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Development of the pellicle and thecal plates following ecdysis in the dinoflagellate *Glenodinium foliaceum*

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Summary. The ultrastructure and development of the amphiesma of the dinoflagellate *Glenodinium foliaceum* was studied using conventional electron microscopy and immunocytochemistry. Ecdysis (shedding of the flagella, the outer two membranes of the cell, and the thecal plates) was induced by centrifugation. The cells were resuspended and the thickening of the pellicle and the development of the new thecal vesicles and plates was studied over a 9 h period. After ecdysis, the thin pellicle which underlay the thecal plates in the motile cells thickens to form a complex structure of four distinct layers: an outer layer of randomly oriented fibrils, a 50 nm layer of fibrils oriented perpendicular to the dense layer, the dense layer which has a trilaminate structure, and a wide inner homogeneous layer. The new thecal vesicles form in these pelliculate cells by the migration of electron translucent amphisomal vesicles over the layer of peripheral microtubules to a position directly under the plasmalemma. The thecal vesicles then flatten and elongate. A discontinuous pellicular layer appears within them. Subsequently, the thecal vesicles widen and are filled with a fibrillogranular substance overlying the pelliculate layer. The thecal plates form on top of this fibrillogranular material. By this time, most cells have escaped from the pellicle and are motile. At first, the outer thecal vesicle membrane is continuous with the inner thecal vesicle membrane at the sutures, but when this connection is broken, the dense pelliculate layers become continuous across the suture as does the inner thecal vesicle membrane. At ecdysis, this membrane becomes the new plasmalemma of the cell. Cells at each stage of pellicle thickening and thecal development were labelled with a polyclonal antiserum raised against the 70 kDa epiplasmic protein of *Euglena acus*. This antiserum labelled both the thecal plates of the motile cells and the inner homogeneous layer of the pellicle of ecdysed non-motile cells. No other amphiesmal structure was labelled, nor was any intracellular compartment.

Keywords: Amphiesma; Ecdysis; *Euglena acus; Glenodinium foliaceum*; Pellicle; Thecal development.

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Abbreviations: PBS phosphate-buffered saline; PIPES piperazine-N,N′-bis[2-ethane sulfonic acid].

Introduction

Many studies have been made in recent years on the unique complex structure enclosing the dinoflagellate cell (for review, see Morrill and Loeblich 1983). This structure was called the theca (Dodge and Crawford 1968, 1970 b; Wetherbee 1975 a) or amphiesma (Schütt 1895, Loeblich 1970). The structure of the amphiesma varies from the simple single layer of flattened vesicles with a thin dense layer below them found in *Oxyrrhis marina* (Dodge and Crawford 1971, Cachon et al. 1987) to the complex amphiesma of many armoured dinoflagellates, whose fused thecal vesicles contain thick ornamented plates underlain by a continuous pellicular layer. The development of the amphiesma of armoured dinoflagellates has been studied in most detail in *Heterocapsa niei* following experimentally induced ecdysis (Morrill 1984). Ecdysis occurs following environmental stress in those dinoflagellate species which already contain a pellicular layer formed within their thecal vesicles (Taylor 1987). The thecal plates are shed and a thickened pellicle forms at the periphery of the resulting ecdysal cyst. A new theca forms beneath the pellicle on the developing motile cell(s) in the cyst. However, a number of questions about amphiesmal development remain unanswered. It is not known, for example, from what structure the thecal plates develop, or how the pellicle thickens at ecdysis. Although the thecal plates are claimed to be composed of cellulose or another

glucose polymer (Loeblich 1984) and the trilaminate layer of the pellicle to contain sporopollenin (Morrill and Loeblich 1981), very little is known about the chemical composition of these two structures.
Recently, several groups have begun to analyse the composition of the cell covering of euglenoids (Dubreuil and Bouck 1985; Bricheux and Brugerolle 1986, 1987) and of ciliates (Williams et al. 1979, Vigues and David 1989). It has been discovered that antibodies raised against (1) specific proteins of the pellicular strips of *Euglena acus*, (2) the epiplasmic layer of the amphiesma of the dinoflagellate *Noctiluca miliaris*, and (3) the epiplasmic layer of the ciliate *Pseudomicrothorax dubius* all cross-react, and all label epiplasmic structures on electron micrographs (Vigues et al. 1987).
In this study, we have used a polyclonal antibody raised against the 70 kDa protein of the pellicle of *E. acus*. We have employed this antibody in a detailed ultrastructural and immuncytochemical study of pellicle and thecal plate formation following ecdysis in the dinoflagellate *Glenodinium foliaceum*. We have demonstrated that two different structures are labelled by the antiserum, the thecal plates of motile cells and the inner homogeneous layer of the pellicle of non-motile cells.

Materials and methods

Cells

Glenodinium foliaceum Stein (CCAP 1116/3) was obtained from the Culture Collection of Algae and Protozoa of the Freshwater Biological Association at Ambleside, U.K. Cultures were grown at 20 °C in an artificial seawater medium, 0–3 of McIntosh and Cattolico (1978), under constant illumination at a light intensity of ≈ 50 µE/m/s.

Electron microscopy

Since centrifugation causes motile cells to shed their thecal plates and form a thick pellicle, cells were grown in 10 ml of growth medium in 30 ml culture tubes and then gently decanted into double strength fixative. The final concentration of fixative was 2.5% glutaraldehyde in 0.1 M PIPES (piperazine-N,N'-bis[2-ethane sulfonic acid]) buffer containing 1 mM $MgSO_4$ and 1 mM EGTA, pH 7.1. The cells were fixed at room temperature for 75 min. After three 15-min rinses in PIPES buffer, the cells were postfixed in 2% osmium tetroxide in the same PIPES buffer for 1 h at room temperature. After 2 washes in PIPES buffer, the cells were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (Spurr 1969). Sections were stained with 2% uranyl acetate for 10 min followed by Reynolds' (1963) lead citrate for 20 min prior to viewing in a Philips EM 200 or a Philips EM 410 electron microscope.

Thecal development study

Eight 10 ml cultures were grown for 4 weeks to stationary growth phase. One culture was kept as a control and 7 cultures were harvested by centrifugation (810 rpm) for 3 min. Inspection showed that all cells were rounded up and non-motile. Each culture of centrifuged cells was resuspended in 10 ml of fresh medium for 0, 0.5, 1.0, 2.0, 4.0, 7.0, and 9.0 h, respectively, and then fixed by the decanting method described above. The non-motile cells, which remained at the bottom of the culture tube after the resuspension medium was decanted into double-strength fixative, were fixed directly in single-strength fixative.

Immunocytochemistry

A polyclonal antibody against the 70 kDa epiplasmic protein of the pellicle of *Euglena acus* was made as follows. Pellicles were isolated from sonicated cells of *E. acus* as previously described (Bricheux and Brugerolle 1986). The pellicular fraction was separated by centrifugation on a discontinuous Percoll gradient. The purified fraction was collected, diluted, and then centrifuged to remove Percoll. Pellicles were then resuspended in Tris buffer. Electrophoresis was performed on an 8 to 15% polyacrylamide gradient according to Laemmli (1970). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250. For antibody production, the 70 kDa protein band was cut out from the gel, equilibrated in phosphate-buffered saline (PBS), then mixed with Freund's adjuvant in liquid N_2 to form an emulsion. The mixture was then injected subcutaneously into a rabbit. Preimmune serum was tested to be certain it contained no natural antibodies against the protein injected.
The labelling studies were made on the same glutaraldehyde- and osmium tetroxide-fixed cultures which were used for the morphological study of thecal development. Pale gold sections of Spurr-embedded cells were cut and mounted on formvar-coated nickel grids. Prior to labelling, the sections were etched with 10% H_2O_2 or by 0.58 M sodium metaperiodate for 20 min and then washed twice in distilled H_2O for 10 min. Grids were then floated successively on drops of the following solutions: 1% bovine serum albumin in PBS, 15 min; antiserum against the 70 kDa epiplasmic protein, diluted 1 : 100, 1 h; PBS 3 times 10 min; protein A-gold (15 nm) diluted 1 : 10, 1 h; PBS 2 times 10 min; distilled H_2O, 1 min. All steps were carried out at room temperature. The grids were then stained with uranyl acetate and lead citrate and viewed in a Philips EM 410 electron microscope operated at 80 kV.
Control studies were performed on cells of *G. foliaceum* which had been centrifuged and resuspended in fresh medium for either 1.0 h

