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Histology of the ascidian *Botryllus schlosseri* tunic: in particular, the test cells

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ABSTRACT

From the tadpole stage we have examined the tunic of *Botryllus schlosseri* and particularly the histology of the test cells.

Three distinct types of cells were recognized. The first, the fusiform, elongated and without filopodia, were distributed along the vessel walls. The second, the fibrocytes, had granular cytoplasm and short pseudopodia. The third, the vacuolated, had cytoplasm, much vacuolated, and long filopodia.

Our findings indicate that the vacuolated cells are the blood morula cells transformed after having migrated through the epidermis or endothelium into the tunic.

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INTRODUCTION

The ascidian *Botryllus schlosseri* has a colonial vascular system composed of a complex network that runs in the common tunic and ends in a crown of vascular ampullae at the periphery of the colony. This system originates in the initial eight ampullae of the larva and expands in a characteristic pattern (Brunetti & Burighel, 1969), following the colony growth.

As in other ascidians, the tunic of *B. schlosseri* is basically made up of a fibrous matrix populated with cells, generically referred to as test cells. In addition to enveloping and supporting the zooids, the tunic plays other important roles, e.g. in budding and in defence. This latter aspect has recently received particular attention because of the immunologist's interest in the phylogeny of immunity (Anderson, 1971; Reddy *et al.*, 1975; Wright & Cooper, 1975; Warr & Marchalonis, 1978; Hildemann, 1979).

When different colonies contact the tunic, Botryllids show an intraspecific self-notself recognition by means of a fusion non-fusion reaction; this latter reaction being accompanied by formation of necrotic zones with repulsion and removal of the colonies (Oka & Watanabe, 1957; Sabbadin, 1962; Oka, 1970). Some aspects of this process have been clarified by Tanaka (1973) and by Tanaka & Watanabe (1973) with the electron microscope and by Mukai & Watanabe (1974), but others are to be yet elucidated, e.g. the effective participation of the different tunic components in the process. For such an investigation a good basic knowledge of the tunic's components under normal conditions is an obvious prerequisite.

Reports on the test cells and on the tunic origin of the various ascidians differ greatly owing to the great variations in the tunic of the different species and to the different conditions (see Godeaux, 1964; Smith, 1970).

In the *B. schlosseri* tunic, De Santo (1968) distinguishes several cell types and suggests for them a possible origin and differentiative line. Based on our observations, the test morphology and the possible relations between the blood cells and the test cells under normal conditions are here reconsidered and discussed.

MATERIALS AND METHODS

Colonies of compound ascidian *B. schlosseri* were cultured in our laboratory, according to the technique of Sabbadin (1960). Specimens at different stages of development were used: from larva to colony composed of numerous adults, arranged in systems within the common tunic (Fig. 1).

All the specimens were fixed in 1.5% glutaraldehyde buffered with 0.2 M sodium cacodylate plus 1.7% NaCl. After post-fixation in 1% OsO_4 in 0.2 M cacodylate buffer, they were dehydrated and embedded in Epon. Thick sections of 1 µm were stained directly or after removal of the plastic matrix.

As a general stain for the tunic the technique was used of Huber *et al.* (1968) with basic fuchsin and methylene blue, and also that of Humphrey and Pittman (1974) with methylene blue, azure II and basic fuchsin. For the cells we used light green with PAS (Pool, 1964), malachite green with PAS, and also Giemsa's stain (Berkovitz *et al.*, 1968). Some sections were stained with the polychromatic staining method of Sato & Shamoto (1973), using basic fuchsin and methylene blue in boric acid solution.

RESULTS

Tunic matrix and cuticle

In transverse section the tunic of *B. schlosseri* appears as an integument enveloping the zooids. It is limited internally by the zooid epidermis and externally by a thin layer of high density, the cuticle (Fig. 2).

The zooid epidermis is a simple epithelium with cells of strongly basophilic cytoplasm containing large vesicles with PAS positive granules and ovoidal nuclei having a nucleolus. In the tadpole larva and oozooid the epithelium is cylindrical.

The tunic is organized like true connective tissue: there are fibres and ground substance in which the cells are located. The ground substance is homogeneous and uniformly distributed, staining pink with basic fuchsin. The fibres are arranged in bundles which stain a darker pink and are parallel with the cortical layer.

