ORGANIZATION OF THE FLAGELLAR APPARATUS AND ASSOCIATED CYTOPLASMIC MICROTUBULES IN THE QUADRIFLAGELLATE ALGA *POLYTOMELLA AGILIS*

DAVID L. BROWN, A. MASSALSKI, and R. PATENAUDE

From the Department of Biology, University of Ottawa, Ottawa, Canada K1N 6N5

ABSTRACT

The organization of microtubular systems in the quadriflagellate unicell *Polytomella agilis* has been reconstructed by electron microscopy of serial sections, and the overall arrangement confirmed by immunofluorescent staining using antiserum directed against chick brain tubulin. The basal bodies of the four flagella are shown to be linked in two pairs by short fibers. Light microscopy of swimming cells indicates that the flagella beat in two synchronous pairs, with each pair exhibiting a breast stroke-like motion. Two structurally distinct flagellar rootlets, one consisting of four microtubules in a 3 over 1 pattern and the other of a striated fiber over two microtubules, terminate between adjacent basal bodies. These rootlets diverge from the basal body region and extend toward the cell posterior, passing just beneath the plasma membrane. Near the anterior part of the cell, all eight rootlets serve as attachment sites for large numbers of cytoplasmic microtubules which occur in a single row around the circumference of the cell and closely parallel the cell shape. It is suggested that the flagellar rootlets may function in controlling the patterning and the direction of cytoplasmic microtubule assembly. The occurrence of similar rootlet structures in other flagellates is briefly reviewed.

The presence of flagellar rootlets, constructed of microtubules in a characteristic grouping and/or striated fibers, originating near flagellar basal bodies, is a common feature of motile algal cells. In some of these algae, for example, *Chlamydomonas* (34) and the zoospores of *Microthamnion* (43), the microtubules comprising the flagellar rootlets appear to constitute the entire cytoplasmic microtubule system. In others, *Ochromonas* (2) and the zoospores of *Schizomeris* (1), large numbers of additional cytoplasmic microtubules appear to attach to the flagellar rootlets. In an earlier series of papers (2, 4, 5) it was shown that in the formation of this attachment in *Ochromonas* the flagellar rootlets, the rhizoplast and kineto-beak fiber in this organism function as nucleating sites (or microtubule-organizing centers, 29) for the initial assembly of cytoplasmic microtubules. These observations were made on cells in which the cytoplasmic microtubule system was regenerating after an exposure to hydrostatic pressure or antimitotic chemicals. To further clarify this proposed function, we suggested that it was important to examine the role of such sites during the normal development of the cytoplasmic microtubule system (e.g. in synchronously dividing cultures), and ultimately to analyze the polymerization capabilities of these structures in an in vitro

system. The flagellate *Polytomella agilis* offers several advantages for these and other studies on the role of cytoplasmic microtubules and microtubule initiation sites in development.

Members of the genus *Polytomella* (Chlorophyceae, Volvocales) are colorless, quadriflagellate unicells (32). The nutritional requirements (46, 47), growth characteristics (36), and life cycle and reproduction (20) of the genus have been examined. In exponentially growing cultures, *P. agilis* is a free-swimming flagellate lacking a cell wall. It grows rapidly in liquid culture (mean generation time of 4–5 h) and can be synchronized by a repetitive temperature cycle (7). The basic structure of the swimming cell has been examined and the presence of numerous cytoplasmic microtubules noted (28). In late exponential and stationary phases of growth, increasing numbers of cells become immotile thick-walled cysts, which, if resuspended in fresh medium, will excyst and release quadriflagellate swimming cells. During the excystment period the complete complement of basal bodies, flagella, and cytoplasmic microtubules is formed (3).

The basic objective of this report is to provide a detailed description of the flagellar apparatus and associated microtubular structures of the free-swimming quadriflagellate stage of *P. agilis*. The structure and organization of the basal body apparatus are correlated with the flagellar activity of the swimming cell, and comparisons are made with the flagellar apparatus and activity of other biflagellate and quadriflagellate algae. It is shown that flagellar rootlets terminating near the basal bodies serve as attachment sites for large numbers of cytoplasmic microtubules. Separate reports will describe the role of these rootlets in initiating the assembly of cytoplasmic microtubules in vivo (see ref. 3 for a preliminary report) and in the in vitro assembly of microtubules from brain microtubule protein subunits (40).

MATERIALS AND METHODS

Cultures

Axenic cultures of *P. agilis* Aragoa were obtained from the Culture Collection of Algae, Indiana University, Bloomington, Ind. (39) and as a gift from Dr. Joseph Moore, California State University at Northridge, Northridge, Calif. Cultures were grown at 25°C in the dark in liquid medium containing 0.1% tryptone, 0.2% yeast extract, and 0.2% sodium acetate. For these studies, cultures were inoculated to give an initial population density of 10^4 cells/ml and the cells were harvested, after approximately 36 h, at a cell density of 2–5 $\times$ 10^6 cells/ml.

Microscopy

LIGHT MICROSCOPY: Photographs of swimming cells were obtained using a Zeiss 1.30 NA planapochromat bright-field objective, Nomarski optics, and a Zeiss Ukatron UN 60, 60 W·s electronic flash. The patterns of flagellar movement were observed in cells swimming in normal medium and in medium containing increasing concentrations of methyl cellulose.

TRANSMISSION ELECTRON MICROSCOPY: Cells were pelleted by gentle centrifugation and fixed at 20°C for 1.5 h in 0.5% glutaraldehyde in 0.05 M sodium phosphate at pH 7.4. The cells were then washed four times in buffer and postfixed for 1.5 h in cold, buffered 1% osmium tetroxide. The cells were dehydrated in a graded acetone series and were infiltrated with Spurr's (38) hard resin mixture which was then polymerized at 60°C for 18 h. Sections were cut with a DuPont diamond knife on a Sorvall Porter-Blum MT-2B ultramicrotome, stained for 7 min in uranyl acetate (5% in 50% ethanol) and for 3 min in lead citrate (33), and examined in an AEI-EM6B electron microscope.

SCANNING ELECTRON MICROSCOPY: Samples for scanning microscopy were prepared by freeze-drying or critical point procedures. For freeze-drying, the cells were fixed for 30 min in 0.5% glutaraldehyde in 0.05 M sodium phosphate at pH 7.4 and washed in distilled water. A thin layer of cells was then spread on a cover glass and rapidly frozen in liquid nitrogen-cooled Freon 12. The cover glass was then transferred to the cooled stage (−80°C) of a Speedyvac Pearse Tissue Dryer Model 1 and dried for 12–16 h. Cells for critical point drying were fixed in glutaraldehyde as described above, washed, and postfixed for 30 min in 1% osmium tetroxide. The cells were then dehydrated in a graded ethanol series, gradually transferred to Freon 113 and finally to Freon 13 (8), and dried in a Bomar SPC 900 critical point apparatus (The Bomar Co., Tacoma, Wash.). Samples prepared by both drying methods were then coated with gold-palladium and examined in a Cambridge Mark 2A Stereoscan (Cambridge Thermionic Corp., Cambridge, Mass.).

IMMUNOFLUORESCENCE: The immunofluorescent staining was carried out in the laboratory of Dr. V. Kalnins, Department of Anatomy, University of Toronto, Toronto, Canada, using antiserum directed against electrophoretically purified chick brain tubulin.[1] Swimming cells were fixed for 30 min in 2% paraformal-

[1] Kalnins, V. I., and L. Subrahmanyan. 1976. Staining of microtubule-containing regions by immunofluorescence with antibodies to electrophoretically purified chick brain tubulin. Manuscript submitted for publication).

dehyde in 0.1 M phosphate buffer at pH 7.0. The cells were then washed several times in phosphate-buffered saline by gentle centrifugation and resuspension, and stained in suspension by the indirect fluorescein-labeled antibody technique (for details on preparation of antiserum and staining methods, see footnote 1).

After the staining, some samples were subjected to vortex agitation. The cells are fragile after a brief paraformaldehyde fixation and some of them lyse, releasing the intact flagellar apparatus. Samples were photographed with a Zeiss microscope equipped with epifluorescence or phase-contrast optics, using Ilford FP4 film developed in Diafine.

RESULTS

General Cell Structure

The external morphology of *P. agilis* is best seen in the scanning electron micrographs (Figs. 1 and 2). The cell shape is basically ovoid, averaging 16 μm in length and 8 μm in width. Four anterior flagella of equal length (11 μm) emerge from depressions below a cruciform papilla (Figs. 1 and 2).

The distribution of organelles in the swimming cell has been described already (28) and will be considered only briefly here. The anterior papilla contains a fine fibrillar network (Figs. 3 and 6–8) and is devoid of larger organelles. The four basal bodies are situated directly below the papilla. A single row of cytoplasmic microtubules occurs just beneath the naked plasma membrane. Mitochondria (or the single mitochondrion, 6) are usually located in the anterior half of the cell near the periphery, and the Golgi bodies surround the central nucleus. The starch-containing plastids generally occupy the posterior half of the cell.

Flagellar Apparatus

TERMINOLOGY: Although the arrangement of basal bodies and their interconnecting fibers is more complex in *Polytomella* than in *Chlamydomonas* (34), there are several similarities; hence, whenever possible, the terminology of Ringo (34) will be used. The arrangement of the basal bodies is best explained by first referring to the diagrammatic reconstruction (Fig. 4). The four basal bodies are organized in two pairs, with each pair having distinctive interconnecting fibers. One of the pairs (termed the A pair) is situated slightly more anterior in the cell than the other pair of basal bodies (termed the B pair).