Table 1. Number of motile cells in the resuspension medium following ecdysis

Culture no.	Recovery time (h resuspended)	Motile cell density (cells/ml)
1	0[a]	2.30×10^4
2	0[a]	0
3	0.5	0
4	1.0	0
5	2.0	2.04×10^2
6	4.0	4.26×10^3
7	7.0	2.87×10^4
8	9.0	2.56×10^4

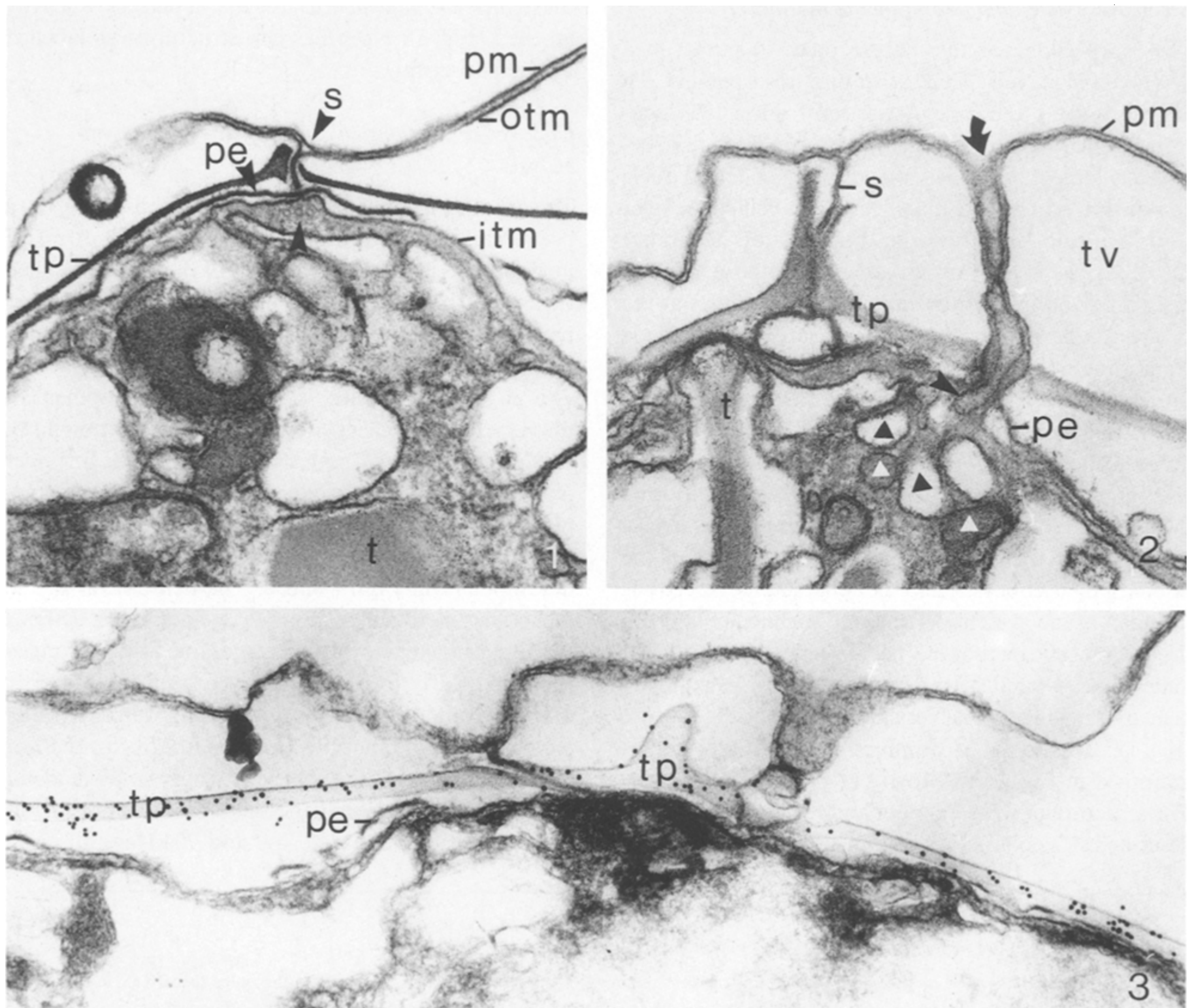
[a] Cultures 1 and 2 were control cultures for the EM study. Culture 1 was not centrifuged. Culture 2 was centrifuged, resuspended in fresh medium and immediately fixed

or 9.0 h. Cells were fixed by gently pouring double-strength fixative into the cultures to give a final concentration of fixative of 3% paraformaldehyde, 1% glutaraldehyde, and 0.2 M glucose in 0.1 M cacodylate buffer, pH 7.2. After a 1 h fixation, the cells were dehydrated in ethanol and embedded in LR White. Unetched sections were labelled by the regime described above either with anti-70 kDa antiserum or with preimmune serum. Although ultrastructural morphology was poor in this preliminary study, the same amphiesmal structures were labelled as in the etched osmicated tissue and no additional labelling was observed. When the antibody was replaced by preimmune serum, no labelling was observed over any amphiesmal or cell structure.

Results

Observations on living cells

The vegetative motile cell of *Glenodinium foliaceum* is a ventrally concave, dorsally convex, flattened cell measuring 35.4 ± 1.8 μm in length and 30.0 ± 2.4 μm in width. When a culture of motile cells is disturbed by shaking or centrifugation, the cells shed their thecal plates (ecdysis), round up and rapidly form a thick pellicle around themselves. The shed thecal plates may



Figs. 1–3. Amphiesma of mature motile cells

Fig. 1. Section through the suture (*s*) region of a 9 h cell. The outer membrane of the thecal vesicle (*otm*) is separated from the inner thecal vesicle membrane (*itm*) by a darkly stained pellicle (*pe*). The thecal plates (*tp*) lie above the pellicle. A band of three microtubules (arrowhead) underlies the inner thecal vesicle membrane at the suture. *pm* Plasmalemma, *t* trichocyst. × 58,000

Fig. 2. Section through a dense vesicle (arrowhead) of the amphisome which crosses the thecal vesicle (*tv*) and empties its contents onto the cell surface (curved arrow). ▲ Amphisomal vesicles with electron translucent contents, △ those with dense contents. *pm* Plasmalemma, *s* suture, *pe* pellicle, *t* trichocyst, *tp* thecal plate. × 58,000

Fig. 3. Late logarithmic phase cell labelled with anti-70 kDa antiserum. The thecal plates (*tp*) are heavily labelled. A discontinuous pellicle (*pe*) is present below the thecal plates. × 58,000

remain intact around the pelliculate non-motile cell, but in this study, they are usually shed during centrifugation. After resuspension in fresh medium, most of the non-motile cells enlarge and divide into two or four cells before emerging from the pellicle as motile cells. Table 1 shows the number of motile cells present in the resuspension medium at each time interval. The first swimming cells were observed at 2 h, with a maximum number being reached at 7 h after resuspension.

Structure of the amphiesma of the motile cell

The morphology of the mature theca is shown in a newly divided cell from a culture resuspended 9 h (Figs. 1 and 2) and in a motile cell from a late logarithmic phase culture (Fig. 3). The suture region between two thecal vesicles is shown in Fig. 1. Each vesicle contains a thin thecal plate. A dense pellicular layer 12–14 nm thick underlies the thecal plates. The outer thecal vesicle membrane lies adjacent to the cell's plasmalemma and infolds to separate the two plates at the suture. Earlier in development the outer thecal vesicle membrane of each thecal vesicle was continuous with the inner thecal vesicle membrane at the suture, but this connection has been broken by the fusion of the dense pellicular layers of the adjacent thecal vesicles. The inner thecal vesicle membrane of the neighboring thecal vesicles has now fused and is continuous over a large part of the cell, except at the two flagella, the discharging trichocysts, and the collared pits. At ecdysis this membrane, which Morrill and Loeblich (1983) call the cytoplasmic membrane, becomes the cell's new plasmalemma. A band of three microtubules lies directly beneath the inner thecal vesicle membrane at the suture (Fig. 1). This type of amphiesmal structure is diagrammed in Fig. 1 a in Morrill (1984).

The structure of what Schnepf and Deichgräber (1972) described as a collared pit is shown in Fig. 2. The membrane of a vesicle with electron dense contents has fused with the plasmalemma and the vesicle's dense material is being released to the outside (Fig. 2). This dense vesicle is part of the amphisome which is described below. Where the discharging vesicle's crosses a thecal vesicle, the outer thecal vesicle membrane is continuous with the inner thecal vesicle membrane at each side of the collared pit (Fig. 2).

When mature motile cells are treated with antiserum against the 70 kDa epiplasmic protein (anti-70 kDa) of the pellicle of *Euglena acus*, the thecal plates are heavily labelled (Fig. 3). No other cell structure was labelled by the antiserum.