The cuticle forms a protective barrier between the tunic matrix and the environment; under light microscopy it appears as a peripheral thickening of the matrix, intensely stained. The cuticle surface is not smooth. Especially in young colonies it is frequently endowed with small folds of different shapes about 0.6-1 µm (Fig. 3). These folds are quite different in size from those described with the electron microscope by Milanesi *et al.* (1978) and by Katow & Watanabe (1978).

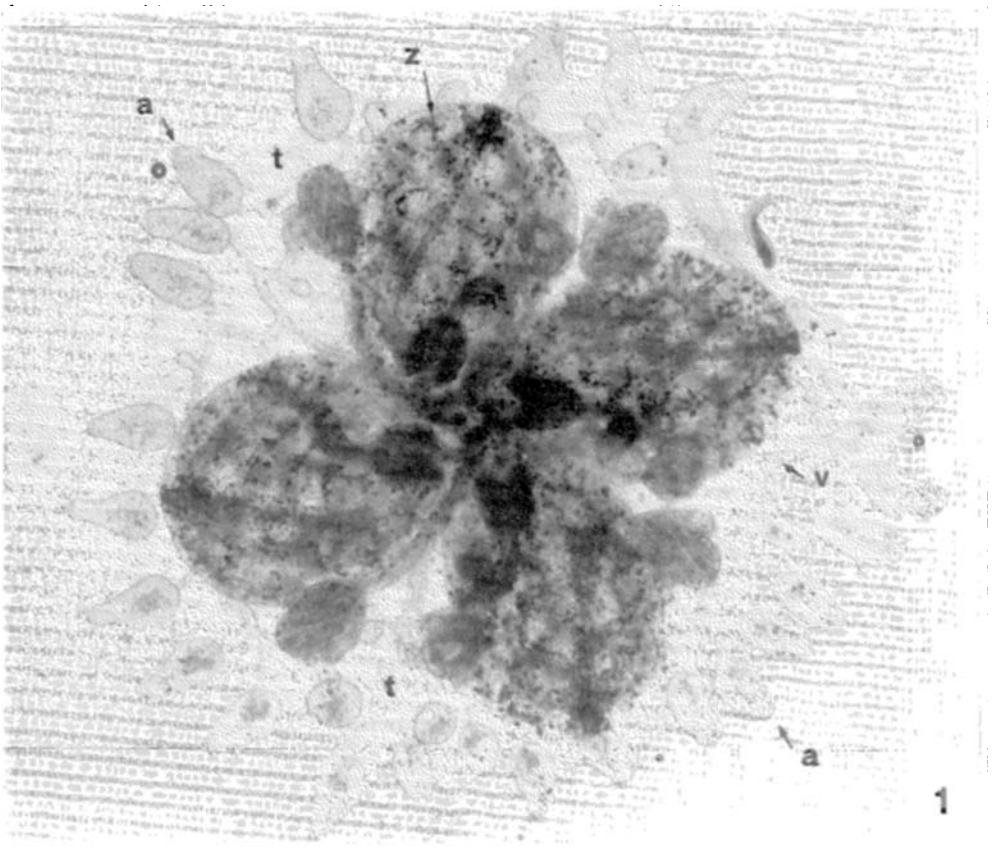
In the tadpole larva the tunic has two layers: a smooth external, which is the embryonic tunic, and a folded inner layer, which is the definitive tunic. During metamorphosis the embryonic tunic breaks up and disappears (Fig. 9).

