

# IMMUNOLOGICAL AND STRUCTURAL EVIDENCE FOR PATTERNED INTUSSUSCEPTIVE SURFACE GROWTH IN A UNICELLULAR ORGANISM

## A Postulated Role for Submembranous Proteins and Microtubules

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### ABSTRACT

The surface complex of *Euglena* has been examined intact and after isolation and purification by the use of mild sonication to disrupt cells. In intact cells the surface complex (pellicle complex) is oriented in a series of parallel ridges and grooves, and possesses among other components a characteristic group of four to seven microtubules. Isolated pellicles retain the ridge and groove pattern but no microtubules are present. Isolates yielded at least three major polypeptides on SDS acrylamide gels; one or more of the polypeptides are postulated to be identical with a submembrane layer present in both intact and isolated pellicles; one polypeptide appears to be in or on the surface membrane. Antibodies directed against the isolated pellicles were conjugated directly or indirectly to fluorescein, latex spheres, or ferritin. In appropriate experiments with these antibody conjugates, it has been found that antigenic sites are immobile and that new antigenic sites (daughter strips) are inserted between parental strips in replicating cells. These results together with direct observation of daughter strips by transmission electron microscopy suggest that surface growth in *Euglena* occurs by intussusception. Microtubules associated with the pellicle complex are postulated to play a role in the development of new daughter strips, and possibly also in cell movements.

Synthesis and insertion of new surface materials are among the universal requirements of replicating or developing cells. Orientation of such materials may be equally important, but it is difficult to detect surface patterns in many cells because of highly mobile or freely diffusible surface components (10, 12, 13, 34). It is clear, however, that subsurface components such as the protein complex spectrin (31, 38) or microtubules (45, 49) may influence the ultimate orientation of some surface sites.

The well-patterned surfaces of some lower flagellates seem particularly well suited for further resolving the questions of how new surfaces are regenerated and oriented during cell duplication, and how their patterns are maintained once formed. In the present study, the surface complex (pellicle) of the unicell *Euglena* has been analyzed by attempting (a) to assess which components of the pellicle are directly involved in maintenance of the ridge and groove patterns, (b) to determine the biochemical composition of the surface complex,

and (c) to follow pellicle replication both by examining directly the replicating pellicle ridges, and by application of immunospecific surface markers during pellicle replication. These studies suggest that euglenoid surfaces replicate by intussusceptive growth, that pellicle form is apparently maintained by a layer of proteinaceous material underlying the plasma membrane, and that microtubules may play a role in the initial deposition and orientation but not the maintenance of the intussuscepted strips. Extensive microtubule cross-bridging further suggests, by analogy with other systems, that microtubules present in the mature pellicle may be associated with surface movements related to metaboly (cell shape changes).

## MATERIALS AND METHODS

### *Growth and Maintenance of Organisms*

Cultures of *Euglena gracilis* strain Z were grown at 25°C in 1-liter flasks containing 300 ml of Cramer and Myer's (8) medium at a continuous illumination of about 300 foot-candles. Cells were generally harvested 4 or 5 days after inoculation when culture density was about  $5 \times 10^6$  cells/ml.

### *Pellicle Isolation and Purification*

Euglenoid pellicles were purified by discontinuous density gradient centrifugation after cavitation of whole cells. The following procedure produced good yields of highly purified pellicle fragments.

Cells from 500 ml of 4- to 5-day old culture (containing  $5 \times 10^6$  cells/ml) were harvested by centrifugation and deflagellated by using either cold shock or a slightly acidic, ethanol-containing medium (46) at 0–4°C. Centrifugation at full speed for 10 min (~1,500 g) in an International clinical centrifuge (International Scientific Instruments, Inc., Mountain View, Calif.) pelleted cell bodies, while flagella remained in the supernate which was discarded. The entire procedure was repeated a total of three times to insure complete deflagellation. Subsequently, the remaining whole cells were suspended in 0.1 M sodium phosphate, pH 7.0, and were fractionated by five successive 15-s sonications at setting 5 on a Branson S125 sonifier (Branson Instruments, Danbury, Conn.). To prevent overheating of the mixture, the procedure was carried out on ice, and a 1-min cooling interval followed each sonication. The entire mixture was centrifuged in a Sorvall RC-2B centrifuge (Dupont Instruments, Sorvall Operations, Newtown, Conn.) with an SS-34 rotor at 3,500 rpm (~1,500 g) for 15 min, thereby producing a pellet consisting of two discrete portions. The upper, bright-green portion containing chloroplasts and other debris was gently rinsed away with phosphate buffer. The lower,

whitish portion of the pellet was resuspended in 2 ml of buffer and distributed in 0.3-ml portions onto 16 ml discontinuous sucrose gradients consisting of layered 75%, 85%, 95%, and 100% wt/vol sucrose in 0.1 M sodium phosphate buffer at pH 7.0. Centrifugation for 18–24 h in a Beckman SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at ~52,000 g at the tube center resulted in the formation of a distinct white band of pure pellicle fractions at the 85%/95% sucrose interface (Fig. 7 a). The band was collected with a Pasteur pipette, and sucrose was removed by dilution of the band with distilled water followed by pelleting of the pellicle fragments at about 50,000 g for 20 min. About 500 µg dry weight of purified pellicles could be obtained from one harvested culture (500 ml).

### *Pellicle Composition*

A run of purified pellicles was carefully divided into two portions. The first portion was further subdivided to measure total proteins colorimetrically (27), total carbohydrates colorimetrically (11), and total weight gravimetrically using vacuum-dried samples in aluminum boats. The second aliquot was twice extracted with chloroform-methanol-water (1:2:0.8). This single-phase mixture was centrifuged to remove precipitate, and the supernate was then made biphasic with further addition of water and chloroform to give a final chloroform-methanol-water ratio of 2:2:1.8. The chloroform phase, containing lipids, was carefully removed, dried, and weighed in an aluminum boat. The aqueous phase was combined with the single-phase precipitate, evaporated to dryness, and weighed in an aluminum boat. The latter preparation is referred to as the lipid-extracted residue. All gravimetric measurements were made with a Cahn electrobalance (Cahn Div., Ventron Instruments Corp., Cerritos, Calif.) calibrated with a 10 mg standard with an accuracy of  $\pm 5$  µg. Lipid extraction essentially followed the procedure of Bligh and Dyer as recommended by Johnson and Davenport (20).

### *SDS Acrylamide Gel Electrophoresis*

To assure complete disaggregation of protein subunits, pellicle fragments were reduced and alkylated as described by Renaud et al. (35). Ultimately, the mixture was dialyzed 12–24 h against SDS gel sample buffer (0.01 M sodium phosphate, pH 7.2, 0.1% SDS, and 0.1% mercaptoethanol). 5% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) were prepared as described by Weber, Pringle, and Osborn (47) in glass tubes 5 mm in diameter and 7 cm in length. Samples containing 50–75 µg of pellicle protein, 5 µl bromophenol blue, and one drop of glycerol in a total vol of 150 µl were applied to the gels. Samples were electrophoresed at 6 mA/gel. After electrophoresis, the gels were removed from the tubes and stained with Coomassie blue, periodic acid-Schiff (PAS), or quantitatively with fast green (14). After destaining, densitometric tracings of fast green-

stained gels were performed on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a linear transport at  $\lambda = 650$  nm. The densitometric scans of fast green-stained gels were quantitated by weighing cutout peak areas on an analytical balance as described by Gorovsky et al. (14).

### Preparation of Anti-Pellicle Antibodies

**IMMUNIZATION:** Mature female rabbits were given a series of four weekly subcutaneous injections of approximately 500  $\mu$ g of isolated pellicles (the first injection in Freund's complete adjuvant and subsequent injections in incomplete adjuvant) followed by a 250  $\mu$ g pellicle booster 2 wk later. Within 5–10 days of the final injection, the rabbits were bled.

**ANTIBODY PURIFICATION:** After deactivation of complement by incubation at 56°C for 20–30 min, rabbit blood serum was dealbuminated by ammonium sulfate precipitation with standard procedures. Total protein in the salt-purified fraction was estimated by the technique of Lowry et al. (27), and anti-pellicle antibody titer and specificity were confirmed by immunodiffusion, immunoelectrophoresis, and complement-fixation tests.

The final product was frozen in 1- to 2-ml portions or used immediately. For most procedures, the globulin preparation as described required no further purification. When necessary, further purification of the globulin fraction of the serum was achieved by DEAE-cellulose separation by batchwise purification of IgG (44).

### Fluorescent Antibody Technique

**FLUORESCENT ANTIBODY PREPARATION:** The purified rabbit anti-pellicle preparation was labeled with fluorescein isothiocyanate (FITC; BBL Division of Becton, Dickinson and Company, Cockeysville, Md.) as described by Kawamura (21). FITC-conjugated goat anti-rabbit immunoglobulin was obtained from Kallestad Laboratories, Inc., Chaska, Minn.

**FLUORESCENT ANTIBODY STAINING:** *Euglena* cells were harvested and rinsed twice with phosphate-buffered saline (PBS) at pH 7.0. 1 ml of rabbit antipellicle antiserum adjusted with PBS to a concentration of 20 mg protein per ml was added to approximately  $10^8$  pelleted *Euglena* cells (from 1 ml of log phase culture). After incubating for 40 min at 37°C, the cells were washed by repeated centrifugation in PBS. The sensitized cells were then reacted with fluorescein-coupled antibody immediately or incubated in PBS for various time intervals from 24 to 48 h and then reacted with fluorescein-coupled antibody (see Table II). Staining was accomplished by adding 0.4 ml of PBS and 0.1 ml of goat antirabbit immunoglobulin-FITC conjugate (8.5 mg protein/ml) to a pellet of sensitized *Euglena* cells. After 40 min of incubation at 37°C, the cells were washed three times with PBS and resuspended in mounting medium (50:50 mixture of PBS and glycerine). Samples were viewed and photographed on a Zeiss

Photomicroscope II equipped with a mercury vapor light source (HBO 200 W/4), an FITC excitation filter, and 0.65 NA Planapochromat objective.

