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Spherical forms of *Trichoplax adhaerens* (Placozoa)

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Summary. Two types of spherical forms of this normally flattened organism appear sporadically in our cultures. Hollow spheres have an outer wall of flagellated ventral epithelium. The large fiber cells protrude into the central cavity which can include a closed compartment of flagellated dorsal epithelium. Cells of the outer wall that withdraw their flagellum and leave the epithelium are phagocytozed by fiber cells. Solid spheres consist of an outer layer of dorsal epithelium and densely packed fiber cells in the interior that may also include a closed compartment of ventral epithelium cells. Closely apposed fiber cells may form special cell contacts or pores connecting the cells.

A. Introduction

The primitive marine metazoon *Trichoplax adhaerens* F.E. Schulze, 1883, is typically a small flattened organism with a dorsal, protective and a ventral, nutritive epithelium enclosing an interspace with branched, mesenchyme-like fiber cells (Grell and Benwitz 1971). The only viable spherical forms described so far are hollow swarmers that are budded off from the dorsal surface (Thiemann and Ruthmann 1988). They are covered on the outside by the flattened cells of the dorsal epithelium and on the inside by columnar cells of the ventral epithelium whose flagella beat within the central cavity. After floating some time they sink to the bottom of the culture dish. Thereafter, they open up and gradually transform into the typical flattened shape as described in the paper cited above. Non-viable spherical forms can arise in old cultures. In this degenerative phase (Grell and Benwitz 1971) the dorsal epithelium loses contact with the fiber cells and the interspace becomes filled with fluid. As the ventral epithelium also detaches from the substrate, these moribund forms float for some time in the culture dish like small balloons.

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The present report concerns two different spherical forms which are not connected with asexual reproduction nor are they to be confused with the moribund, balloon-like, floating spheres that appear in old cultures. Instead, the hollow as well as the solid spheres we observed are always at the bottom of the culture dish and show no signs of degeneration. Both forms have distinctive features and diverge in characteristic ways from the usual body plan of *T. adhaerens*.

B. Materials and methods

Trichoplax adhaerens was cultured as described by Grell and Benwitz (1971).

The ability to carry out pinocytosis was tested with 0.25% cationized ferritin in sea water for 60 min. Specimens were fixed immediately after incubation.

For transmission electron microscopy (TEM), the samples were fixed by a two-step procedure. The solution for prefixation (45 min, pH 7.2) contained 5% glutaraldehyde, 150 mM PIPES, 50 mM HEPES, 40 mM EGTA, 30 mM KCl and 30 mM $MgCl_2$. After a brief washing in the above mentioned buffer mixture, the samples were postfixed with 1% OsO_4 and 0.7% $K_3Fe(CN)_6$ in 50 mM PIPES buffer for 10 min. After washing in distilled water, they were incubated for 1 h in 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with lead citrate for 5 min and observed in a Philips EM 410 electron microscope at 60 or 80 kV.

For scanning electron microscopy (SEM), the samples were fixed for 45 min in a mixture containing 5% glutaraldehyde and 5% paraformaldehyde in 100 mM PIPES buffer (pH 7.0). After washing in distilled water they were transferred to a millipore filter moistened with poly-L-lysine. Excess water was removed with filter paper put below the millipore filter which was then, together with the adhering samples, fixed for another 5 min in the buffered aldehyde mixture. After washing they were dehydrated in ethanol and subjected to critical point drying. Following this, they were sputtered with gold and studied in a ISI Super III A scanning electron microscope at 15 kV.

C. Results

Both hollow and solid spheres are between 120 and 200 µm diameter and appeared sporadically in our cul-

tures. The conditions favoring their appearance and their mode of formation are unknown. Although flagellated, both types lie generally motionless at the bottom of the culture dish, but the hollow ones are capabable of a slow rotation which is considerably enhanced when touched by a glass capillary. No motion of the solid spheres has been observed.

1. Hollow spheres

The typical morphology of hollow spheres is shown in Figs. 1, 2. The sphere in Fig. 1 has a total diameter of 145 μm and a wall thickness of approximately 23 μm. Its outer border is formed by a densely packed layer of slender flagellated cells which correspond to the ventral epithelium of flattened *Trichoplax*. Like the latter, they are capable of pinocytosis as indicated by the uptake of cationized ferritin (Fig. 3). The inner part of the wall is composed of large fiber cells, occasionally in several layers on top of each other. They are losely arranged without special cell contacts and protrude more or less into the cavity. There are no flagellated cells in the interior. Compared to normal *Trichoplax*, two cell types are missing, i.e., the flattened cells of the dorsal epithelium and the gland cells which are normally interspersed among the flagellated cells of the ventral epithelium.

Since the hollow type of sphere is completely enclosed by cells connected by belt desmosomes, as is the ventral epithelium of flattened *Trichoplax* (Ruthmann et al. 1986), the tightness of these cell junctions could be tested by injecting dye into the cavity. If a small amount of dye (e.g., Giemsa) is injected with a 3 μm capillary, the cavity can be stained selectively. However, the staining disappears almost completely within 15 min. When larger amounts are injected, the dye stuff leaks diffusely all over the wall of the sphere. Pressure, which would enlarge or burst the sphere, can not be built up. Since the spheres remain intact, the dye must have passed to the outside by way of the intercellular spaces and the belt desmosomes, which, in the absence of tight junctions, are the only terminal intercellular structures in *Trichoplax* epithelia.

