

## FLAGELLAR ELONGATION AND SHORTENING IN *CHLAMYDOMONAS*

### III. Structures Attached to the Tips of Flagellar Microtubules and Their Relationship to the Directionality of Flagellar Microtubule Assembly

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#### ABSTRACT

Two structures on the distal ends of *Chlamydomonas* flagellar microtubules are described. One of these, the central microtubule cap, attaches the distal ends of the central pair microtubules to the tip of the flagellar membrane. In addition, filaments, called distal filaments, are observed attached to the ends of the A-tubules of the outer doublet microtubules. Inasmuch as earlier studies suggested that flagellar elongation *in vivo* occurs principally by the distal addition of subunits and because it has been shown that brain tubulin assembles *in vitro* primarily onto the distal ends of both central and outer doublet microtubules, the presence of the cap and distal filaments was quantitated during flagellar resorption and elongation. The results showed that the cap remains attached to the central microtubules throughout flagellar resorption and elongation. The cap was also found to block the *in vitro* assembly of neurotubules onto the distal ends of the central microtubules. Conversely, the distal filaments apparently do not block the assembly of neurotubules onto the ends of the outer doublets. During flagellar elongation, the distal ends of the outer doublets are often found to form sheets of protofilaments, similar to those observed on the elongating ends of neurotubules being assembled *in vitro*. These results suggest that the outer doublet microtubules elongate by the distal addition of subunits, whereas the two central microtubules assemble by the addition of subunits to the proximal ends.

**KEY WORDS** flagella · microtubules · microtubule assembly · *Chlamydomonas* · membranes

It has been shown by pulse labeling and light and electron microscope autoradiography that flagella (21, 22) and flagellar microtubules (34) assemble *in vivo* principally by the addition of subunits to their distal ends. These observations were made

by amputating the flagella of certain protozoans and algae, allowing them to regenerate their flagella to half-length, and then adding a radioactive precursor, such as tritiated leucine, to the cells until flagellar regeneration was completed. After autoradiography, most of the incorporated radioactive precursor was observed to be in the distal (tip) half of the flagellum (21, 22) or flagellar microtubules (34), indicating that elongation oc-

curred by distal addition of subunits; however, there was always some labeled precursor (ca. 20–35%) incorporated into the proximal half of the flagellum or flagellar microtubules (21, 22, 34) even after prolonged chases with the unlabeled precursor. It was not known whether this proximal label represented microtubule assembly by the proximal addition of subunits, or whether the label was present in some microtubule-associated structures added after microtubule assembly (see reference 34).

With the development of methods for the in vitro assembly of brain tubulin (3, 33), it was found that brain tubulin would assemble onto flagellar microtubules in vitro (1, 2) and that the assembly occurred principally onto the distal ends of the flagellar outer doublet and central microtubules (1, 2). However, if the tubulin concentration was sufficiently high, neurotubules also assembled onto the proximal, nonfavored ends of the flagellar microtubules but at a rate five to eight times slower than the assembly onto the distal ends (2). The relevance of this in vitro proximal assembly to flagellar assembly during regeneration in vivo was unknown, as no information was available on the concentration of tubulin in the cells being used. However, the possibility of proximal assembly occurring in vivo was considered to be small because, in addition to the fact that the distal ends of both the central and outer doublet microtubules were favored for the in vitro assembly of brain tubulin, the outer doublet microtubules do not have free proximal ends but are extensions of the basal body microtubules. On the other hand, the proximal ends of the two central microtubules are free, ending in or just above the basal cup (axostome) of the basal body (9, 20). Therefore, the possibility could not be excluded that the central microtubules assembled in vivo from their proximal ends at high local tubulin concentrations even though their distal ends were favored for the in vitro assembly of brain tubulin.

In this study, it is shown that the distal ends of the two central microtubules insert into a cap, called the central microtubule cap, which is attached to the distal tip of the flagellar membrane. It is also shown that the cap remains attached to the central microtubules during flagellar regeneration and resorption and that the cap prevents the addition of brain tubulin subunits onto the distal ends of the central microtubules. In addition, the results in this report show that the A-tubules of the outer doublet microtubules of full-grown fla-

gella terminate in a pair of filaments, called distal filaments, which apparently do not prevent the distal assembly of brain tubulin onto the A-tubules of the outer doublets. Observations of the distal ends of the outer doublet microtubules during flagellar regeneration show them frequently to terminate in sheets of protofilaments, similar to those observed on the ends of brain microtubules being assembled in vitro (7, 10, 15, 16, 27). These results suggest that the flagellar outer doublet microtubules elongate by the distal addition of subunits, whereas the central microtubules elongate by the proximal addition of subunits.

A preliminary report of some of the data presented in this paper has been published (8).

## MATERIALS AND METHODS

### Cell Culture

*Chlamydomonas reinhardtii* (strain 21 gr) were grown in 125-ml flasks to a cell density of  $0.8\text{--}1.0 \times 10^8$  cells/ml in medium I of Sager and Granick (25). The cells were grown with constant aeration at 25°C on a cycle of 13 h of light and 11 h of dark. Cells were harvested by centrifugation in 50-ml conical centrifuge tubes (IEC Model HN centrifuge, rotor number 958, 350 g, 3–5 min, room temperature) and were resuspended in fresh culture medium. The cells were aerated during the experiment either by bubbling air through the medium or by stirring the culture with a magnetic stirring bar.

