Release of Intact Microtubule-capping Structures from *Tetrahymena* Cilia

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Abstract. The distal ends of ciliary microtubules are attached to the membrane by microtubule-capping structures. The capping structures are located at the sites of tubulin addition and loss in vivo and may be part of the regulatory system that directs ciliary and flagellar microtubule assembly. This study describes conditions for the release and stabilization of microtubule capping structures as a first step in their purification. Two types of capping structures, the distal filaments and the central microtubule caps, are selectively and independently released from the axoneme by $CaCl_2$ and $MgCl_2$ but not by $MgSO_4$, $ZnCl_2$, NaCl, KCl, or KI. The release of the caps and filaments is specific for Ca^{+2}, Mg^{+2}, and Cl^- and is not simply a function of ionic strength. The capping structures are released without major disruption of the axonemal structure. In addition to providing a means to purify and identify the cap and filament components, these results suggest ways in which their binding to the axoneme may be modulated during periods of microtubule growth or shortening. This report also reveals that the distal filaments are composed of two separable components, a small bead inserted into the end of each A-tubule and a "Y"-shaped plug and filament that slips through the bead.

THE molecular events that occur at the ends of microtubules are likely to control the assembly and functions of microtubules in vivo. Most, if not all, microtubule assembly in vivo occurs by tubulin addition to the plus end of each microtubule. For example, during mitosis, the assembly and disassembly of the plus ends of microtubules at the kinetochore is thought to play a major role in the generation or regulation of chromosome movements (Heideman and McIntosh, 1980; Euteneur and McIntosh, 1981; Telzer and Haimo, 1981; Mitchison et al., 1986; Gorbsky et al., 1987). Microtubule assembly and disassembly also occurs at the distal tips of flagellar microtubules in vivo (Johnson and Porter, 1968; Rosenbaum et al., 1969; Witman, 1975), and in vitro assembly studies established that, similar to kinetochore microtubules, the plus ends of flagellar microtubules are at the distal tips (Binder et al., 1975; Snell et al., 1974; Allen and Borisy, 1974; Bergen and Borisy, 1980). The plus ends of each of the ciliary microtubules are capped and bound to the membrane. The distal tips of each of the A-tubules are capped by distal filaments, composed of a plug structure inserted into the microtubule lumen and filaments that link the plugs (and A-tubules) to the membrane. The central microtubules are capped by a central microtubule cap, composed of plug structures inserted into the lumen of each central microtubule and a complex structure linking the plugs (and central microtubules) to the membrane (Dentler, 1980, 1981, 1984, 1987; Dentler and LeCluyse, 1982*b*;

LeCluyse and Dentler, 1984). These capping structures are firmly attached to ciliary and flagellar microtubules throughout their assembly and disassembly in vivo (Dentler, 1980; Portman et al., 1987). Although capped microtubules are able to elongate in vivo, the caps block the addition of tubulin subunits in vitro (Dentler and Rosenbaum, 1977; Dentler and LeCluyse, 1982*a*), which suggests that there must be cytoplasmic signals that regulate the accessibility (addition or loss) of tubulin to capped microtubules in vivo.

To understand the regulatory events that occur at the ends of microtubules, we have chosen to study the microtubule-capping structures of *Tetrahymena* cilia. In this study, we have examined the solubility and stability of the ciliary microtubule caps, and report that the distal filament plugs and central microtubule caps can be selectively dissociated from the intact axoneme. These results are significant in that they reveal that the binding of the caps and filaments to the axoneme may be under different cellular controls. Moreover, they suggest methods by which structurally intact caps and filaments can be purified and functionally analyzed.

Materials and Methods

Isolation of Axonemes

Ciliary axonemes were isolated from *Tetrahymena thermophila*, a non-mucous-secreting clone (SB911) of strain b (formerly *T. pyriformis* syngen 1) (Orias et al., 1983), by the dibucaine method (Thompson et al., 1974) as described below. For large quantities of cilia, 9–12 liters of cells were grown at room temperature to stationary phase in 2% (wt/vol) protease pep-

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The Journal of Cell Biology, Volume 107, (No. 6, Pt. 1) Dec. 1988 2259–2269   2259

tone containing 0.1 mM FeCl₃ and 0.0025% (wt/vol) penicillin-streptomy-cin. Approximately 150 g (wet wt) of cells were harvested, washed twice, and concentrated in 400 ml of 2% (wt/vol) proteose peptone by filtration in a Pellicon unit (Millipore Continental Water Systems, Bedford, MA). The concentrated cells were transferred to a 1-liter erlenmeyer flask and were deciliated by vigorous swirling in the presence of 1 mg/ml dibucaine. The cells were diluted fourfold with 2% proteose peptone and the deciliated cell bodies were pelleted by centrifugation at 4,420 g_{max} (5,000 rpm; JA 10 rotor; Beckman Instruments, Inc., Palo Alto, CA) for 7 min at 4°C (all sub-sequent steps are at 4°C). The cilia remaining in the supernatant fluids were pelleted by centrifugation at 17,700 g_{max} (10,000 rpm; JA 10 rotor; Beck-man Instruments, Inc.) for 35 min. The cilia were resuspended in 100 ml of cilia wash buffer ([CWB][1] 50 mM Pipes, pH 7.10 with KOH, 3 mM MgSO₄, 0.1 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid, 250 mM sucrose, 1 mM dithiolthreitol, and 1 mM phenyl-methylsulfonyl fluoride) and pelleted by centrifugation at 17,400 g_{max} (12,000 rpm; JA 20 rotor; Beckman Instruments, Inc.) for 10 min. The cilia were resuspended and centrifuged (usually twice more) until the preparation was judged to be >99% cilia, as observed by phase microscopy. The ciliary membrane was dissociated at 4°C for 20 min with 1% NP-40 in CWB. The demembranated axonemes were washed twice in CWB by centrifugation at 17,400 g for 10 min. The final preparation of axonemes was resuspended in CWB at a final concentration of 1 mg/ml, determined by the Coomassie Blue dye-binding assay (Bradford, 1976).

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1. *Abbreviation used in this paper*: CWB, cilia wash buffer (50 mM Pipes, pH 7.10 with KOH, 3 mM MgSO₄, 0.1 mM ethylene glycol bis [β-amino-ethyl ether]-N,N,N',N'-tetraacetic acid, 250 mM sucrose, 1 mM dithiolthre-itol, and 1 mM phenylmethylsulfonyl fluoride).