The fiber cells are capable of phagocytosis. We found unidentified protists with tubular mitochondria as well as ingested epithelial cells included in their cytoplasm (Figs. 4, 5). The latter become dislodged from the outer epithelium of the sphere and withdraw their flagella (Fig. 6). There are always a number of epithelial cells with small nuclei within the thick wall of the sphere that have lost contact with the outer medium. However, their derivation from the covering epithelium is evident since they may still contain vesicles with pinocytosed cationized ferritin. Close to the interior cavity, these epithelial cells seem to fall prey to the fiber cells. Recently phagocytosed cells are in a vacuole while material taken up some time ago, such as the protozoon in Fig. 4, is in the concrement vacuole which always contains partly digested residues (Fig. 5, 7). In Fig. 5, phagocytosis was still progressing when the cell was fixed as the vacuole is not closed (arrow). The vacuoles around the prey may fuse secondarily. Thus, Fig. 7 shows at least four epithelium cells in a single vacuole, three identifiable by their nuclei, the fourth by a striated flagellar rootlet.

In some of the spheres the outer epithelium appears thinned out. Cell fragments are in the interior and the number of fiber cells is quite small without signs of phagtocytosis. Such spheres may be degenerating.

Still other hollow spheres contain flattened cells connected by belt desmosomes that form an apparently closed compartment within the central cavity. Their flagella extend towards the inside of the compartment. *Pyrenomonas* cells, which are used as food for *Trichoplax*, are occasionally enclosed in this space. The cells forming this inner compartment correspond in all respects to the dorsal epithelium cells of flattened *Trichoplax*. They are in contact with extensions of fiber cells (Fig. 8) as is the case in normal, flattened *Trichoplax*.

2. Solid spheres

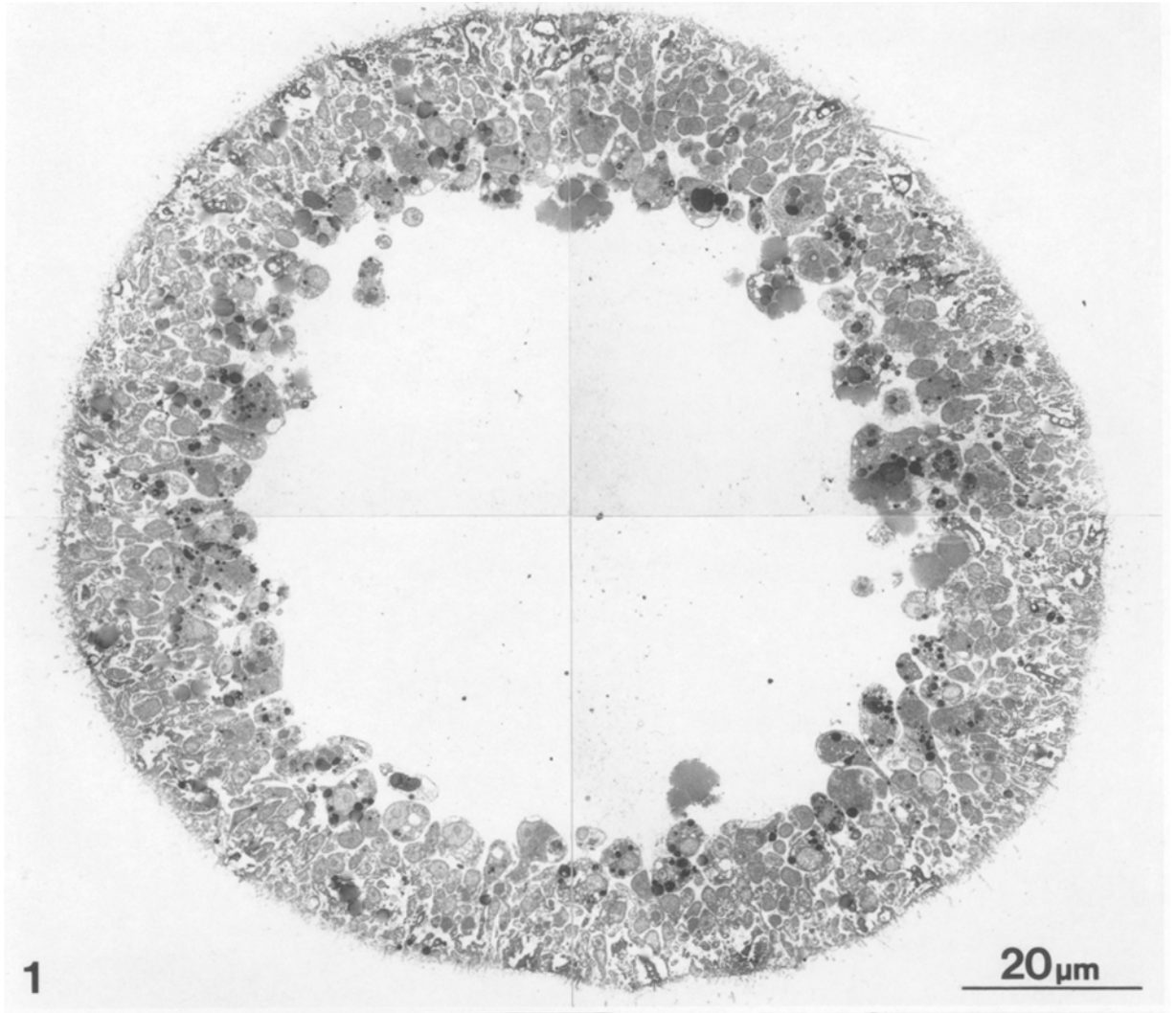
Aside from some very electron-dense, possibly degenerating cells of unknown origin, the interior of solid spheres is completely occupied by large cells (Fig. 9) that can be identified as fiber cells based on their peculiar mitochondrial complexes and the presumably endosymbiotic bacteria (Fig. 12, 13). The outer border of the spheres is formed by sparsely flagellated cells. They seem to correspond to the dorsal epithelium although their small nuclei are flattened and not located in a proximal cytoplasmic part that, in normal *Trichoplax*, extends into the interspace. These outer cells are unable to take up cationized ferritin as are the cells of the dorsal epithelium of *Trichoplax*. Occasionally, there are some gaps in the outer covering of the sphere (Fig. 9).