### Preparation of Axonemes for Electron Microscopy

To attach flagella to the electron microscope grids, the grids were treated with poly-L-lysine by a modification of the method of Mazia et al. (18). Electron microscope grids were coated with a Formvar film, shadowed with carbon, and glow-discharged for 1–2 min in a vacuum evaporator. The glow-discharged films were then overlaid with a solution of 0.1% poly-L-lysine in water for 5–10 min, washed under a stream of double-distilled water, and air dried. A drop containing *Chlamydomonas* cells was then applied to the grid and the grid was drained by touching it to filter paper. The grid was then inverted over a drop of deflagellation solution (5 mM MgSO<sub>4</sub>, 0.35 mM DTT, 0.5 mM EDTA, 10 mM HEPES, pH 7.5, 0.005–0.05% Nonidet P-40 [Shell Chemical Co., N. Y.]) for 5–20 s at room temperature. The concentration of detergent and the time of extraction varied with the concentration of cells and various *Chlamydomonas* mutants; extraction conditions were adjusted so the minimum treatment, which resulted in the removal of cell bodies from the flagella (and from the grid) and the dissolution of most of the flagellar membranes without disruption of the structures at the flagellar tips, was used. After detergent treatment, the grid was rinsed with 2–5

drops of 2% aqueous uranyl acetate and air dried. A procedure similar to this has recently been used by Sale and Satir (26) to visualize the organization of radial spokes in *Tetrahymena* cilia.

### Flagellar Resorption and Regeneration

*Chlamydomonas* were induced to resorb their flagella by the high-salt method developed by Lefebvre et al.<sup>1</sup> in which the flagella could be synchronously shortened from an average length of 12–14 to 2–3  $\mu\text{m}$  in 3 h. Flagellar regeneration subsequently was induced by harvesting the cells and resuspending them in fresh medium. Samples of cells were taken at intervals during flagellar resorption and regeneration and were treated as described above to observe the flagellar microtubules. In addition, samples of cells were fixed in 2% glutaraldehyde in polymerization mixture (PM) and their flagellar lengths were measured with a Zeiss phase microscope equipped with an ocular micrometer. An average of 50 flagella were measured at each time-point.

### Isolation of Brain Tubulin and Assembly of Neurotubules onto Flagellar Axonemes

Tubulin was purified from brains of 1- to 3-day-old chicks by two cycles of assembly and disassembly in vitro in a PM containing 0.1 M PIPES pH 6.9, 2 mM EGTA, 1 mM GTP, 1 mM  $\text{MgSO}_4$  as previously described (3, 6, 27–29). The final microtubule pellet was depolymerized at 4°C and centrifuged for 1 h at 100,000  $g$  at 4°C to obtain a supernate (S-2) containing tubulin. To assemble brain tubulin onto the flagellar microtubules, drops of the S-2 were placed on parafilm strips in Petri dishes. *Chlamydomonas* were then attached to grids and treated with Nonidet P-40 as described above to remove the flagellar membranes and cell bodies. The grids with the attached axonemes were then inverted over the drops containing S-2 and the drops warmed to 25°C. The grids were removed at 1-min intervals, rinsed in warm PM (25°C), and negatively stained with uranyl acetate.

## RESULTS

When *Chlamydomonas* were placed on polylysine-coated electron microscope grids and then negatively stained, both the cell bodies and their flagella were attached to the grids (Fig. 1A). If the attached cells were first treated with a low concentration of detergent for a few seconds before negative staining, the cell bodies usually were removed and some or all of the flagellar membranes were dissolved (Fig. 1B). Both the duration of treatment and the concentration of detergent necessary

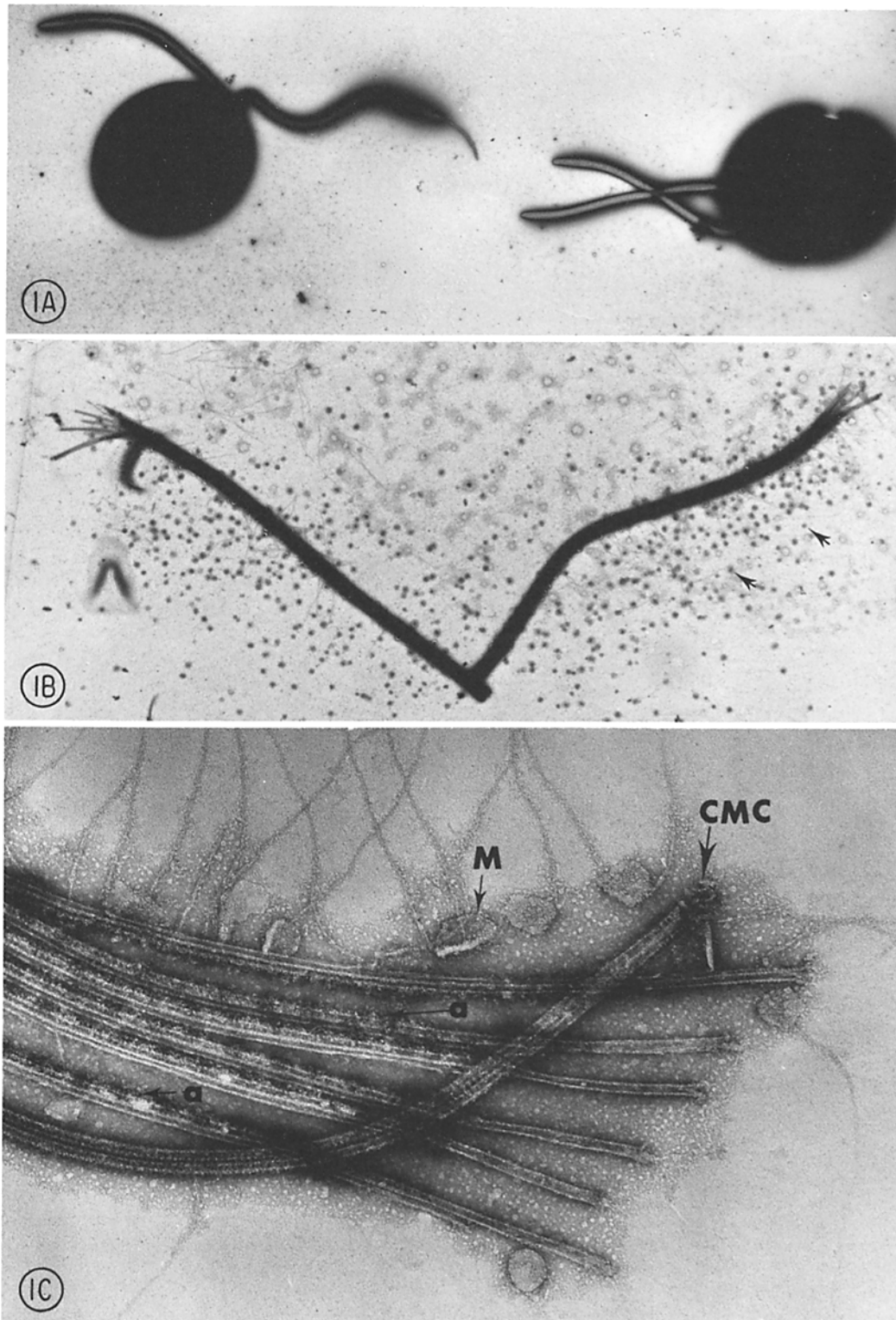
<sup>1</sup> Lefebvre, P., J. Jarvik, and J. L. Rosenbaum, unpublished results.