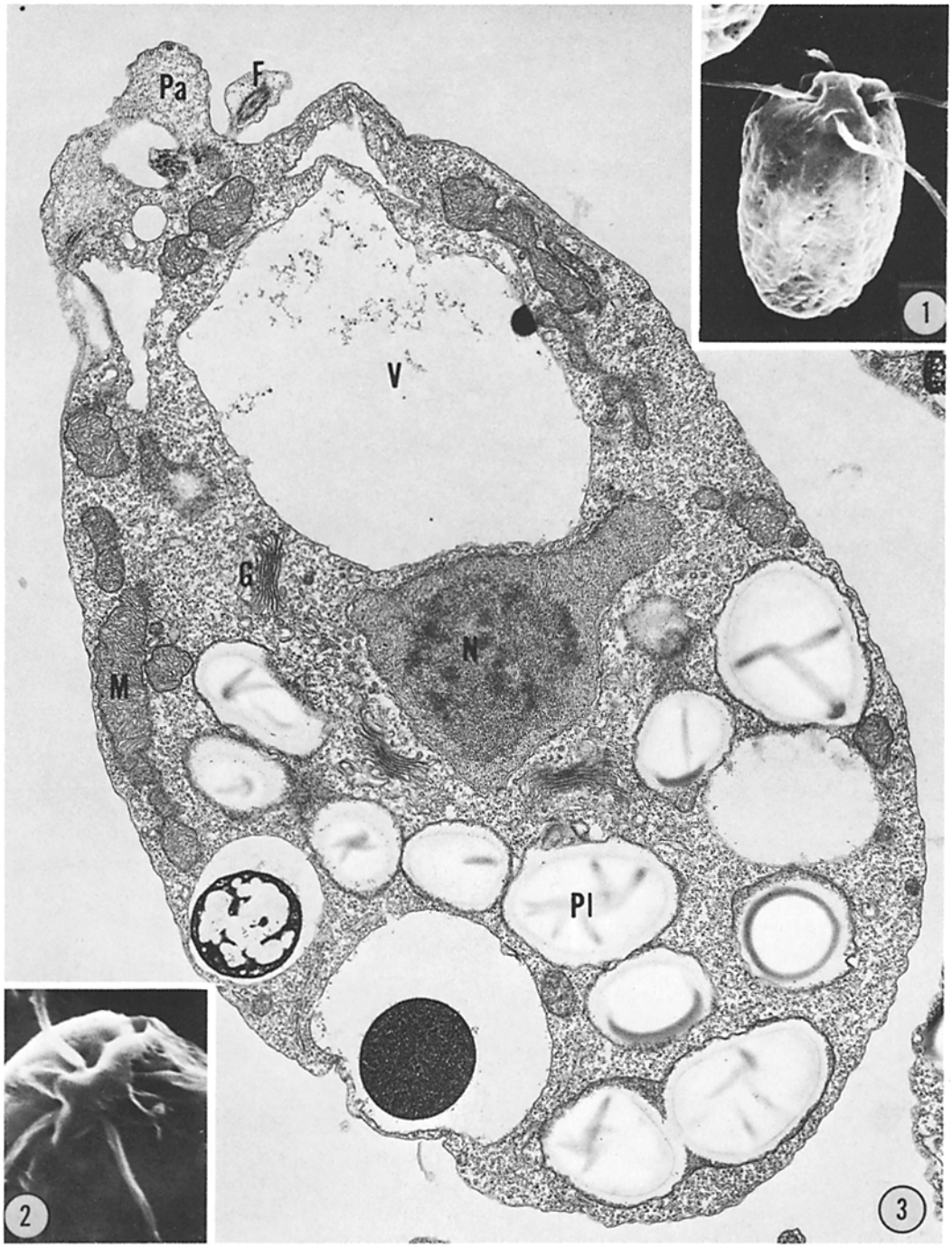
FLAGELLAR MOTILITY: The most frequently observed forward motion of *P. agillis* is a straight line path, with the anterior end of the cell facing in the direction of movement (Fig. 5 *a–e*). The four flagella do not beat synchronously (Fig. 5 *a–c*), but there is good coordination between the opposite pairs of flagella. Both pairs show the breast stroke type of movement described for *Chlamydomonas* (34), with one pair sweeping farther back along the cell at the end of the power stroke (Fig. 5 *b*), although occasionally all four flagella appear in almost the same position of power (Fig. 5 *d*) or return strokes (Fig. 5 *e*). The cells can reverse direction and swim backwards for short periods, with the four flagella trailing and beating in synchrony (Fig. 5 *f*).

BASAL BODIES: The basal bodies of the A pair are inserted at an angle of 110°–120° to one another, with their proximal ends almost touching (Figs. 6 and 7). They are connected near their tops (distally) by a large distal, striated fiber (Fig. 6) and proximally by two smaller striated fibers (Figs. 6, 19, and 20). Both the distal and proximal striated fibers are constructed of fine, 40–50 Å, filaments. The distal striated fiber, seen in longitudinal views taken at right angles (Figs. 7 and 8) and in surface views (Figs. 14 and 15), is about 450 nm long, 200 nm wide, and 50 nm thick. The striation pattern appears to consist of seven, equally spaced major cross striations and an undetermined number of less prominent striations. The smaller proximal striated fibers (200 nm long, 80 nm wide, 40 nm thick) also have a repeating cross striation pattern (Fig. 20).

The B pair of basal bodies are situated slightly deeper in the cell, posterior to the A pair. They insert at a wider angle of about 150° (Fig. 8) and their proximal ends are farther apart than those of the A pair (Figs. 18 and 19). A single nonstriated fiber connects the B basal bodies at their proximal ends (Fig. 8). This fiber consists of a bundle of thin (80–90 Å) parallel filaments (Fig. 9) and is seen as a cluster of dots in cross section below the A basal bodies in Figs. 6 and 7.

As far as we can tell, there are no fibers directly connecting A basal bodies to B basal bodies. However, the four basal bodies are held together by connections to rootlets which terminate between adjacent A and B basal bodies (see below).

The basal bodies of either the A or B pair are not in a directly opposite arrangement, and we refer to them as two nearly opposite pairs (Table I). By this we mean that if a line is drawn longitudinally through the center of one basal body of a pair (Fig. 18) it does not pass through the center of the other



FIGURES 1-2 Scanning electron micrographs showing the external morphology of the cell and the four flagella emerging from depressions below the cruciform papilla. Fig. 1, ×2,500; Fig. 2, ×6,000.

FIGURE 3 Median longitudinal section through the cell showing the distribution of organelles. *Pa*, papilla; *F*, flagellum; *M*, mitochondrion; *G*, Golgi apparatus; *V*, vacuole; *Pl*, plastids with starch grains; *N*, nucleus. ×12,500.

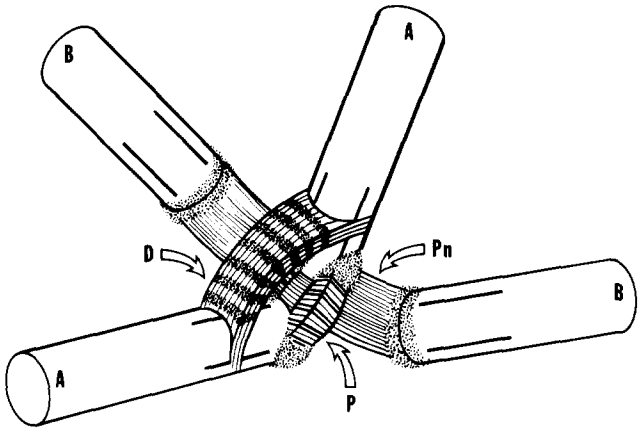


FIGURE 4 Schematic diagram of the arrangement of the four basal bodies and their interconnecting fibers. *A*, basal body of the A pair of flagella; *B*, basal body of the B pair of flagella; *D*, distal striated fiber of the A pair; *P*, proximal striated fiber of the A pair; *Pn*, proximal nonstriated fiber of the B pair.

basal body. The asymmetry has certain phyletic implications (see Discussion).

The Basal Body-Rootlet Complex

ORGANIZATION OF ROOTLETS

SERIAL SECTIONS: The organization of the rootlet system has been reconstructed from serial sections cut at both right angles (transverse sections) and parallel (longitudinal sections) to the long axis of the cell. The overall arrangement is shown diagrammatically in Fig. 10 as it would appear in a view looking down on the anterior part of the cell. The flagellar rootlets are of two structural types: one consists of four microtubules in a 3 over 1 pattern (microtubule rootlet), and the other of a striated fiber over two microtubules (compound rootlet). One rootlet of each type terminates between adjacent basal bodies for a total of eight rootlets (numbered 1 to 8) in the cell. The exception is the compound rootlets numbered 1 and 5 in which the striated portion appears continuous between the closely spaced A basal bodies. To aid the reader in interpreting the electron micrographs, all the transverse sections through the basal body region are printed as if viewed from the anterior of the cell, and the basal bodies of the A and/or B pair are labeled.

One example of serial sections through the basal body region is shown in Fig. 11 *a–e*. Since the rootlets terminate near the proximal end of each basal body (Figs. 16 and 18), and since the two pairs of basal bodies are situated at different depths in the cell, no single section shows all the rootlets. However, it is possible in these and other serial sections to identify all eight rootlets (numbered in Figs. 11 *b–e*).