The fiber cells below the covering cells are much more numerous and more densely packed than in any other form of *Trichoplax*. Unlike normal flattened *Trichoplax*, there is scarcely any interspace and the dense packing of the cells leads to intercellular contacts which have not been observed so far. Thus, Fig. 10 shows two cells that are in contact over a large area. Adjacent cell membranes are separated by a fairly constant space of 50 nm width that is spanned by cross-connections with a periodicity of about 40 nm. This fairly regular lattice is bisected by what appears, in sections, as a central

Fig. 1. Section through a hollow sphere of 120 μm diameter. Note the large fiber cells protruding into the interior. The outer wall of slender epithelium cells is densely flagellated

Fig. 2. Scanning electron micrograph showing the interior of hollow spheres. Note the palisade-like arrangement of the epithelium cells forming the wall (*short arrow*) and fiber cells protruding into the interior (*arrows*)

Fig. 3. Slender ventral epithelium cells forming the outer wall. *F* flagellum; *N* nucleus. The dark vesicles, enlarged in the *inset*, are filled with cationized ferritin taken up by pinocytosis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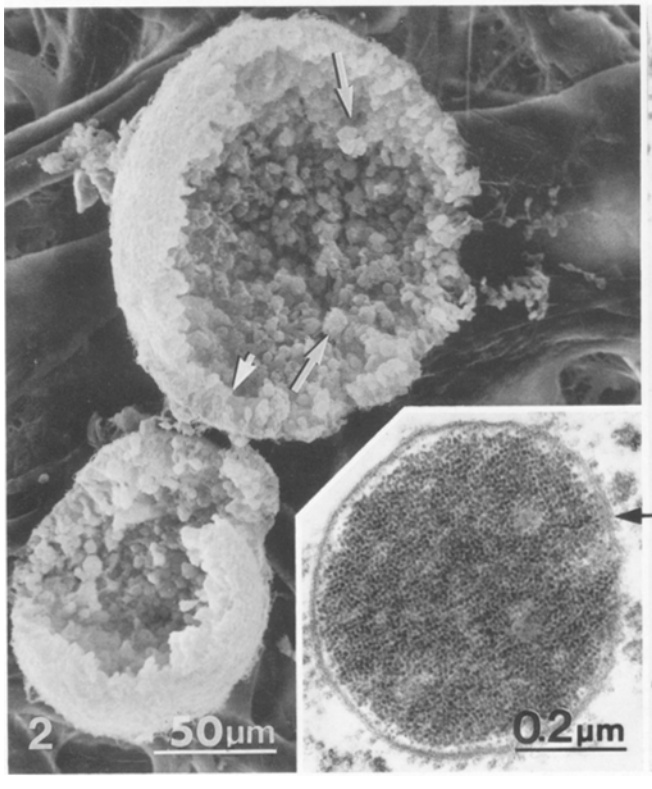
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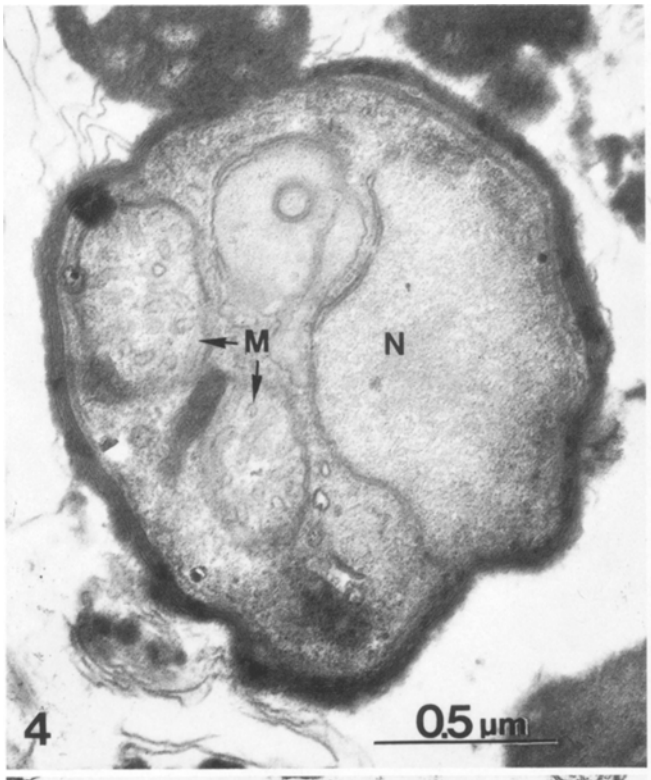
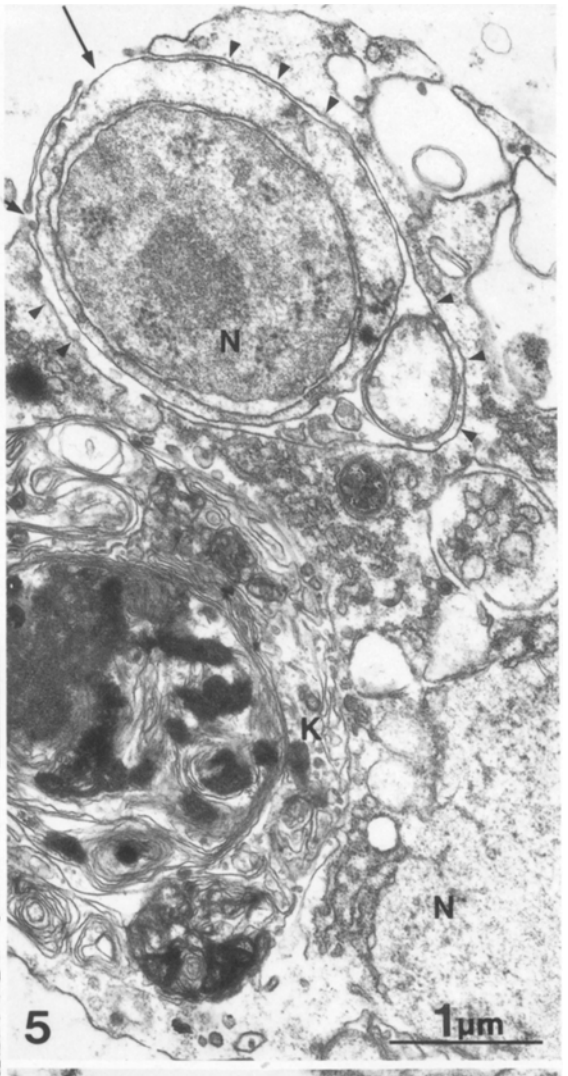
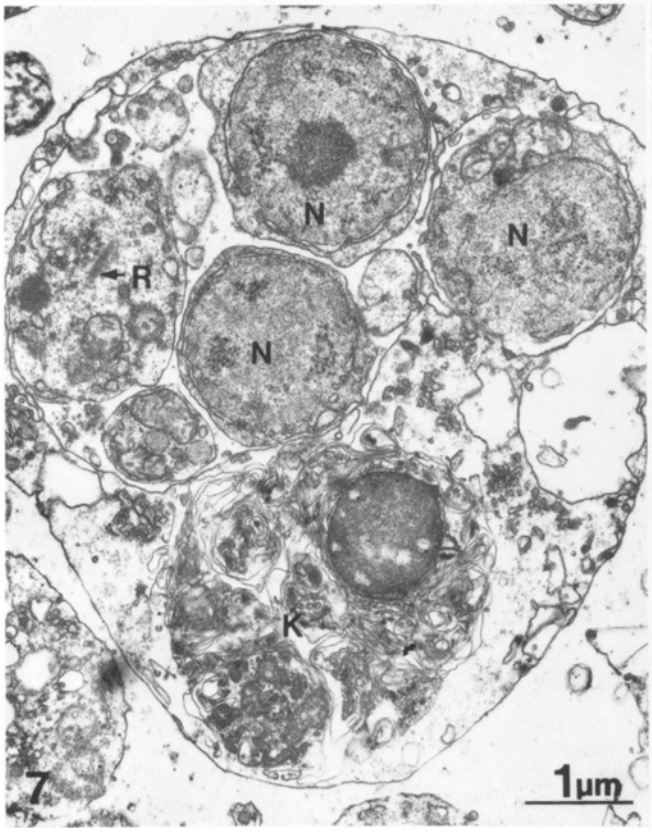






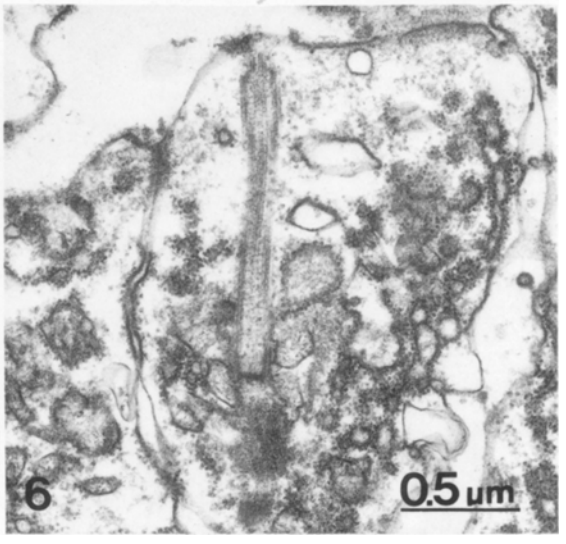


Fig. 4. Protozoon phagocytozed by a fiber cell. *M* mitochondria with tubular cristae; *N* nucleus

Fig. 5. Phagocytosis of an epithelium cell that is almost (*arrow*) completely enclosed in a vacuole (*arrowheads*). *N* nuclei of epithelium and fiber cell; *K* lamellar remnants in its concrement vacuole