Release of Caps and Filaments

The central microtubule cap and distal filaments were released from the axo-nemes by the addition of small aliquots of concentrated salt solutions (from stocks made 0.1–1.0 M in CWB). The salt concentrations and times of incu-bation are noted in the text. The concentration is reported as the total amount of salt added, not as a calculated free ion concentration. The pH of the buffer varied between 6.6 and 7.3 in the presence of added salts but the capping structures remained intact and attached to axonemes over a pH range of 6.4–7.4. The concentration of salt required for release of the caps and filaments was dependent upon the protein concentration, so the axo-nemes were always resuspended at a concentration of 1 mg/ml.

Electron Microscopy

For negative staining, each axoneme sample was applied for 30 s to a freshly glow-discharged carbon-formvar–coated grid, rinsed with two drops of dis-tilled water, followed by three drops of freshly prepared 2% uranyl acetate. Each grid was incubated on the last drop of uranyl acetate for 30 s before blotting with filter paper. For thin sectioning, pellets of axonemes were fixed, embedded, and thin sectioned for electron microscopy as previously described (Dentler et al., 1975).

The release of the caps and filaments from the axoneme was monitored in two ways. For the quantitation of the stability of caps and filaments in salt solutions with time (Tables I and II), samples were negatively stained, examined in a transmission electron microscope, and scored as either " A": intact caps and filaments are attached to the axoneme; "F": intact caps and filaments are removed from the axoneme and are lying free on the grid; or "S": caps and filaments are not found on the axoneme or on the grid and
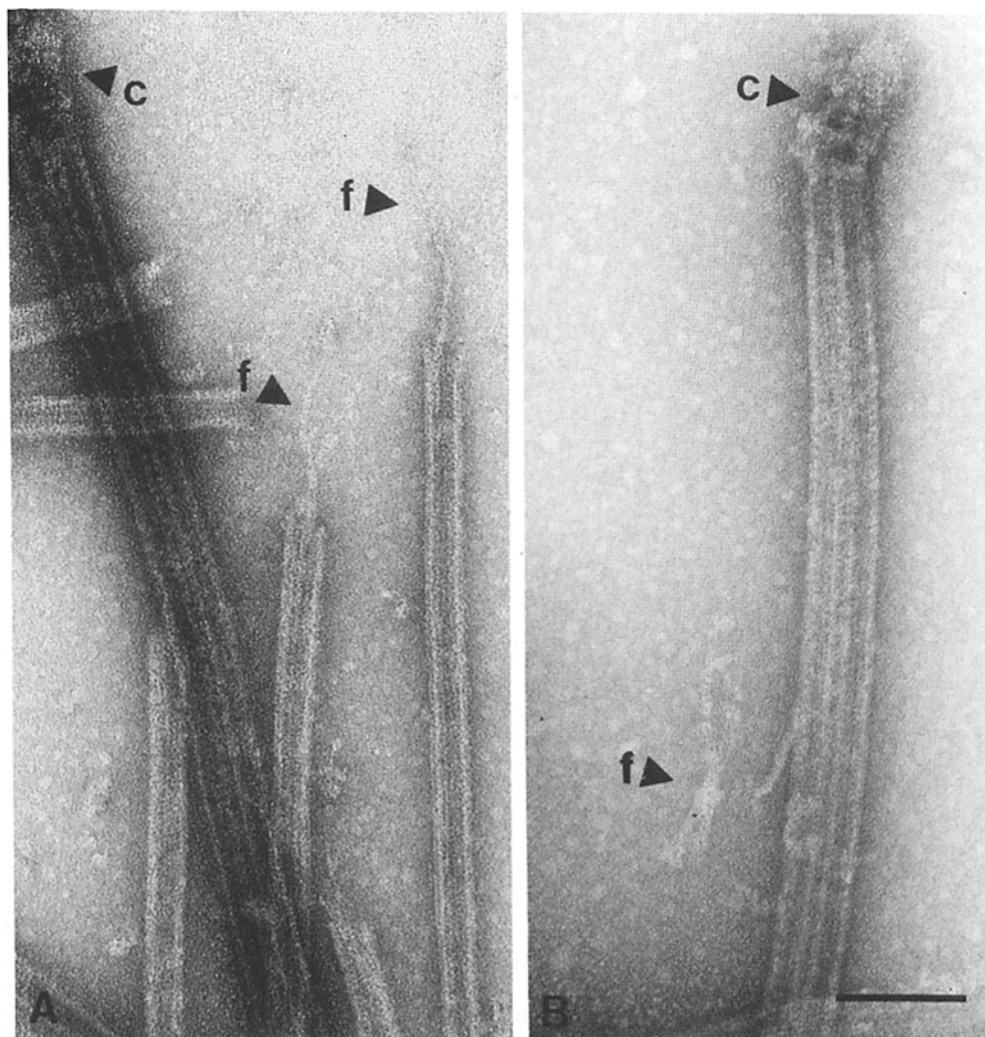
Figure 1. Structure of the dis-tal filaments and central micro-tubule caps of *Tetrahymena* axonemes. (*A*) The distal fila-ments (*f*) are composed of a pair of filaments that insert into the lumen of each A-tu-bule by a carrot-shaped plug that is surrounded by a small 9-nm-diam bead structure. When the filaments are re-leased by mechanical shear (*B*) the 9-nm-diam bead sur-rounds the paired filaments. The central microtubule cap (*c*) lies at the end of the two central microtubules and is composed of a large beadlike structure that attaches to the cytoplasmic surface of the ciliary membrane and two plate structures that attach to the plugs that insert into the lumen of each central pair microtubule. Bar, 100 nm.
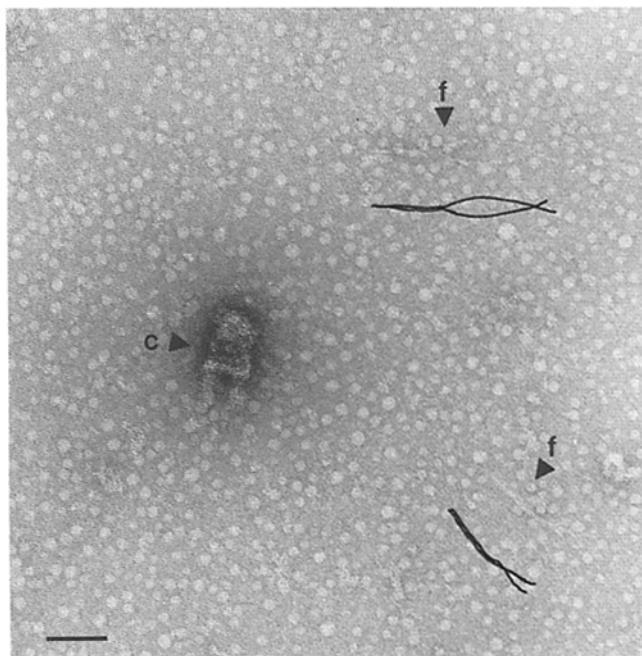
Figure 2. Comparison of the distal filaments and central microtubule caps released by MgCl₂. The central microtubule caps are structurally intact and contain a large bead, two disks, and associated plug structures that are morphologically indistinguishable from those observed on the intact axoneme. The MgCl₂-released distal filaments frequently are frayed to reveal finer filamentous structures similar to those in *Chlamydomonas* flagella (Dentler, 1980). Note that the 9-nm bead structure of each distal filament has been extracted. Bar, 100 nm.