Changes in amphiesmal structure accompanying ecdysis

The cells in Figs. 4–7 are all 0 h control cells. They have been centrifuged, quickly resuspended into 10 ml of medium and immediately fixed. All the cells have shed their thecal plates and their outer two membranes either partially or completely and have developed thickened pellicles of complex structure. Several cells (Figs. 4 and 7) even show the earliest stages of the development of the new thecal vesicles (described in the next section). In Fig. 4, the thecal plates have been shed, but over part of the cell the broken remnants of the former plasmalemma and the outer thecal vesicle membrane are still present. The pellicle shows a similar degree of development in Figs. 4 and 6. The dense layer of the pellicle has increased to 20 nm in thickness. Just exterior to the dense layer is a 50 nm layer of fine fibrils arranged perpendicularly to the dense layer (seen more clearly in Figs. 10 and 12). Outside this organized fibrillar layer is a wide layer of randomly oriented fine fibrils. Interior to the dense layer of the pellicle, an inner homogeneous, or finely granular, layer has formed. This homogeneous layer is heavily labelled by anti-70 kDa antiserum

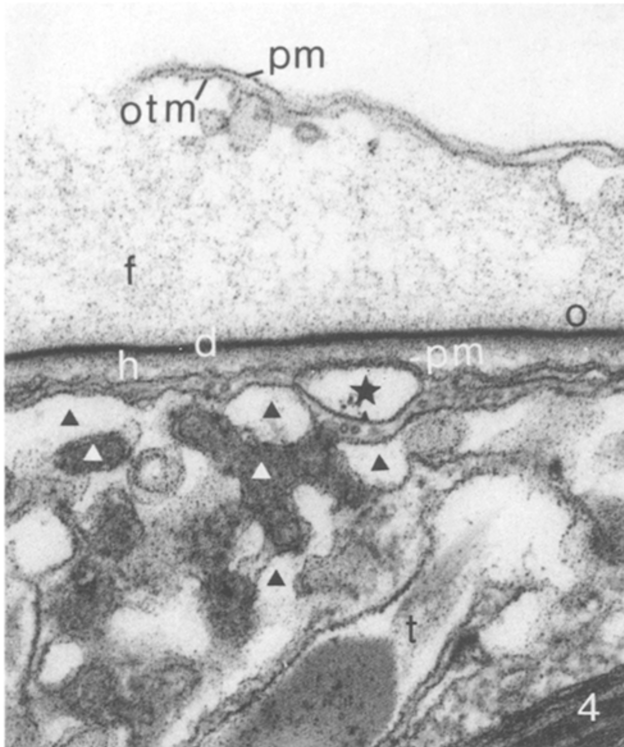
Figs. 4–7. Cells which are in the process of ecdysis or have just ecdysed

Fig. 4. 0 h cell which has shed its thecal plates, but has broken remnants of the old plasmalemma (*pm*) and the outer thecal vesicle membrane (*otm*) remaining. Outside the dense layer (*d*) of the pellicle is an organized layer (*o*) of perpendicular fibrils and a layer of randomly oriented fibrils (*f*). The pellicle has also developed a narrow inner homogeneous layer (*h*). An electron translucent amphisomal vesicle (★) has migrated over the microtubules to lie just beneath the new plasmalemma. Other amphisomal structures are labelled as in Fig. 2. *t* Trichocyst. × 48,700

Fig. 5. 0 h cell labelled with anti-70 kDa antiserum. The lifted up thecal plates (*tp*) are labelled as is the inner homogeneous layer (*h*) of the pellicle. The other layers of the pellicle are unlabelled. The outer two membranes have been shed. × 28,600

Fig. 6. Ecdysed non-motile cell (0 h). The pellicle is slightly more developed than in Fig. 4, but the same four layers (labelled as in Fig. 4) are present. An extended sheet of microtubules is present beneath the plasmalemma (*pm*). × 63,700

Fig. 7. 0 h non-motile cell with a less well-developed pellicle, although the dense layer (*d*) is prominent. A large amphisomal vesicle (★) with electron translucent contents has migrated over the microtubules to the cell surface. Other amphisomal structures are labelled as in Fig. 2. × 63,700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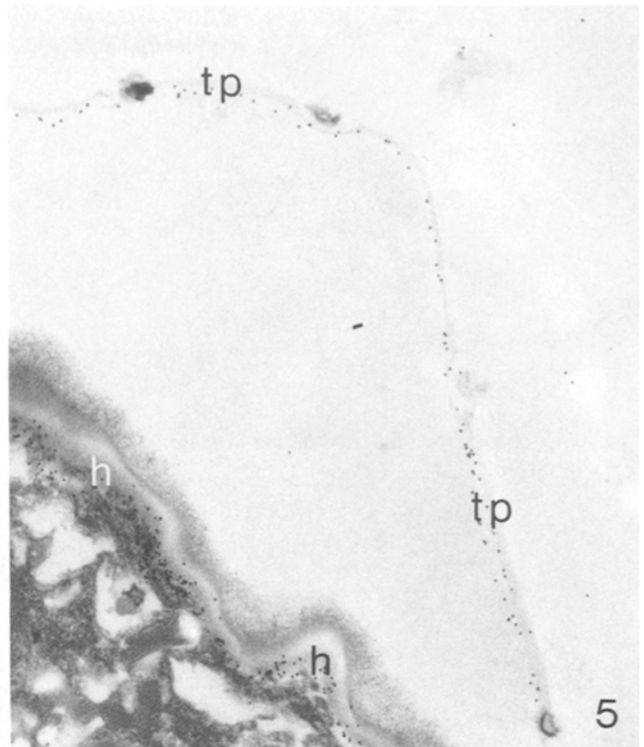
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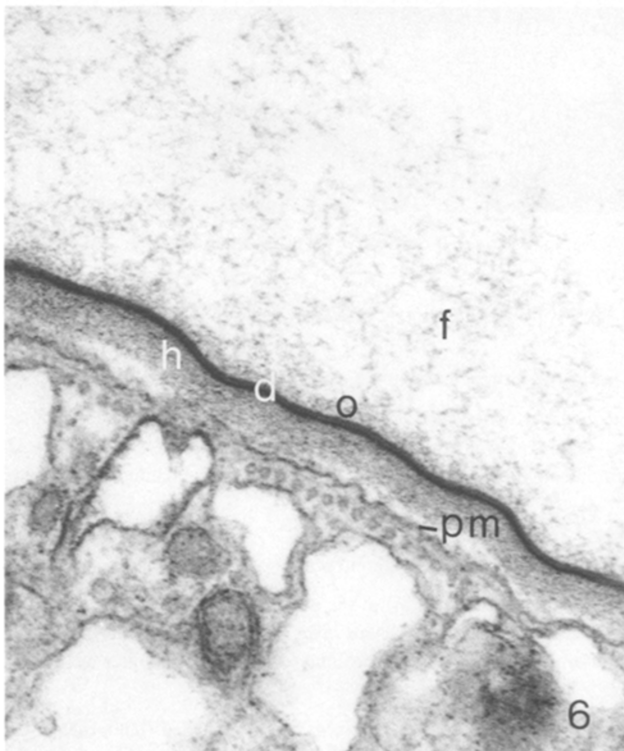
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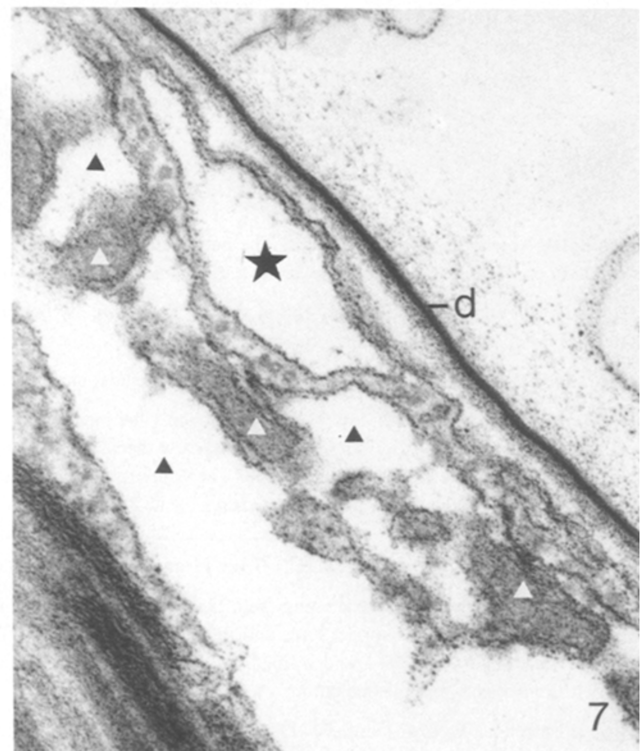
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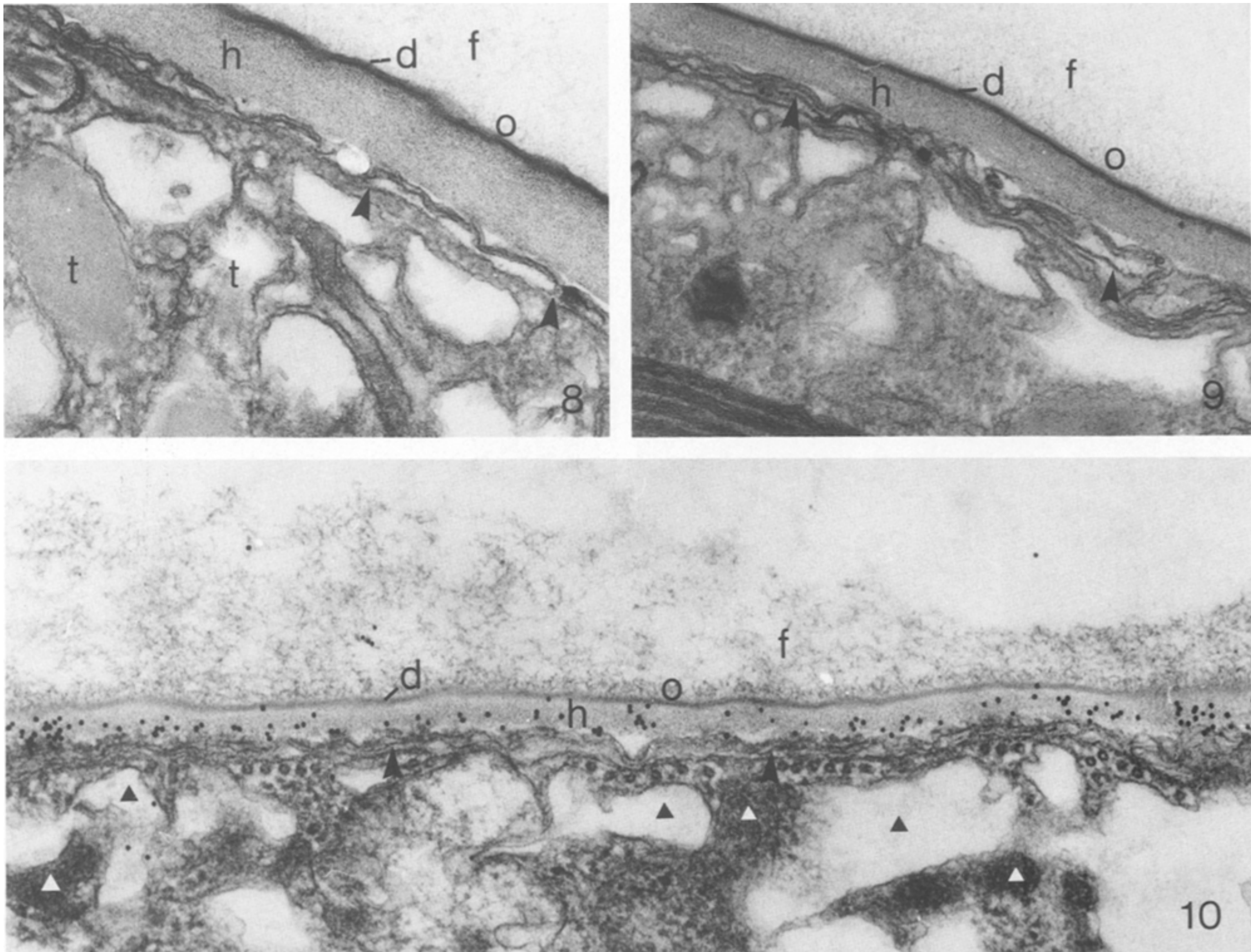