The following controls were run to test for nonspecific binding of immunoglobulins at the cell surface: for nonspecific binding of rabbit serum on the pellicle surface, *Euglena* cells were initially treated with heterologous rabbit antisera (at 20 mg protein/ml), and subsequently incubated with goat antirabbit-FITC conjugate as indicated previously; for nonspecific binding of goat antiserum at the cell surface, cells were incubated only in PBS before treatment with goat antirabbit-FITC conjugate. Finally, to evaluate binding specificity of goat antirabbit immunoglobulins, blocking experiments were undertaken. *Euglena* cells initially sensitized with rabbit anti-pellicle antibodies were incubated with unconjugated goat anti-rabbit immunoglobulins followed by a second incubation with goat anti-rabbit-FITC conjugate. Further confirmation of binding specificity was obtained from mixed blocking control tests: sensitized *Euglena* were mixed (1:1) with sensitized *Euglena* cells treated with unconjugated goat anti-rabbit antibodies. The entire mixture was then incubated with goat anti-rabbit-FITC conjugates. FITC conjugated directly to rabbit anti-pellicle antibodies was also used in procedures similar to those described above.

### Antibody/Latex Sphere Cell Surface Labeling

A latex labeling method adapted from LoBuglio et al. (26) and D. S. Linthicum (reference 25 and personal communication) was used to localize, by scanning EM, immunoglobulins directed against the pellicle surface. Lytron 622 latex spheres 0.23  $\mu$ m in diameter were a generous gift from the Monsanto Company, Des Plaines, Ill. The latex content of the stock solution was determined by drying an aliquot to a constant weight at 60°C. The stock solution (370 mg/ml) was diluted as needed to 1 mg/ml by using 0.2 M sodium phosphate buffer (pH 9.2). The following procedure produced reproducible, specific cell surface labeling.

Cells were harvested and rinsed twice with PBS at pH 7.0. 1 ml of rabbit anti-*Euglena* pellicle antiserum adjusted with PBS to a concentration of 20 mg protein/ml was added to approximately  $10^8$  *Euglena* cells (from 1 ml of log phase culture). After incubation for 40 min at 37°C, the cells were washed twice by centrifugation and resuspension in PBS. The sensitized cells were then fixed for latex sphere labeling or incubated in PBS for 24–28 h. Labeling was accomplished by a combined fixation and first-step coupling reaction. Sensitized cells were first treated with 0.5% glutaraldehyde in PBS (pH 7.0) for 1 h at room temperature. Excess glutaraldehyde was rinsed from the cells by alternating centrifugation and resuspension in 0.2 M sodium phosphate buffer (pH 9.0). The second stage of the coupling reaction was accomplished by incubation of the cells with 1 mg of

latex spheres in phosphate buffer (pH 9.2) for 1 h with gentle agitation at room temperature. Cells were subsequently rinsed twice by sedimentation with phosphate buffer (pH 9.2) and postfixed in 0.5% glutaraldehyde in the same buffer for 20 min at 26°C. Finally, cells were rinsed three times in double-distilled water by successive cell sedimentations, lyophilized, and coated with gold/palladium. To test for nonspecific binding of rabbit serum on the pellicle surface, cells were initially treated with heterologous rabbit antisera (at 20 mg/ml), and subsequently fixed and treated with latex spheres as discussed previously. To test for nonspecific binding of latex spheres at the cell surface, cells were incubated in PBS only before fixation and incubation with latex spheres.

### *Ferritin-Antibody Cell Surface Label*

**PREPARATION OF FERRITIN-CONJUGATED IgG ANTIBODY:** Glutaraldehyde was used to conjugate DEAE-purified IgG antibodies with ferritin, according to a technique adapted from D. S. Linthicum (personal communication). Ferritin (6× crystallized, Grand Island Biological Co., Grand Island, N. Y., glutaraldehyde (8% aqueous, Polysciences, Inc., Warrington, Pa.), and purified IgG prepared as described above were used in the molecular ratio of 3 IgG: 2 ferritin:600 glutaraldehyde. Continuous stirring without bubbling or foaming was maintained for 1 h at room temperature. To a 3-ml mixture, 0.3 ml of 0.1 M  $(\text{NH}_4)_2\text{CO}_3$  was added followed by incubation on ice for 15–20 min to prevent additional cross-linking with glutaraldehyde. The reaction mixture was then dialyzed against 0.1 M  $(\text{NH}_4)_2\text{CO}_3$  for 2–3 h at 4°C and subsequently dialyzed exhaustively against PBS (pH 7.0). Finally, large polymeric aggregates for IgG, ferritin, or both, were removed by centrifugation at 10,000 g for 20 min; pelleted aggregates were discarded, while the supernate was collected for further purification. The conjugate was applied to a 7 cm × 25 cm Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column previously equilibrated with PBS (pH 7.0). 1-ml fractions were collected and read at both 440 and 280 nm. The first peak eluted represented aggregated material and was discarded, the leading shoulder of the second peak contained the ferritin-IgG conjugate, and the trailing shoulder had free ferritin and free IgG which were also discarded. The ferritin-IgG conjugate preparation was concentrated and tested against SDS solubilized pellicle by using immunoelectrophoresis and immunodiffusion.

**FERRITIN-ANTIBODY LABELING OF THE CELL SURFACE:** For pellicle labeling, ferritin conjugated directly to rabbit antipellicle antibodies was used. Cells rinsed twice in PBS were incubated with 0.5 ml of ferritin-antibody conjugate (containing about 3.6 mg ferritin) for 40 min at 37°C. After incubation, cells were rinsed in PBS and processed for transmission electron microscopy. To test for specificity of the surface bind-

ings, cells were first incubated with 1 ml of unconjugated rabbit anti-pellicle antibody (12 mg/ml, 40 min, 37°C) and subsequently incubated with the ferritin/anti-pellicle conjugate. Such control samples were also processed further for transmission electron microscopy.

### *Light Microscopy*

A 1.3 NA planapochromat Zeiss bright-field objective was used with Nomarski optics for photographing *Euglena* cells. Flash photographs of swimming and dividing cells were taken with Zeiss microflash illumination of about 0.3–0.5 ms duration. Images were recorded on Kodak Panatomic X film which was developed in Diafine (Acufine, Inc., Chicago, Ill.).

### *Transmission Electron Microscopy*

Cells were pelleted by centrifugation and resuspended in 4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer at a pH of 7.0 for 1.5 h at room temperature. The cells were then recentrifuged, and the glutaraldehyde solution was replaced with cold phosphate buffer. After four buffer rinses, cells were postfixed for 1.5 h or overnight in cold 1% osmium tetroxide in phosphate buffer. Sequential dehydration in acetone was followed by infiltration with Spurr's (43) hardest epoxy mixture. After 24 h of polymerization at 60°C, the blocks were sectioned with a DuPont diamond knife on a Reichert OmU3 ultramicrotome. After staining with uranyl acetate and lead citrate (36), the sections were observed and photographed in a Hitachi 11B or 11E electron microscope.

### *Scanning Electron Microscopy*

Whole cells and isolated pellicle fragments were prepared for scanning electron microscopy essentially as described by Guttman and Styskal (16). Samples were lyophilized for 2 h at –80°C in a Pearse tissue dryer (Edwards High Vacuum Corp., Grand Island, N. Y.) and were then vacuum-coated with gold or gold/palladium. The specimens were examined on a Cambridge Stereoscan Mark IIA electron microscope at an accelerating voltage of 20 kV.

## RESULTS

### *Light Microscopy*

In *E. gracilis*, shape changes occur by a process generally known as metaboly. Cell shapes may vary from elongate to ovate or even spherical. In log-phase cultures the elongate form predominates and characteristically possesses two flagella extending through the anterior reservoir region, with only a single flagellum emerging (Fig. 1 a and b). Numerous paramylon granules are apparent

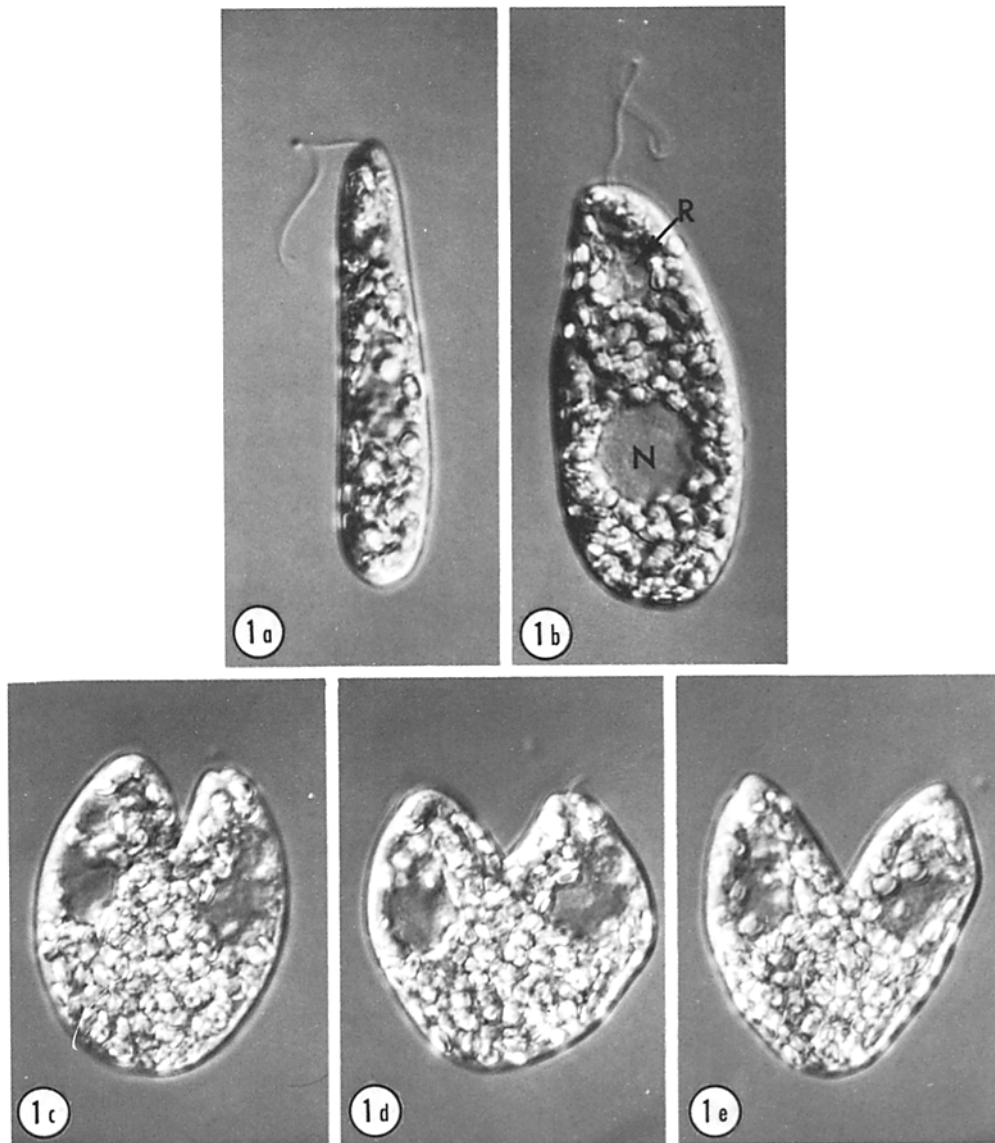


FIGURE 1 (a) Swimming *Euglena* cell illustrating elongate form and emergent flagellum oriented backward from its anterior attachment. Granules throughout the cell are starch (paramylon). Pellicular strips cannot be seen in whole cells in this species. (b) Slightly flattened cell with prominent nucleus (*N*) and reservoir (*R*). (c-e) Dividing cell undergoing longitudinal furrowing in an anterior to posterior direction. Nomarski interference contrast.  $\times 1,600$ .

throughout the cytoplasm. Cells divide by longitudinal fission in an anterior-to-posterior direction (Fig. 1 c-e). The pellicle strips cannot be seen with phase, Nomarski, or bright-field optics in intact cells of this species of *Euglena*.

