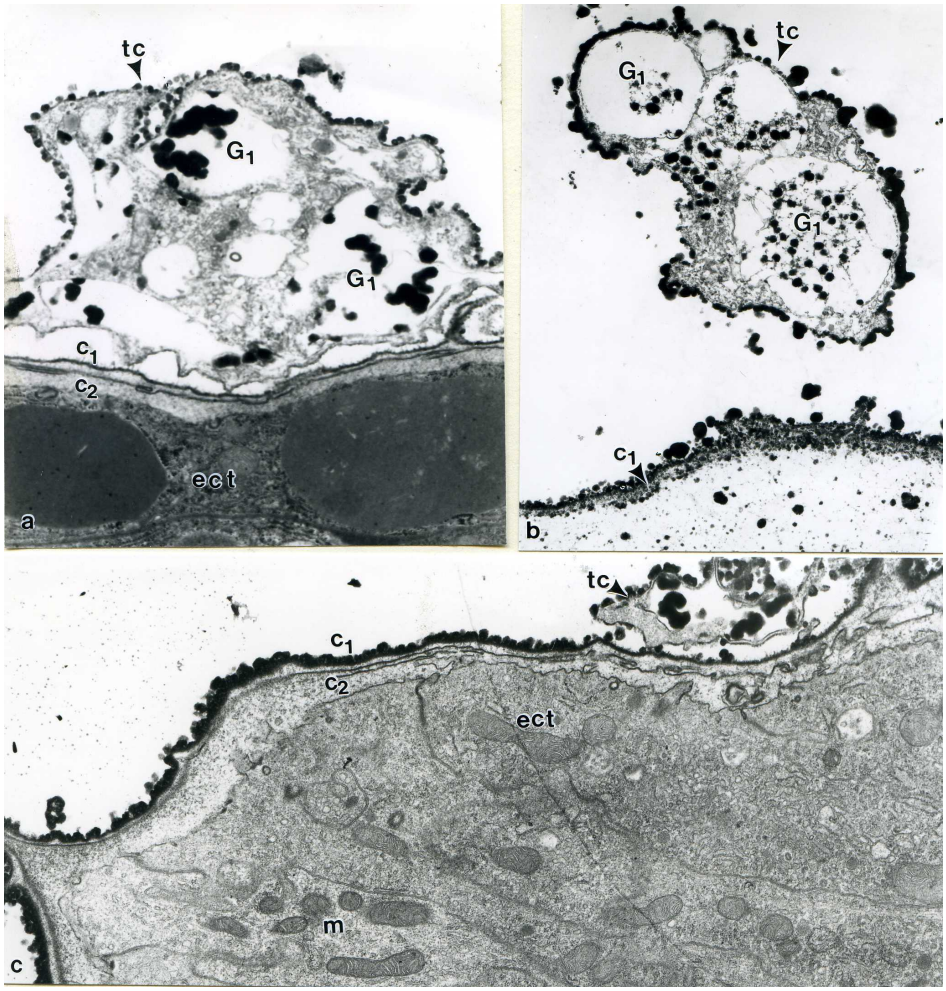


**Fig. 4** (**a, b**) Larva fixed in glutaraldehyde-tannic acid. The reaction is positive, as visible in highly electron-dense granular particles, inside breaking granules of test cells, on the surface of the outer membrane of test cells and (**c**) above the outer cuticular layer $C_1$ of the larval test, where the reaction products settle forming a continuous layer. (a) 18.000x; (b) 14.000x; (c) 20.000x.

## 4. Discussions

In some Ascidian species during the larval stage test cells adhere to the test surface on which they deposit their secretion products, thus contributing to the differentiation of this coat that encloses the larva entirely. Test cell secretion on the test surface in some Ascidian species is not limited to ornaments but includes glycosaminoglycans, substances proteoglycanic in nature. According to the literature, the release of glycosaminoglycans on the test surface renders the larva hydrophilic and thus able to swim being immersed in water. After releasing the ornaments, test cells of type I ascidians detach from the test at larval hatching. In other species known as type II (Ascididae and Cionidae) test cells do not secrete ornaments, but an amorphous substance which could have the role of making them adhere to the larval test [1-6]. What is the function played by test cells of swimming larvae belonging to the Ascididae and Cionidae families? In order to answer this question, ultrastructural and cytochemical investigations have been carried out on test cells of *A. malaca* swimming larvae. The ultrastructural observations have evidenced that these cells show an amoeboidic behaviour as they mainly adhere to the $C_1$ cuticular layer of the test by means of finger-like evaginations of their membrane similar to pseudopodes. Contact between test cells and the outer cuticular layer of the test can also occur through a large number of small wedge-shaped protrusions of the test cell membrane. The amoeboidic behaviour of test cells and their consequent shape modifications could be explained by the presence in their cytoplasm of a network of microfilaments of actin. It is generally agreed upon that actin microfilaments besides contributing to cytoplasm support, take also part in cell motion. The motion features of test cells and their movements on the test surface have been described in several ultrastructural investigations by TEM [2, 11]. The amoeboidic behaviour of test cells in ascidian larvae has been evidenced by SEM investigations by Lübbering *et al.* [12] during the initial stages of test formation in *Halocynthia roretzii*. Our present ultrastructural investigations have also shown that in swimming larvae of *A. malaca* the ultrastructure of test cells is still typical of cells metabolically active. The nucleus presents eterochromatine clusters and euchromatine regions dispersed in the nucleoplasm; besides mitochondria, in the cytoplasm can be observed a Golgi complex actively involved in synthesis and large granules. Our present observations have evidenced that some of these granules progressively empty their contents turning into vacuoles containing scarce residues of electrondense material.