Figs. 8–10. Non-motile pelliculate cells. The pellicles have an inner homogeneous layer (*h*), a dense layer (*d*), an organized layer (*o*) of fibrils oriented perpendicular to the dense layer, and an unorganized fibrous layer (*f*)

Fig. 8. 2 h cell with flattened empty thecal vesicles lying directly beneath the plasma membrane. Arrowheads mark the two ends of one such vesicle. *t* Trichocyst. × 47,450

Fig. 9. 7 h cell. An interrupted pellicular layer (arrowheads) is now present in the flattened thecal vesicles at the cell's periphery. × 65,700

Fig. 10. 0 h cell labelled with anti-70 kDa antiserum. The inner homogeneous layer of the thick enclosing pellicle is labelled. An interrupted pellicular layer (arrowheads) is present in the narrow thecal vesicles underlying the plasmalemma. Microtubules in groups of 2 to 6 lie directly beneath the inner thecal vesicle membrane. The contents of the dense amphisomal vesicles (△) appear granular after etching with sodium metaperiodate. ▲ Clear amphisomal structures. × 57,850

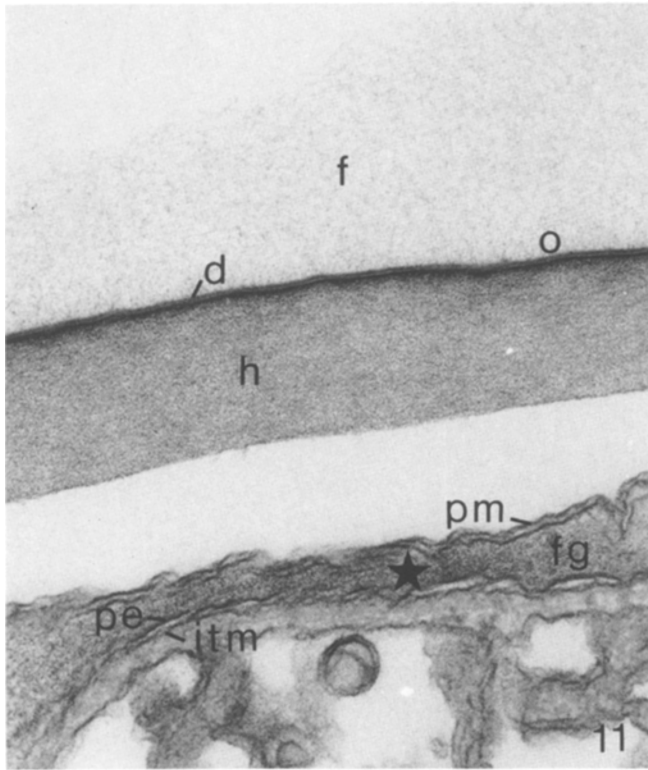
Figs. 11–14. Non-motile pelliculate cells (Figs. 11 and 12) and motile cells (Figs. 13 and 14) at different stages of thecal development

Fig. 11. 2 h cell in process of separating from the mature pellicle. The inner homogeneous layer (*h*) of the pellicle is now very thick, the dense layer (*d*) has a trilaminate appearance, and the outer fibrous layer (*f*) and the inner organized fibrous layer (*o*) are clearly demarcated. The thecal vesicle (★) has widened and is filled with a dense fibrillogranular material (*fg*) overlying the pellicular layer (*pe*). *itm* Inner membrane of the thecal vesicle, *pm* plasmalemma. × 67,600

Fig. 12. 1 h cell labelled with anti-70 kDa antiserum. The wide inner homogeneous layer (*h*) of the pellicle is heavily labelled. The other layers of the pellicle are marked as in Fig. 11. A flattened thecal vesicle with a discontinuous pellicle (arrowhead) lies beneath the plasma membrane (*pm*). × 62,400

Fig. 13. 9 h motile cell with thecal plates (*tp*) forming on top of the fibrillogranular material (*fg*) which overlies the pellicle (*pe*). The pellicle is continuous under the suture (*s*), so the outer membrane of the thecal vesicle (*otm*) is not continuous with the inner thecal vesicle membrane (arrowheads). *pm* Plasmalemma. × 63,700