#### *Electron Microscopy*

Electron microscopy of whole and sectioned *E. gracilis* cells essentially confirmed earlier studies by Sommer (40) and others (22, 23, 29, 30, 41) that

the cell surface complex or pellicle consists of a series of strips which complete two–three turns as they pass helically around the cell (Fig. 2). In transverse section, the surface displays alternating ridges and grooves of fairly constant dimensions (Fig. 3). At the anterior end of the cell, the 0.2- to 0.4  $\mu\text{m}$ -wide pellicle strips (40–50 or more) curve over the mouth of the canal and continue into the canal proper where their individual integrity is lost.

In agreement with previous observations (40), the surface of the cell (including both ridge and groove regions) is limited by a continuous tripartite cell membrane 85–120  $\text{\AA}$  thick with no apparent external coating. Just beneath the membrane is a 100–120  $\text{\AA}$  thick layer of electron-dense material (Fig. 3). The pellicle ridges display a notch on one side (Fig. 3), which is oriented toward the posterior end of the *Euglena* cell, in agreement with earlier observations (22, 41). Sommer (40) reported the presence of 3–5 (usually 4) microtubules each about 250  $\text{\AA}$  in diameter, in the region of the notch running parallel along each pellicular ridge in *E. gracilis*. The present study showing 4–7 microtubules in each ridge is more closely in agreement with the findings of Guttman and Ziegler (17). Two of the microtubules (no. 2, 3 of Figs. 3 and 6) were located immediately adjacent to the notch, one was located in the posterior third of the ridge (no. 1 of Fig. 3, 6), and the remaining 1 to 4 microtubules (no. 4, 5, 6, and 7 in Figs. 3 and 6) were found in the region of the pellicular groove. Microtubule 3 was frequently obscured by electron-dense material (cf. also, reference 1), but in favorable longitudinal sections was seen to possess numerous lateral appendages extending in several directions (Figs. 4 and 5). A characteristic tubular cisterna of the endoplasmic reticulum was always present throughout the length of each pellicle strip (40, Fig. 4). Evidence for fibrous material connecting the notch region of the pellicle with the overlying electron-dense layer clearly present in isolates (Fig. 8) could be seen only with difficulty in whole cells. Finally, one or two small, electron-dense rods were observed in favorable sections passing through the notch region (Fig. 6), and a series of striated fibers running perpendicular to the strips were localized just beneath the cell membrane of the groove area where adjacent pellicle strips meet (Figs. 4 and 6).

Just before and during cytokinesis, the entire cell or parts of the cell display alternating large and small pellicle ridges. Cleavage of the mother

cell begins after replication of the canal and reservoir and results in two daughter cells connected only by a narrow bridge of cytoplasm. Since pellicle replication proceeds gradually from anterior to posterior, it is possible to localize the earliest stages in the initiation of the daughter strips (Fig. 10). The first appearance of new strips seems to involve an upwelling of portions of parental pellicle complex particularly the area associated with microtubules no. 4 and 5. Images of subsequent development (Fig. 11) reveal fewer than usual (often one) or no microtubules associated with the parental groove region, and two to three microtubules present in each presumptive daughter strip. Thus, circumstantial evidence strongly suggests that parental microtubules no. 4 and 5 or no. 5 and 6 become microtubules no. 1 and 2 of the daughter strips. The variable number of microtubules (usually 2 to 4) associated with the grooves of nonreplicating surfaces (Fig. 3) may then represent stages of replenishment or of anticipation of microtubules for use in subsequent daughter strips.

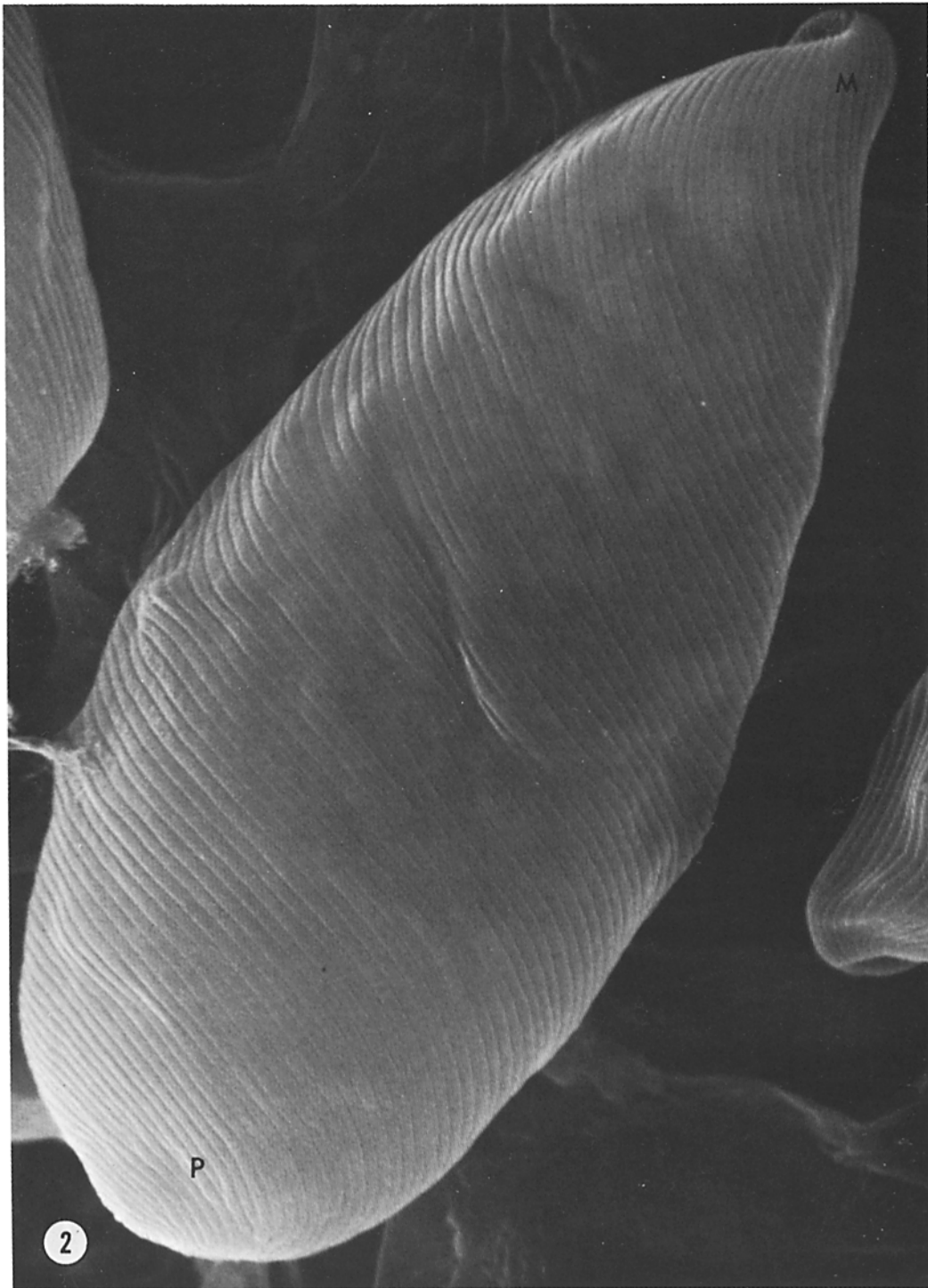
#### *Purified Pellicle*

Scanning electron microscopy revealed that lyophilized pellicle fractions consist of nearly pure fragments, each composed of 10–20 pellicular strips (Figs. 7 *b* and *c*). In transverse section, these fragments appear as rosettes of curled pellicle pieces (Figs. 8, 8'). Each rosette seems to consist primarily of an 85–120  $\text{\AA}$  tripartite plasma membrane and an underlying 100–120  $\text{\AA}$  layer of dense material (Fig. 8'). A fiberlike material appears to connect ridge and groove regions as well as adjacent notched regions of the pellicle (Figs. 8, 8'). No microtubules are present in isolated pellicles. Longitudinal sections also consistently revealed the presence of fiberlike material with regular periodicity (Fig. 8'').