An additional feature of this set of serial sections is the presence of two forming basal bodies (Figs. 11 *c–e*). Before mitosis, a daughter basal body forms at right angles to each of the parental basal bodies. Flagella remain attached to the parental basal bodies throughout cell division, and the newly formed basal bodies generate flagella before cytokinesis. The flagellar rootlets pass from the proximal ends of each basal body to the cell surface and extend at least part of the length of the cell. The compound rootlets have been observed only in the anterior quarter of the cell, but the three closely associated tubules of the microtubule rootlet can be identified in transverse sections at least to the level of the nucleus (Fig. 27) and may persist to the cell posterior.

IMMUNOFLUORESCENCE: The use of an antibody directed against chick brain tubulin has allowed us to observe the extent of the rootlet system by immunofluorescence. In the intact cell (Fig. 12 *a*), the flagella and four basal bodies fluoresce brightly and a less intense background fluorescence is apparent in the cytoplasm. In samples lysed by agitation (Fig. 12 *b*), some of the cell contents are released from the cell, leaving a "ghost" which has retained the normal cell shape. Some of the flagellar rootlets clearly extend the length of the cell.

Since the rootlets consist of only a few microtubules (two to four), it has not been possible to detect them or demonstrate their length in intact or

partially lysed cells by phase microscopy (Fig. 12 *d*). In the same samples, some cells have lysed to release the entire flagellar apparatus as a unit (Fig. 12 *c*). Both the compound rootlets (containing two microtubules) and the microtubule rootlets (consisting of three or four microtubules) are retained and can be visualized by immunofluorescence microscopy. The fluorescent material seen as aggregates or faint lines between, and sometimes on, the rootlets may represent remnants of the cytoplasmic microtubules and does not fluoresce in samples treated with normal preimmune serum (Fig. 12 *f*).

Detailed Structure of the Rootlets

The substructure of the rootlets and their association with the basal bodies are illustrated in transverse and longitudinal sections through the basal body region. Fig. 13 is a transverse section through the cruciform papilla just anterior to the basal bodies. Although the papilla contains only a network of fine filaments, numerous cytoplasmic microtubules are observed just beneath the plasma membrane in the ridges at the base of the papilla (Fig. 13). In sections slightly lower in the cell (Figs. 14 and 15), the distal striated fiber connecting the A basal bodies is seen in surface view, and, running beneath it, the striated portion of the compound rootlets 1 and 5. The striation pattern of the compound rootlet consists of repeating groups of three closely spaced dark lines with a center-to-center spacing of 300 Å. The two tubules subtending the striated part of this rootlet (Fig. 15) terminate near the proximal end of a basal body (Figs. 16–18) so that, in longitudinal sections

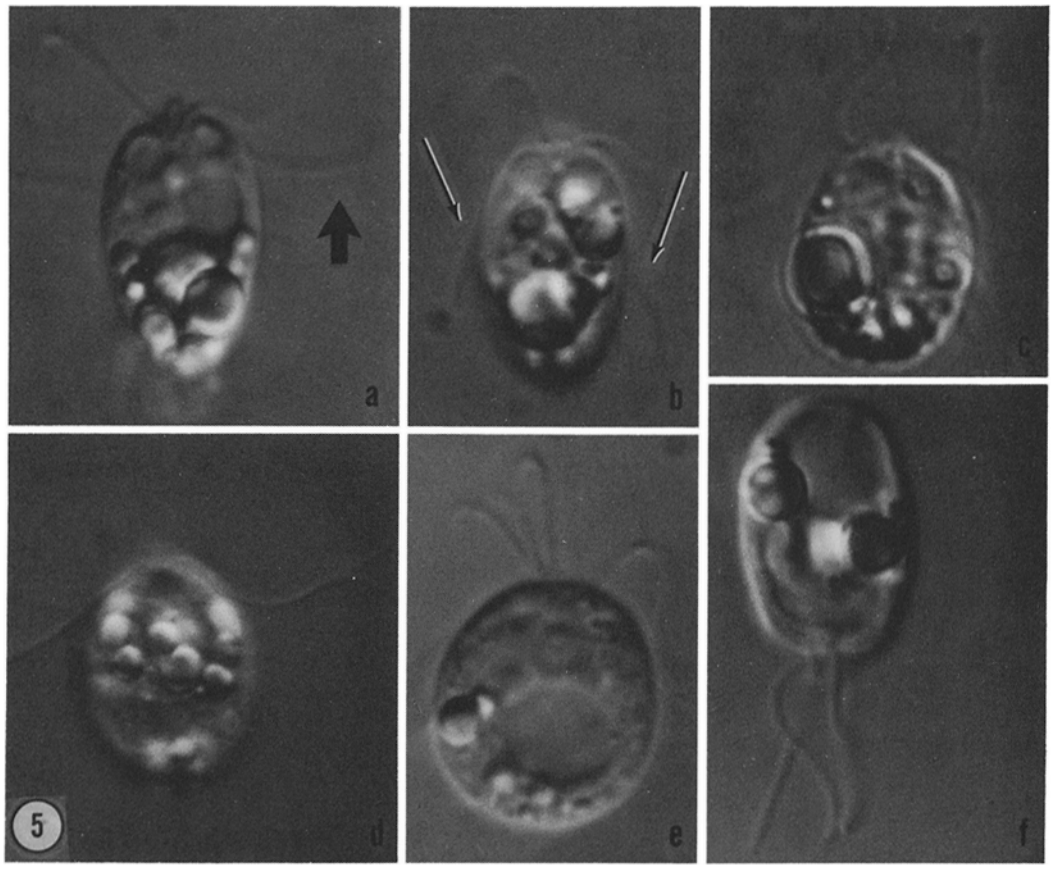
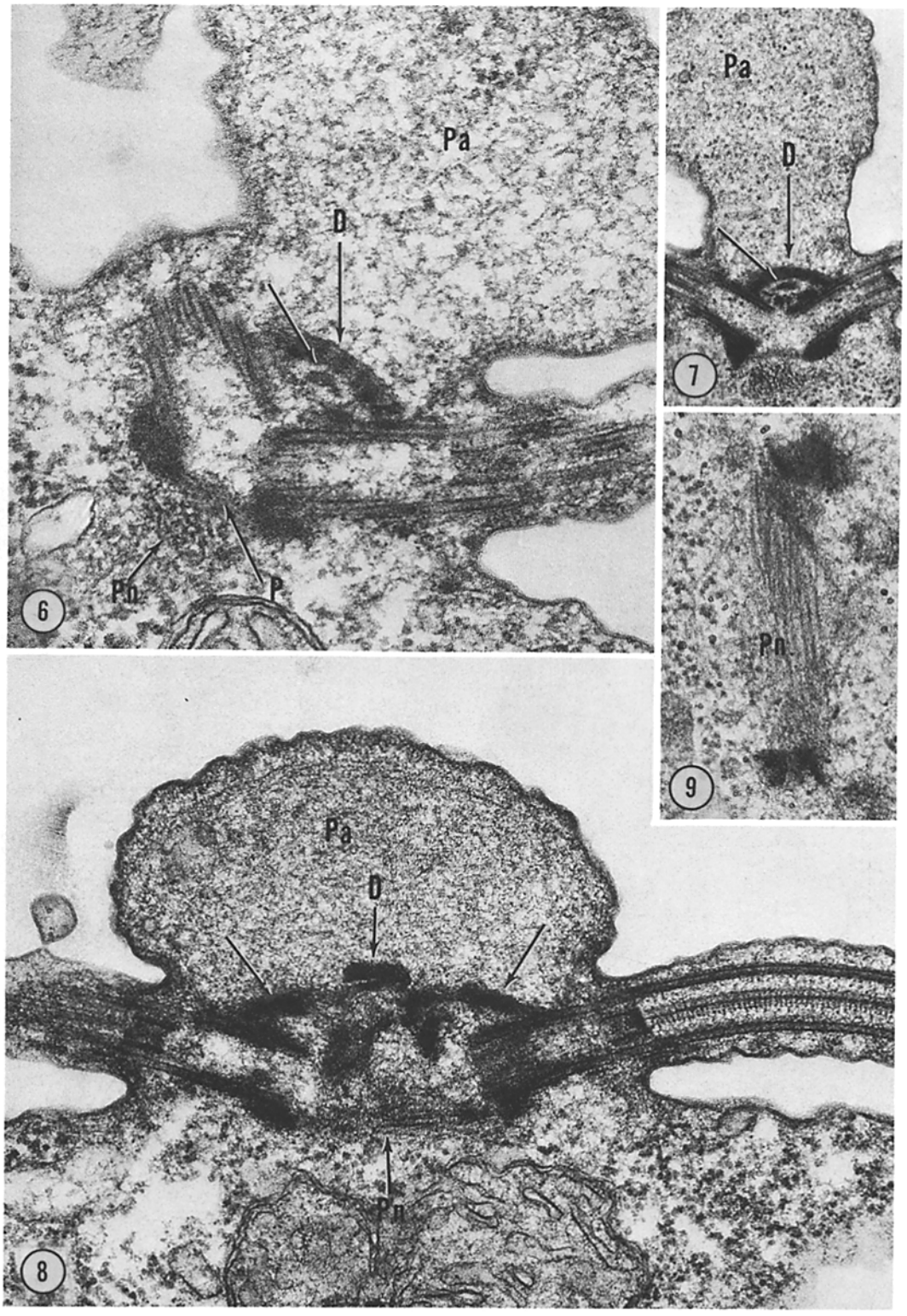