Fig. 14. Late logarithmic phase cell labelled with anti-70 kDa antiserum. The newly forming thecal plates (*tp*) are labelled, but the fibrillogranular material (*fg*) they lie on top of is not. *pe* Pellicle. × 67,600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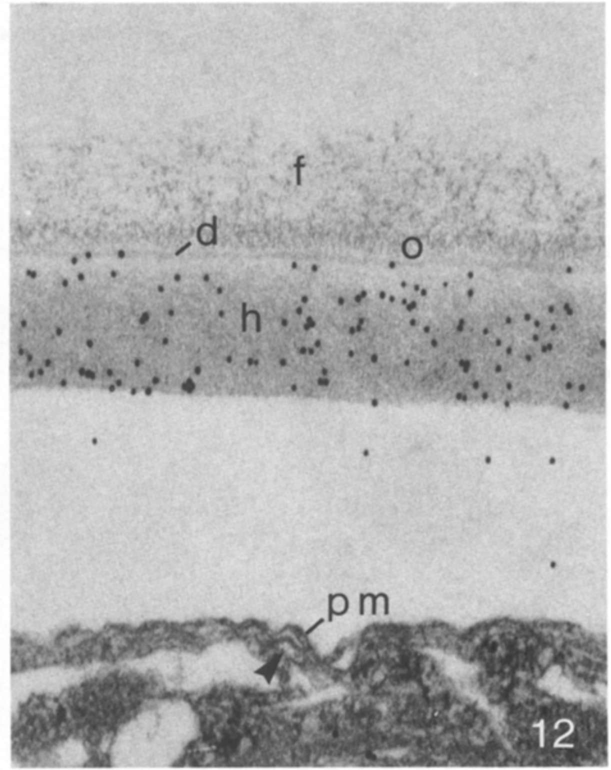
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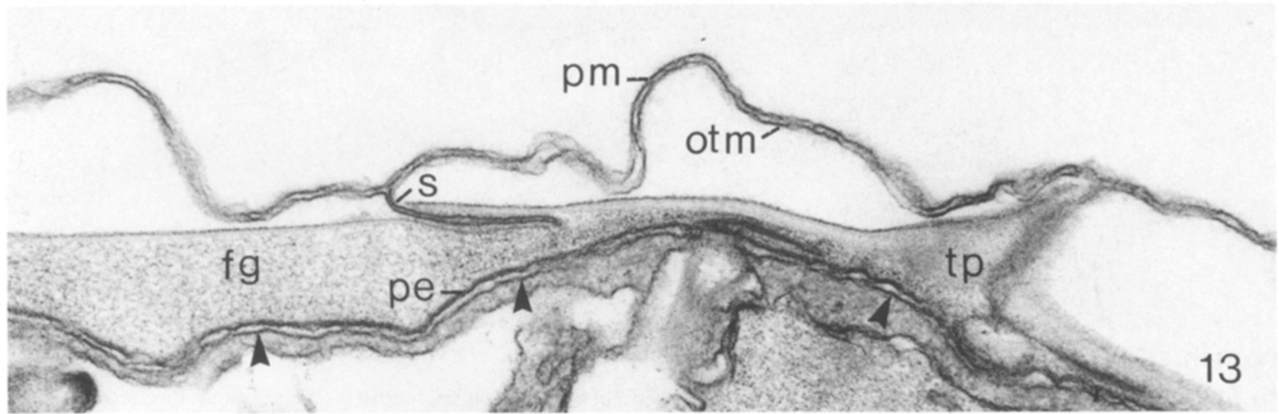
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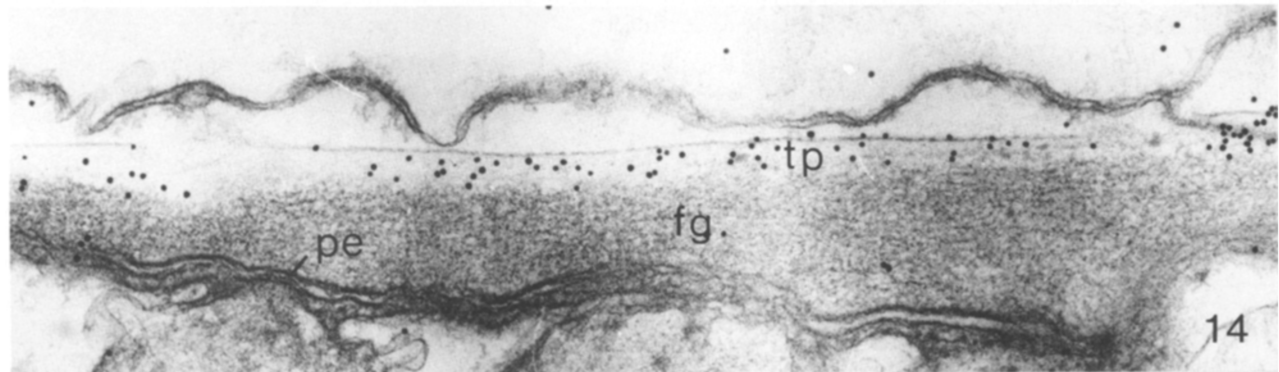
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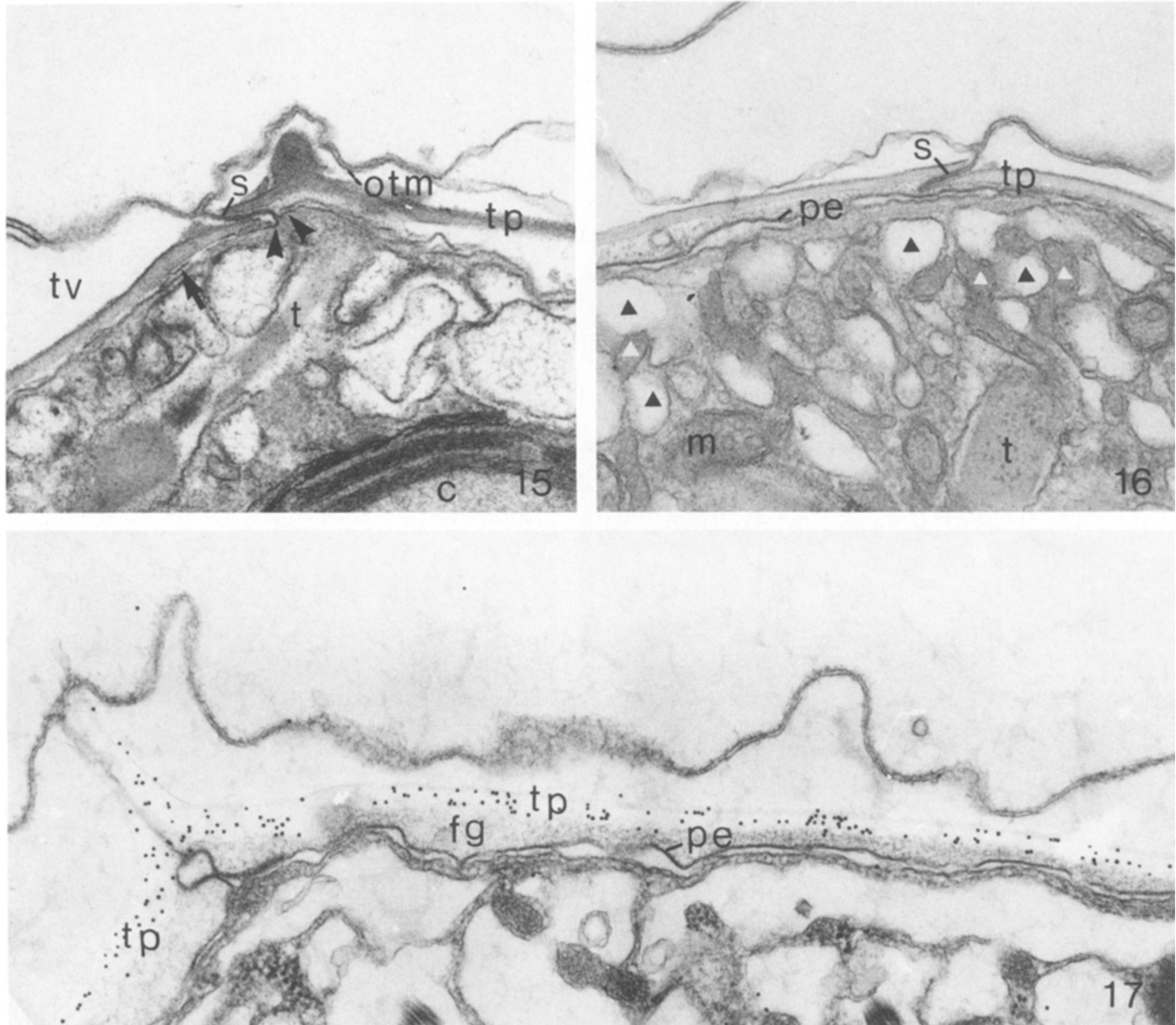

Figs. 15–17. Motile cells with well developed thecal plates

Fig. 15. 9 h cell with adjacent thecal vesicles (*tv*) completely separated from each other at the suture (*s*). The outer membrane (*otm*) of each thecal vesicle is continuous across the suture with the inner membrane of the thecal vesicle (arrowheads). A discontinuous pellicle (arrow) is present under the thecal plates (*tp*). *c* Chloroplast, *t* trichocyst. × 67,500

Fig. 16. 9 h cell with an incomplete suture. The pellicle (*pe*) is continuous across the suture (*s*) and the thecal plates (*tp*) are separated from the pellicle by a small amount of fibrillogranular material. *m* Mitochondrion, *t* trichocyst, ▲ clear amphisome vesicles, △ dense amphisome vesicles. × 47,450

Fig. 17. Late logarithmic phase cell labelled with anti-70 kDa antiserum. The thecal plates (*tp*) are heavily labelled, but the fibrillogranular material (*fg*) directly underlying the plates is unlabelled. *pe* Pellicle. × 47,450

(Fig. 5). In Fig. 5, the thecal plates (distinguished by their labelling by anti-70 kDa antiserum) have lifted up during ecdysis, but still form a layer of plates around the pelliculate non-motile cell. The former plasmalemma and outer thecal vesicle membrane appear to be lost.