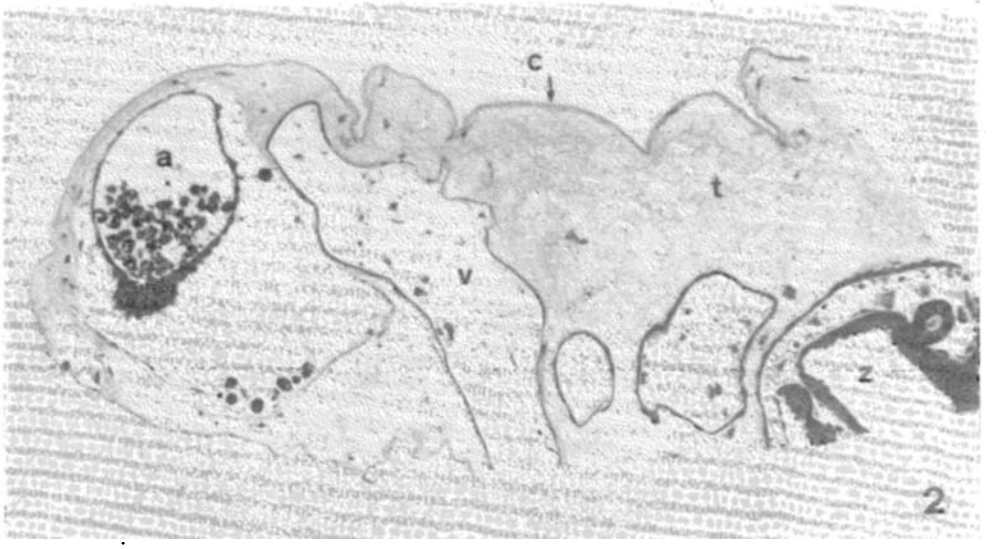
Tunic cells

Three types of cells are distributed throughout the matrix: fusiform, fibrocytic, vacuolated. The fusiform cells are usually found along the vessel walls (Fig. 4). The ovoid nucleus has no nucleolus and the thin cytoplasm is homogeneous. The cells have no pseudopodia, and are always very close to, but separate from, the vessel walls.

Fig. 1. — A young colony of *B. schlosseri* seen *in vivo* from the ventral surface. The zooids, embedded in the common tunic, are connected by a branching network of blood vessels ending in peripheral ampullae (a). t: tunic, v: vessel, z: zooid. × 25.

Fig. 2. — Section of a peripheral region of a colony showing vessels (v) running in the tunic (t) and the external cuticle (c). a: ampulla, z: zooid. Methylene blue-basic fuchsin. × 250.





The fibrocytes are widespread and mainly surrounded by the fibrils of the ground substance. These elongated cells, varied in size, have small conical and irregularly shaped pseudopodia at their extremities, and a large roundish nucleus with a nucleolus. Their PAS positive cytoplasm contains small round granules but no vacuoles (Figs. 5 and 6).

The vacuolated cells are more numerous than the other cells within the tunic. They are characterized by the presence of a great number of vacuoles, each containing a single round granule with great affinity for osmium (Figs. 7 and 8). The large roundish nucleus, usually in the middle of the cell body, contains uniformly distributed granular chromatin. The vacuolated cells have numerous thin and long filopodia, radiating in all directions from the cell-body. When free in the abundant matrix, these cells become stellate, but in a scanty tunic matrix, they become elongated, parallel the cuticle, with filopodia only at their extremities (Fig. 9).

The vacuolated cells found in the tunic are very similar in morphological characteristics and staining affinity to the morula cells present in the blood lacunae, and described in *B. schlosseri* by Milanesi & Burighel (1978), but with the difference that the blood morula cells are without filopodia. In this study we have noticed several blood morula cells closely applied to the base or scattered between the epithelial cells of the epidermis and the tunic vessels (Fig. 9).

In the tunic some vacuolated cells could be observed showing various degenerative stages, such as pyknosis, broken cytoplasmic membranes and loss of filopodia.