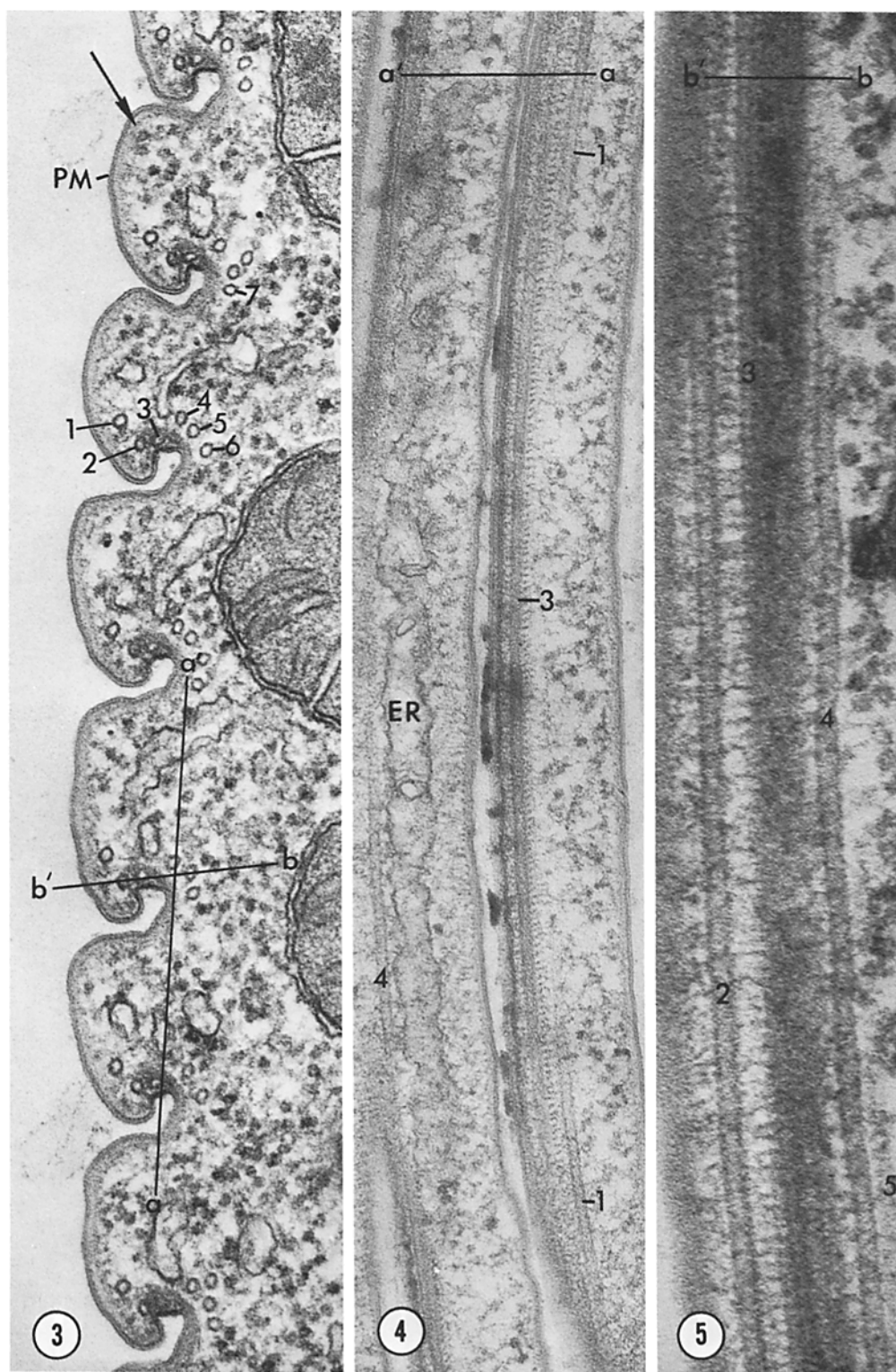
It is especially significant that the pellicle retained its basic conformation of alternating ridges and grooves even when isolated completely from potentially supportive cytoplasmic material including the underlying microtubules.

#### *Biochemical Characterization of the Pellicle*

Estimates of total lipids, carbohydrates, and proteins of purified pellicles are tabulated in Table I. Colorimetric (27) determination of proteins



**FIGURE 2** Scanning electron micrograph of freeze-dried, gold/palladium-coated cell. Note helical coiling of pellicular strips in one to two gyres around the cell. The pattern terminates abruptly at the posterior end (*P*), whereas at the anterior end the individual strips gradually lose their integrity after passing through the mouth (*M*) into the canal and reservoir (not shown)  $\times 6,650$ .





proved to be unreliable, presumably because of interfering materials in the isolates, and generally gave values two–three times the total pellicle weight. Hence, total protein was calculated by subtracting estimates of lipids and carbohydrates from the total dry weight of isolated pellicles, and these values were checked by comparing weights obtained from lipid extracted residue minus carbohydrates. Protein to lipid ratios were never less than 3 and averaged somewhat higher. These values suggest that much if not all the submembrane material is protein. The relatively high carbohydrate values are difficult to explain in view of the absence of any visible surface layer and of the apparent absence of contaminating starch grains. Further work to define the nature of the carbohydrates is in progress.

SDS polyacrylamide gel electrophoresis of reduced and alkylated pellicle samples separated approximately 10 polypeptide bands (Fig. 9). Three of the bands, characterized by mol wt of approximately 25,000, 40,000 and 80,000 (Fig. 9), appeared to be major constituents of the pellicle. Quantitation of the gel proteins indicated that the three major bands comprised nearly 50% of the total polypeptides present. PAS-staining of the gels showed only trace amounts of carbohydrate present in the predominant polypeptide bands.

#### Localization of Cell Surface Antigens

Purified pellicle proved to elicit rabbit antibody readily. Therefore, three types of surface markers were used in conjunction with anti-surface immunoglobulins for various levels of resolution. Ferritin, latex spheres, and fluorescein conjugated to the antibodies were used as visual markers for localization of pellicle surface antigens with transmission electron, scanning electron, and fluorescent light microscopy, respectively.

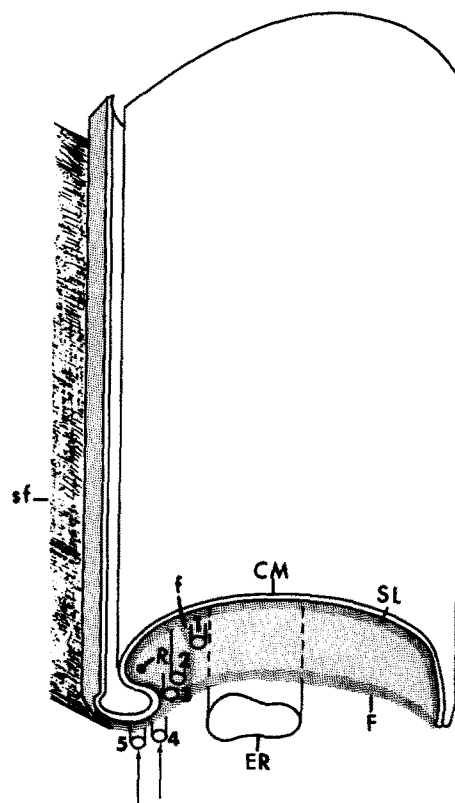


FIGURE 6 Diagrammatic representation of the repeating portion of the pellicle in *E. gracilis*. ER, endoplasmic reticulum; *f*, interconnecting fiber; *F*, traversing fiber; and *CM*, cell membrane; *R*, rodlet; *SL*, submembrane layer; 1, 2, 3, 4, 5, microtubules.

Pellicle antigens were localized by incubating cells with ferritin-conjugated rabbit anti-pellicle antibodies. Both transverse section and tangential views of the pellicle indicate association of ferritin-conjugated antibodies with the ridge surface, and

FIGURE 3 Section perpendicular to the pellicle strips, illustrating components of the pellicle complex. Microtubules are numbered as in Fig. 6. Note variable number of microtubules associated with groove region, dense material associated with microtubule no. 3, and the electron-dense layer (arrow) underlying the plasma membrane (*PM*). One or more cisternae of endoplasmic reticulum is also present in each ridge area *a'-a*, *b'-b*, approximate orientation of Figs. 4 and 5.  $\times 85,000$ .

FIGURE 4 Section tangential to pellicle ridges. Microtubules are numbered as in Fig. 3, and are exposed at different levels due to the curved nature of the pellicle. Numerous lateral extensions with a periodicity of about 210 Å can be seen on microtubule no. 3. Note continuous ER cisterna running parallel to the strip.  $\times 75,000$ .

FIGURE 5 Portion of microtubule no. 3 with lateral extensions. Comparison of Figs. 4 and 5 suggest that the extensions on no. 3 extend around the entire microtubule.  $\times 122,000$ .

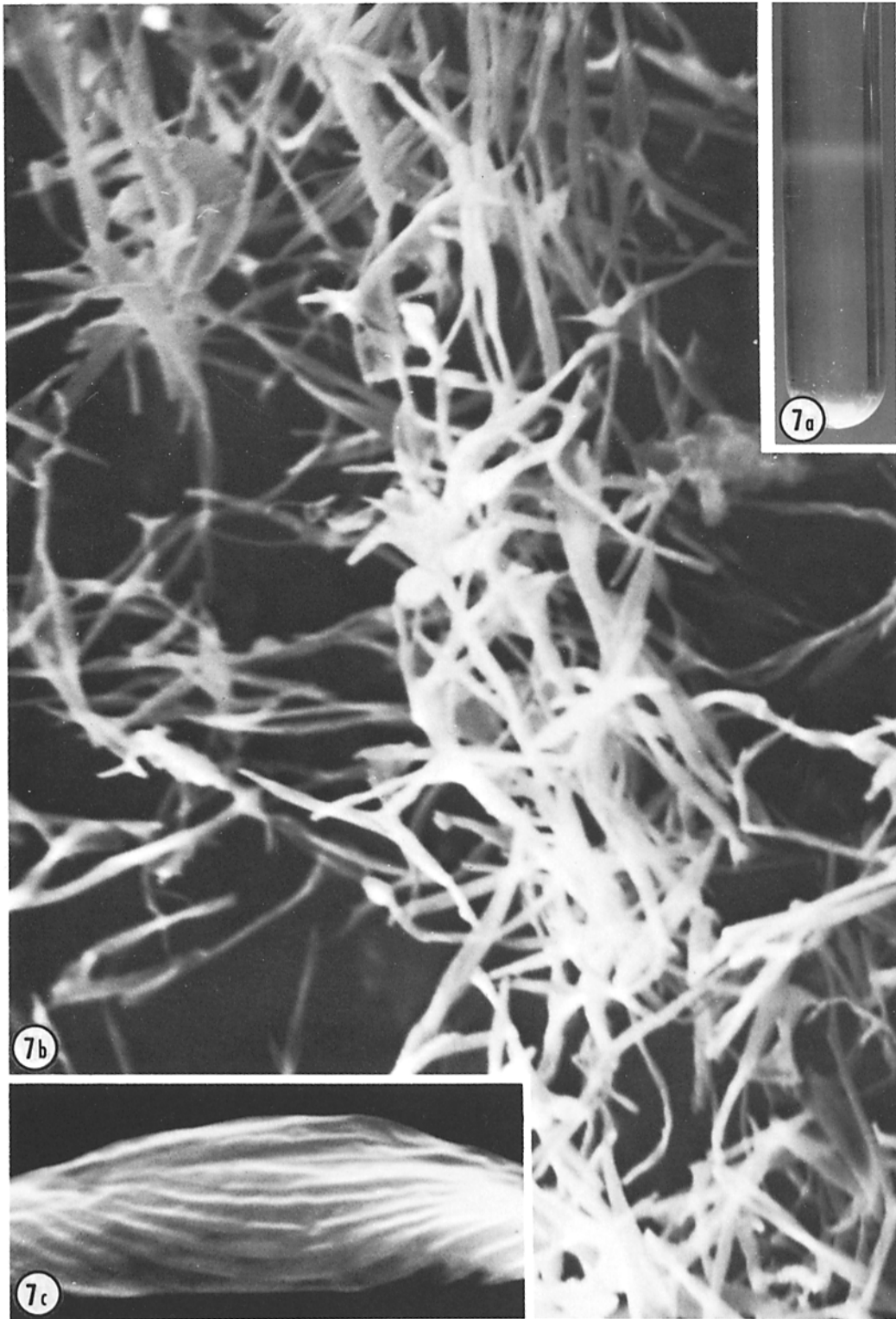


FIGURE 7 (a) Sonicated cell fragments centrifuged to equilibrium, illustrating layering of pellicle portions at the 85–95% (wt/vol) sucrose interface. (b and c) Sample of purified pellicle fragments from gradient similar to that in a. A variable number of strips remain associated with one another and tend to coil laterally, but maintain longitudinal stiffness. (c) Detail of isolated strip. (b)  $\times 2,400$ ; (c)  $\times 8,000$ .