Development of the thecal vesicles and new thecal plates following ecdysis

Mahoney (1984) has described an organelle in *G. foliaceum* consisting of closely appressed membrane-limited tubules and vesicles, which she has named the

amphisome. In its simplest form, the amphisome consists of an ordered array of closely packed membrane-bound tubules, in which tubules with electron dense contents alternate with those with electron translucent contents. The volume of the amphisome in cells which are forming new thecal vesicles and plates is 10 times that in mature motile cells (Mahoney 1984), and most of the amphisome is found at the surface of the cell (Fig. 4). The amphisome here has an irregular structure, tubules and vesicles of similar content having fused with each other, so that irregular membrane-bound structures containing dense material (Fig. 4) alternate with irregular membrane-bound structures containing electron translucent material (Fig. 4). The amphisomal vesicles with dense interiors can secrete their contents to the cell surface via collared pits (Fig. 2). Some of the amphisomal vesicles with translucent interiors become the new thecal vesicles. Figure 6 shows that these vesicles lie interior to the row of microtubules which underlie the plasmalemma of the non-motile pelliculate cell. Figure 7 shows that an electron translucent vesicle of the amphisome has migrated to the cell surface, probably by sliding over the microtubules and displacing them from their former position just beneath the plasmalemma. A similar vesicle is seen between the plasmalemma and the microtubules in Fig. 4.

The next step in the formation of thecal vesicles is a flattening and elongation of the new thecal vesicle. A flattened vesicle is seen at the right in Fig. 4. Empty flattened thecal vesicles lie just underneath the plasmalemma in the pelliculate cell in Fig. 8. This cell is 2 h post-ecdysis. Note that the inner homogeneous layer of the pellicle has become very thick and that the dense layer of the pellicle can be seen to have the trilaminate structure observed by other authors. The first structure to appear in the flat thecal vesicles is a discontinuous dense pelliculate layer (Fig. 9). The new pellicle is 10 nm thick. Figure 10 shows a cell at the same stage as Fig. 9 labelled with anti-70 kDa antiserum. Only the inner homogeneous layer of the outer thickened pellicle is labelled. The microtubules underlying the new flattened thecal vesicles are especially prominent in this cell. They are no longer present in extended rows, but are organized in bands of 2 to 6.

In the 2 h cell in Fig. 11, several things have happened. The cell body has moved away from the pellicle. Note the prominent trilaminate nature of the dense layer of this mature pellicle. The developing thecal vesicle has now widened considerably and a dense fibrillogranular material fills the entire thecal vesicle above the pellicular layer. In the 1 h cell in Fig. 12, the cell has also separated from the pellicle, although the flattened thecal vesicles present in this cell are poorly resolved. The four layers of the pellicle, however, are especially clearly resolved. The entire inner homogeneous layer of the pellicle is heavily labelled by anti-70 kDa antiserum.

We did not obtain any micrographs showing the emergence of motile cells from the pellicle. Figures 13–17 are all motile cells which have discarded the old pellicle. In Fig. 13, the fibrillogranular material overlying the pellicular layer in the thecal vesicle has become less dense and a thecal plate appears to be condensing out of this material. Labelling with anti-70 kDa antiserum (Fig. 14), however, shows that this impression is erroneous. The thecal plates form on top of this fibrillogranular material. Only the forming plates, not the fibrillogranular material, are labelled by the antiserum (Fig. 14).

The cells in Figs. 15–17 have wide thecal vesicles with mature thecal plates. Very little of the fibrillogranular material remains between the plates and the pellicle. In the 9 h cell in Fig. 15, the thecal vesicles are still separate. The outer thecal vesicle membrane of each of the adjacent thecal vesicles is continuous with the inner thecal vesicle membrane at the suture (Fig. 15). The pellicle is interrupted at the suture. In the 9 h cell in Fig. 16, the pellicle has become continuous across the suture and the inner thecal vesicle membranes of the neighbouring vesicles have fused. A microtubule sectioned longitudinally is seen under this membrane. Amphisome vesicles, trichocysts and mitochondria are seen in the cytoplasm. Figure 17 shows a similar motile cell labelled by anti-70 kDa antiserum. Only the thecal plates are labelled. The fibrillogranular material underlying the plates is not labelled, nor is the continuous pellicle.

Discussion

Chemical composition of the amphiesma

The most detailed studies of the chemistry of the amphiesma have been made on *Heterocapsa niei* (Loeblich 1970) and *Peridinium westii* (Nevo and Sharon 1969). In each case, the thickened pellicles as well as the thecal plates were probably present in the thecal preparations analyzed (Loeblich 1984). Nevo and Sharon (1969) found 95% of the theca to be a polymer of D-glucose. Both $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ glycosidic linkages were present. Loeblich (1970) found that the organic component of his thecal preparations consisted of 84.4% glucan (glucose was the only sugar detected), 3.5% lipid, 2.3–3.9% protein and the remainder unknown.

Loeblich (1970) found that the thecal plates stained blue with iodine-KI and H_2SO_4 and purplish with the zinc-chlor-iodide reaction, tests which indicate the presence of cellulose. However, two different cellulose solvents failed to solubilize the thecal plates unless the preparations were boiled in HCl first. However, cellulase did partially digest the thecal plates. Loeblich (1984) concluded that the thecal plates and the inner layer of the pellicle consist of glucose polymers in either an amorphous or low crystalline state since structures resembling cellulose fibers are not seen in electron micrographs.

The chemistry of the pellicle of *H. niei* and a variety of other dinoflagellates has been studied by Loeblich (1970) and Morrill and Loeblich (1981). These authors believe that the very resistant layer of the pellicle which is not solubilized by hot ethanolamine corresponds to the trilaminate layer (what we call the dense layer of the pellicle in *G. foliaceum*). They suggest that this layer consists of the plant terpenoid, sporopollenin. Staining studies (Morrill and Loeblich 1981) showed that the pellicle of many species also contains a cellulose-like layer. This was especially evident in *Peridinium balticum*, a symbiont-containing dinoflagellate closely related to *G. foliaceum*. A wide blue-stained "cellulose" layer was observed at the inner surface of the pellicle. Thus, the two structures that are labelled by anti-70 kDa antiserum in *G. foliaceum*, the thecal plates and the inner homogeneous layer of the pellicle, have been shown to consist largely of a glucose polymer with at most only a small amount of protein present. We believe our antiserum is recognizing an epitope shared by a protein(s) in the thecal plates and the inner layer of the pellicle of *G. foliaceum* and the 70 kDa protein of the epiplasm of *Euglena acus*. We feel certain that the antiserum is not recognizing a carbohydrate epitope. Bricheux and Brugerolle (1986) have shown that the 70 kDa protein is not a glycoprotein by treatment of polyacrylamide gels of *E. acus* pellicles by the method of Eckhardt et al. (1976). The 70 kDa protein did not fluoresce under UV light, although the 140 and 64 kDa glycoproteins of the plasmalemma did. The 80 and 86 kDa epiplasmic proteins from *E. gracilis* (Dubreuil and Bouck 1985), which have been shown to be closely related to the 70 and 80 kDa epiplasmic proteins of *E. acus* (Bricheux and Brugerolle 1987), have also been shown not to be glycoproteins, for they are not stained on gels treated by periodic acid Schiff (Rogalski and Bouck 1980).

As an additional control, we determined that anti-70 kDa antiserum did not recognize higher plant cellulose. In glutaraldehyde-fixed, LR White-embedded cells of *Pisum sativum*, labelled with anti-70 kDa and protein A-gold, no labelling was observed over the cell walls.