Fig. 6. Epithelium cell that has completely withdrawn its flagellum

Fig. 7. Fiber cell that has engulfed at least four epithelium cells. *N* nuclei of three cells; *R* remnant of a flagellar rootlet; *K* concrement vacuole. The digestive vacuoles around the engulfed cells have become confluent

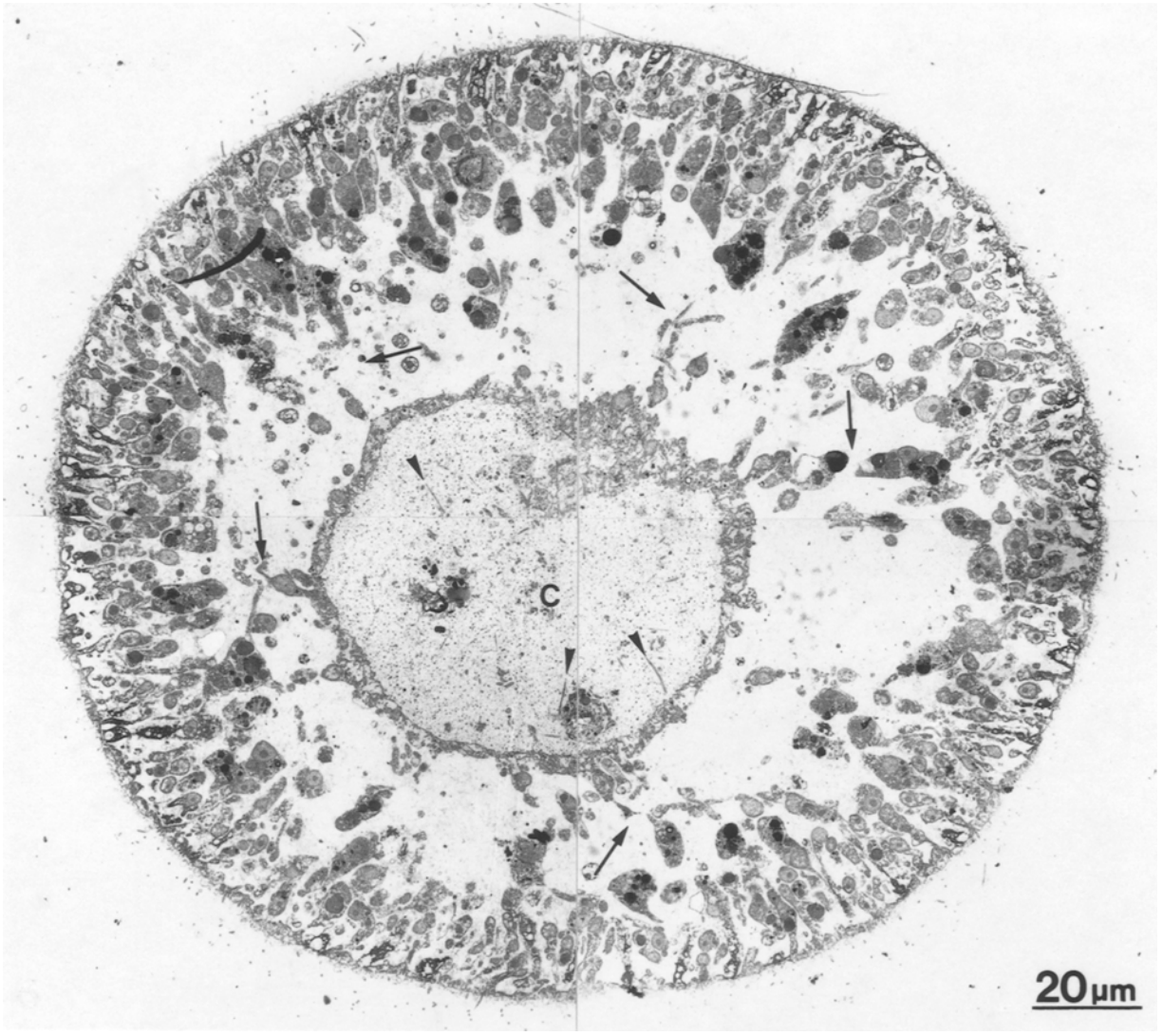


Fig. 8. Hollow sphere with an interior compartment (*C*) formed by flattened cells whose flagella (*arrowheads*) project into the interior. *Arrows* denote connections of fiber cell extensions with the flattened cells

dense line. In other cases, the intercellular space is reduced to about 20 µm, more irregular and for the most part filled with diffuse material (Fig. 11). A pore of 60 nm width seems to connect the cytoplasm of both cells. Another striking feature of the fiber cells in solid spheres are the unusually prominent Golgi systems whose lamellae are filled with a dense product (Fig. 12). Binucleated cells are not rare (Fig. 13). Many fiber cells contain very dense phagocytozed cells of unknown origin.

Some of the solid spheres were found to contain an inner hollow compartment of flagellated cells (Fig. 14). These form an epithelium of columnar cells like the ventral epithelium of flattened *Trichoplax*.