FIGURE 5 *a–f* Flash photomicrographs of swimming *Polytomella* illustrating the patterns of flagella beat. One of the pairs appears to beat farther back along the cell (arrows, *b*). All the cells are swimming in the direction of the large arrowhead (*a*). ×2,000.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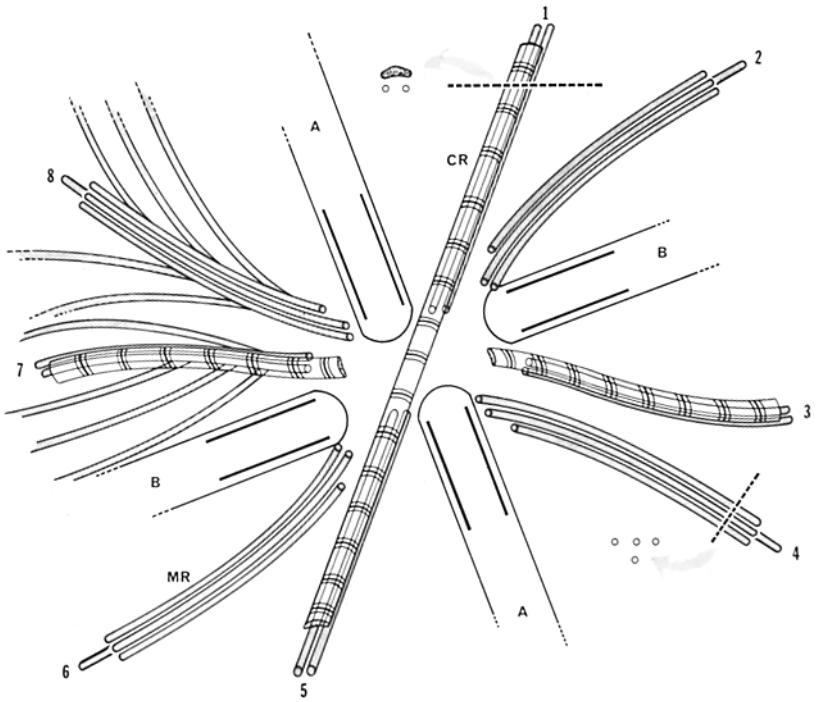


FIGURE 10 Diagrammatic representation of the structure and arrangement of the eight flagellar rootlets. The rootlets are arbitrarily labeled 1 to 8 to serve as reference points in the following electron micrographs. *A*, A flagella; *B*, B flagella; *CR*, compound rootlet; *MR*, microtubular rootlet. Cytoplasmic microtubules are shown attached to only two of the rootlets (7 and 8).

through the center of the A basal bodies, only the striated part of the rootlet is seen (Figs. 6 and 7). In similar sections farther from the center, the two tubules are evident in cross section below the striated part of the rootlet (Figs. 24 and 25). The other two compound rootlets (numbered 3 and 7, Fig. 10) are of similar construction, but both the striated and tubular portions terminate near the proximal end of a basal body. In all sets of serial sections, it has been possible to detect the continuity of rootlets 1 and 5 but the continuity of rootlets 3 and 7 was never observed.

One microtubule rootlet terminates near the proximal end of each basal body on the side opposite a compound rootlet. All four of these rootlets consist of four microtubules in a 3 over 1 pattern (Fig. 23). As a microtubule rootlet approaches the basal body, one of the tubules in the

FIGURES 6-7 Longitudinal sections through the A pair of basal bodies. Fig. 6 shows the striations of the distal fiber (*D*), which are not apparent in the median section as seen in Fig. 7. *Pa*, papilla; *P*, proximal striated fiber; *Pn*, proximal nonstriated fiber. The arrows indicate the part of the compound rootlets which is continuous between the A basal bodies. Fig. 6, ×79,000; Fig. 7, ×34,000.

FIGURE 8 Longitudinal section through the B pair of basal bodies showing the proximal nonstriated fiber (*Pn*) and the distal striated fiber of the A pair (*D*) in cross section. The dense material indicated by the arrows represents the attachment points of rootlets (see text). ×55,000.

FIGURE 9 Surface view of the single nonstriated fiber (*Pn*) which connects the proximal ends of the B pair of basal bodies. ×61,000.

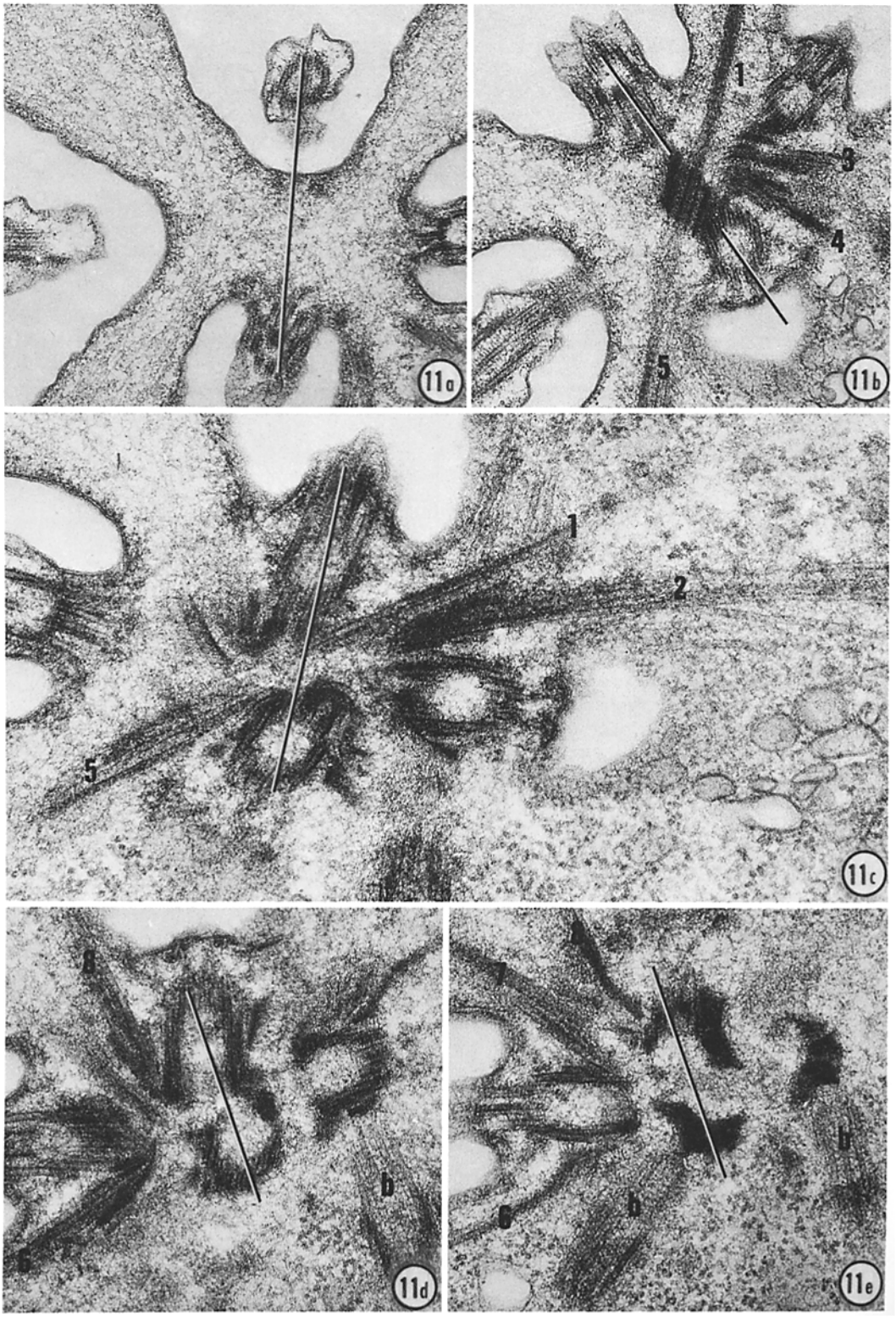


FIGURE 11 *a–e* Serial, nonadjacent, cross sections passing down through the papilla and basal body region showing portions of all eight rootlets (numbered). The line on each micrograph passing over the A pair of basal bodies is included to orient the viewer. Forming basal bodies (*b*) are present in *c–e*. (a) ×33,000; (*b*) ×37,000; (*c*) ×53,000; (*d*) ×47,000; (*e*) ×45,000.