We have tried to do immunoblots in order to identify the peptide(s) giving the antigenic reaction observed on the thecal plates and on the inner homogeneous layer of the pellicle. Cells were centrifuged to induce ecdysis, then a fraction enriched in thecal plates was collected by centrifugation. The non-motile pelliculate cells were then submitted to a brief sonication to break the cells, then to a gentle centrifugation to recover the pellicular fraction. Both fractions were solubilized by SDS and β-mercaptoethanol prior to electrophoresis. However, no proteins were recovered on Coomassie Blue-stained gels, nor on Western blots. Possibly peptides were present in each fraction but were inaccessible to detergents. Loeblich (1970) proposed that the cellulose of the thecal plates and pellicle might be a tight structure very difficult to disorganize. We believe that the sectioning of the structures and the etching of sections before treatment by antiserum could have unmasked the peptide epitope recognized by the anti-70 kDa antiserum.

Origin of the thecal vesicles from translucent amphisomal vesicles

Until now the origin of thecal vesicles in dinoflagellates has remained a mystery. Morrill (1984) in her study of thecal development in *Heterocapsa niei* observed a row of tiny vesicles under the plasmalemma and suggested that these fused with each other to form a thecal vesicle. She did not say how these tiny vesicles originated. We have shown in *G. foliaceum* that the large amphisomal vesicles with electron translucent contents migrate over a sheet of microtubules to the cell surface, thereby displacing the microtubules from their position just under the plasma membrane.

We have given the name amphisome to a distinctive structure in dinoflagellates in which membrane-bound tubules, vesicles, or large irregular structures with electron translucent contents are appressed against similarly-shaped membrane-bound structures with electron dense contents. How this complex structure develops from an organized array of tubules in which dense tubules alternate with translucent tubules is described in detail elsewhere (Mahoney 1984). We have named this structure the amphisome because the amphiesmal vesicles develop from its electron translucent vesicles and also because it is made up of *both* electron dense

and translucent structures. The amphisome has been observed in a number of other species of dinoflagellates and called by a variety of names. It has been particularly well illustrated by Wetherbee (1975 a, b) in cells of *Ceratium tripos* which are forming new theca. He noted that elongate vesicles (the dense amphisomal vesicles) are separated from each other by fenestrated ER (the electron translucent amphisomal vesicles). In cysts of *Crypthecodinium cohnii*, the amphisome is prominent. Dense amphisomal elements were termed convoluted cisternae and the translucent amphisomal vesicles were called electron transparent vacuoles (Pokorny and Gold 1973). The latter contained small particulate inclusions not present in *G. foliaceum*. Similarly in *Peridiniopsis berolinense*, the vacuoles containing small crystals which underlie the thecal vesicles are the equivalent of the translucent amphisomal vesicles of *G. foliaceum*. The dense amphisomal vesicles in this species are called subthecal vesicles (Wedemayer and Wilcox 1984). At the cell surface of *Dinophysis* (Lucas and Vesk 1990), dilated ER (the translucent amphisomal vesicles) lies appressed against dense mucocysts. The dense material in these mucocysts shows various differentiations including striations, but they are clearly more differentiated forms of the dense amphisomal vesicles of *G. foliaceum*. They also empty their contents onto the cell surface in the same manner as the dense amphisomal vesicles do (Fig. 2).

Development of the thecal plates

During thecal plate development in *G. foliaceum*, the first structure to appear in the flattened thecal vesicles is a discontinuous pellicle. This has also been observed in *H. niei* by Morrill (1984). As the thecal vesicle widens, dense fibrillogranular material appears above the pellicle, and later thecal plates form on top of this fibrillogranular material. Mahoney (1984) hypothesized that the thecal plates formed out of this material, for usually only small amounts of fibrillogranular material underly the mature thecal plates (Figs. 16 and 17). However, we no longer believe this happens, for an antiserum to the 70 kDa protein of the epiplasm of *Euglena acus* labels the thecal plates, but not the fibrillogranular material nor any intracellular structure. We believe all three structures, pellicle, fibrillogranular material, and thecal plates, assemble from soluble precursors that cross the thecal vesicle membrane. We have never observed any cytoplasmic structure fusing with the thecal vesicle and emptying its contents into it. Both Wetherbee (1975 a, b) and Dürr (1979 a, b) observed that elongate vesicles (i.e., the dense amphisomal vesicles) fuse with the thecal vesicles in *Ceratium tripos, Gonyaulax polyedra*, and *Peridinium cinctum*. Both authors note that this occurs only at the sutures. We see dense amphisomal vesicles emptying their contents to the outside (Fig. 2), never into a thecal vesicle. We cannot explain the discrepancy. Dürr (1979 a, b) also claimed that multivesicular bodies and dense rounded bodies (i.e., dense amphisomes) fused with the thecal vesicles, but their micrographs of this are unconvincing.

Role of microtubules in thecal development

We have observed that in newly formed pelliculate cells, extended rows of microtubules underlie the plasmalemma, whereas after the new thecal vesicles have formed, the microtubules underlying them are present in groups of 2 to 6. As long ago as 1970, Dodge and Crawford (1970 a) noted that microtubules were present under the inner thecal membrane in groups of 2 to 7 in *Ceratium hirundinella*, but a sheet of 300 microtubules underlay the immature theca of the anterior horn. Dürr (1979 a) noted that the microtubular layer became looser during development of the amphiesma of *Gonyaulax polyedra*. Schnepf et al. (1989) made similar observations for *Gymnodinium aeruginosum*. Roberts et al. (1988) has elegantly described by fluorescence and EM microscopy the sub-thecal microtubular cytoskeleton of motile cells of *Amphidinium rhynchocephalum*. Our results (Figs. 4 and 7) have shown that a new thecal vesicle is formed by the migration of a translucent amphisomal vesicle over a sheet of microtubules to the cell surface. The movement of the amphisomal vesicle may be driven by a molecular microtubular motor. This may be premature speculation, but there seems little doubt that the microtubules play a role in thecal development as well as in the ultimate shaping of the motile cell.

Are the epiplasmic structures of euglenoids, dinoflagellates, and ciliates evolutionarily related?

The pellicle of *Euglena acus* consists of the plasma membrane plus a thick underlying epiplasmic layer, which is organized into overlapping strips. Bricheux and Brugerolle (1986) isolated the epiplasmic layer of *E. acus* free of the plasma membrane and showed that it consisted of three main polypeptides with molecular weights of 70, 80, and 180 kDa. Antisera raised against all three polypeptides cross reacted with each other, and all the antisera labelled the epiplasmic layer of the pellicle in electron micrographs. Digestion of the 70

and 80 kDa proteins showed they were very similar, and they were postulated to be the major epiplasmic proteins (Bricheux and Brugerolle 1986). Vigues et al. (1987) showed that antiserum against the 80 kDa epiplasmic protein of *E. acus* cross reacted with a 220 and 140 kDa protein of the amphiesma of the dinoflagellate *Noctiluca scintillans* and with a 78 kDa doublet and a 64 kDa polypeptide of the epiplasm of the ciliate *Pseudomicrothorax dubius*. Similarly, antisera raised against the 220 kDa protein of *Noctiluca* and against whole epiplasms of *P. dubius* cross reacted with the 70 and 80 kDa epiplasmic proteins of *E. acus* and with the corresponding epiplasmic proteins of each other.

At the electron microscope level, anti-80 kDa antiserum from *E. acus* labelled the epiplasmic layer subtending the alveoli in the ciliate *P. dubius* and what was termed the epiplasm of *Noctiluca scintillans* (Vigues et al. 1987). The ultrastructure of the amphiesma of this dinoflagellate, now called *N. miliaris*, has recently been elucidated by Melkonian and Höhfeld (1988). It consists of a plasmalemma underlain by amphiesmal vesicles which are empty but for some honeycomb-like material. A finely granular pellicular layer, which varies from 20 to 800 nm in thickness, lies immediately underneath the amphiesmal vesicles. It is this pellicular layer that labels with anti-80 kDa antiserum from *E. acus*.

In thecate dinoflagellates like *G. foliaceum* and *H. niei*, the pellicle is formed inside the thecal vesicles and only thickens when the thecal plates and outer membranes are shed at ecdysis. Only the inner homogeneous layer of the pellicle of the ecdysal cyst of *G. foliaceum* was labelled by anti-70 kDa antiserum from *E. acus*. In texture, also, this layer looks like the pellicle of *Noctiluca miliaris*, so they appear to be fundamentally similar structures, although one is intracellular and one extracellular. It is interesting that the thecal plates are also labelled by anti-70 kDa antiserum.