to remove the flagellar membrane varied with the number of cells attached to the grid and the particular strain of *Chlamydomonas* used. After detergent treatment, most of the flagellar axonemes remained intact except for the distal tips, which were slightly splayed (Fig. 1B, C). The structure of the microtubules is similar to that reported by others (1, 2, 13, 31): dynein arms were attached along the A-tubule of each outer doublet and the projections attached to the central microtubules were often superimposed to form an apparent helical coil (see references 5 and 31). Examination of the tips of the axonemes at higher magnification (Figs. 1C, 2, 3) revealed structures at the ends of the microtubules that had not been observed before in negatively stained axonemes. These included a caplike structure on the distal ends of the central pair microtubules and short filaments projecting from the distal ends of the A-tubules of the outer doublet microtubules.

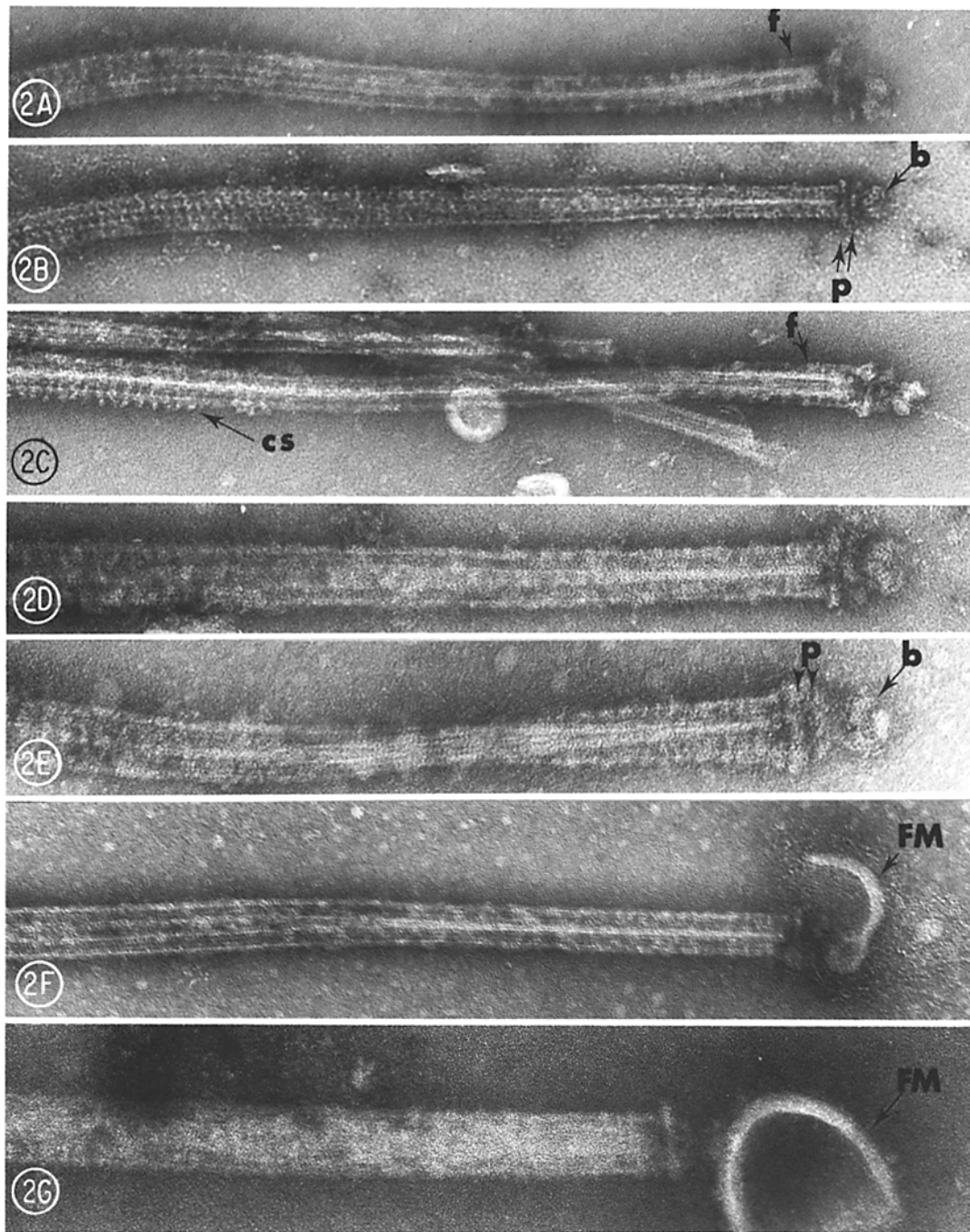
### The Central Microtubule Cap

The cap at the distal end of the central pair microtubules, the *central microtubule cap*, was composed of two plates oriented perpendicular to the long axis of the microtubule (Figs. 1C, 2). The most proximal plate, at which the central microtubules terminated, measured approximately 68 nm  $\times$  10 nm, whereas the slightly smaller distal plate measured approximately 48 nm  $\times$  10 nm. The plates were separated by a 9- to 12-nm clear space, although the plates were often observed to be attached to each other by small filaments. A spherical bead, approximately 50 nm in diameter, was observed at the distal end of the cap. The bead may be derived from or closely associated with the flagellar membrane because it is attached to the membrane after gentle detergent treatment but is absent after extensive detergent treatment. Although the plates were slightly more resistant to detergent than was the bead, neither plates nor beads were observed with the extraction times and detergent concentrations used by others (1, 2) to isolate and demembranate flagellar axonemes.