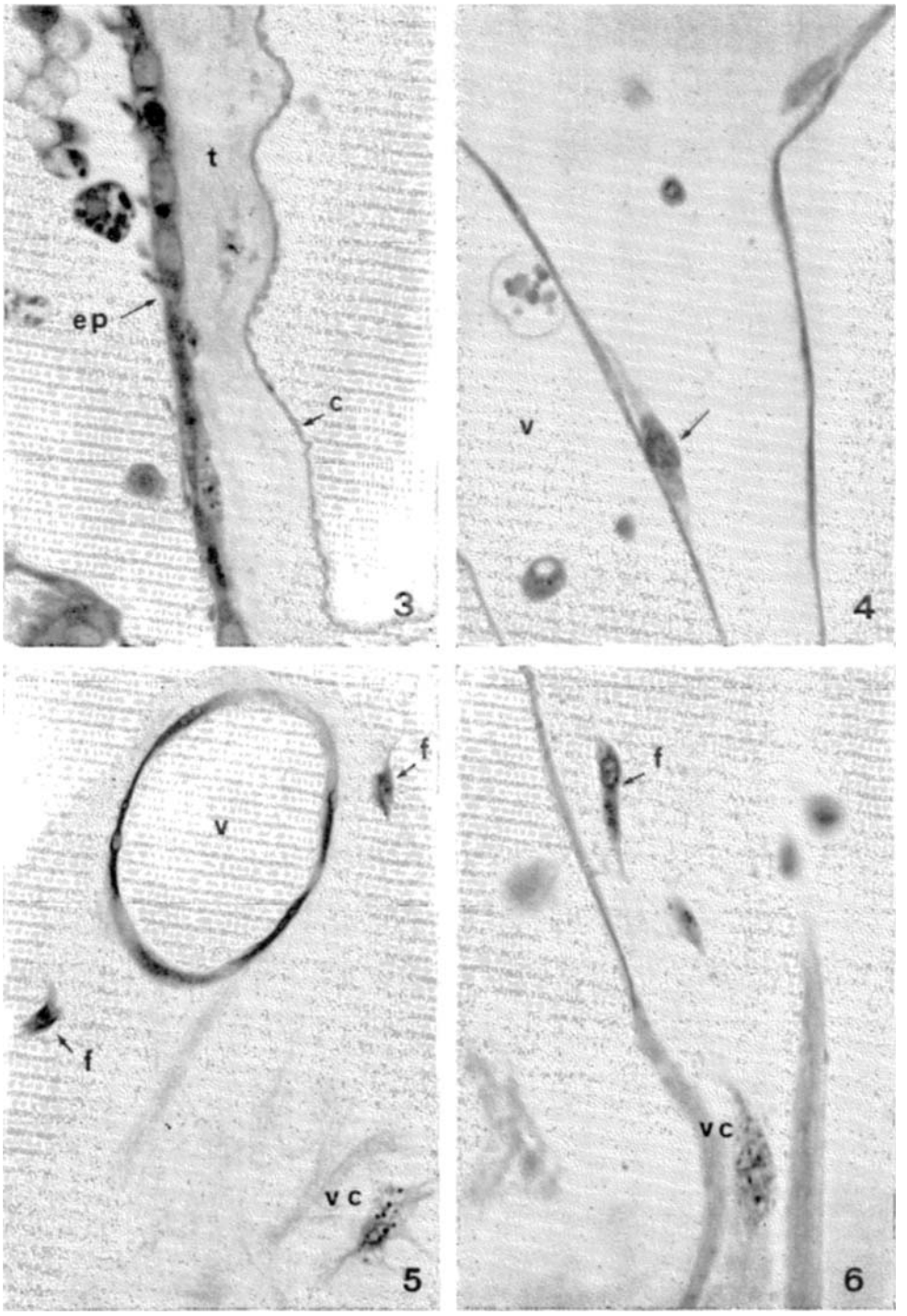
Test vessels and ampullae

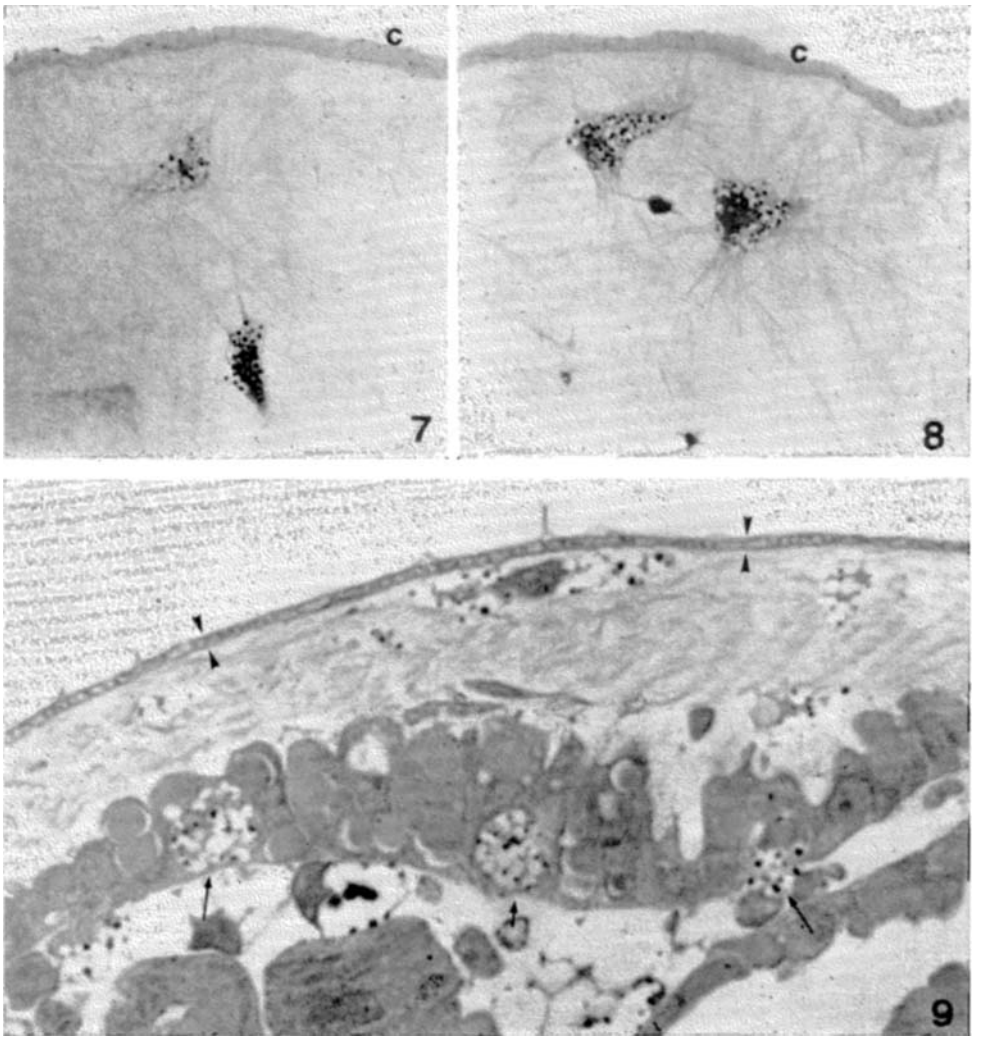
Histological sections show the vessels with a simple flattened epithelium running in the tunic. Because of its function, this epithelium is considered to be an endothelium, continuous with the epidermis of the zooids. The endothelial epithelium arises initially as an evagination of the epidermis in both the tadpole and the developing buds. It becomes a true barrier without break in continuity and separates the blood from the tunic. At the level of the ampullae it shows a particular differentiation. Indeed in the ampullae two regions are recognizable: the proximal part and the apical part which juts into the tunic (Figs. 10, 11 and 12). In the proximal part the epithelium is cubical with cells having an ovoid nucleus with a small quantity of chromatin, whereas at the level of the cap the cells are columnar and about twice (8 μm) as long as the former. The large nucleus of the cap cells stains strongly with malachite green and is always located basally; the cytoplasm is dense and homogeneous with several small PAS positive granules in the apical region of the cells (Fig. 11).

DISCUSSION

The tunic is the peculiar covering of tunicates. No other zoological group has a similar envelope; it has been considered as a "mesenchyme periphérique" (Brien, 1937), or a "conjonctif physiologique" (Pérès, 1948). The physical characteristics (consistence, thickness) and also the distribution of the vessels and cells in the tunic of various ascidians are quite different (Godeaux, 1964; Goodbody, 1974) and they have tended to increase the con-

Figs. 3-6. — Sections of tunic of *B. schlosseri* colonies. Figure 3 shows the folded cuticle and the epidermis (ep), which delimitates internally the tunic (t). Different cell types are recognized in Figures 4-6: a fusiform cell (arrow) (Fig. 4), tunic fibrocytes (f) with short pseudopodia and vacuolated cells (vc) (Figs. 5 and 6). v: vessels. Fig. 3: PAS - malachite green; Figs. 4 and 6: Giemsa; Fig. 5: methylene blue - azure II - basic fuchsin. Figs. 3-6, × 1 600.