Figure 3. High magnification montage of distal filaments released by MgCl₂. The distal filaments are composed of a tapering plug structure (*p*), two filaments (*f*), and a bead (*b*) at the junction of the filaments and the plug. When distal filaments are attached to microtubules, the bead marks the distal end of the A-tubule. The bead does not appear to be necessary to maintain the Y-shaped structure of the distal filament because filaments that have lost the bead (*D–F*) retain the characteristic Y shape. The distal filaments and the plug structure occasionally unravel to reveal a bundle of finer filaments (*D, F*). Bar, 100 nm.

are presumed to be soluble. To be placed in the A, F, or S category, >75% of the axonemes in each preparation had caps or filaments that were either attached, freed, or solubilized.

For quantitation of caps and filaments in the presence of various concentrations of different ions, axonemes were incubated in the salt solutions for 15 min at 4°C, negatively stained as described above, and scored for the presence or absence of central microtubule caps and distal filaments. Grids were coded and scored "blind" to minimize bias by the observer. Greater than 100 axonemes were observed for each time point and solution variable. The data is expressed independently for both the central microtubule cap and the distal filaments as:
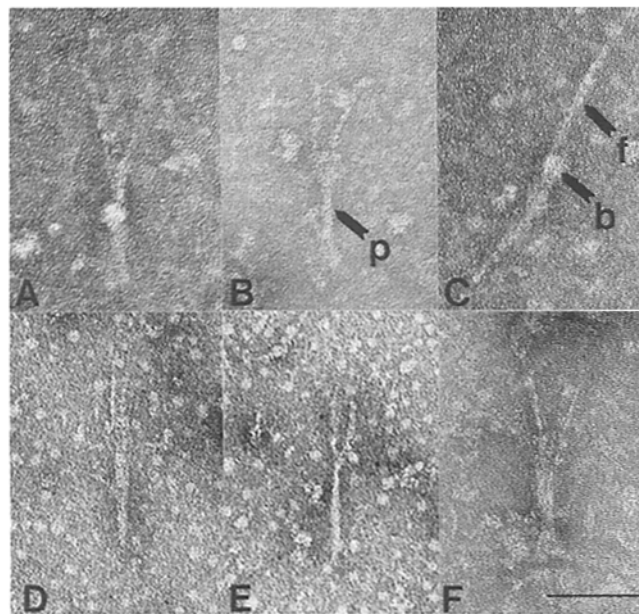
$$\frac{(n \text{ of axonemes in the presence of salt with caps or filaments}) \times 100}{n \text{ of axonemes in CWB with caps or filaments.}}$$

Results

Structure of the Central Microtubule Cap and Distal Filaments

The distal ends of the outer doublet A-tubules and the pair of central microtubules are capped by two morphologically distinct structures (Fig. 1). The central microtubule cap is composed of a 45-nm-diam spherical bead that is attached to two plate- or disk-shaped structures. The proximal plate is attached to the ends of each of the central pair microtubules by peg-shaped plugs (also see Figs. 2 and 4, and Dentler, 1984).

In contrast, the distal filaments are composed of a 70-nm-long plug structure that is inserted into the distal end of each

A-tubule and two 100–120-nm-long, 4-nm-diam filaments that link each plug and A-tubule to the membrane (Figs. 1–3). A 9-nm-diam bead is attached at the junction between the filaments and the plug (Figs. 1 and 3). When the distal filaments are firmly attached to the A-tubules, the bead is stuck into the end of the microtubule, giving a rounded appearance to the microtubule end (Dentler and Rosenbaum, 1977; Dentler, 1980). The bead is not essential to maintain the integrity of the distal filaments, since both the plug and filaments retain a characteristic Y structure with the loss of the bead (Figs. 2 and 3). The bead appears somewhat like the slip on a bolo tie and may be important for the attachment of the filaments or plugs to the ends of the microtubule.

The distal filaments are less tightly attached to the axoneme than the central microtubule cap, and can be released from some axonemes by mechanical shear (Fig. 1). The released distal filaments are morphologically similar to the intact structure, with the exception that the two distal filaments frequently splay into several smaller 1–2-nm filaments (Fig. 1).

Stability of the Caps and Distal Filaments

One approach for determining the composition and function of the capping structures is to isolate intact caps and distal filaments. A necessary prerequisite for their isolation is to understand the stability of the structures on the isolated axoneme. We initially lowered the ionic strength of the buffer to
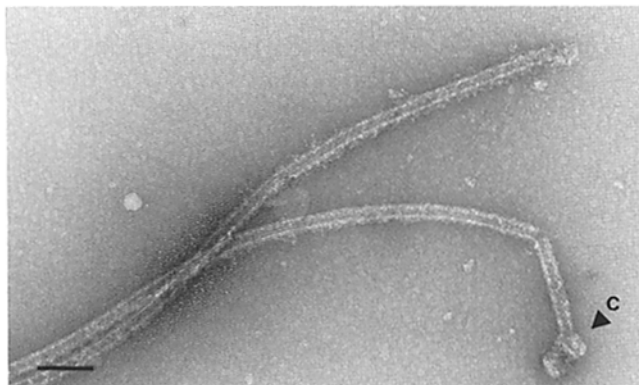
Figure 4. Stability of the central microtubule cap in low ionic strength buffers. Axonemes were dialyzed for 24 h against 1 mM Tris, 0.1 mM EDTA, and negatively stained. The central microtubule cap is stable and remained attached to those central microtubules that did not dissociate during dialysis. Bar, 100 nm.