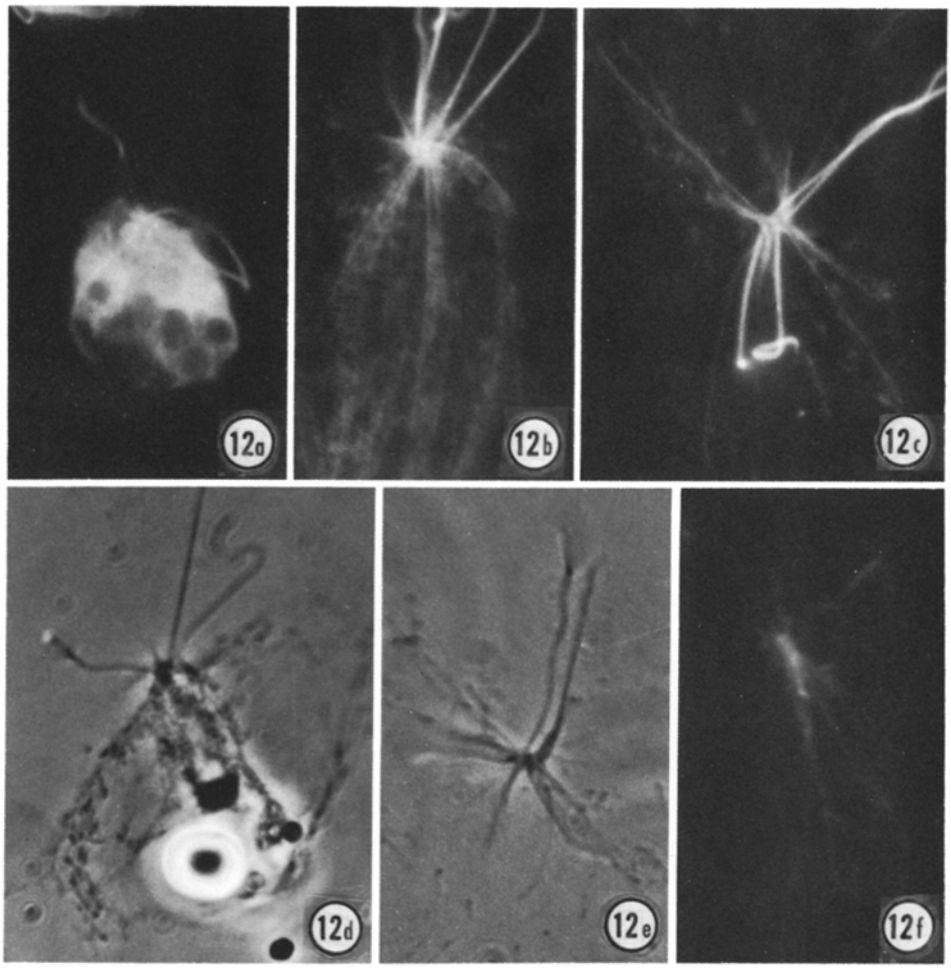
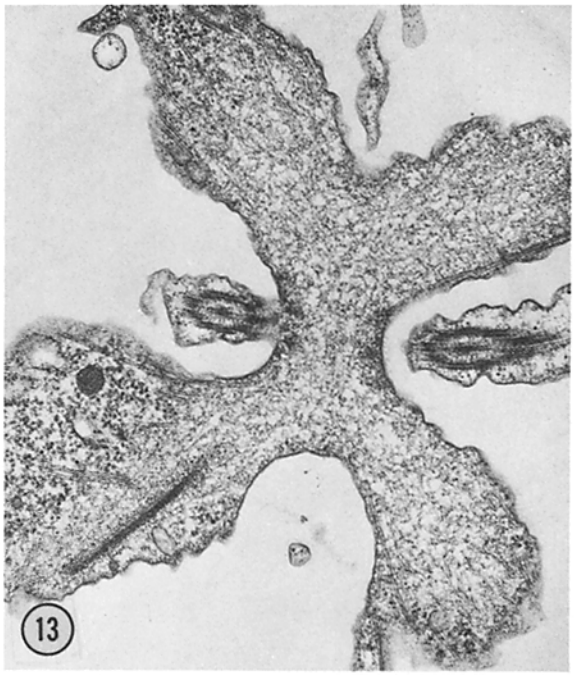


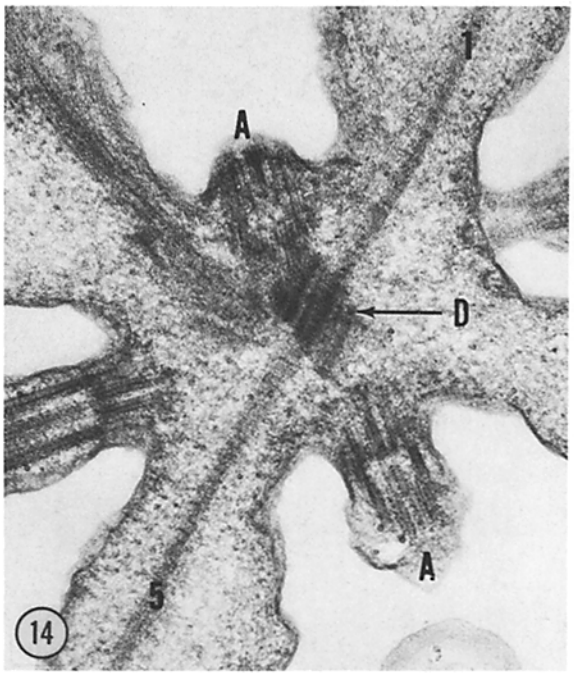
FIGURE 12 *a–f* Immunofluorescence (*a–c*) and phase microscopy (*d*, *e*) of intact and partially lysed cells. (*a*) The four flagella and their basal bodies are evident. ×1,500. (*b*) Partially lysed cell "ghost" showing the four brightly fluorescing flagella (top) and the rootlet system extending to the posterior. ×3,000. (*c*) The intact basal body-rootlet complex with attached flagella free from the cell. ×3,000. (*d*) Partially lysed cell with phase optics (compare to *b*). ×2,000. (*e*) Free basal body-rootlet complex with rootlets barely visible. ×2,000. (*f*) Free complex similar to that in (*c*) but treated with normal preimmune serum and printed on high contrast paper. The film exposure time was identical for (*c*) and (*f*), and when these figures were printed, using the same grade of paper and exposure time, no structure could be seen in (*f*). ×3,000.

row of three ends to leave a 2 over 1 arrangement (Fig. 26). The remaining three tubules end closer to the basal body at, or very near, the same point.

One compound rootlet and one microtubule rootlet terminate between each set of adjacent A and B basal bodies (Figs. 16, 18, 21, and 24) and diverge in passing to the cell surface. In transverse sections, therefore, if one of the rootlets is seen in cross section the other appears in oblique view (Fig. 24). The rootlets appear to be firmly attached to the basal bodies, and by lateral association link the adjacent basal bodies (Fig. 16). Two observations support this suggestion. (*a*) No other connections are observed between A and B basal bodies, yet the entire basal apparatus can be isolated as a unit. (*b*) In cells exposed to hydrostatic pressure (Brown, unpublished), the four basal bodies become separated in the cytoplasm but each still has two rootlets attached to the proximal end.

As the rootlets approach the basal body region,





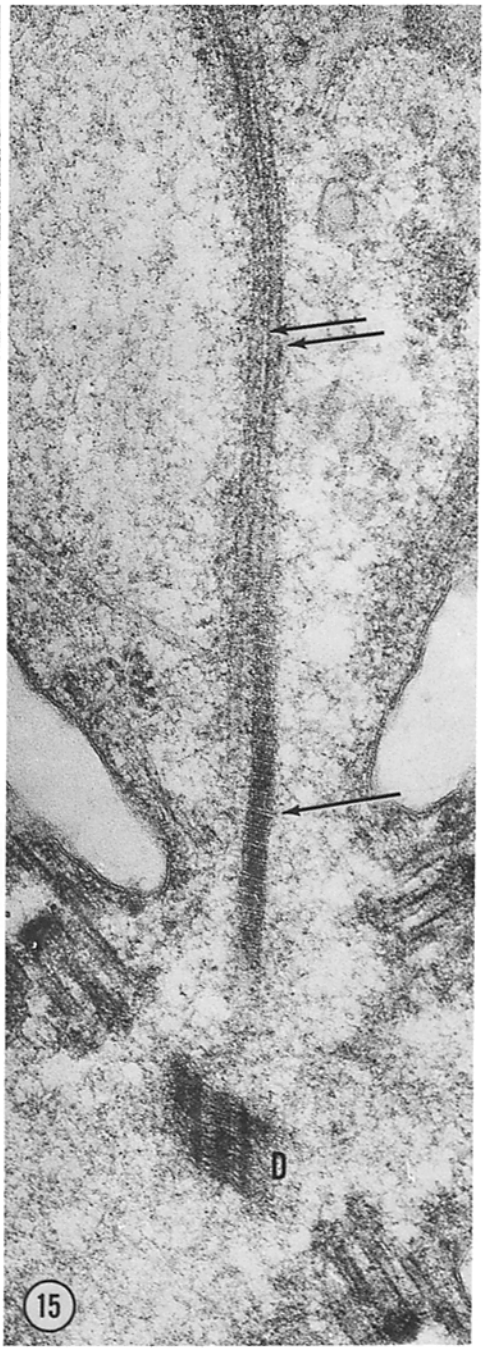


FIGURE 13 Cross section through the cruciform papilla region anterior to the basal bodies. ×26,000.

FIGURE 14 Cross section through the papilla region posterior to that in Fig. 13 showing the distal striated fiber (*D*) in surface view and the striated part of the compound rootlet (1–5) running beneath it. ×46,000.

FIGURE 15 Cross section at the same level as that in Fig. 14. The two parts of the compound rootlet; the striated part (arrow) and the two subtending microtubules (double arrow) are indicated. *D*, distal striated fiber. ×69,000.