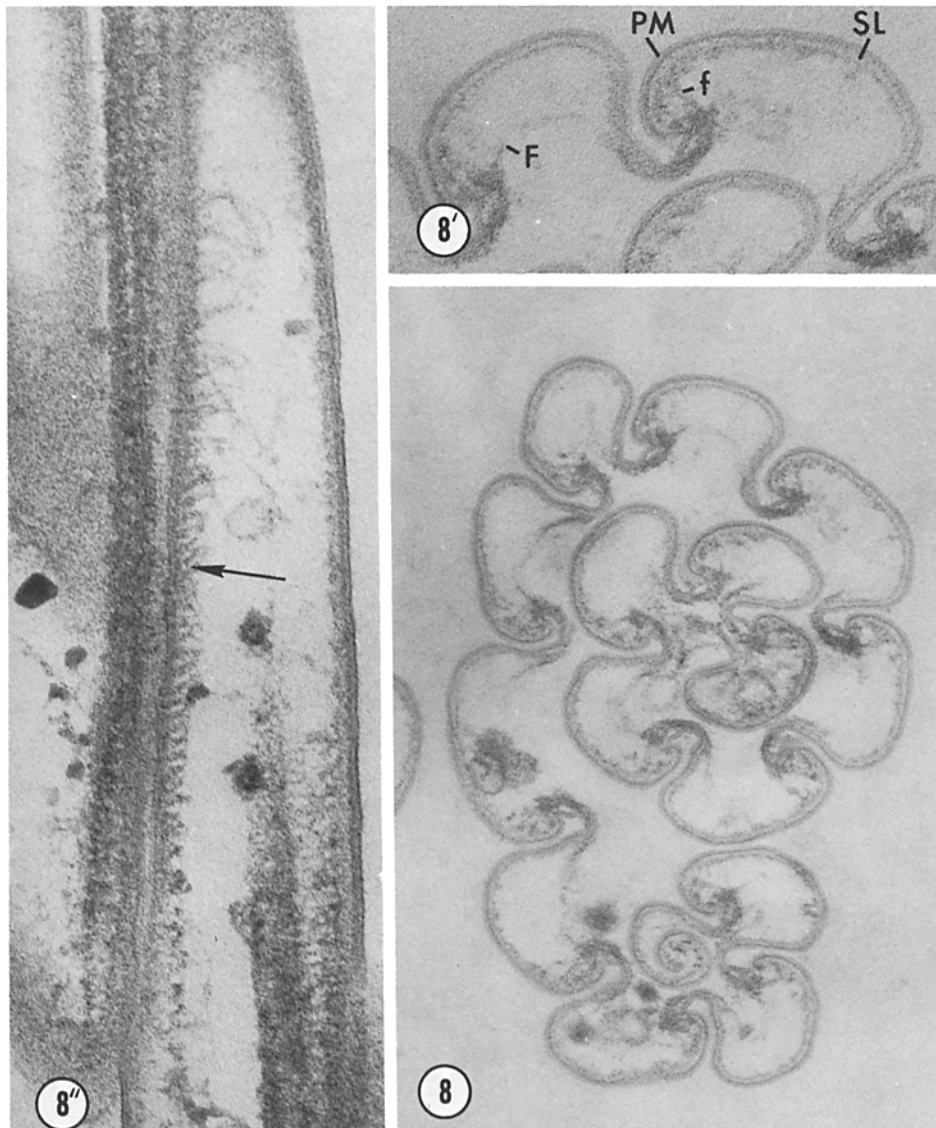


FIGURE 8 Transverse section through pellicle isolate from Fig. 7. Note maintenance of ridge and groove pattern and absence of microtubules, ER, or most other cytoplasmic material. Tendency of isolates to curl laterally is apparent in this micrograph. (8') Enlarged portion of Fig. 8 to show details of pellicle. Plasma membrane (PM), submembraneous layer (SL), traversing fiber (F), and interconnecting fiber (f) are still present. (8'') Longitudinal profile of isolate illustrating retention of material with regular periodicity (arrow). Such material may be remnants of lateral appendages extending from microtubule no. 3 to the membrane area. (8)  $\times 75,000$ ; (8')  $\times 120,000$ ; (8'')  $\times 90,000$ .

generally not with the pellicle groove (Figs. 15 and 16). The large ferritin-antibody complex may prevent localization in the groove region.

#### *Cell Surface Development*

Under our culture conditions, cells usually underwent asynchronous division once every 20–24

h. When cells were coated with antibody, the division cycle seemed somewhat slowed.

Reaction of surface-specific antiserum-coated cells with glutaraldehyde and latex spheres (cf. Materials and Methods) produced surfaces labeled with spheres which clearly delineate the contours of the helically coiled pellicle strips (Fig. 12). Cells

TABLE I  
Estimates of Total Lipids, Carbohydrates, and Proteins of Purified Pellicles

Isolate no:	I		II		III		Average
	Wt	Percent of total	Wt	Percent of total	Wt	Percent of total	
	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	
Total dry weight	348	100	576	100	468	100	100%
Carbohydrates	60	17.2	88.8	15.4	100	19.1	17.2%
Lipid	40	11.5	120	21	48	10.3	14.2%
Lipid-extracted residue	248	71.3	428	74.1	340	72.7	72.7%
Total recovered (lipid and residue)	288	82.8	548	95.1	388	82.9	86.9%
Protein calculated (Total minus lipids and carbohydrates)	248	71.3	367	63.8	320	68.4	67.8%
Protein/lipid	6.2		3.1		6.7		5.3

initially reacted with rabbit anti-pellicle antibody and allowed to incubate in buffer for 24–48 h, however, showed quite different latex distribution patterns upon labeling with the antibody/glutaraldehyde/latex (Fig. 13). Latex-labeled pellicle strips can be observed to alternate more or less regularly with unlabeled pellicle strips. From these studies, replication of antigenic sites would appear to proceed by insertion and growth of new (unlabeled) sites between the old (labeled) sites.

As seen in sectioned cells, latex beads were localized in close proximity with the surface membrane. Although the relatively large size of the beads made precise localization difficult, the beads seemed to be associated with the elevated pellicle ridge surfaces (Fig. 14). This fairly localized binding may be due to more stable binding of the relatively large spheres to the flatter regions of the pellicle. Cells treated initially with PBS or nonspecific rabbit serum showed little or no subsequent binding of beads by the usual reaction series. Beads were, however, effectively bound to isolated pellicle strips which were treated with pellicle specific antibody.

Use of fluorescein-labeled antibodies confirmed the surface developmental patterns observed with latex spheres. Enhancement of the fluorescent surface label was affected by piggyback immunochemical labeling whereby sites on the pellicle surface were first reacted with rabbit anti-pellicle gamma globulins. The rabbit gamma globulin was in turn localized by marking with FITC-conju-

gated goat anti-rabbit antibodies (see Table II). Thus, pellicle sites were indirectly labeled with fluorescein dye. When cells labeled by this process were observed immediately, overall green-fluorescent staining was observed in most cells. In contrast, cells initially labeled with rabbit anti-pellicle antibody and allowed to incubate for 24–48 h showed different patterns upon counter-labeling with goat anti-rabbit antibody. Fluorescent-labeled helically coiled pellicle strips could be distinguished alternating with nonfluorescent strips as seen in Figs. 17 and 17'. Presumably, old pellicle material initially labeled with rabbit anti-pellicle antibody would still counterstain with goat anti-rabbit-FITC, but any "new" pellicle sites would not be labeled with rabbit anti-pellicle antibody and would remain unstained. Fig. 17 represents a cell which presumably has undergone one pellicle replication, thus exhibiting labeling of every second strip. Fig. 17' seems to show labeling of every fourth strip, a condition which can be explained by a pellicle replication, cell division, and a second pellicle replication preceding the division recorded in this figure. Although the cultures studied were not synchronized for simultaneous cell division, and a number of cells appeared not to divide (probably due to effects of the surface antibody coating), the most prevalent staining pattern after 24 h of incubation was that of narrowly spaced strips as in Fig. 17, while the predominant staining pattern after 48 h was that of widely spaced strips as in Fig. 17'. A further point