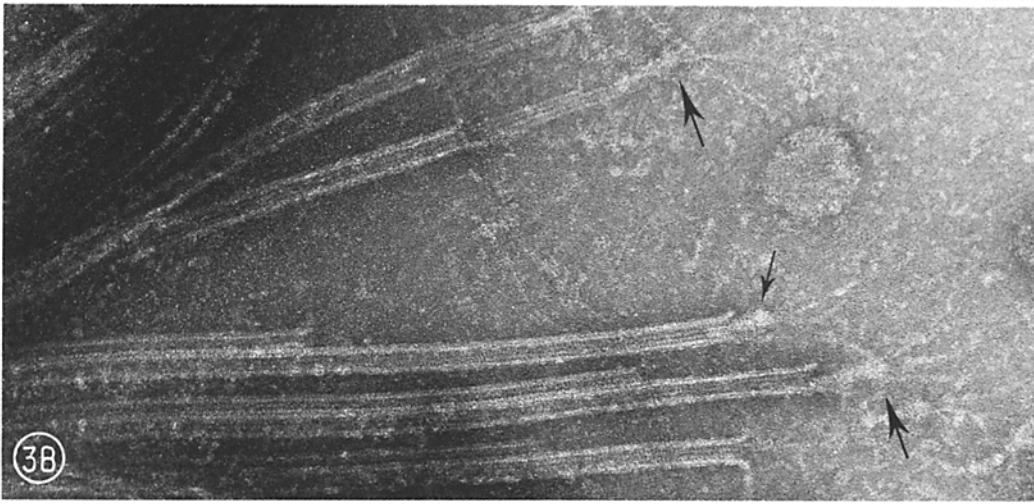
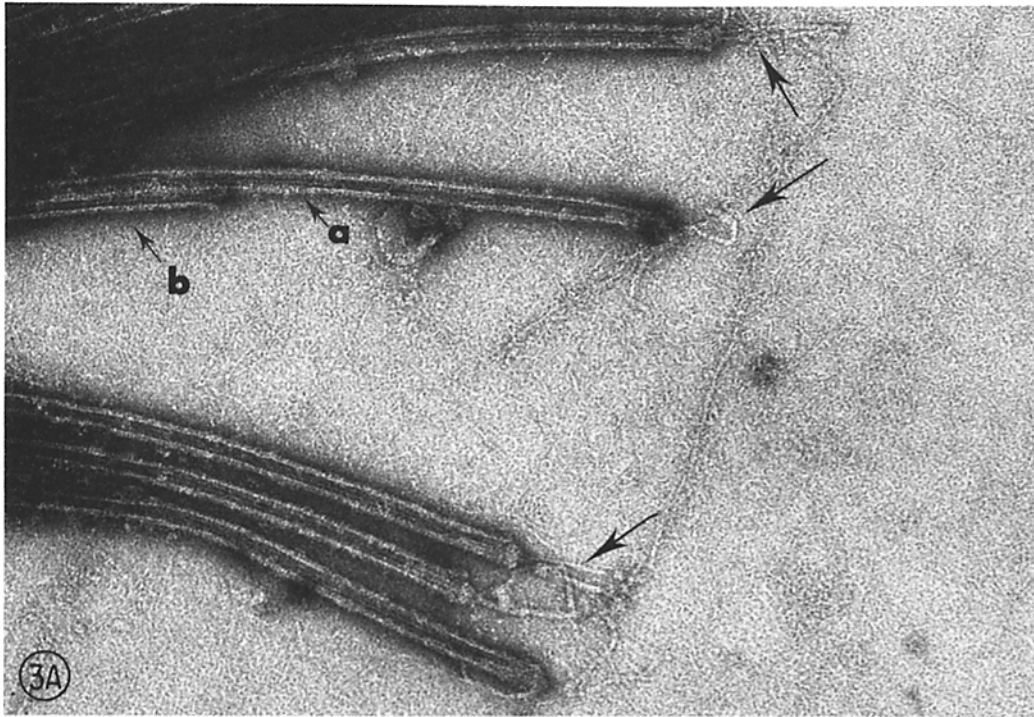
In addition to the plates and the bead, a helical filament, 2.3 nm in diameter, was occasionally observed attached to the proximal plate of the central microtubule cap. This filament wound proximally around the central microtubules for a distance of 60–90 nm (Fig. 2) and was only observed under conditions of extremely gentle detergent extraction. The 2.3-nm filament was distinct from the projections that are attached along most



**FIGURE 1** Attachment of *Chlamydomonas* cells to Formvar films, demembration of their flagella, and negative staining with uranyl acetate. (A) *Chlamydomonas* cells attached to a poly-L-lysine-coated Formvar film.  $\times 5,000$ . (B) Flagellar axonemes after detergent treatment (see Materials and Methods). Cell bodies become detached from the axonemes and from the Formvar film; fragments of the flagellar membrane (arrows) dot the background. The axonemes remain intact for most of their length and are slightly splayed at their distal tips.  $\times 8,000$ . (C) Distal tip of a detergent-treated flagellum. Patches of flagellar membranes (M) with attached mastigonemes lay near the axoneme. Six outer doublet microtubules with dynein arms (a) attached to the A-microtubule lie under the central pair microtubules which terminate at their distal ends in the central microtubule cap (CMC).  $\times 72,000$ .