D. Discussion

The sporadic occurrence of spherical forms in an otherwise flattened organism with a pronounced dorsoventra-

Fig. 9. "Solid" sphere bounded by a thin layer of flattened cells. The surface is sparsely flagellated (*arrows*)

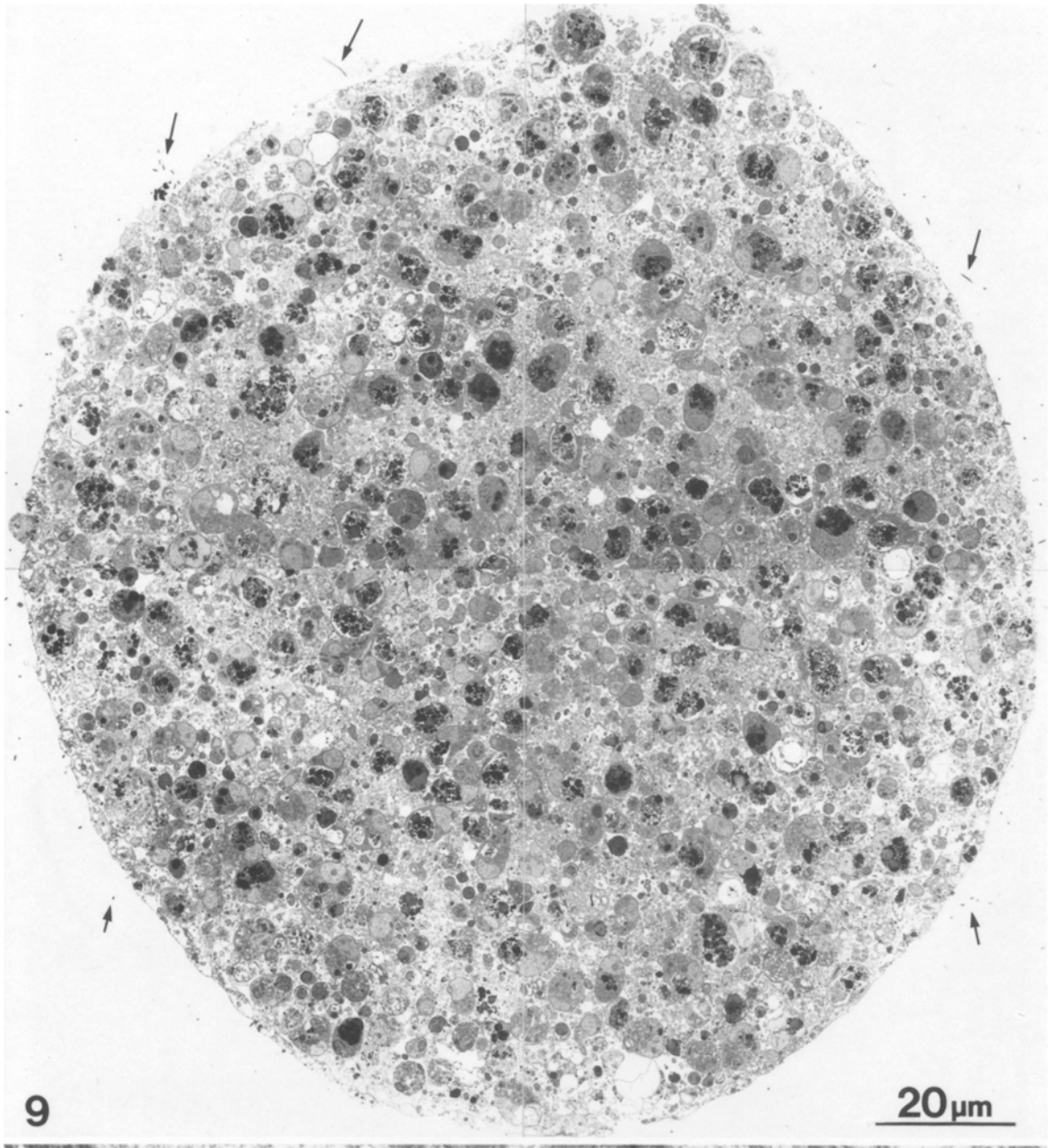
Fig. 10. Regular cross-connections (*arrowheads*) between the membranes of adjacent fiber cells. *Arrow* indicates central dense material. *M* mitochondrion

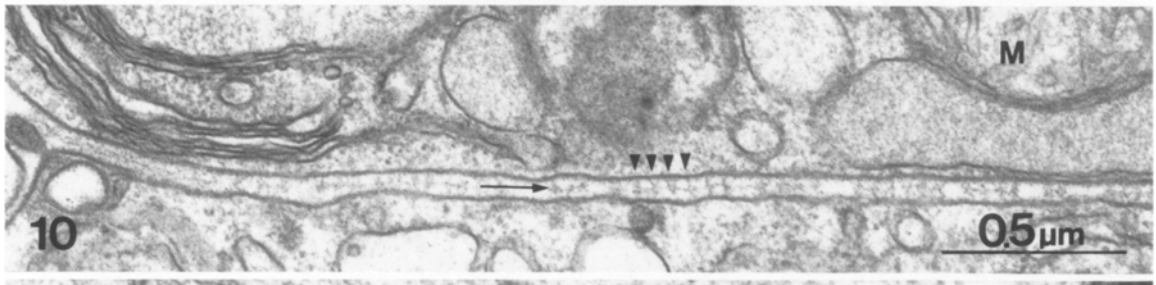
Fig. 11. Adjacent fiber cells. Note pore (*arrowhead*) connecting both cells. *N* nucleus; *double arrowhead* nuclear pore