gently dissociate the ends of the microtubules and (hopefully) release the distal filaments and central microtubule cap. However the central microtubule cap remains structurally intact even after 24-h dialysis against Tris-EDTA (Fig. 4). Dialysis also strips the axoneme of dynein arms and partially disassembles the central microtubules. In most cilia, the cap is attached to only one of the central pair microtubules but it is impossible to tell if the cap is preferentially attached to either the C1 or C2 microtubule of the central pair. Identification of distal filaments attached to A-tubules is difficult because the axonemes are partially dissociated during dialysis and washing and the distal ends of the A-tubules often are frayed. Free distal filaments disappear among the background of particulate material dissociated from the axoneme.

Since some axonemal structures can be extracted by raising the salt concentration, we suspended demembranated axonemes (that contained caps and distal filaments) in CWB to a final concentration of 1 mg/ml and added aliquots of NaCl, KCl, or $MgSO_4$ (in CWB) to the axonemes to make a final concentration of 100 mM salt. The axoneme suspension was gently mixed for 2 min on ice and negatively stained for electron microscopy. The results from these experiments are shown in Fig. 5. These concentrations and combinations of Mg^{+2}, Na^+, K^+, SO^{-4}, and Cl^- ions did not remove or solubilize the caps or distal filaments during the 2-min incubation. Both capping structures appear to be intact and are stable for short periods of time in moderate salt concentrations.

In most cases, >600 mM NaCl or KCl, or 400 mM $MgSO_4$ is needed to release the central microtubule caps. The fate of the distal filaments at these higher ionic strengths was not determined because the axonemes often were broken and it was difficult to identify the distal tips of the A-tubules.

The caps and filaments are stable and remain attached to axonemes in up to 50 mM KI and 10 mM $ZnCl_2$. Higher concentrations of KI were not used because the microtubules started to disintegrate. $ZnCl_2$ could not be used above 10 mM because it formed a precipitate in cilia wash buffer.

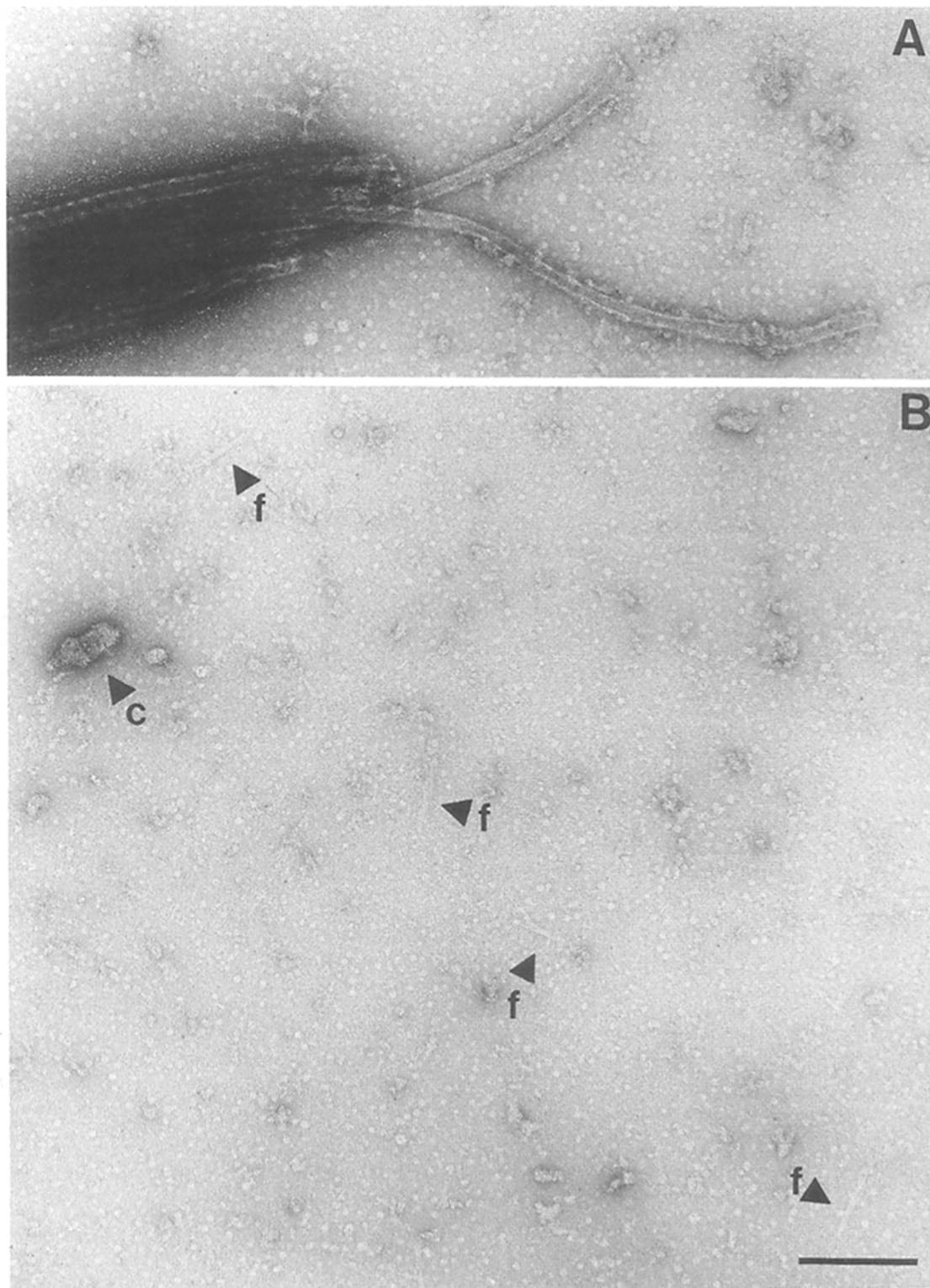### Release of the Central Microtubule Cap and Distal Filaments by $MgCl_2$