**FIGURE 2** Individual pairs of central microtubules after detergent treatment of the flagellum. The 6.5-nm central sheath (*cs*) extends proximally (to the left in these micrographs) along the central microtubules. The central microtubule cap at the distal ends of the central microtubules is composed of two plates (*p*) and a bead (*b*). A 2.3-nm filament (*f*) attaches to the proximal plate and winds proximally around the two central microtubules; this is most evident in Fig. 2A and C. A "bare zone" appears between the distal end of the 6.5-nm sheath (*cs*) and the proximal end of the 2.3-nm filament (*f*). A fragment of the flagellar membrane (*FM*) is attached to the bead on the central microtubule cap in Fig. 2F and G. (A)  $\times 100,000$ . (B)  $\times 80,000$ . (C)  $\times 80,000$ . (D)  $\times 132,000$ . (E)  $\times 160,000$ . (F)  $\times 120,000$ . (G)  $\times 172,000$ .



of the length of the central microtubules and that often appear superimposed to form a 6.5-nm diameter sheath wound about the central microtubule. A "bare zone" was routinely observed between the end of the 6.5-nm central microtubule projections and the 2.3-nm filament. Although the length of the bare zone varied somewhat with the extent of detergent treatment, the 6.5-nm projections terminated, on the average, 550 nm from the distal tips of the central microtubules. The length of the bare zone varied from 100 to 1,000 nm.

#### *Distal Tips of the Outer Doublet Microtubules*

In addition to the central microtubule cap, the tips of the A-tubules of the outer doublet microtubules appeared to end in short filaments (*distal filaments*; Fig. 3A). These filaments were generally paired, with two at the end of each A-tubule, and were approximately 4.2 nm in diameter and approximately 95 nm long. Preliminary results (Fig. 3B, C) suggest that the filaments form part of a pluglike structure which is inserted into the distal tip of the A-tubule of each of the outer doublets. Filaments were never observed on either of the central microtubules, or on the B-tubules of the outer doublets.

#### *Analysis of the Axonemal Tip Structures during Flagellar Elongation and Shortening*

Earlier work had shown that *Chlamydomonas* flagella and flagellar microtubules assembled primarily by the addition of subunits to the distal tips during flagellar regeneration (21, 22, 34) and that flagellar resorption probably occurred by the disassembly of the microtubules from the tip (14). In addition, brain tubulin was shown to assemble preferentially onto the distal ends of pieces of

flagellar axonemes in vitro (1, 2). Because of these observations, it was of interest to observe the fate of the flagellar tip structures described in this report during flagellar regeneration and resorption.

As shown in Table I, the caps were observed on the ends of the central pair microtubules with essentially equal frequency before resorption (96% of the central pair microtubules were capped) and during resorption (an average of 82% of the central microtubules were capped). Similarly, when the flagella were examined during regeneration (Table II), an average of 93% of the central microtubules were capped. In both the resorption and regeneration experiments, there was no trend toward a greater or lesser frequency of caps at different stages in elongation or shortening. These results show, therefore, that the caps on the central microtubules were present during all stages of flagellar assembly and disassembly. Furthermore, when *Chlamydomonas* cells were deflagellated and the flagella observed during regeneration, all of the central microtubules had caps attached to their distal tips (data not presented here). Inasmuch as the caps were removed

TABLE I  
*Analysis of the Central Microtubule Cap during Flagellar Resorption*

Time during resorption	Flagellar length	Central microtubules with caps	Central microtubules without caps
min	$\mu\text{m}$		
0	13	25	1
120-140	5	18	5
180-215	2.5	10	1

*Chlamydomonas* were placed on grids at different times during flagellar resorption, the flagellar membranes removed as described in Materials and Methods, and the number of central pair microtubules with or without caps assessed.

FIGURE 3 Distal tips of the outer doublet microtubules after detergent treatment of the flagellum. (A) Distal filaments (arrows) attached to the distal tips of A-tubule (*a*) of each outer doublet; B-tubule is indicated by *b*.  $\times 120,000$ . (B) Distal tips of the outer doublets of regenerating flagella. Distal filaments (large arrows) project from a pluglike structure which lies along the protofilament sheets. The plug is partially inserted into the A-tubule of one of the outer doublets (small arrow).  $\times 120,000$ . (C) Distal tip of a regenerating flagellum. Sheets of protofilaments (*S*, see also Fig. 3B) extend distally from the ends of each A-tubule of each outer doublet. In contrast, the central microtubule cap remains intact and appears firmly attached to the central microtubules. Distal filaments (large arrowheads) can be seen to lie in association with the protofilament sheets. Flagellar membrane fragments are observed in the background.  $\times 100,000$ .

TABLE II  
Analysis of the Central Microtubule Cap during  
Flagellar Regeneration

Flagellar length $\mu\text{m}$	Central microtubules with caps	Central microtubules without caps
2-4	26	4
5-7	50	1
8-11	10	1

*Chlamydomonas* with resorbed flagella were placed in fresh medium to permit flagellar regeneration, and the number of central pair microtubules with or without caps was determined at different times during regeneration.

when the flagella were amputated and because the caps were present as the flagella regenerated, the caps must have been one of the first flagellar structures to be assembled.