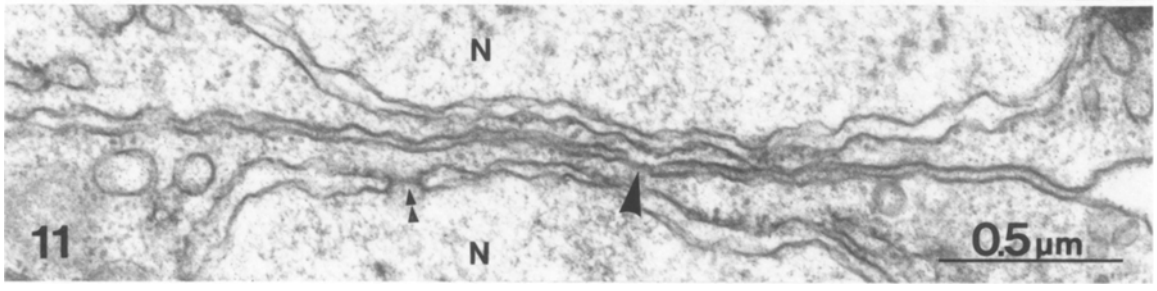
Fig. 12. Fiber cell showing highly active Golgi apperatus (*G*). *N* nucleus; *M*, *V* mitochondrial complex consisting of mitochondria and associated vesicles

Fig. 13. Binucleated (*N*) fiber cell with characteristic endosymbiotic bacteria (*B*)

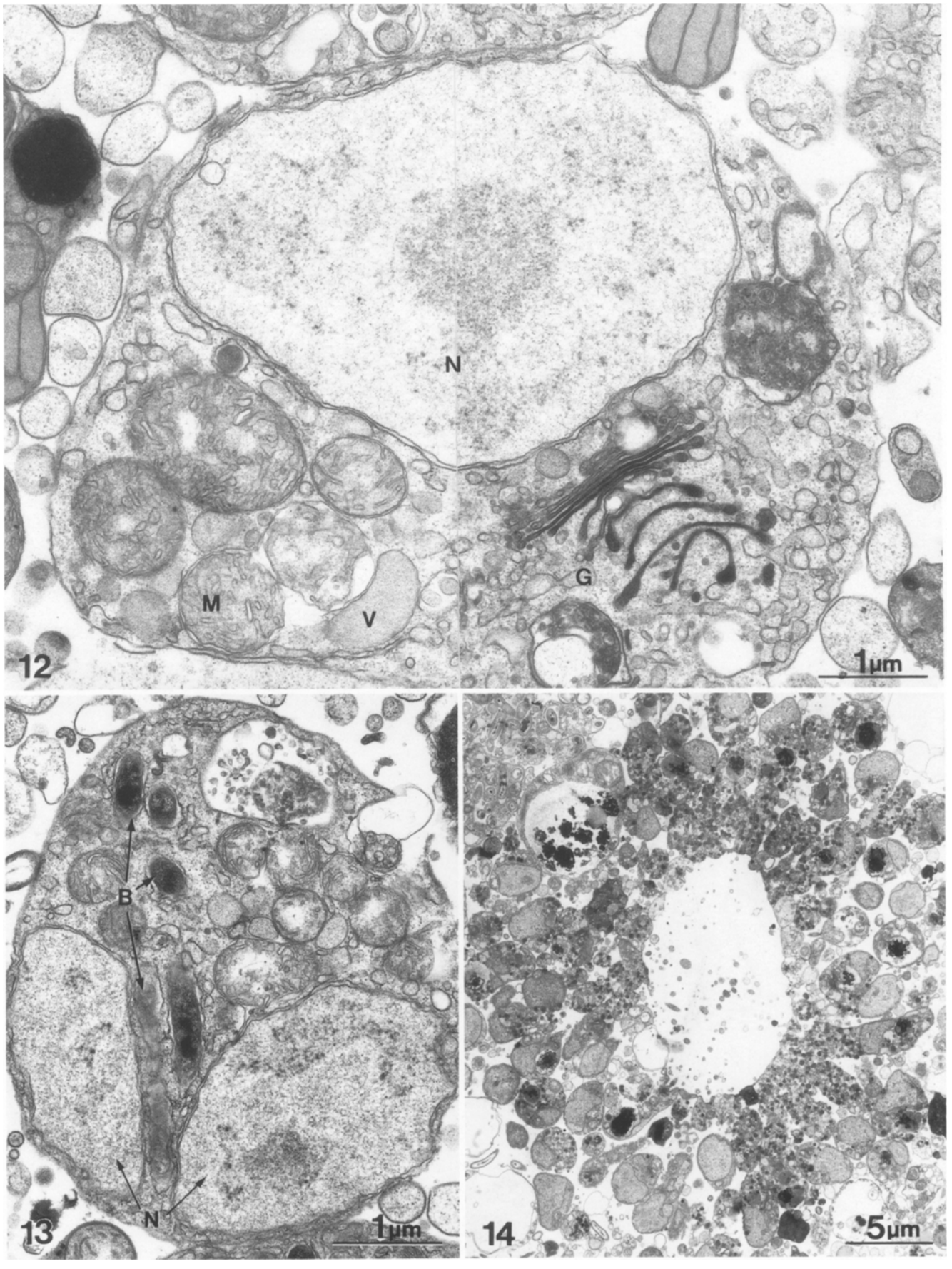
Fig. 14. Hollow compartment of flagellated cells within a solid sphere







Figs. 9–11



Figs. 12–14

lity indicates unexpected morphogenetic potentials that are realized in the absence of one or the other type of epithelial cells. Hollow (type A) spheres are essentially devoid of a dorsal epithelium while solid spheres (type B) lack a ventral epithelium. It seems that both epithelia are essential for the maintenance (or establishment) of the flattened body shape.