In contrast to the experiments described above, a 2-min incu-
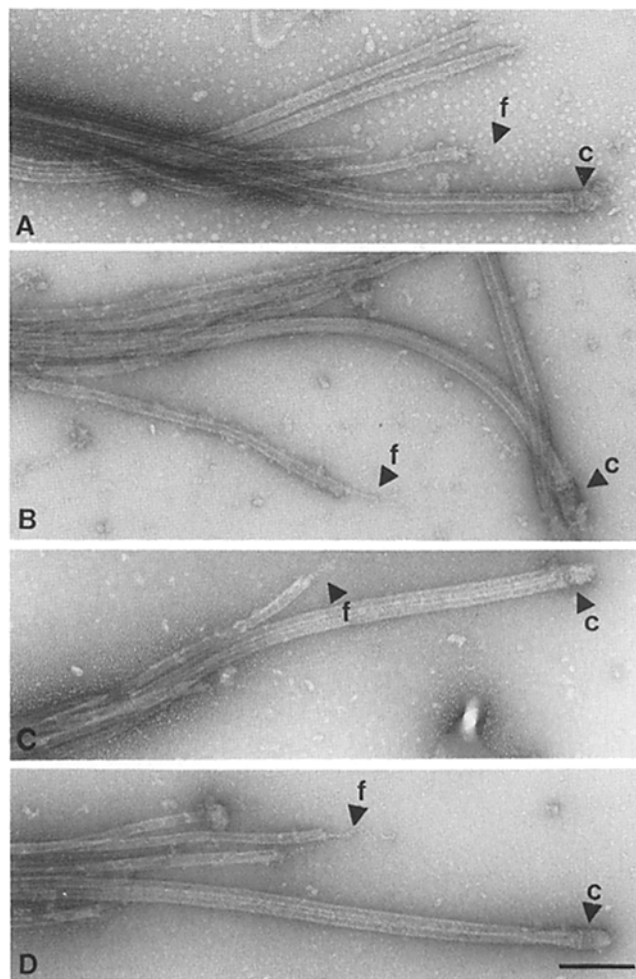
Figure 5. Stability of the caps and filaments in NaCl, KCl, and $MgSO_4$. Axonemes were incubated for 2 min with CWB (*A*), 100 mM NaCl (*B*), 100 mM KCl (*C*), or 100 mM $MgSO_4$ (*D*), and then negatively stained. Both capping structures appear to be intact and are stable for short periods of time in moderate salt concentrations. Bar, 200 nm.

bation of axonemes in the presence of 100 mM $MgCl_2$ effectively releases both central microtubule caps and distal filaments (Figs. 2, 3, and 6). To enrich for the released capping structures, the preparation was centrifuged for 10 min at 17,400 g_{max} and supernatants and resuspended pellets were negatively stained (Fig. 6). The released caps are structurally intact and morphologically indistinguishable from those observed on the intact axoneme (Figs. 2 and 6). The distal filaments are mostly intact but the 9-nm bead at junction of the plug and the filament often is lost (Figs. 2 and 3). Each of the filaments unravels into smaller filaments that either spread out in the $MgCl_2$ or are partially dissociated during specimen preparation. After a 60-min incubation in 100 mM $MgCl_2$, the distal filaments are completely solubilized and not found in the supernatants or pellets of the preparations.

After 1 h in 100 mM $MgCl_2$, the caps are partially broken into component structures (Fig. 7): the large bead, two plates, and smaller plugs are separated from each other, but remain attached by previously unobserved filaments that link the cap components together. In addition to the two smaller

Figure 6. Release of the central microtubule cap and distal filament plugs with 100 mM $MgCl_2$. Axonemes were made 100 mM in $MgCl_2$, gently mixed for 2 min, and pelleted (see text). Pelleted axonemes are free of both distal filaments and central microtubule caps (*A*), but the supernatant (*B*) contains both intact caps (*c*) and intact distal filaments (*f*). Frequently, the 9-nm bead attached to the distal filaments (Figs. 1 and 3) is lost from the filaments during this treatment. Bar, 200 nm.

plugs that insert into the lumen of the central pair microtubules, a 10-nm-diam bead that attaches to the proximal plate (see Dentler, 1984) is present. Frequently, the small bead disappears in these salt-extracted preparations, indicating that it is more labile than the two proximal plugs.

The distal filaments are more labile than the caps and are less firmly attached to axonemal microtubules. In preparations incubated in 75 mM $MgCl_2$ for 2 min, only the distal filaments are released and recovered in the supernatant (Fig. 8 *B*). Interestingly, the caps are attached to the two central
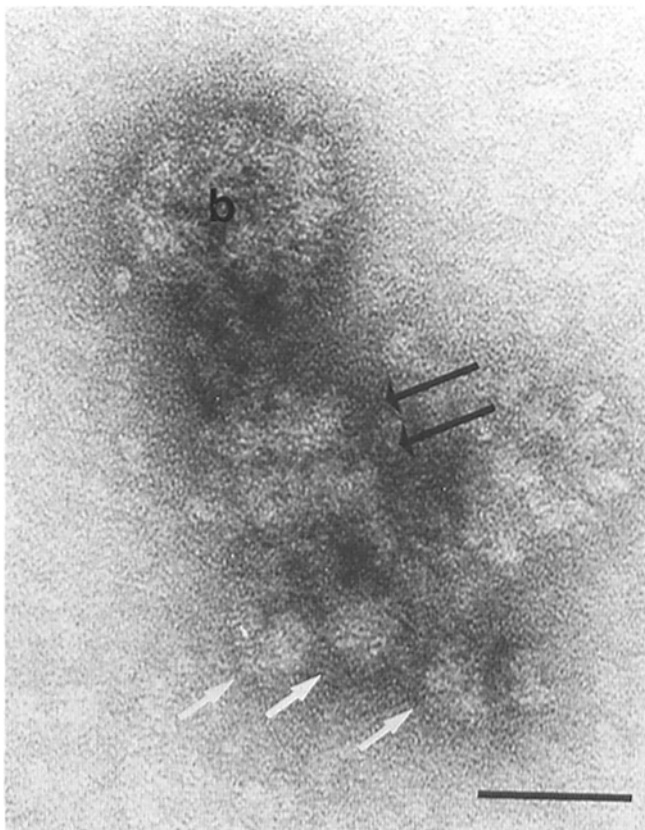
Figure 7. Extraction of the central microtubule cap in 100 mM MgCl₂ for 60 min. The large bead (*b*), two plates or disks (*black arrows*), and smaller plugs (*white arrows*) are separated from one another but are still held together by previously unobserved filaments. In addition to the two plugs that would ordinarily insert into the lumen of the central pair microtubules, a 10 nm bead is observed (*white arrows*). The cap is gradually disintegrating in the presence of MgCl₂ and the filamentous structures that link the plates and beads together can be seen to stretch somewhat. From this, as well as other micrographs, it is apparent that the cap is a complex structure presumably composed of several polypeptides. Bar, 50 nm.

pair microtubules of the intact axonemes that were pelleted by centrifugation (Fig. 8 *A*).