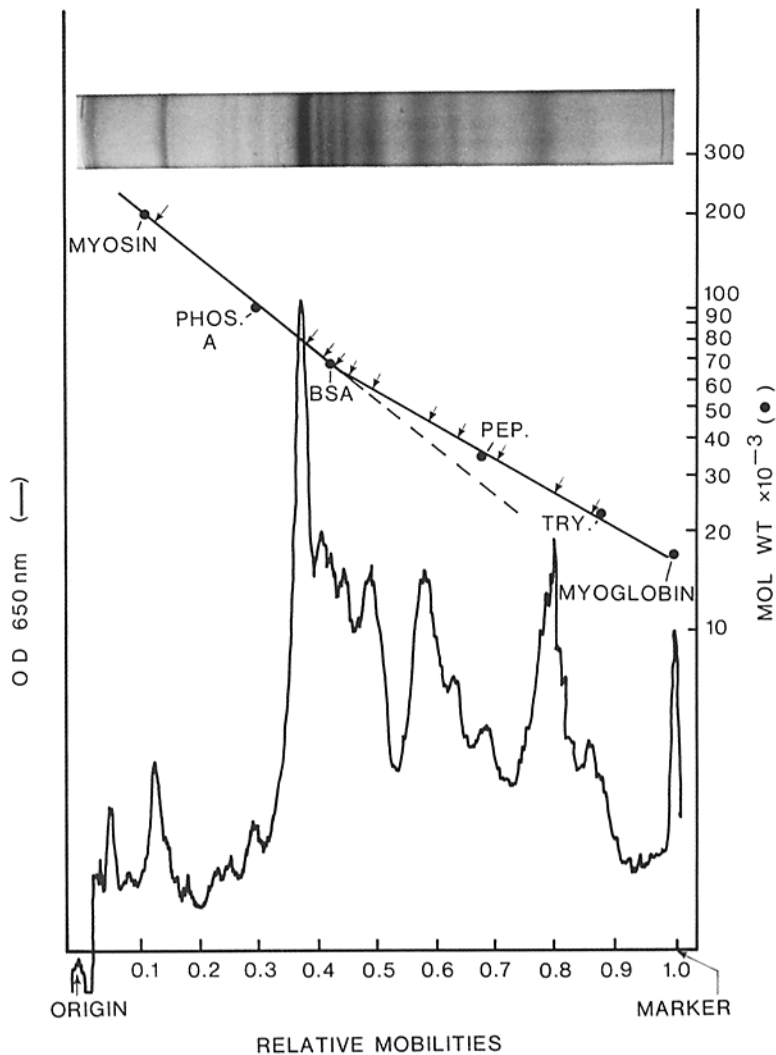
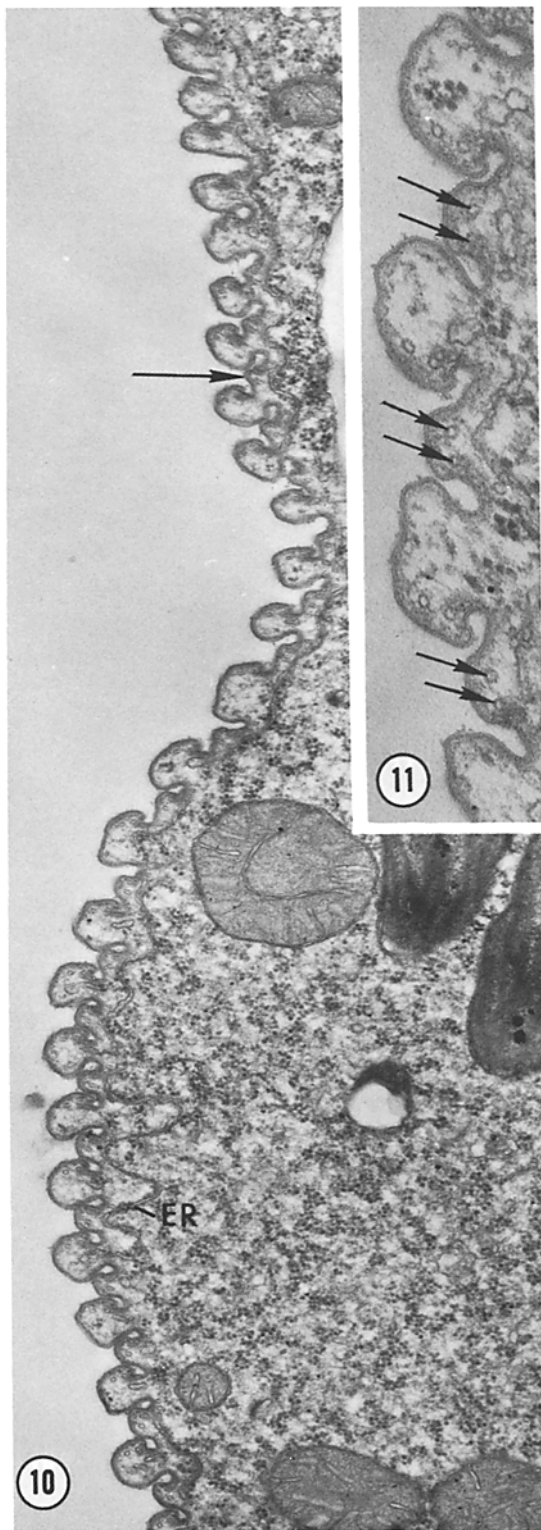


FIGURE 9 SDS acrylamide gel and corresponding scan of dissociated, reduced, and alkylated proteins prepared from purified pellicles. Origin at left. Arrows on plot of mobilities of standards with known molecular weights indicate approximate molecular weights of principal pellicle polypeptides.

of interest is the similarity in labeling of both daughter cells in Fig. 17'—thus suggesting equal distribution of new strips in both cells. Several controls were run to insure specificity of the antibody-surface and antibody-antibody reactions: (a) *Euglena* were incubated with nonimmune rabbit serum and counter-labeled with goat antirabbit-FITC, and the cell body showed no evidence of fluorescence (as expected), though there was some nonspecific binding to the flagellum. (b) Pellicle fragments were incubated with rabbit anti-pellicle

antibody followed by goat antirabbit-FITC conjugate. Fluorescence of strips was clearly apparent. (c) Rabbit antipellicle labeled cells were subsequently incubated with unconjugated (i.e., non-fluorescent) goat antirabbit antibody before incubation as usual with goat anti-rabbit-FITC. With this treatment, fluorescent binding was successfully blocked. Mixed-blocking experiments also gave approximately 50% labeled and 50% unlabeled cells as expected. These results suggest that new pellicle strips are indeed formed by intercalary



growth rather than by a division of pre-existing strips and further suggest that antigenic sites do not migrate during pellicle replication under the experiment conditions.

## DISCUSSION

The cell surface of euglenoid flagellates such as *E. gracilis* is generally characterized by a series of parallel helically coiled strips, collectively called the pellicle complex. Among the general properties of these complexes are (a) the ability to retain the characteristic euglenoid surface architecture, (b) mediation of cell shape changes, and (c) capacity for self-maintenance and replication during growth and division. Since these are probably basic properties of many kinds of cell surfaces, a full understanding of pellicle behavior may have implications in other kinds of surface activities.

### *Pellicle Structure and Biochemical Composition*

Structural findings in the present report essentially confirm and extend those of several earlier studies on euglenoid surfaces examined *in toto* (7, 16, 22, 23, 29, 30, 40). Basically, the complete pellicle complex consists of (a) the 85–120 Å cell membrane which overlies the series of alternating ridges and grooves, (b) a 100–120 Å electron-dense layer subjacent to the membrane, (c) four–seven subsurface microtubules arranged parallel to each strip, (d) portions of endoplasmic reticulum, and (e) a series of fibers traversing the individual pellicle strips.

In the studies reported here, the pellicle is defined to include only the structures present in our purified isolates (i.e. surface membrane, electron-dense layer, and traversing fibers). It is evident that these components are sufficient to maintain mature pellicle form and integrity even when the pellicle is separated from potentially

FIGURE 10 Replicating pellicle. Anterior of cell at bottom of micrograph. Note progressive upwelling of new strips, with the least developed near arrow and the larger daughter strips towards cell anterior. Association of endoplasmic reticulum (ER) with new strips is also evident.  $\times 32,000$ .

FIGURE 11 Enlarged portion of replicating pellicle. At least two microtubules (arrows) are associated with each daughter strip even in their earliest detectable condition.  $\times 75,000$ .



**FIGURE 12** Scanning electron micrograph of nonreplicating cell coated with antibody directed against pellicle and latex beads. Tendency for beads to become attached along the pellicle strips is evident and highlights the helical pellicle pattern.  $\times 4,500$ .