The spheres described in this paper are not to be confused with the nearly spherical moribund forms that appear in the so-called degenerative phase (Grell and Benwitz 1971) in old cultures. In these, the dorsal epithelium has detached from the underlying tissue, the large fluid-filled space giving the whole organism a balloon-like appearance. The spheres we are concerned with do not show such signs of degeneration. As in flattened *Trichoplax*, the ventral epithelium cells are capable of pinocytosis and the fiber cells of phagocytosis. In fiber cells of type B spheres, the Golgi apparatus shows signs of intense activity and nuclear divisions must have taken place as there are many binucleated cells.

The occasional inclusion of *Pyrenomonas* cells in the hollow interior offers a clue how spheres of type A may have developed. This cryptomonad, used as a food organism, is often seen to adhere to slime extruded at the dorsal surface of *Trichoplax*. It may be assumed that, possibly due to a lag of growth in the dorsal epithelium, the ventral epithelium "rolls up" and fuses to enclose *Pyrenomonas* cells in the central cavity. The remains of the dorsal epithelium which are also enclosed (c.f. Fig. 8) may gradually degenerate giving rise to spheres that consist only of fiber cells surrounded by cells of the ventral epithelium. The latter show the typical palisade shape of the ventral epithelium and they are able to take up cationized ferritin, a unique property of ventral epithelium cells (Ruthmann et al. 1986). Phagocytosed foreign cells, such as the protozoon with mitochondria of the tubular type shown in Fig. 4, must have been taken up via the dorsal surface before transformation to the hollow sphere. This type of transepithelial cytophagy has been demonstrated by Wenderoth (1986) who fed dead yeast cells to *Trichoplax*.

Transepithelial phagocytosis, in addition to the uptake of extrasomatically digested material by the ventral epithelium (Grell and Benwitz 1971), may play a significant role in the nutrition of *Trichoplax* since remnants of *Pyrenomonas* cells, such as starch granules, thylacoids and ejectisomes, are commonly found in the concrement vacuoles of fiber cells. However, an autophagic uptake of epithelium cells by fiber cells has not been observed before. It seems significant that evidently only cells that have left the epithelial sheet fall prey to fiber cell phagocytosis. We have no indication why these cells should withdraw their flagellum and leave the epithelium. Since similar phenomena, including autophagy, have not been observed in normal flattened *Trichoplax*, we feel certain that this is not a normal form of cellular turnover.

One important question regarding the degree of functional integration of an organism is whether it can build up and maintain an interior milieu of its own. Tightness of the terminal cell junctions, in the case of *Trichoplax* the belt desmosomes since tight junctions are absent (Ruthmann et al. 1986)), would seem to be a prerequisite for maintaining a constant internal environment. We had previously found (unpublished) that colloidal lanthanum hydroxide could penetrate beyond the belt desmosomes of *Trichoplax* fixed for electron microscopy. The finding of hollow spheres permitted a test with living cells using dye injection, which showed that the belt desmosomes are leaky junctions. Unless special conditions prevail in the spheres, this would mean that the sea water has access to the interspace of normal *Trichoplax*. This conclusion is in agreement with the fact that isolated fiber cells can be observed for hours in sea water (Thiemann and Ruthmann 1989) as long as there is sufficient oxygen. The internal environment of *Trichoplax* does therefore not seem to be constant although it may differ from sea water due to the presence of large molecules to which the belt desmosomes may be impermeable.

Type B spheres were shown to consist essentially of a densely packed mass of fiber cells surrounded by flattened cells with the characteristic features of a dorsal epithelium. Small interior cavities lined by ventral epithelium as shown in Fig. 14 were very rarely found. A somewhat similar appearance has been noted after experimental dispersal of *Trichoplax* (see Ruthmann and Terwelp 1979). Reaggregation led to spheres bounded by the flattened cells of the dorsal epithelium. The interior cell mass of densely packed fiber cells contained closed compartments lined by flagellated cells of the ventral epithelium and others lined by flagellated cells of the dorsal epithelium. The two cell types were, however, not intermingled in the same compartment. In the present case, we have no indication how the spheres arise and how they might acquire hollow inclusions of ventral epithelium. One might consider the possibility that type B spheres are aberrant formations akin to the so-called swarmers. The latter are 35–80 μm large, spherical and with a central cavity lined by ventral epithelium (Grell 1974). They are budded off at the dorsal surface of *Trichoplax* (see Thiemann and Ruthmann 1988) and serve for dissemination of the species. An abnormal multiplication of fiber cells and the failure to include or differentiate ventral epithelium could conceivably give rise to type B spheres.

The unusually dense packing of the fiber cells in type B spheres might be responsible for peculiar cell contacts that are not observed in flattened *Trichoplax* where these cells form a loose meshwork in the space between the dorsal and the ventral epithelium. The pores we observed between neighboring cells may either be secondary formations or they may be due to an incomplete cytokinesis.

We do not know what conditions favor the formation of spheres. Neither the population density nor the age of the cultures seem to play any role. Of several cultures set up at the same time, they may appear in only one. Sometimes no spheres are seen for weeks. Type A (hollow) spheres are noted more often than those of type B. This may be due to their longevity as individual spheres have been observed for more than two weeks. Their ultimate fate is not known. There are no signs that either type can give rise again to normal, flattened *Trichoplax*.

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