Release of the Central Microtubule Cap and Distal Filaments by CaCl₂ and MgCl₂

The combination of calcium and chloride ions is more effective at dissociating caps and filaments from the axoneme than a combination of magnesium and chloride ions. Axonemes were resuspended to 1 mg/ml in CWB, incubated in the various concentrations of MgCl₂ and CaCl₂, negatively stained, examined by transmission electron microscopy, and were scored for the presence of central microtubule caps and distal filaments attached to the A- and central microtubules. The results (Fig. 9) show that both types of capping structures are released with CaCl₂ and MgCl₂ but that CaCl₂ is much more effective at lower concentrations. In each case, the distal filaments are more easily dissociated from the A-tubules than the caps from the central microtubules.

Stability and Solubility of the Caps and Filaments in CaCl₂ and MgCl₂

To develop a preparative technique for isolating the caps and distal filaments, it is necessary to determine the minimum salt concentration necessary for their release and subsequent solubilization. Aliquots of the suspension were made 0–100 mM in MgCl₂ or 0–50 mM CaCl₂ and negatively stained after 2, 10, 30, or 60 min. Negatively stained samples were examined by electron microscopy and were scored for the presence of intact capping structures. The results, in Tables I and II, showed that the caps are intact and attached to the central microtubules for up to 60 min in 25 mM MgCl₂ or 1 mM CaCl₂. Intact caps are released from the microtubules within 30 min in 50 mM MgCl₂ or 10 min in 75 mM MgCl₂ and found scattered on the formvar film. The caps remain intact for up to 60 min in 100 mM MgCl₂ but are solubilized within 30 min in 25 mM CaCl₂. The distal filaments are more soluble than the caps and are released from microtubules within 10 min in 50 mM MgCl₂ or 10 min in 5 mM CaCl₂.

To verify that CaCl₂ and MgCl₂ preferentially released the capping structures and did not result in any noticeable release of other axonemal structures, axonemes were extracted for 15 min with either 10 mM CaCl₂ (to preferentially release distal filaments) or 75 mM MgCl₂ (to release both caps and distal filaments, see Fig. 9), pelleted, fixed, and sectioned. As shown in Fig. 10, extracted axonemes are virtually identical to the control axonemes (incubated in CWB) and contain dynein arms, radial spokes, and central microtubules. The CaCl₂ and MgCl₂ extraction, therefore, selectively releases the capping structures. Although this extraction might have provided supernatants of pure capping structures, examination of the supernatants by thin sections (not shown) and negative staining (Fig. 11) reveals the presence of a few detergent-resistant membrane vesicles and central microtubules in addition to the distal filaments and central microtubule caps. Further purification of the capping structures will be necessary before biochemical studies of the capping structures can be accomplished, particularly because it only takes a small quantity of central microtubule fragments to swamp the preparation with thousands of tubulin molecules. Positive identification of the individual components that comprise the capping structures must await the development and characterization of monospecific antisera and/or the stabilization of the capping structures and their purification to homogeneity.