Observations were also made on the outer doublet distal filaments during flagellar resorption and regeneration. As shown in Fig. 3, the distal tips of the A-tubules of the outer doublet microtubules ended with either distal filaments (Fig. 3A), sheets of protofilaments similar to those observed on the growing ends of neurotubules (Figs. 3B, C; 7, 10, 15, 16, 27) or, occasionally, squared-off ends. The quantitation of these outer doublet tip structures at different stages during flagellar resorption and regeneration, as compared to controls which were not induced to regenerate or resorb their flagella, is shown in Table III: 89% of the A-tubules of the outer doublets of "full-grown" flagella<sup>2</sup> have distal filaments. By contrast, during flagellar regeneration there is a substantial increase in the percentage of A-tubules that terminate in sheets of protofilaments (ca. 70%), whereas during flagellar resorption the A-tubule tips tend to remain similar to the tips of fully grown flagella. To minimize the possibility that the protofilament sheets observed during flagellar regeneration were created by harsh treatment of the outer doublets (see references 17 and 32), the only outer doublets quantitated were ones from flagella in which the highly detergent-sensitive central microtubule cap was intact. In all cases (full-grown, resorbing, and regenerating), a minority of the A-tubules had even or squared-off ends. The results show that the A-tubules of the outer doublet microtubules, which ended in distal filaments in full-grown and resorbing flagella,

<sup>2</sup> The "full-grown" flagella of these vegetative *Chlamydomonas* cells grow very slowly during interphase (22).

ended predominantly in sheets of protofilaments during flagellar regeneration; these protofilament sheets are also characteristic of neurotubules which are elongating in vitro (7, 10, 15, 16, 27). It is important to note that 22% of the regenerating outer doublets had distal filaments attached. This result suggests that the filaments were present throughout flagellar elongation but that they are more loosely associated with the ends of growing outer doublets than with the ends of fully grown outer doublets.

#### *In Vitro Assembly of Brain Tubulin onto Flagellar Microtubules*

The observations of the terminal structures of the axonemal microtubules during flagellar regeneration and resorption suggested that during flagellar growth in vivo (a) central microtubule assembly was occurring either by the distal insertion of tubulin subunits under the central microtubule cap or by the addition of subunits to the proximal ends of the central microtubules, above the basal body and that (b) the outer doublet microtubules were probably being assembled by the distal addition of subunits as had already been suggested by the autoradiographic experiments of others (21, 22, 34). It had been demonstrated earlier that brain tubulin could be assembled onto pieces of flagellar microtubules, and that this assembly occurred primarily onto the distal ends of both central and outer doublet microtubules (1, 2). Therefore, experiments were carried out to determine whether neurotubules would assemble onto the distal ends of central microtubules if the central microtubule cap

TABLE III  
Analysis of the Distal Tips of Flagellar Outer Doublet Microtubules during Flagellar Elongation and Shortening

	Filaments	Sheets	Even ends
No treatment	82 (89%)	1 (1%)	9 (10%)
Resorption	15 (45%)	12 (36%)	6 (18%)
Regeneration	27 (22%)	88 (70%)	10 (8%)

The distal ends of outer doublet microtubules from untreated *Chlamydomonas* and from cells with resorbing or regenerating flagella were analyzed for the presence of terminal structures. In an effort to control for the effect of detergent treatment (see Results), outer doublet microtubules were scored only on axonemes whose central microtubule cap was still intact because the cap was the terminal structure most sensitive to removal by detergent treatment.



were still present as well as onto the ends of the A-tubules of the outer doublets if the distal filaments were present. The results (Fig. 4, Table IV) show that the presence of the central microtubule cap blocked the assembly of brain tubulin onto the distal ends of the central microtubules, while at the same time the neurotubules assembled readily

onto the A-tubules of the outer doublets. Moreover, in certain preparations in which the central microtubule cap had been lost during detergent treatment of the flagella, the distal ends of the central microtubules became competent to nucleate the assembly of neurotubules.

Finally, in a few cases, only part of the central

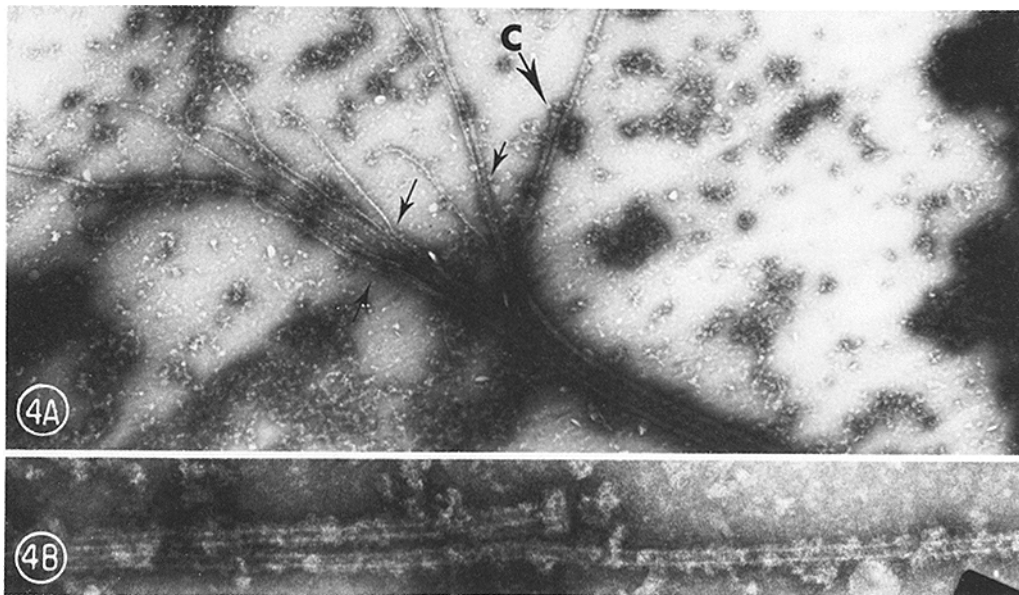


FIGURE 4 Detergent-treated flagellum incubated with brain tubulin. (A) Neurotubules are assembled onto the ends of each of the outer doublet microtubules (arrows) whereas no neurotubules are assembled onto the capped central microtubule (C).  $\times 24,000$ . (B) Higher magnification of region in Fig. 4A. The central microtubule cap remains attached to one central microtubule and blocks neurotubule assembly whereas the uncapped central microtubule nucleates a neurotubule.  $\times 52,000$ .