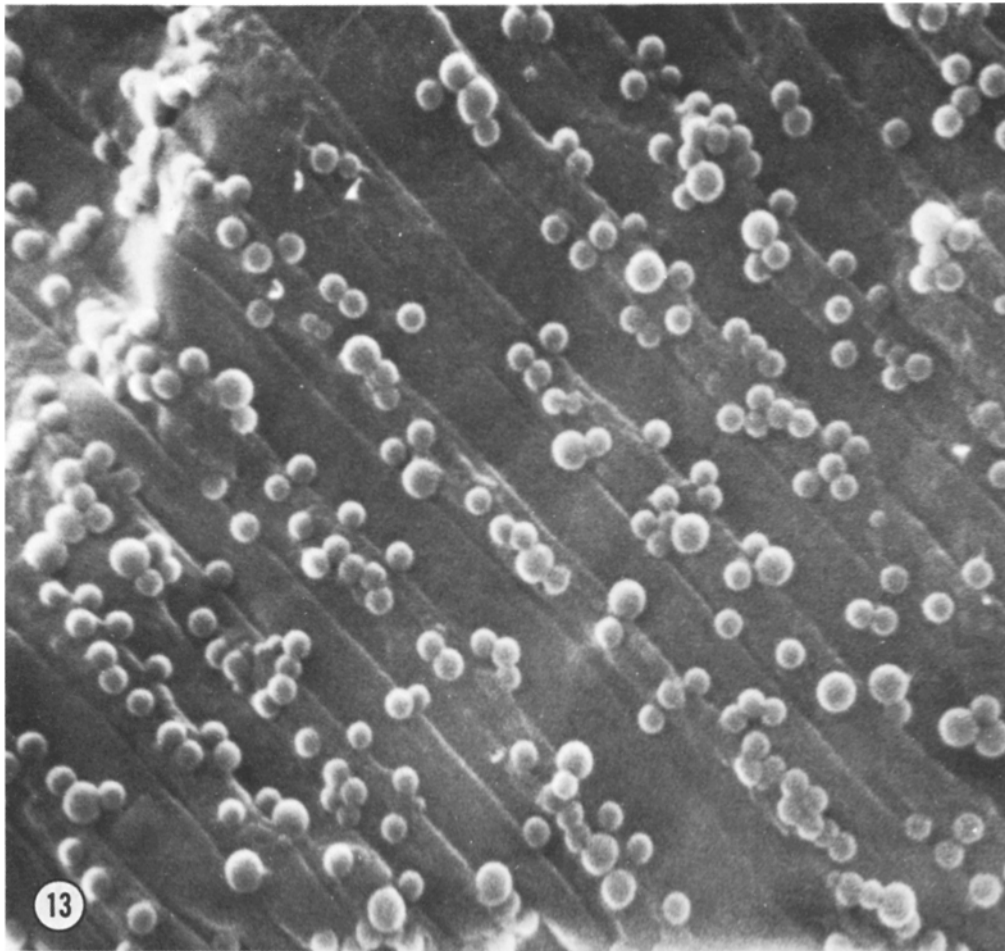
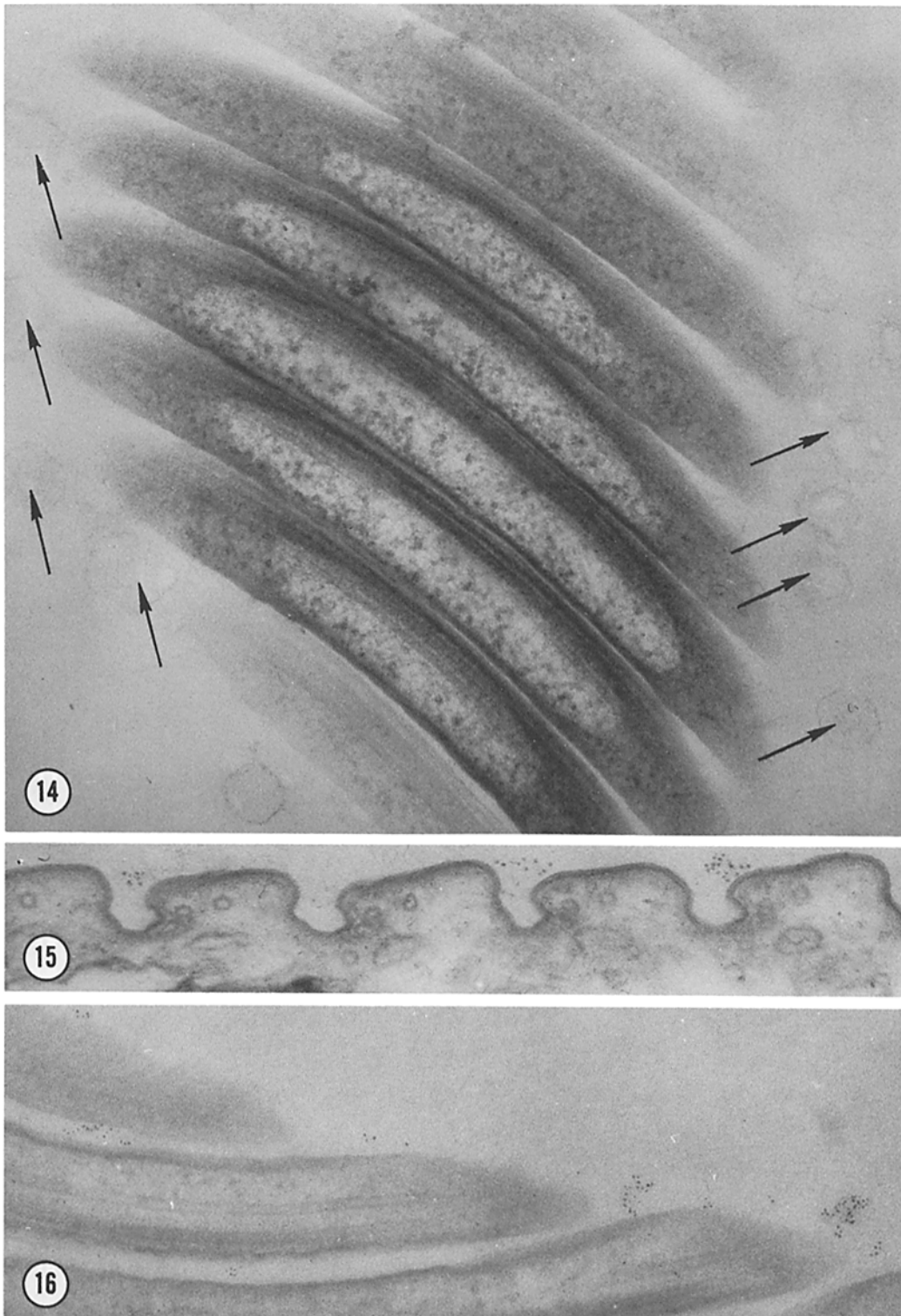


FIGURE 13 Latex bead distribution after a cell such as that in Fig. 12 has undergone one replication after coating with anti-pellicle antibody and subsequently in alternate strips. Intervening strips are presumably those newly replicated and therefore free from pellicle antibody.  $\times 17,000$ .

supportive cytoplasmic material including underlying microtubules. Further, antigens distributed in the pellicle surface membrane seem to be essentially immobile since there does not appear to be substantial diffusion of immunologically labeled antigenic sites during pellicle replication. It is possible, in the experiments reported here, that the divalent antibody itself may cause such immobility by cross-linking reaction sites. However, in adult human erythrocytes the submembranous spectrin protein complex may restrict mobility of membrane components (31, 38), and the presence of a well-defined and closely adhering submembranous (proteinaceous, see below) layer in *Euglena* pellicle suggests that a mechanism conferring stability to

the membrane antigens and form to the pellicle may be present here as well. Regardless of whether the parental antigens are immobilized by antibody in the experiment or by submembrane proteins, it seems clear from both morphological and immunological evidence that new antigens are added between parental strips. No evidence for random surface antigen insertion was detected (associated with diffuse overall labeling), for the pattern after one or several cell replications was consistently of alternating parental and new pellicle strips. Furthermore, initially the entire cell was labeled with FITC-antibody, suggesting that all areas of the surface were accessible to the antibody complex, and that there was no preferential labeling of





**FIGURE 14** Tangential section through cell coated with latex beads as in Fig. 12. Note near alignment of spheres with strips (arrows).  $\times 53,000$ .

**FIGURES 15-16** Transverse and tangential sections, respectively, of pellicle reacted with ferritin-conjugated, antipellicle antibody. Somewhat preferential binding to one portion of the pellicle strip is apparent. (Fig. 15)  $\times 85,000$ . (Fig. 16)  $\times 65,000$ .

TABLE II  
*Use of Fluorescein-Labeled Antibodies To Confirm Surface Developmental Patterns Observed with Latex Spheres*

<i>Euglena</i> plus specific pellicular rabbit antiserum (20 mg ml <sup>-1</sup> , 40 min at 37°C)		Pattern expected:		
		Growth by intussusception	Diffuse growth	Growth of whole new half
0 h incubation in PBS →	Incubate with FITC goat anti-rabbit (8.5 mg ml <sup>-1</sup> )	Full label all cells	Full label all cells	Full label all cells
24 h incubation in PBS →	Incubate with FITC goat anti-rabbit	Alternate strips labeled	All strips labeled but less intense	Half cells full label, half cells unlabeled
48 h incubation in PBS →	Incubate with FITC goat anti-rabbit	Greater separation of labeled strips	All strips labeled but still less intense	Most cells unlabeled, some full label

parental surfaces when the antibody complex was first applied. Confirmation of the FITC-antibody labeling pattern with the higher resolution of the scanning electron microscope to resolve latex bead localization adds additional support to the intussusceptive origin of new pellicular antigens in this organism.

The biochemical composition of the *Euglena* pellicle may ultimately provide some indication of how the pellicular complex functions. Barras and Stone (4) reported that isolated *Euglena* pellicle consisted of 80% protein, 11.6% lipid, and 6.4% or 17% carbohydrate (depending on method of analysis), though the absolute purity or structure of the isolate was not clearly shown. Since this and earlier studies indicate that the euglenoid pellicle is largely protein, it has been speculated that the strips may contain a protein of the fibrous elastic group, such as keratin, to account for their flexibility and elasticity (24).

Analysis of the highly purified pellicle isolates obtained in the present study confirms and extends these earlier studies of pellicle composition. In the present analysis, consistently high protein to lipid ratios were obtained although not as high as those obtained by Barras and Stone (4). The significance of these differences must await a more detailed analysis of the carbohydrates and the development of a direct protein assay not influenced by other pellicle materials. Nonetheless, since the protein to lipid ratios of most eucaryotic plasma membranes vary from about 1.0–1.5 (15), it seems reasonable to conclude that the additional proteins (ratios > 3) in *Euglena* pellicle contribute to the submembrane layer. It is also of interest that lipid extracted pellicle fragments for the most part retain their original form, whereas pepsin (1%) rapidly solubi-

lizes similar fragments (unpublished observations). These results strongly suggest that it is protein(s) which stabilize and give form to the intact pellicle.

Further analysis of the proteinaceous components on SDS acrylamide gels after reduction and alkylation revealed the presence of a number of polypeptides with three species comprising about 50% of the total. These three major bands appear to represent proteins with mol wt of about 25,000, 40,000 and 80,000, while the minor bands represent polypeptides ranging in mol wt from 25,000 to greater than 200,000. Whether the protein constituents of the pellicle, such as the dominant components of mol wt 25,000, 40,000 and 80,000, may be contractile in nature remains to be determined.

While a large proportion of the total material present in the pellicle was seemingly associated with the submembrane layer, preliminary experiments designed to remove the immunogenic component by precipitation with antibody obtained against pellicle suggested that a protein of ~75,000 mol wt is the major immunogenic component of the pellicle. By localization with ferritin-conjugated anti-pellicle antibody, this antigen is found to be a membrane-associated protein accessible on the outer pellicular region.

#### *Microtubules and Their Possible Relationship to Pellicle Development and Metabolic Movement*

In agreement with previous reports (1, 16, 40, 41), microtubules were found running parallel to the ridge of each pellicular strip. From our observations of isolated *Euglena* pellicle fragments, it is apparent that microtubules are not required for maintenance of mature pellicle integrity and form.

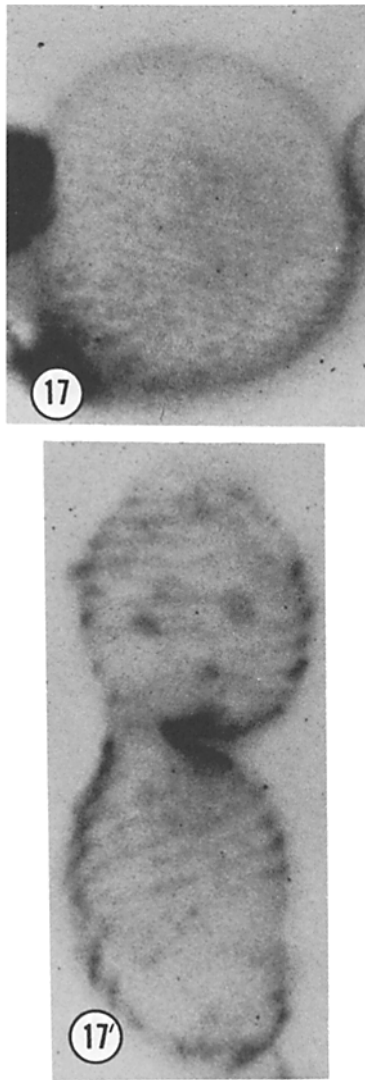


FIGURE 17 The distribution of FITC-conjugated antibody after one pellicle replication (Fig. 17) and several replications (Fig. 17'). Dividing cell in Fig. 17' indicates that both daughter cells receive equal amounts of old and new strips in a quasi-semiconservative manner. Prints reversed to show fluorescent (dark bands) more clearly.  $\times 1,300$ .