Discussion

Previous studies from this laboratory identified two morphologically distinct microtubule-capping structures, the central microtubule caps and the distal filaments, at the distal ends of the central pair and outer doublet microtubules, in virtually every eukaryotic cilium and flagellum (reviewed in Dentler, 1981, 1987, 1989). Both the caps and the distal filaments are complex structures yet both contain similar attachment sites to the microtubules. The central microtubule caps have two stubby plugs that insert into the lumen of each of the central microtubules. Distal to the microtubules, the plugs are bound to several plate structures that are linked to each other by short pillars topped by a large sphere that is tied to the membrane by thin filaments (Dentler, 1980, 1984), giving
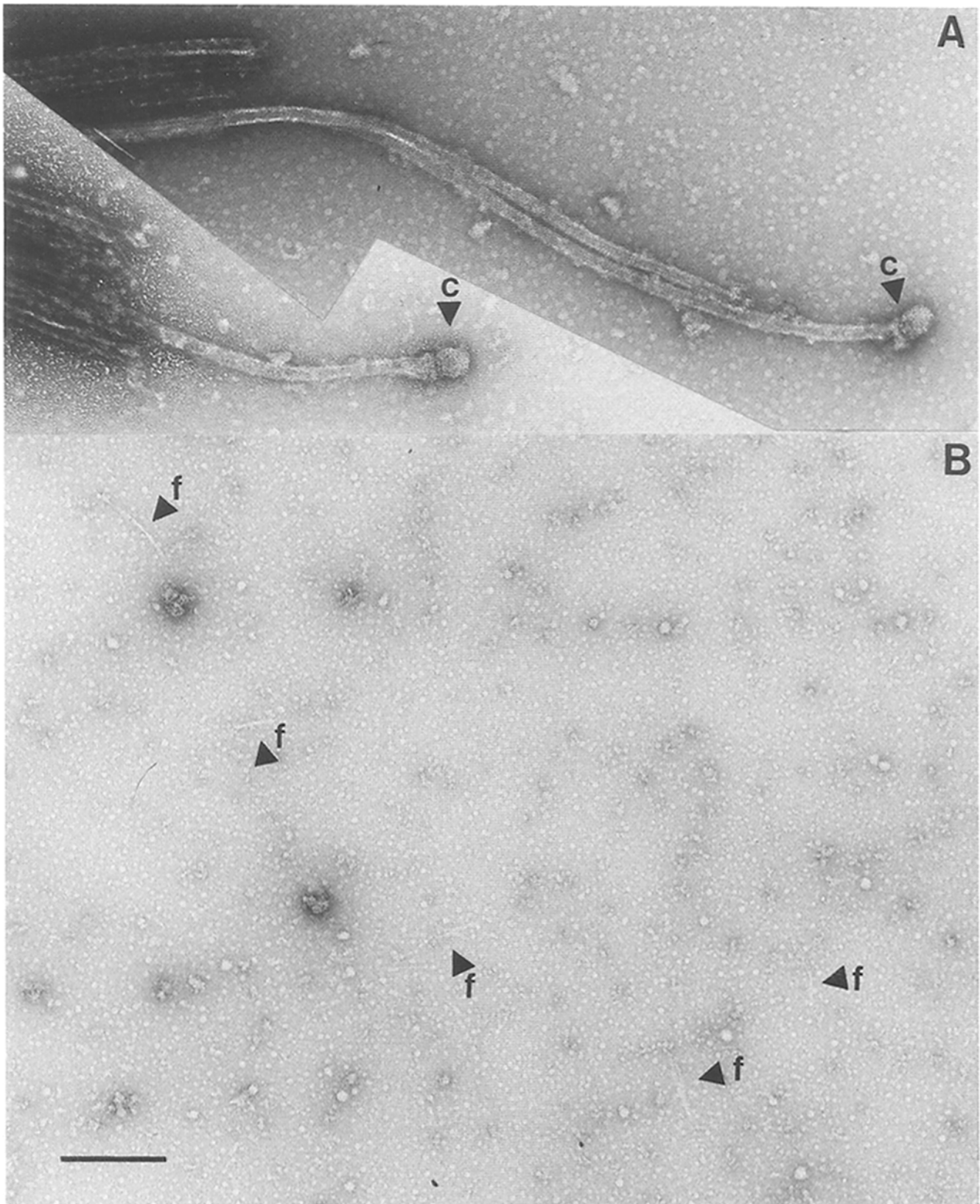
Figure 8. Release of the distal filament plugs with 75 mM MgCl₂. After a 2-min incubation in 75 mM MgCl₂, axonemes were pelleted in the microfuge (2 min at ~12,000 *g*) and the pellets (*A*) and supernatant (*B*) were examined by negative staining. Under these conditions, only the distal filaments are released from the axonemes and recovered in the supernatant fluids. The central microtubule caps remain attached to the axoneme and are pelleted. Again, the distal filaments have lost the small 9-nm bead associated with the in vivo structure. Bar, 200 nm.
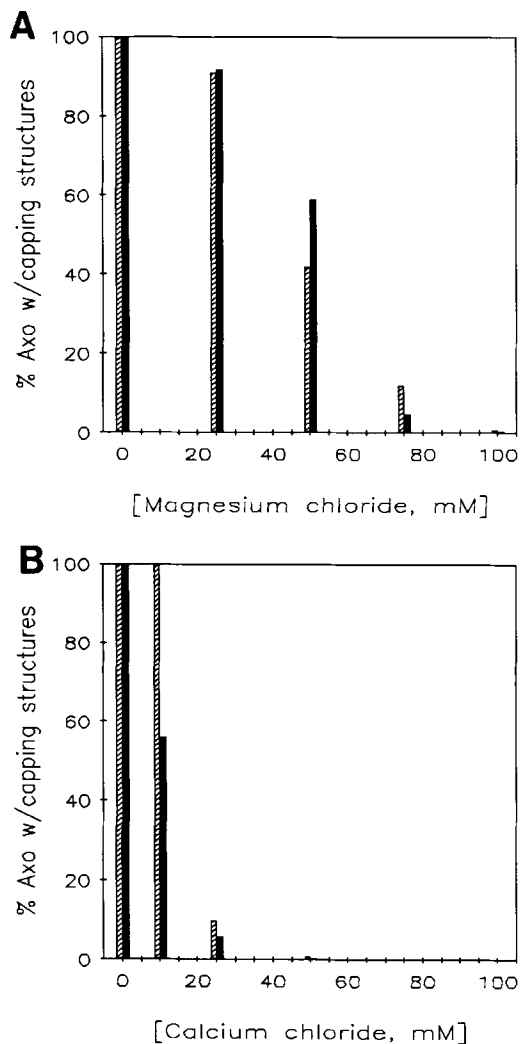
Figure 9. Release of capping structures with MgCl2 and CaCl2. For quantitation of the attachment of capping structures, aliquots of concentrated salt solutions in CWB were added to a solution of axonemes (1 mg/ml) in CWB. The axonemes were incubated on ice for 15 min and then negatively stained for electron microscopy and quantitated as described in Materials and Methods. Greater than 100 axonemes for each salt concentration were scored for the percentage of axonemes with distal filaments and central microtubule caps. The presence of central microtubules caps is indicated by hatched bars and the distal filaments by solid bars. (A) Release of the central microtubule caps and distal filaments with MgCl2. A threefold higher concentration of MgCl2 than CaCl2 was needed to remove >90% of both caps and filaments from the intact ciliary axoneme. (B) Release of the central microtubule caps and distal filaments with CaCl2. Greater than 90% of the axonemes have no caps or filaments after a 15-min incubation in 25 mM CaCl2.

the whole capping structure the appearance of something one might find on a tiered wedding cake. Similar to the central microtubule cap, the distal filaments contain a distal plug structure that is inserted into each A-tubule, although the plug is longer and more tapered than the central microtubule plugs. The filament portion of the distal filament is composed of two tapering filamentous structures that extend along the cytoplasmic face of the ciliary membrane. The fila-

Table I. Stability of Caps and Filaments in MgCl2

|	0 mM	25 mM	50 mM	75 mM	100 mM
Central microtubule cap					
2 min	A	A	A	A	F
10 min	A	A	A	F	F
30 min	A	A	F	F	F
60 min	A	A	F	F	F
Distal filament/plugs					
2 min	A	A	A	F	F
10 min	A	A	F	S	S
30 min	A	A	F	S	S
60 min	A	A	S	S	S

ments are linked to the membrane by short bridges (Dentler, 1980). Each of the filaments is composed of an array of still finer filaments (see Figs. 1 and 3) and the entire structure (plug + filament portion) is assembled into a Y, with a small bead wrapped around the junction of the filaments with the plug. The bead may be necessary to tether the distal filaments to the distal ends of the A-tubules; however, it is not necessary to hold the filaments together since free distal filaments frequently lose the bead structure but maintain their characteristic Y structure. Clearly the capping structures are complex and are likely composed of several different polypeptides.