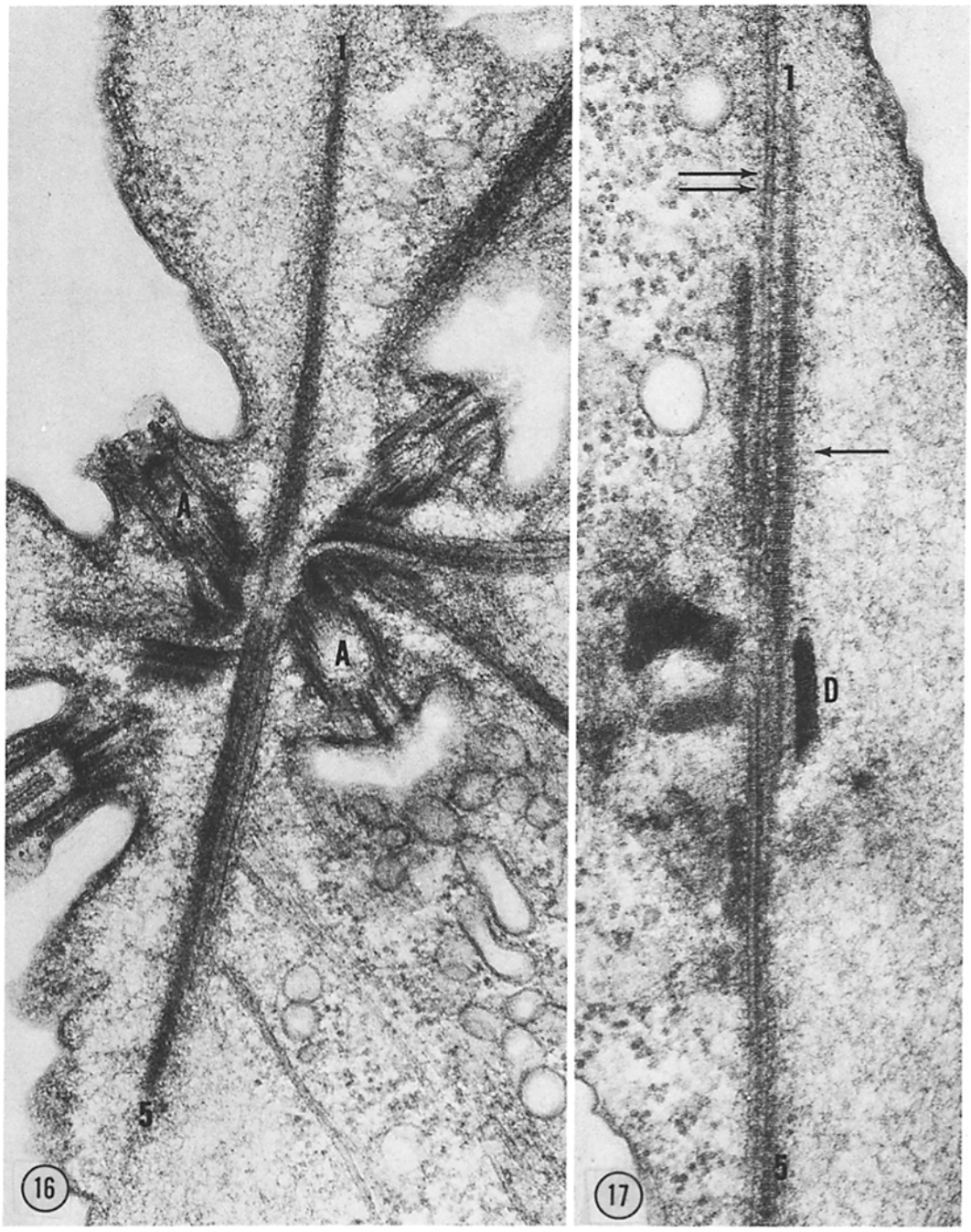


FIGURE 16 Cross section through the basal body region posterior to Fig. 14 showing portions of several rootlets. Cytoplasmic microtubules clearly attach to the microtubular part of the compound root (1-5). ×52,000.

FIGURE 17 Section approximately at right angles to Fig. 16 showing details of the compound rootlet (1-5). Arrow, striated part; double arrow, subtending microtubules; *D*, distal striated fiber. ×64,000.

they are surrounded by a dense-staining amorphous material (Figs. 23–26) which can also be seen running parallel to the rootlets (Fig. 17). This material may function in interconnecting or attaching rootlets to basal bodies, but it could also simply obscure finer attaching fibers. However, it may have a very different function (see below).

Cytoplasmic Microtubules

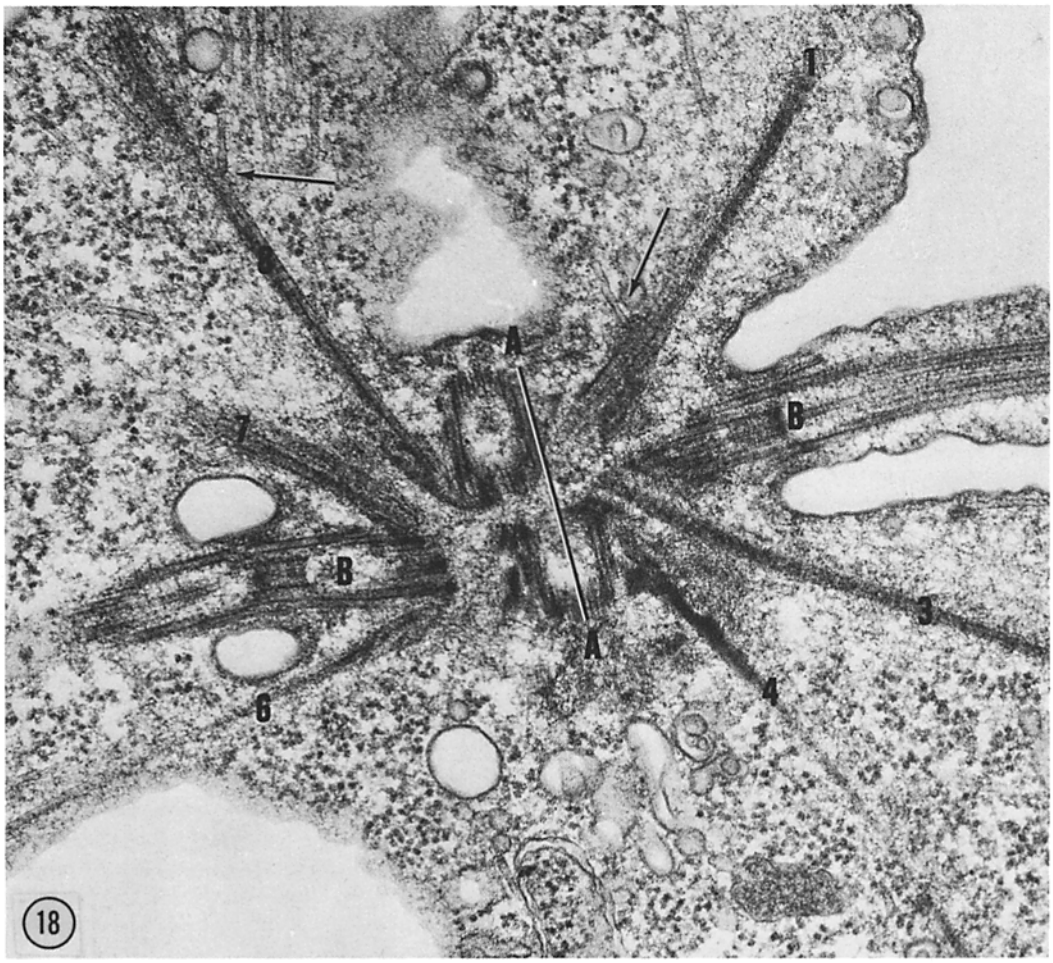
All eight basal body rootlets serve as attachment sites for large numbers of cytoplasmic microtubules (Figs. 11 *c*, 15, 16, and 18). These microtubules diverge at an angle from the rootlet toward the cell surface and occur in a single row around the circumference of the cell just beneath the plasma membrane. The site(s) of attachment of the cytoplasmic microtubules appears specific. Tubules have not been observed to terminate on the striated fiber of the compound rootlets. All microtubules attach to the side of the rootlets and all terminate very close to, but not directly on, a rootlet tubule (Figs. 15, 16 and 18). It appears that the cytoplasmic microtubules insert on the amorphous electron-dense material surrounding the rootlet tubules. As the rootlets approach the basal bodies, the number of attached microtubules increases and the dense material is most prominent. The juncture of the rootlets and basal bodies is often obscured by this material. At points in the posterior part of the cell, the dense material is not observed around the rootlet extensions (Fig. 27), nor are cytoplasmic microtubule attachments observed.

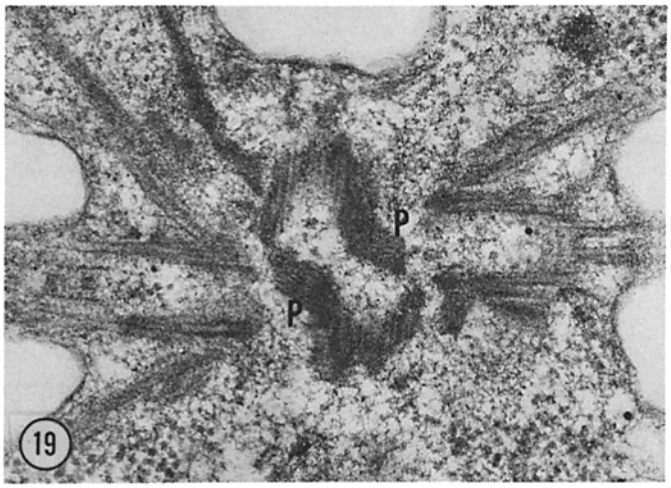
DISCUSSION

Cell Motility

P. agilis normally swims in straight, helical, or curved paths with its anterior, flagella-bearing end facing in the direction of movement. The most frequently observed swimming path is a straight line, and the average speed of swimming is up to 220 μm/s (14). The flagellar activity during forward movement has been analyzed by high-speed cinephotomicrography (13), and some correlations with our observations on the structure of the flagellar apparatus can be made. Gittleson and Jahn (13) point out that two flagella always appear to beat on one side of the cell and two on the other side. In all views in which all four flagella are seen, the two on the same side of the cell are adjacent flagella (in our terminology, one A flagellum and one B flagellum). One of the two flagella on each side of the cell beats farther back along the cell than the other. Since the B basal bodies are set deeper in the cytoplasm, it seems likely that these are the B flagella (Fig. 5 *b*). Gittleson and Jahn also examined the beat frequency of the individual flagella and found that a flagellum which beats farther back along the cell has a beat frequency of 7.1 times per second and that the other flagellum beats 33.3 times per second. We suggest that the slower flagellum is a B flagellum and the faster an A flagellum. This difference in beat frequency would account for our observation that there is good coordination between opposite flagella but usually a lack of synchrony between the A and B pairs of flagella.