TABLE IV

*Relationship between the Presence of Distal Terminal Structures on the Flagellar Microtubules and the Assembly of Neurotubules onto the Distal Ends of the Flagellar Microtubules*

Condition of the distal ends of the flagellar axoneme	Number of distal axoneme ends observed in the described condition	Number of distal axoneme ends with assembled neurotubules	Number of distal axoneme ends without assembled neurotubules
Outer doublet microtubules, terminal filaments present	21	21	0
Central microtubules, central microtubule cap present	11	0	11
Central microtubules, central microtubule cap absent	6	6	0
Central microtubules, central microtubule cap present on one microtubule and absent on the other	4	All four had one central microtubule with assembly of a neurotubule and one central microtubule without assembly of a neurotubule	

Cells were attached to grids, the flagellar membranes removed with detergent, and the grids with attached axonemes incubated with brain tubulin (see Materials and Methods for incubation conditions). The assembly of neurotubules onto both the central pair and the outer doublet microtubules was analyzed.

microtubule cap was removed so that only one of the two central microtubules was covered; in these cases, the central microtubule without the cap nucleated neurotubule assembly, whereas no assembly was detected onto the central microtubule with the cap attached (Fig. 4, Table IV). That the tubulin subunits were not inserting under the cap and assembling onto the ends of the central microtubules was indicated by the fact that the position of the cap in relation to the tips of the outer doublet microtubules remained the same during assembly of the neurotubules onto the outer doublets. Moreover, the transition between flagellar microtubules and neurotubules could be easily distinguished by means of the criteria discussed previously (2).

## DISCUSSION

### *Structures Associated with the Distal Ends of the Flagellar Microtubules*

The ultrastructure of cilia and flagella has been extensively studied but the distal ends of the central and outer doublet microtubules have not been well characterized, in part, because of the difficulty in locating the ciliary and flagellar tips in thin-sectioned material. In spite of this, there have been several reports of amorphous material at the distal ends of the central microtubules (20, 21, 23, 24), the most detailed one being that by Ringo (20), who described a caplike structure at the distal ends of *Chlamydomonas* central microtubules. He showed that the central microtubules ended in the more proximal of two plates and that the distal plate appeared to be attached to the tip of the flagellar membrane (20). The central microtubule cap described in this report from observations of negatively stained flagella is similar to that shown in Ringo's micrographs. It is possible that the bead observed at the distal tip of the central microtubules in negatively stained preparations is equivalent to the flagellar membrane tip because the more distal of the plates of the central microtubule cap is attached to the flagellar membrane, as observed in Ringo's thin sections (20). Additional evidence that the bead is derived from the flagellar membrane is its sensitivity to detergent extraction and the occasional observation, in preparations in which the flagellar membrane was only partially dissolved, that pieces of the membrane are continuous with the bead. Recently, a cap has been observed to be attached to the central pair micro-

tubules in *Tetrahymena pyriformis* cilia.<sup>3</sup> In isolated, detergent-extracted, and negatively stained cilia, the cap appears virtually identical to that reported here for *Chlamydomonas*. When viewed in thin section,<sup>4</sup> however, the bead appears to be a distinct structure which is more tightly bound to the distal plate of the central microtubule cap and less tightly bound to the ciliary membrane. Although the nature of the bead is, at present, incompletely defined, it is clear that the central microtubules are bound to the ciliary and flagellar membranes by the cap. The cap is, therefore, one of the few structures that have been shown to attach microtubules directly to cell membranes. It is of interest to note that the nuclear membranes of the fungus *Uromyces* contain a structure composed of several dense plates (called the nucleus-associated organelle) from which the microtubules of the mitotic spindle project (12). It will be interesting to determine whether other microtubules that appear to terminate at membranes are attached by similar structures (for example, in the nuclear membrane plaques of other fungi [see reference 11] and yeast [19]).

Ringo's thin sections also showed, in addition to the central microtubule cap, dense material that appeared to join the central microtubules to each other near their distal tips. In the negatively stained preparations described here, this dense material was not evident, but the central microtubules did appear to be very closely associated with each other near their tips in contrast to the region more proximal from the tips (ca. 100 nm) where the central microtubules separated. The 2.3-nm filament which is attached to the central microtubule cap and which appears to wind around the distal portions of the two central microtubules may bind the two central microtubules together near their tips. Alternatively, the filament may be composed of projections from the dense material shown in Ringo's micrographs which, when viewed from certain angles, appear to form a filament. This may be similar to the "helical filament" that appears to wind around the central microtubules; when closely examined, this "filament" is found to be composed of discrete projections attached to the central microtubules (5, 32).

The negatively stained preparations also showed a bare zone along the central microtubules in which no central sheath was apparent. This bare

<sup>3</sup> W. L. Dentler, unpublished results; W. Sale and P. Satir, in press.

<sup>4</sup> W. L. Dentler, unpublished results.

zone extended for variable lengths in different preparations, but it generally ended between 100 and 1,000 nm from the distal tip of the central microtubules. The apparent central sheath filament (see references 5 and 31) is approximately three times the diameter of the 2.3-nm filament attached to the central microtubule cap; the two filaments are, therefore, distinct and not continuous with each other.

The A-tubules of the outer doublets terminated in a pair of filaments, which we call the distal filaments. The filaments appear to be continuous with a pluglike structure that is inserted into the tips of the A-tubules of the outer doublet microtubules. Other data<sup>5</sup> have shown that these plugs and distal filaments are present in *T. pyriformis* cilia and that they may attach the distal tips of the A-tubules of outer doublets to the ciliary membrane. Although the role of these structures in flagellar microtubule assembly is unknown, they appear to be associated with the distal tips of growing as well as fully grown outer doublets (Table III), although they are observed less frequently in the growing microtubules. Inasmuch as the growing ends of the outer doublet microtubules are fragile and often appear as sheets of protofilaments, the plugs and filaments may only be loosely associated with them and are released from the microtubules by the detergent treatment; by contrast, the plugs and filaments attached to fully grown microtubules appear to be more resistant to detergent treatment and, therefore, may be more tightly associated with them.