Figs. 7 and 8. — The micrographs illustrate vacuolated cells with long filopodia radiating into the tunic. c: cuticle. Methylene blue-basic fuchsin. × 1 200.

Fig. 9. — Section of a tadpole larva: the micrograph indicates the possibility that morula cells (arrows) can pass through the epidermis. Externally (arrowheads), the folded cuticle is still covered by the embryonic tunic. Sato & Shamoto's (1973) method. × 1 600.

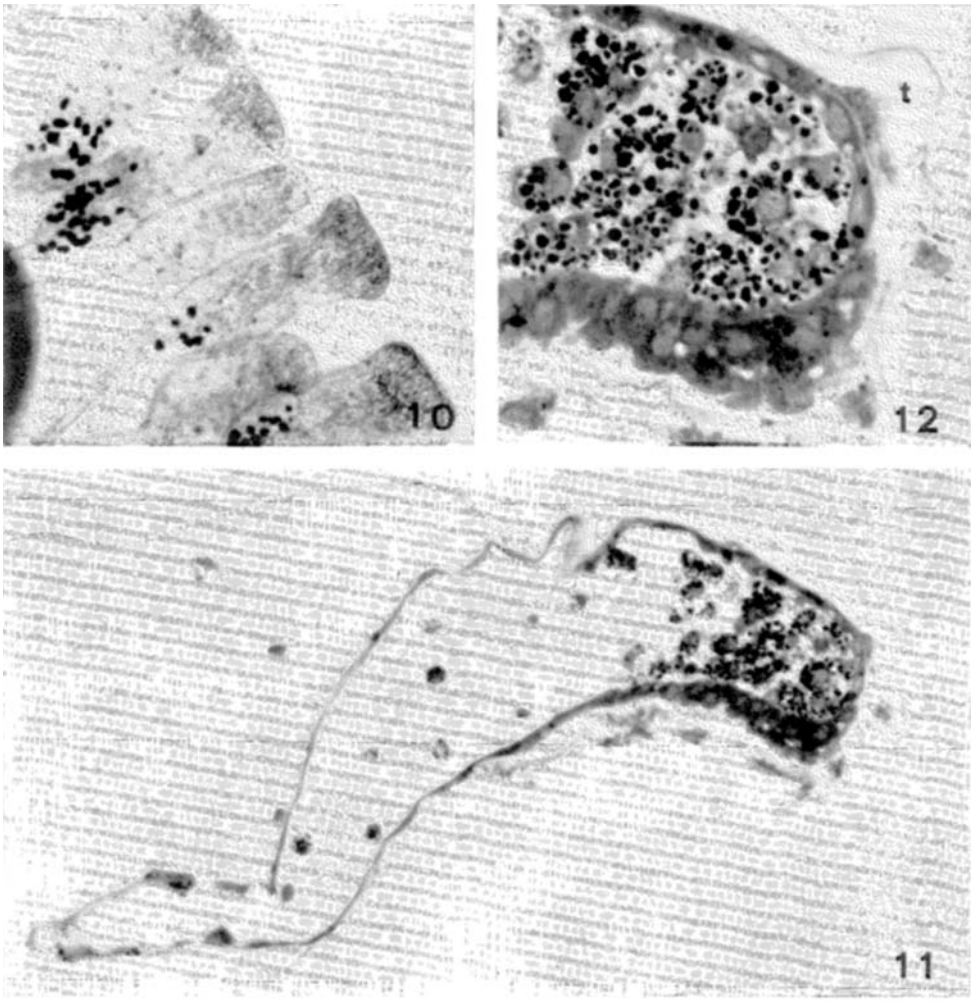


Fig. 10. — Peripheral ampullae of *B. schlosseri* seen *in vivo*. × 80.

Fig. 11. — Sagittal section of an ampulla showing the flat endothelial cells in the proximal part and the columnar cells of the apical cap. PAS - light green. × 550.

Fig. 12. — Detail of Fig. 11. Numerous granules are recognizable in the columnar endothelial cells. The ampullar lumen distally is full of blood cells. × 1 200.

troversial queries about the tunic. One important question is on the origin of the tunic cells. The hypothesis that in the embryo these cells could arise from the test cells of the eggs has been abandoned.