This type of breast stroke-like coordination between two flagella (of either the A or B pairs) which beat in opposite directions at the same time is identical to that observed in the biflagellate *Chlamydomonas*. Ringo (34) suggested that the distal striated fiber in *Chlamydomonas* might provide this coordination by either initiating or conveying the initiation of the flagellar beat. The same suggestion could be advanced to explain coordination in *Polytomella*. The A pair of flagellar basal bodies have interconnecting distal and proximal fibers very similar to those in *Chlamydomonas*, and the flagellar movement appears identical. The B flagella in *Polytomella* are also interconnected by a large proximal fiber which might function in coordination. It may be significant here that in the closely related quadriflagellate *Carteria*, the four basal bodies are not linked in opposite pairs and there is no coordinated breast stroke movement (19). The recent demonstration (17) that the isolated flagellar apparatus (consisting of two flagella with basal bodies and accessory structures) of *Chlamydomonas* when reactivated in vitro shows coordinated movement of the two flagella lends some support to Ringo's suggestion. However, it is still not clear which accessory structures, the distal fibers, the proximal fibers, or the microtubule rootlets, are responsible for the coordination. The similarity in size between the filaments comprising distal and proximal fibers and actin filaments has been pointed out (19), and it is tempting to speculate that these fibers have some contractile function. Now that methods are available to isolate and purify the basal bodies with their interconnecting fibers from *Chlamydomonas*





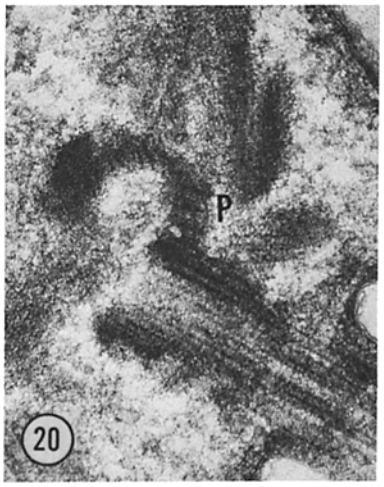
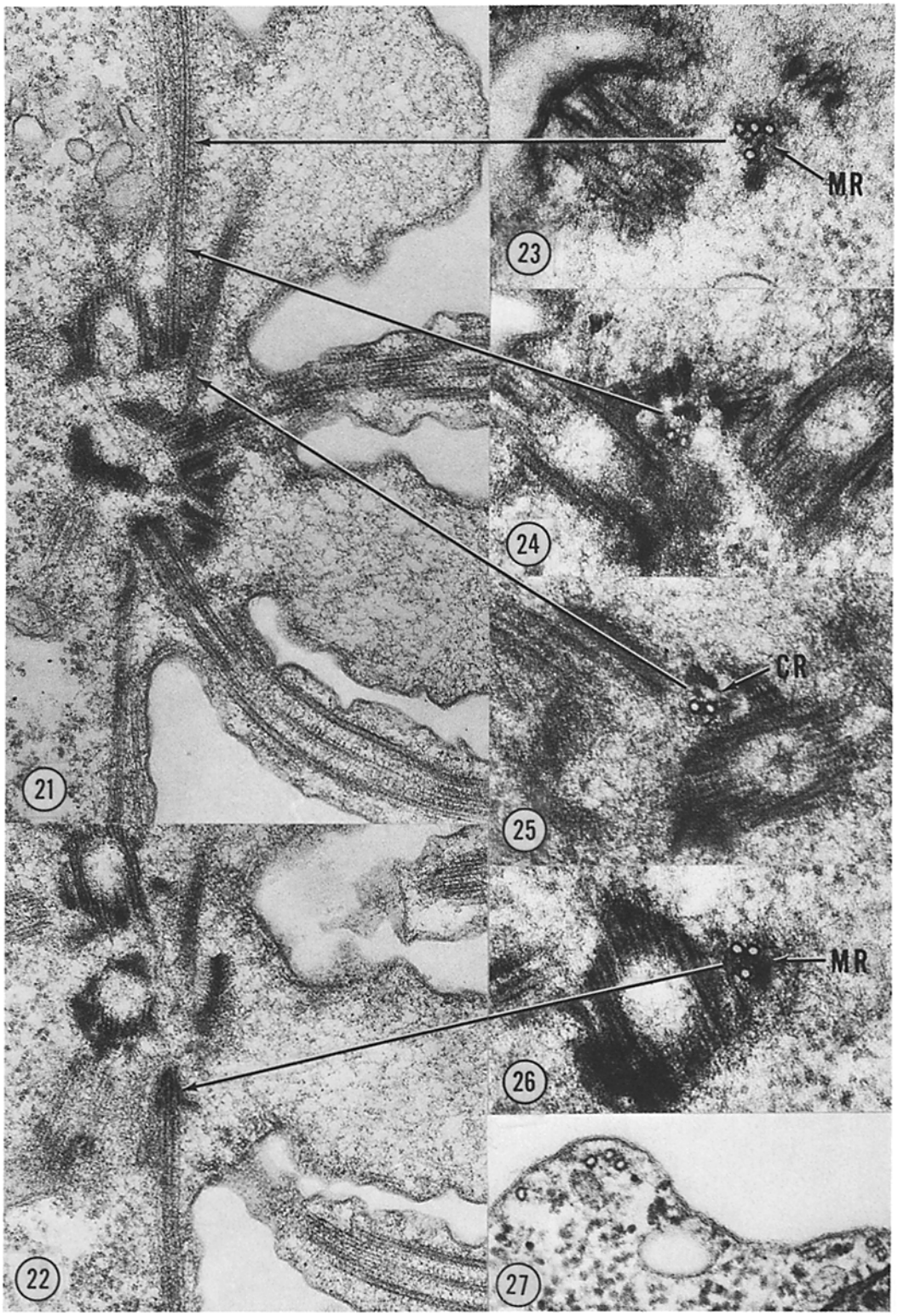


FIGURE 18 Cross section through the basal body region showing all four basal bodies. Note that the *A* pair is at a different level (more anterior) than the *B* pair and that the basal bodies of a pair are not directly opposite each other (line through *A* pair). Six of the eight rootlets and the cytoplasmic microtubules which contact (arrow) the rootlets are seen. ×52,000.

FIGURE 19 Section through the basal body region showing the two proximal striated fibers (*P*) connecting the A basal bodies. ×59,000.

FIGURE 20 Oblique section through the A basal bodies to show the striation pattern of one of the proximal striated fibers (*P*). ×61,000.



FIGURES 21–27 Detailed structure of the rootlets and rootlet endings. Figs. 21 and 22 are two serial, nonadjacent sections showing in surface view the termination of rootlets near the basal bodies. Figs. 23–26 show the microtubule and compound rootlets as they appear in cross sections cut in the areas indicated by the arrows running between the figures. Fig. 27 is a cross section of the cell at the level of the nucleus showing the three closely associated tubules of a microtubule rootlet. *MR*, microtubule rootlet; *CR*, compound rootlet. Figs. 21 and 22, ×45,000; Figs. 23–27, ×80,000.

(15, 37) and from *Polytomella* (40), it should be possible to determine whether actin- or myosin-like components are present.

Phylogenetic Considerations

The phyletic significance of the fine structure of the flagellar apparatus in the motile cells of algae and primitive land plants has been recognized for some time (c.f. the review of Manton, 23). The structure of the mitotic and cytokinetic apparatus has proven most useful in analyzing evolutionary trends in the green algae (for a complete review, see 31). Pickett-Heaps (and others) has recognized a "class" of green algae (including *Chara*, *Nitella*, *Coleochaete*, and *Klebsormidium*) that he has termed the "bryophytan" type which clearly resembles the primitive land plants in the structure of the mitotic spindle and cytokinetic apparatus. *Chaetosphaeridium* (26) and *Trentepohlia* (16) will also probably be added to this "bryophytan" class. The presence of a multilayered structure (a complex flagellar rootlet) associated with the basal bodies of the motile cells of organisms in this class and in the motile sperm of primitive plants adds further support to the suggestion that this group of organisms is very likely closely related to the green algal progenitor(s) of the land plants (1, 26, 31).