#### *The Relationship of Terminal Microtubule Structures to Flagellar Microtubule Assembly and Disassembly*

Because earlier studies clearly demonstrated that most flagellar microtubule assembly during flagellar regeneration in vivo occurred by addition of subunits to the distal tip (21, 22, 34), and because in vitro assembly studies showed that brain tubulin assembled primarily onto the distal ends of the central and outer doublet microtubules (1, 2), it was surprising to find that the central microtubule cap was present throughout flagellar resorption and regeneration. These results suggested that the central pair microtubules might assemble by the addition of subunits to their proximal ends. This suggestion is not unreasonable because the pulse-labeling studies of Rosen-

<sup>5</sup> W. L. Dentler, unpublished results.

baum and Child (21) showed that up to 35% of newly incorporated flagellar protein was assembled onto the proximal portions of regenerating flagella. This proximal label could have been due, in part, to the proximal assembly of the central microtubules. Additional support for this possibility is the result showing that the presence of the central microtubule cap blocks the assembly of brain tubulin subunits onto the distal ends of the central microtubules.

In contrast to the central microtubules, the A-tubules of the outer doublet microtubules were capable of nucleating the assembly of brain tubulin. This indicates either that the plugs and filaments were not blocking the assembly of neurotubules or that the plugs and distal filaments were extracted during polymerization of the neurotubules. Further experiments are being carried out to determine the fate of the plugs and distal filaments during neurotubule assembly onto outer doublets.

#### *Significance of the Terminal Microtubule Structures in Relation to the Directionality of Flagellar Microtubule Assembly*

It seems quite clear at this time that the preferred directionality of assembly of both the outer doublet and central microtubules of the flagellum is in the proximal to distal direction because in vitro studies have shown that the rates at which neurotubules assemble onto the distal ends of both outer doublet and central microtubules are five to eight times greater than the rates of their assembly onto the proximal ends (2). The results reported here showed that the neurotubules assembled onto the distal ends of both outer doublet and central microtubules unless the central microtubule cap was present, in which case assembly occurred only onto the outer doublet microtubules. Inasmuch as the central microtubule cap was present during flagellar regeneration, this strongly suggests that the central microtubules assemble in vivo from their nonfavored or proximal ends. These proximal ends usually are observed to terminate in or slightly distal to the basal cup in the transition region between the basal body and flagellum in *Chlamydomonas* (20) and therefore appear as free ends in thin-sectioned material. In cilia and flagella of other cells, the central microtubules terminate in the transition region at the axostome, a dense amorphous granule (9).

More direct evidence for the proximal assembly of the central microtubules could be obtained by incubating flagellar axonemes in high concentrations of brain tubulin and determining whether neurotubules assembled principally onto the distal ends of the outer doublets and only onto the proximal ends of the central microtubules if their distal ends were blocked by the central microtubule cap. Although this was attempted, conditions sufficient to fray the axonemes to expose the proximal ends of the central microtubules removed the central microtubule caps at the distal ends.

Because both the central microtubules and the central microtubule cap were attached to the Formvar film in the studies in which brain tubulin was assembled onto the flagellar microtubules, it is possible that the cap blocked neurotubule assembly only because it was held in place on the grid and was not free to move at the distal end of an assembling microtubule as might occur in vivo. In attempts to obtain central microtubules and associated caps that were not attached to grids, either flagella were lysed on the air-water interface of a solution (4) which contained a low concentration of detergent and brain tubulin, or flagella were isolated, demembrated, and then incubated with brain tubulin. Unfortunately, in both types of experiments the caps were removed by the treatments.

Other methods are currently being used to approach this problem. Paralyzed mutants (pf) which lack the two central microtubules can be mated with wild-type cells to form a dikaryon which possesses four flagella, two motile and two paralyzed (30). In certain of these matings, the two paralyzed flagella of the dikaryon regain their motility (30) and their central microtubules<sup>6</sup> within minutes of dikaryon formation without resorption or regeneration of flagella. Because of this, it is possible to study the directionality of the assembly of the central microtubules independently of the outer doublet microtubules. Preliminary experiments have already shown that a pf mutant that lacks the two central microtubules still contains a central microtubule cap on the tip of the flagellar membrane and that, as the central microtubules are assembled after fusion with the wild type, they are associated with the central microtubule cap. Further studies on the directionality of this assembly are in progress.

The possibility that the central microtubules of the flagellum assemble by proximal addition of

<sup>6</sup> W. L. Dentler, unpublished results.

tubulin while the outer doublets assemble by distal addition presents some interesting problems concerning the mechanism of flagellar microtubule assembly in vivo. For example, it is known that the flagellum beats normally during regeneration and that the central microtubules are necessary for this beating. Therefore, both the central and outer doublet microtubules must elongate at nearly the same rate. This may require that the tubulin concentration at the proximal growing ends of the central microtubules be at least five to eight times higher than the tubulin concentration at the growing distal tips of the outer doublet microtubules, based on the difference in rates of assembly of neurotubules in vitro onto the proximal and distal ends of flagellar microtubules (2). Because the site of synthesis of tubulin is in the cytoplasm and because tubulin presumably must be transported to assembly sites on the flagellar axonemes, it is possible that the local concentration of tubulin is much higher near the proximal portion of the axoneme than near the distal tip. In this regard, it will be interesting to determine whether the basal cup or axostome contains a high concentration of tubulin. An alternate possibility is that microtubule-associated proteins similar to those shown to effect the rate of neurotubule assembly in vitro (28) are also present in vivo and that some of these serve to promote the assembly of the central microtubules in the proximal or nonfavored direction.

Finally, it should be mentioned that the possible reversed directionality of assembly of the flagellar outer doublet and central microtubules in vivo is probably not related to the control of flagellar elongation because mutants that lack the two central microtubules regenerate by kinetics similar to those of the wild-type cells that have central microtubules. Furthermore, both mutant and wild-type cells elongate to the same final length (22).

This research was supported by National Institutes of Health grants NS 10907 and GM 14642 (J. L. Rosenbaum) and National Institutes of Health grants GM 05178 and RR 0737 (W. L. Dentler).

Received for publication 15 December 1976, and in revised form 5 May 1977.

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