Recent molecular studies of the sequence of the 18S and 28S ribosomal RNAs have shown that dinoflagellates are closely related to ciliates (Herzog and Maroteaux 1986, Baroin et al. 1988, Lenaers et al. 1989). However, the euglenoids arose much earlier in evolution and are not closely related to either ciliates or dinoflagellates (Sogin et al. 1986). Thus it will be very interesting to determine if the proteins of the epiplasm of the ciliate *P. dubius* and of the pellicle of *Noctiluca* and the thecal plates and the inner homogeneous layer of the pellicle of *G. foliaceum* are indeed related to the 70 and 80 kDa proteins of the pellicle of *E. acus*. Structural proteins like tubulin and actin are evolutionarily highly conserved. Recently, Bouck and coworkers have sequenced the 80 and 86 kDa epiplasmic proteins of *Euglena gracilis*, which are the equivalent of the 70 and 80 kDa epiplasmic proteins of *E. acus*, and have shown that the two proteins share significant homology, and each has a series of more than 30 similar internal 12 amino acid repeats (Marrs et al. 1991). The sequences of these two proteins are unlike any known protein, and Bouck proposes to name them articulins. We have seen that the epiplasmic proteins of ciliates, dinoflagellates and euglenoids are immunologically related to each other (Vigues et al. 1987). Also the epiplasmic layers of ciliates and euglenoids and the thecal plates and inner homogeneous layer of the pellicle of *G. foliaceum* can appear very similar in electron micrographs, all having a finely granular texture of moderate electron density. Thus, it is possible that although dinoflagellates and ciliates have evolved much later than euglenoids, they possess in their amphiesma and epiplasm, proteins similar to the articulins of euglenoids.

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References

Baroin A, Perasso R, Qu L-H, Brugerolle G, Bachellerie J-P, Adoute A (1988) Partial phylogeny of the unicellular eukaryotes based on rapid sequencing of a portion of 28S ribosomal RNA. Proc Natl Acad Sci USA 85: 3474–3478

Bricheux G, Brugerolle G (1986) The membrane cytoskeleton complex of Euglenoids. I. Biochemical and immunological characterization of the epiplasmic proteins of *Euglena acus*. Eur J Cell Biol 40: 150–159

– – (1987) The pellicular complex of Euglenoids II. A biochemical and immunological comparative study of major epiplasmic proteins. Protoplasma 140: 43–54

Cachon J, Cachon M, Boillot A, Brown DL (1987) Cytoskeletal and myonemal structures of dinoflagellates are made of 2–3 nm filaments. Cell Motil Cytoskeleton 7: 325–336

Dodge JD, Crawford RM (1968) Fine structure of the dinoflagellate *Amphidinium carteri* Hulbert. Protistologica 4: 231–242

– – (1970 a) The morphology and fine structure of *Ceratium hirundinella* (Dinophyceae). J Phycol 6: 137–149

– – (1970 b) A survey of thecal fine structure in the Dinophyceae. Bot J Linn Soc 63: 53–67

– – (1971) Fine structure of the dinoflagellate *Oxyrrhis marina*. I. The general structure of the cell. Protistologica 7: 295–304

Dubreuil RR, Bouck GB (1985) The membrane skeleton of a unicellular organism consists of bridged, articulating strips. J Cell Biol 101: 1884–1896

Dürr G (1979 a) Elektronenmikroskopische Untersuchungen am Panzer von Dinoflagellaten I. *Gonyaulax polyedra*. Arch Protistenkd 122: 55–87

– (1979 b) Elektronenmikroskopische Untersuchungen am Panzer von Dinoflagellaten II. *Peridinium cinctum*. Arch Protistenkd 122: 88–120

Eckhardt AE, Hayes CE, Goldstein IJ (1976) A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. Anal Biochem 73: 192–197

Herzog M, Maroteaux L (1986) Dinoflagellate 17S rRNA sequence inferred from the gene sequence: evolutionary implications. Proc Natl Acad Sci USA 83: 8644–8648

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lenaers G, Maroteaux L, Michot B, Herzog M (1989) Dinoflagellates in evolution. A molecular phylogenetic analysis of large subunit ribosomal RNA. J Mol Evol 29: 40–51

Loeblich AR III (1970) The amphiesma or dinoflagellate cell covering. In: Yochelson EL (ed) Proceedings of the North American Paleontological Convention, Chicago, Illinois, vol 2, part G. Allen Press, Lawrence, Kansas, pp 867–929

– (1984) Dinoflagellate physiology and biochemistry. In: Spector DL (ed) Dinoflagellates. Academic Press, New York, pp 299–342

Lucas IAN, Vesk M (1990) The fine structure of two photosynthetic species of *Dinophysis* (Dinophysiales, Dinophyceae). J Phycol 26: 345–357

Mahoney DG (1984) The fine structure of the endosymbiont-containing dinoflagellate *Peridinium foliaceum*. MSc thesis, McGill University, Montrèal

Marrs JM, Levasseur PJ, Bouck GB (1991) Molecular and cellular basis for cell form in *Euglena*. J Phycol 27s: 47

McIntosh L, Cattolico RA (1978) Preservation of algal and higher plant ribosomal RNA integrity during extraction and electrophoretic quantitation. Anal Biochem 91: 600–612

Melkonian M, Höhfeld I (1988) Amphiesmal ultrastructure in *Noctiluca miliaris* Suriray (Dinophyceae). Helgoländer Meeresunters 42: 601–612

Morrill LC (1984) Ecdysis and the location of the plasma membrane in the dinoflagellate *Heterocapsa niei*. Protoplasma 119: 8–20

– Loeblich AR III (1981) The dinoflagellate pellicular wall layer and its occurrence in the division Pyrrhophyta. J Phycol 17: 315–323

– – (1983) Ultrastructure of the dinoflagellate amphiesma. Int Rev Cytol 82: 151–180

Nevo Z, Sharon N (1969) The cell wall of *Peridinium westii*, a non cellulosic glucan. Biochim Biophys Acta 173: 161–175

Pokorny KS, Gold K (1973) Two morphological types of particulate inclusions in marine dinoflagellates. J Phycol 9: 218–224

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212

Roberts KR, Farmer MA, Schneider RM, Lemoine JE (1988) The microtubular cytoskeleton of *Amphidinium rhynchocephalum* (Dinophyceae). J Phycol 24: 544–553

Rogalski AA, Bouck GB (1980) Characterization and localization of a flagellar-specific membrane glycoprotein in *Euglena*. J Cell Biol 86: 424–435

Schnepf E, Deichgräber G (1972) Über den Feinbau von Theka, Pusule und Golgi-Apparat bei dem Dinoflagellaten *Gymnodinium* spec. Protoplasma 74: 411–425

– Winter S, Mollenhauer D (1989) *Gymnodinium aeruginosum* (Dinophyta): a blue-green dinoflagellate with a vestigial, anucleate, cryptophycean endosymbiont. Plant Syst Evol 164: 75–91

Schütt F (1895) Die Peridineen der Plankton-Expedition. Ergebnisse Plankton-Exped Humboldt-Stiftung 4: 1–170

Sogin ML, Elwood HJ, Gunderson JH (1986) Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proc Natl Acad Sci USA 83: 1383–1387

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43

Taylor FJR (1987) Dinoflagellate morphology. In: Taylor FJR (ed) The biology of dinoflagellates. Blackwell, Oxford, pp 24–91

Vigues B, David D (1989) Demonstration of a 58 kd protein as a major component of the plasma membrane skeleton in the ciliate *Entodinium bursa*. Protoplasma 149: 11–19

– Bricheux G, Metivier C, Brugerolle G, Peck RK (1987) Evidence for common epitopes among proteins of the membrane skeleton of a ciliate, an euglenoid and a dinoflagellate. Eur J Protistol 23: 101–110

Wedemayer GJ, Wilcox LW (1984) The ultrastructure of the freshwater, colorless dinoflagellate *Peridiniopsis berolinense* (Lemm.) Bourrelly (Mastigophora, Dinoflagellida). J Protozool 31: 444–453

Wetherbee R (1975 a) The fine structure of *Ceratium tripos*, a marine armored dinoflagellate II. Cytokinesis and development of the characteristic cell shape. J Ultrastruct Res 50: 65–76

– (1975 b) The fine structure of *Ceratium tripos*, a marine armored dinoflagellate III. Thecal plate formation. J Ultrastruct Res 50: 77–87

Williams NE, Vaudaux PE, Skriver L (1979) Cytoskeletal proteins of the cell surface in *Tetrahymena*. I. Identification and localization of major proteins. Exp Cell Res 123: 311–320