However, since a pair of microtubules accompanies the elevation of new pellicular ridges, microtubules may be involved in an early stage in the formation of new pellicular strips.

It is also, of course, possible that the microtubules may be moving coincidentally with the new strips. Unfortunately, it is difficult to test the two alternatives since the mature euglenoid mi-

cro-tubule system is apparently insensitive to colchicine (37) and hydrostatic pressure.<sup>1</sup>

The direct involvement of microtubules in the mediation of cell shape has been established in a variety of cells (see 32), including other unicellular flagellates. In *Ochromonas*, for example, the microtubules form a cytoskeletal network which is apparently responsible for the pyriform shape of these cells (5), since exposure of cells to colchicine or hydrostatic pressure causes microtubule disassembly and a correlative loss of cell shape (6). Here, microtubules are clearly required for maintenance as well as construction of cell form, whereas in *Euglena* pellicular microtubules appear not to be associated with maintenance of pellicular organization when fully formed. The fact that microtubules are present in the ridges of mature cells suggests that they may be associated with other cell functions as, for example, cell movements.

Euglenoid metaboly has been described by Arnett and Walne (2) to have three forms: (a) simple surface fluctuations, (b) anterior and posterior conicalization (distention), and (c) axial deformation. The exact relationship between pellicle structure and control of cell shape remains obscure, although a number of explanations have been offered ranging from sliding of adjacent interlocking strips (24) to cytoplasmic flow within the confines of an elastic pellicle (Pringsheim, 1956, in 18). The function of microtubules in metabolic shape change through interaction with a quasi-muscular layer of fibrous protein found underlying the pellicle has also been suggested (1-3). Still another suggestion is that microtubules are more directly involved in metaboly, by functioning as contractile units mediating shape changes (19).

Several observations from the present study suggest a possible interaction between fibrous material and microtubules in the pellicle which may be related to metaboly. First, an interesting feature of isolated pellicle strips is the inherent elasticity of the pellicle pieces; this elasticity is evident as the pieces curl upon themselves, generally laterally when separated from the underlying (Fig. 7 b and c) cytoplasm. The curling of pellicle pieces becomes especially apparent in sectioned material viewed with transmission electron microscopy (Fig. 8). The presence of a 100-120 Å electron-dense layer of material underlying the cell

<sup>1</sup> Hofmann, C., and G. B. Bouck. Unpublished observation.

membrane provides a structural basis for a rationale that the apparent sideways tension applied to the pellicle membrane and the resultant curling might be mediated through this membrane-associated layer. In addition, transverse fibers appear connecting the notch region of the pellicle with the overlying dense layer as well as running along the basal region of each pellicular strip (Fig. 6). Finally, some pellicular microtubules seem to display filaments or cross bridges (Fig. 4 and 5)

similar to those reported in other microtubular systems (9, 28, 48); these cross bridges are, therefore, presumably positioned to provide force for the sliding of microtubules relative to one another.

For shape control in *Euglena*, the microtubular cross bridges may allow longitudinal contraction and expansion of the cell by a sliding of pellicle-associated microtubules with respect to one another or structures anchored to the cell membrane. The submembranous, transverse, and basal fibers

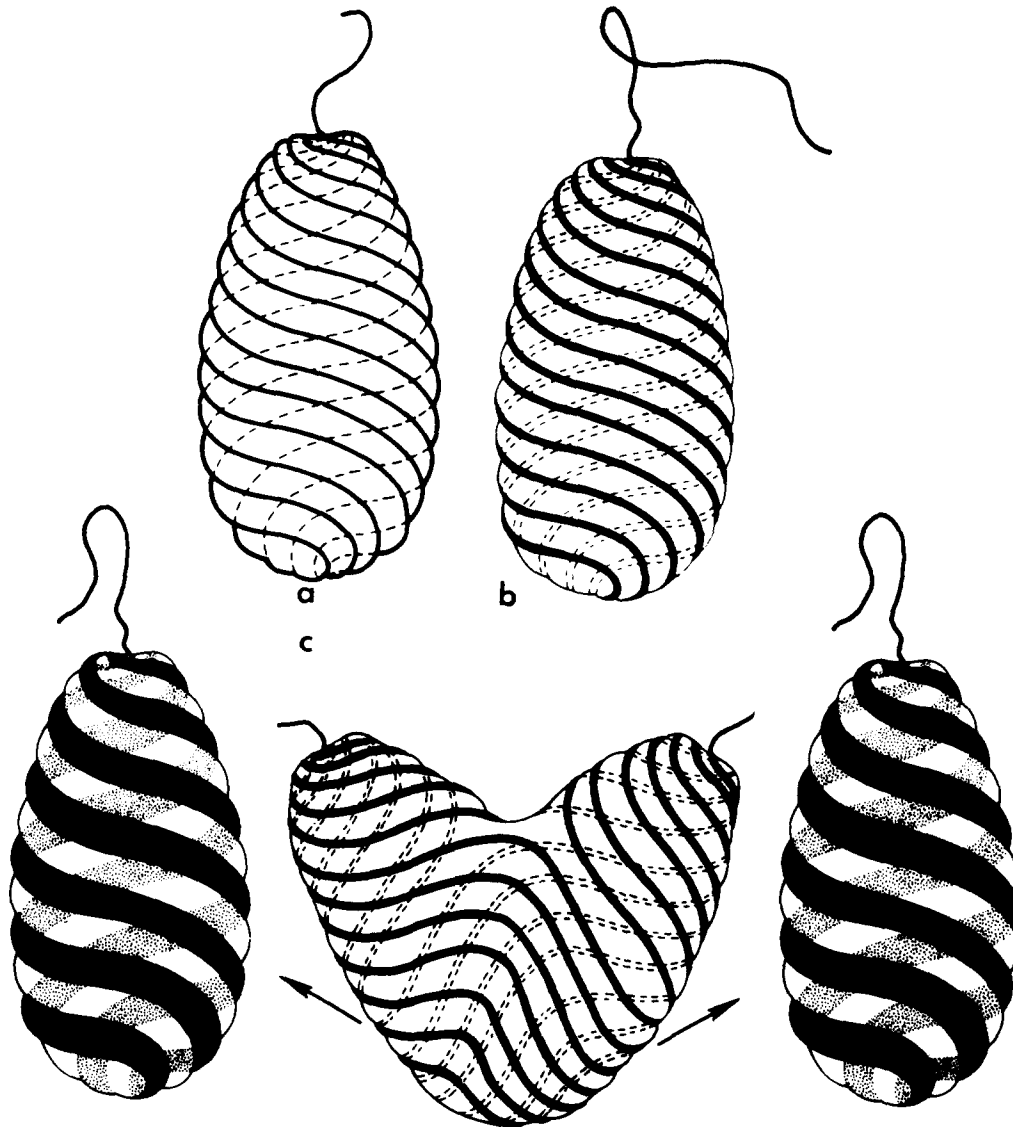


FIGURE 18 Schematic representation of probable intussusception of daughter strips between parental strips. Separation of two daughter cells begins at the anterior region of the cell and apparently follows the helical coil of two adjacent strips. Daughter cells probably rotate as they separate.

may account for sideways contraction and expansion of the pellicle strips. As a working hypothesis, contraction of pellicle fibers may be conceived as maintaining the usual spindle-shaped form of the cells, while sliding of longitudinal microtubules may be responsible for the shortened more spherical forms exhibited by metabolic cells (for a variation of this idea, cf. Arnott and Smith, reference 1). The suggested mechanism could explain cell shape control separately in both longitudinal and transverse directions, though both are undoubtedly closely interrelated.

### *Immunological Evidence for Intussusceptive Surface Replication*

It is evident that in order to maintain the normal number of euglenoid pellicle complexes in succeeding generations, a duplication of pellicle strips and their component structures (fibers, microtubules, endoplasmic reticulum) must take place before cytokinesis. Evidence is presented in this report that this duplication appears to take place by growth of new strips between old ones rather than by any form of lateral division of existing strips. An early suggestion of intercalary strip growth was made by Pochmann (33), although details of Pochmann's model are not consistent with current evidence. Duplication presumably begins in the anterior "mouth" or cytostome region, as in *Astasia* (41, 42), and progresses posteriorly. After formation of a longitudinal furrow, cytokinesis also appears to begin at the anterior end of the cell between the replicated mouths and proceeds toward the posterior, along a pellicle striation. As a consequence of this division, each daughter cell would receive an equivalent portion of new and old strips. Neither daughter pellicle is entirely new, since each daughter cell receives a distinctive portion of parent pellicular surface (Fig. 18).

The use of fluorescein, latex spheres, and ferritin, provided a method for directly testing whether new and old strips appear in each daughter cell by localizing pellicle surface antigens with fluorescent light, scanning, and transmission electron microscopy studies, respectively (Table II). Each method has its limitations and virtues, but in combination they provide information in different ways. For example, fluorescent light microscopy allowed rapid observations of large numbers of labeled cells, while scanning microscopy afforded higher resolution of latex-labeled cell surfaces. The use of ferritin as a marker for transmission

electron microscopy provided precise localization of pellicle antigenic sites, but was not practical for examining large numbers of cells or large areas of cell surface.

Using these various markers in appropriate experiments, it appears that new antigens, and here, new pellicle strips grow by intussusception in the precytokinetic cell. Eventually, each daughter cell receives, in a quasi-semiconservative manner, half old and half new pellicle complexes. The quasi-semiconservative inheritance pattern is seen as an alternation of fluorescent and nonfluorescent or latex-coated and uncoated strips. Such results lend strong evidence that new pellicle strips are formed *de novo*. No evidence for paired labeled and unlabeled daughter cell products from replication and nonconservative division or for complete diffuse labeling of daughter cells from even diffusion of antigen sites was found. The stable *Euglena* pellicle surface is distinctive as compared with the fluid cell surfaces of lymphocytes and other cell systems (12, 13, 38, 39), and hence offers unique advantages for studies of cell surface development. The results reported here suggest that at least some cell surfaces are strongly conserved through cell replication.

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