The use of the structure of the flagellar apparatus and that of the associated flagellar rootlets as characteristics in the phylogenetic analysis of other green algae has been less successful, and in many cases confusing, mainly because too few organisms have been studied, and in those that have been studied many of the descriptions are incomplete. Probably the most complete study to date is that of the biflagellate *Chlamydomonas* reported by Ringo (34). Ringo described the detailed structure of the striated fibers interconnecting the basal bodies, and showed that four flagellar rootlets, each consisting of four microtubules in a 3 over 1 pattern, were present in a cruciate arrangement. The cruciate arrangement of four rootlets is a common feature of chlorophycean algae (for a review, see 25), exclusive of the "bryophytan" type, and has been used as one characteristic in differentiating this group of Chlorophyceae from the green algae of the class Prasinophyceae. This interpretation appears to have been premature, as the prasinophyte *Pyramimonas* (27) has a cruciate arrangement of four rootlets, two consisting of two microtubules and two of four microtubules in a 3 over 1 pattern. Stewart et al. (41) and Moestrup (26) have recently questioned the validity of the class Prasinophyceae on the basis of other fine structural criteria (e.g. structure of spindle and cytokinetic apparatus, presence of flagellar scales).

With the limited amount of evidence available in 1967, Ringo (34) noted the basic similarity in the flagellar apparatus of biflagellate, quadriflagellate and some multiflagellate (e.g. *Oedogonium*) algal cells and suggested that the quadriflagellate and multiflagellate apparatus may be "polymers" of a basic biflagellate unit as is found in *Chlamydomonas*. However, in the quadriflagellate *Carteria*, which is usually considered most closely related to *Chlamydomonas*, the structure of the flagellar apparatus is quite different than that of *Chlamydomonas* (19). In the quadriflagellate algae examined so far, the flagellar apparatus in *Polytomella* most closely resembles that of *Chlamydomonas* but it certainly does not represent a doubling of the basic biflagellate unit. The A pair of basal bodies with their distal and proximal striated fibers is nearly identical to the flagellar apparatus in *Chlamydomonas*, whereas the B pair shows little similarity. Birkbeck et al. (1) have suggested that there may be a closer affinity between the Chaetophorales (e.g. *Schizomeris*) and the Oedogoniales.

Several of the quadriflagellate green algae examined (see Table I for a partial list) appear to have the four basal bodies arranged in two L-shaped pairs which are in a mirror image relationship (the "ulotrichalean" type of Mattox and Stewart, 24). The fibers interconnecting the basal bodies are not detailed for most of these organisms, but in *Schizomeris* it appears that the adjacent basal bodies are linked by distal and proximal fibers. The four basal bodies in *Polytomella* also superficially appear to be two pairs in a mirror image relationship (Figs. 10, 18 and 19). However, serial sectioning clearly shows that opposite flagella are linked in pairs. The positioning of the flagella, with one pair situated more anterior in the cell than the other, has also not been noted for other quadriflagellates, although this may have been missed if adjacent serial sections were not examined.

The two types of flagellar rootlets found in *Polytomella*, one consisting of four tubules in a 3 over 1 pattern and the other of a striated fiber over two tubules, have been described for other flagellate green algae. Usually there are four rootlets in

TABLE I

Summary of the Flagellar Apparatus Structure in Selected Green Flagellates

Organism	No. of flagella	Basal body arrangement	Basal body connections	Flagellar rootlets (cruciate)	Author(s)
Chlamydomonas	Two	Opposite	Distal and proximal links	Four of 4 tubules	Ringo (34)
Carteria-Group I	Four	Ring	Adjacent basal bodies linked	Four of 4 (to 7) tubules	Lembi (19)
Stigeoclonium	Four	Two L-shaped pairs mirror image	Adjacent linked*	Four (two of 5 tubules, two of 2 tubules + striated fiber)	Manton (22)
Enteromorpha	Four	"	?	?	Evans + Christie (11)
Pseudendoclonium	Four	"	?	Four-?	Mattox +
Trichosarcina	Four	"	?	Four-?	Stewart (24)
Schizomeris	Four	"	Adjacent linked	Four of 5 tubules	Birkbeck et al. (1)
Urospora	Four	"	?	Four of 9 tubules + four striated (Rhizoplasts)*	Kristiansen (18)
Polytomella	Four	Two nearly opposite pairs	Opposite linked	Eight (four of 4 tubules, four of 2 tubules + striated fiber)	Present paper
Pyramimonas (Prasinophyceae)	Four	Ring (?)*	Complex-opposite and adjacent links	Four (two of 4 tubules two of 2 tubules)	Moestrup + Thomsen (27)

* Our interpretation of the published micrographs. See text for discussion.

a cruciate arrangement (Table I). In *Polytomella*, six of the eight rootlets are arranged in a bilateral symmetry on either side of the compound rootlets numbered 1 and 5 in Fig. 10. The four microtubule rootlets do appear in a cruciate arrangement in surface view (as shown in the diagram, Fig. 10), but the two attached to the A basal bodies are situated more anterior in the cell than the two attached to the B basal bodies.

Asymmetry of motile cells is generally considered to be a primitive characteristic in the green algae, and the radial symmetry as in *Chlamydomonas* or *Schizomeris*, both of which have four equal rootlets in a cruciate arrangement, is presumed to be a derived characteristic. In comparison to these organisms, *Polytomella* exhibits considerable asymmetry in the structure of the flagellar appratus, and also has the primitive characteristics (31) of a completely closed spindle and cytokinesis by furrowing (unpublished results). On the basis of the structure of the flagellar apparatus, we can make only some very tentative conclusions at this time. *Polytomella* seems to be one of the more primitive Volvocales, and appears to be closely related to *Chlamydomonas*. The appearance of the four basal bodies in surface view as two L-shaped pairs in a mirror image relationship does resemble the arrangement in other quadriflagellates, but the type of basal body interconnection in most of these is unknown. It seems likely, though, that the structure of the flagellar apparatus will prove to be an increasingly important characteristic in phylogenetic analysis as detailed information on more flagellates becomes available.

A phylogenetic consideration of a much broader nature is the conservation of microtubule protein (20). Several studies have shown that antisera directed against tubulin from sea urchin sperm flagella (44), bovine brain (12), and chick brain (footnote 1) cross-react with microtubules in very diverse animal species. Our observation that antibodies produced against chick brain tubulin react with microtubule structures (flagellar axonemes, basal bodies, rootlets and spindle) in a primitive phytoflagellate extend the idea that tubulin is highly conserved. This method should be useful in visualizing microtubule systems in other flagellates.

Cytoplasmic Microtubule System

Cytoplasmic microtubules have been shown to have a cytoskeletal function in the development and maintenance of cell asymmetry in a variety of

organisms (see Roberts, 35, for a recent review). In *Chlamydomonas*, in which relatively few cytoplasmic microtubules are observed, this type of cytoskeletal function is less obvious, and these tubules may play a greater role in anchoring or stabilizing the flagellar apparatus (34). However, cytoplasmic microtubule systems are well developed in many of the flagellated algal cells, particularly in those lacking cell walls. In the multiflagellate zoospores (30) and sperm (10) of *Oedogonium*, there is a proliferation in the number of microtubule rootlets, which extend the length of the cell and presumably function as a cytoskeleton. In *Polytomella*, and in the naked zoospores of many green algae (e.g. *Enteromorpha*, *Schizomeris*, *Urospora*, Table I), the number of rootlets remains relatively low and large numbers of additional microtubules are present, which appear to attach to the flagellar rootlets and underlie the plasma membrane.

The correlation between cytoplasmic microtubule distribution and the development of cell shape is especially evident in asymmetric flagellated cells, and many examples could be cited from all classes of algae that have motile cells. *Ochromonas* is a particularly clear example (2, 4, 5). In this unicellular Chrysophycean alga, the characteristic asymmetry is mediated by two sets of cytoplasmic microtubules which are initiated at two distinct sites in the cell at different times during development of cell shape. Both of these sites are flagellar rootlets, the rhizoplast (a striated rootlet) and the kineto-beak fiber (a microtubule rootlet), and each site is related to the development and maintenance of specific portions of the asymmetric cell form.

The existence of a "structure" which could control the spatial and temporal distribution of microtubules by controlling the initiation of microtubule assembly has been postulated several times [for example, the microtubule organizing center of Pickett-Heaps (29); the nucleating site of Tilney and Goddard, (42)]. Basal bodies are highly structured assembly sites for axoneme microtubules of cilia and flagella. The pattern and orientation of microtubules in the axoneme appears to result, at least in part, from the distal assembly of tubulin subunits onto the existing (pattern of) tubules of the basal body, and this can be partially mimicked in vitro (37). What established that pattern of tubules in the basal body is still not clear, but this may also be a nucleated assembly (15). More commonly, however, the sites (i.e. organizing centers) that are presumed to be associated with microtubule assembly consist of an amorphous electron-dense material that has no obvious structural organization [e.g. the polar bodies of the mitotic spindle in *Oedogonium* (9); the granular aggregate in homogenates of surf clam eggs which will polymerize asters and spindles in vitro (45)].

A more complex type of organizing center appears to result from an ordering of the amorphous material (that described above) onto some other structure to provide a template which could control the patterning and direction of microtubule assembly. The rhizoplast and kineto-beak fiber of *Ochromonas* are examples of this type of organizing center (for a more complete discussion see Brown and Bouck, ref. 4). We suggest, on the basis of the structural information that cytoplasmic microtubules terminate on amorphous material associated with rootlets, that the flagellar rootlets in *Polytomella* are further examples of structured organizing centers. We have demonstrated that these organizing centers appear to initiate microtubule assembly in vivo and in vitro (3, 40). This system should be useful in analyzing the mechanism of spatial and possibly temporal control of microtubule assembly.

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