As far as the functional role played by the Golgi of test cells is concerned, the ultrastructural investigations have shown that its activity consists in the formation of vesicles inside which medium electrondense material can be sometimes evidenced. The same type of vesicles is found inside evaginations of the cytoplasm membrane of test cells and in the shape of multivesicular complexes, delimited by a membrane, inside the larval test in the space between the cuticular layers $C_1$ and $C_2$. Between the two layers medium electron-dense material, probably released by the golgian secretory vesicles, can be noted. From these data we infer that the adhesion of test cells on the test of the *A. malaca* larva does not occur by surface contact, but is due to protruding pseudopodes that wedge into the test through the cuticular layer of the larval test itself. Through the wedging of pseudopodes into the larval test, test cells are able to release inside it substances carried by golgian vesicles. It is hypothetized that these substances are very likely for the adhesion of test cells to the larval test. The protrusion of pseudopodes and their passage through the cuticular layer of the larval test has been described also by Sato *et al.* [13] in *Ciona intestinalis* larvae. In ascidians the formation of adhesive substances by the Golgi during the larval stage is a process well documented in the literature. In the anterior region of ascidian swimming larve, for instance, are present adhesive organs known as palps, through wich larvae can fix to a substrate and start metamorphosis. Ultrastructural investigations reported in the literature have evidenced that the adhesive substance secreted by the palps is synthesized by the very active Golgi found in collocytes, a cell type present in these adhesive organs, and has been identified as a glycoproteic substance [14-20]. All the above Authors agree on the relationship between the glycoproteic nature of the secretion and its adhesive function.

As to the functional role played by the material originated by the progressive emptying of the granules present in test cells of *A. malaca* larvae, our results agree with the data reported in the literature. The ultrastructural observations have confirmed the absence of ornaments in the vacuoles of test cells and on the test surface of *A. malaca* swimming larvae. However, our cytochemical investigations have evidenced that the contents of the emptying granules released by test cells and deposited on the test surface consist of glycosaminoglycans, i.e. proteoglycan substances. The cytochemical technique used to evidence the presence of glycosaminoglycans at the ultrastructural level is based on fixation in glutaraldehyde-tannic acid. Tannic acid, used in electron microscopy as a supplementary fixation agent, forms complexes with proteins and heavy metals and produces a highly electron-dense precipitate insoluble in dehydratation solvents. Morevoer, tannic acid can be used both before and after treatment with osmium tetroxide. The treatment with tannic acid before post-fixation with osmium tetroxide allows cell structures to be well preserved whereas the glutaraldehyde-tannic acid mixture is effective in preserving intercellular glycosaminoglycans [21]. In *A. malaca* swimming larvae fixed in glutaraldehyde-tannic acid, the reaction has given positive results visible as highly electrondense spherical particles evidenced inside the vacuoles of test cells, on the outer membrane of the latter cells and on the larval test surface where the reaction product settles as a continuous layer. The interpretation that can be made of our ultrastructural and cytochemical observations is that the functional role played by test cells consists in pouring the contents of the emptying granules, as glycosaminoglycans, on the outer surface of the larval test. In agreement with the data reported in the literature, we believe that the functional meaning of glycosaminoglycan

 

deposition on the larval test surface is that of render it hydrophilic and thus allow the larva to swim being immersed in water. Various investigations reported in the literature have confirmed the presence of glycosaminoglycans in the vacuoles of ascidian test cells. The deposition of glycosaminoglycans by test cells on the larval test has been described by Sato and Morisawa in *C. intestinalis* [22]. These authors have shown that in late swimming larvae the cuticle is positive to alcian blue whereas test cells, which were previously positive, at this stage reduce their reactivity (i.e. are negative to alcian blue) because they have already deposited their secretion product made of glycosaminoglycans on the test surface. In Cloney's opinion [3], glycosaminoglycans are negatively charged molecules and for this reason they have hydrophilic properties. In fact, these molecules act as anions and could attract water molecules forming a sort of hydrophilic coating on the larval tunic thus allowing the larva to be immersed in water.
In conclusion, the results of our present investigations have contributed to a further understanding of the functional role played by test cells during the genesis of the larval tunic in Ascidians. In agreement with the data reported in the literature, we believe that glycosaminoglycan deposition on the larval test renders the *A. malaca* larva hydrophilic and thus able to swim being immersed in water.

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