According to Pérès (1948) the tunic is colonized by epidermal cells which "glissent" from the epidermis into the tunic. According to other authors, cells arriving by diapedesis from the blood can enter into the tunic through the epidermis (Seeliger, 1893). Cloney & Grimm (1970) with the electron microscope have shown in *Amaroucium constellatum* that at metamorphosis there is a massive movement of granulocytes from the blood into the tunic and that the passage through the epidermis is an emigration of "transcellular" type. We also have noted numerous images suggestive of the blood cells entering into the tunic through the epidermal and endothelial epithelium. Once in the tunic the cells undergo transformation and lose their reproductive capacity: mitoses were not seen previously in other ascidians (Godeaux, 1964; Stievenart, 1971) or in *B. schlosseri* during this study. Hence it appears that all the test cells reaching the tunic come from the outside; although it is not to be excluded that some test cells can abandon the tunic and penetrate the hoemocel (Smith, 1970).

In the tunic of *B. schlosseri* there are three kinds of specific cells: fusiform, fibrocytic and vacuolated. The fusiform cells of homogeneous cytoplasm and without pseudopodia are usually distributed along the vessel walls. The fibrocytes are smaller with granular cytoplasm and pseudopodia, and of unknown function. The vacuolated cells are the third type. Their most remarkable features are highly vacuolated cytoplasm and numerous long filopodia radiating from the cell-body. These cells correspond to the "filopodial cells" studied *in vivo* by Izzard (1974) which are able to move through the tunic matrix by the motile activity of their filopodia. From their nuclear appearance, number and form of vacuoles, and granules present, the vacuolated cells resemble the blood morula cells of Sabbadin (1955) and of Milanesi & Burighel (1978). We have encountered numerous images showing a close connection between the blood morula cells and the epidermis or endothelium, and suggesting the strong possibility that the morula cells gain the tunic by "transcellular emigration" as described by Cloney & Grimm (1970). In the tunic the blood morula cells produce the characteristic filopodia and become the test vacuolated cells.

We found only three types of cells in all the tunics of *B. schlosseri* observed, in both young and adult. On the contrary, De Santo (1968) describes several cell types in the tunic: the nephrocytes and the granulocytes, which are peculiar blood cells, and the sheath, spindle, vacuolated and bladder cells. The sheath, spindle and vacuolated cells correspond exactly to our fusiform, fibrocytic and vacuolated cells respectively; and the bladder cells are the vacuolated cells in degeneration.

According to De Santo (1968) the vacuolated cells originate from the sheath cells through the spindle cell stage. In our opinion the three types of test cells have independent differentiation, for the three types are clearly differentiated from each other; intermediate forms of the three cell types were never encountered. In particular, as above reported, we consider that the vacuolated cells are the blood morula cells migrated into the tunic.

Test cells morphologically corresponding to the vacuolated cells are present also in the tunic of other ascidians, where they have been variously named, for example: "compartment cells" (Freeman, 1970), "stellate cells" (Saint-Hilaire, 1931) and "vanadocytes" (Webb, 1939).

It has been claimed that the test cells may have a role in the defensive mecha-

nism. Anderson (1971) has shown that the vanadocytes are able to encapsulate foreign bodies inserted into the tunic of *Molgula manhattensis*. In addition, Tanaka (1973) states that the "brown cells", which may be regarded as corresponding to both vanadocytes and morula cells, participate in the immune response in *Botryllus primigenus*. Indeed, in the case of the non-fusion reaction between incompatible colonies, "brown cells" together with granular amoebocytes are the main components of the cell clusters formed during this reaction.

The test vacuolated cells were also suggested as responsible for the production of the tunic matrix (Endean, 1955, 1960; Kalk, 1963; Smith, 1970). The vacuolated cells cannot be excluded from such a role, but the main responsibility for the tunic secretion is the epithelium underlying the tunic, as has been suggested also for other ascidians (Deck *et al.*, 1966; Smith, 1970; Wardrop, 1970). In *B. schlosseri* this epithelium secretes PAS positive granules, related probably to tunic production, as claimed also by De Santo (1968). The histology of the ampullar cap cells indicates that they represent an elective region for tunic production, as also noticed by Katow & Watanabe (1978) in *B. primigenus*.

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