The tight association between the capping structures and the distal ends of the flagellar microtubules must be modulated in vivo during periods of microtubule growth. The caps and filaments are dissociated from the axoneme in vitro by high salt (e.g., ∼600 mM NaCl or KCl), indicating that ionic bonds are responsible for their association with the microtubules. However, the release of the capping structures is not strictly a function of ionic strength since much lower concentrations (50-75 mM) of MgCl2 or (1-25 mM) CaCl2 will release the capping structures from the ends of the microtubules. In addition, release of the caps and filaments is dependent on the extraction time, suggesting that there may be a rate-limiting enzymatic process for release. The release of the distal filaments occurs at significantly lower CaCl2 concentration than does the release of the central microtubule caps, implying that they may be under separate control in the living cell and may respond to a different set of regulatory factors. This is not surprising, since there is abundant evidence to show that both the rate and final extent of

Table II. Stability of Caps and Filaments in CaCl2

	0 mM	1 mM	5 mM	25 mM	50 mM
Central microtubule cap					
2 min	A	A	A	A	F
10 min	A	A	A	F	F
30 min	A	A	F	S	S
60 min	A	A	S	S	S
Distal filament/plugs					
2 min	A	A	A	S	S
10 min	A	A	F	S	S
30 min	A	F	F	S	S
60 min	A	S	S	S	S
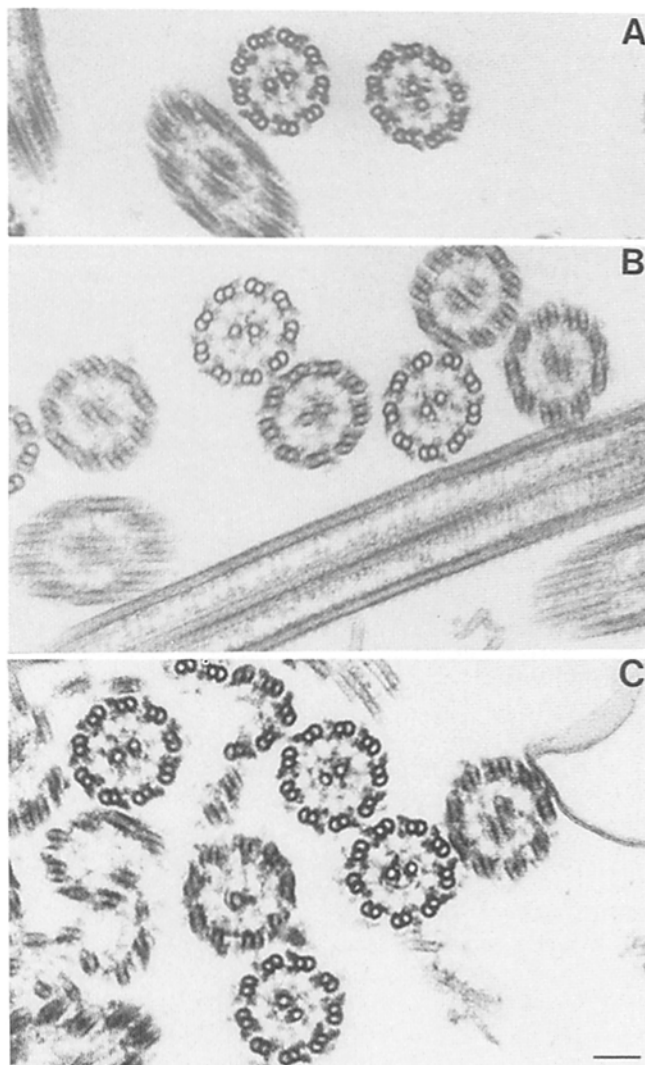
Figure 10. Structural integrity of extracted axonemes revealed in thin sections by electron microscopy. Axonemes were resuspended in 10 mM CaCl$_2$ (*A*) or 75 MgCl$_2$ (*B*) for 15 min on ice, and then pelleted and fixed for electron microscopy. Axonemes extracted with CaCl$_2$ or MgCl$_2$ contain the same percentage of dynein arms, spokes, and central tubules as do the untreated control axonemes (*C*), indicating that only the capping structures are selectively solubilized. Bar, 0.1 µM.

microtubule assembly is regulated at the level of individual central and doublet microtubules in vivo (see Dentler, 1981, 1987; Tamm and Tamm, 1988).

It is clear that many questions remain about the role of Ca^{+2}, Mg^{+2}, and Cl$^-$ in the association of the capping structures with the ends of microtubules. Because CaCl$_2$ was more effective than MgCl$_2$ at much lower concentrations, it seems likely that Ca^{+2} is specifically associated with the in vivo signal for cap and filament release. Concentrations of CaCl$_2$ as low as 1 mM were able to dissociate and solubilize the distal filaments (see Table II). The free calcium concentration at 1 mM total CaCl$_2$ is not known because we do not know to what extent the axonemes at 1 mg/ml buffered the free ion concentration. Based on the effects of Ca^{+2} on the direction and motility of extracted and reactivated cilia and flagella, the free Ca^{+2} concentration in vivo is probably

in the micromolar range (see Dentler, 1980). Micromolar free Ca^{+2} may not be able to dissociate the caps and filaments in vitro but may still play an important regulatory role in vivo. The interaction between the caps and filaments with the microtubule ends need only to be modulated during periods of ciliary growth such that tubulin dimers or small oligomers gain access to the microtubules. The caps and filaments probably stay tenaciously associated with the microtubules because the free Ca^{+2} levels are below the threshold for their complete dissociation from the microtubules.

The studies of capping structures outlined here are directed toward understanding microtubule assembly in vivo, with the expectation that the regulation of the association of ciliary caps and growing or disassembling microtubules will help our understanding of the assembly of cytoplasmic microtubules whose plus ends are associated with membranes or kinetochores. Although the caps attached to the plus ends of ciliary and flagellar microtubules are the first capping structures to be described, caps and capping proteins are not unique to these organelles. A capping structure has been found at the distal tip of a bacterial flagellum (Ikeda et al., 1985). The bacterial cap is at the site of in vivo flagellar assembly, is present during flagellar assembly, and limits the addition of flagellin to the flagellum. The bacterial flagellum (which consists of a single 10-nm tube composed of flagellin) lacks a membrane, which indicates that the cap must be tightly bound to the growing end of the bacterial flagellum. Moreover, capping proteins are associated with the ends of actin filaments and may be involved with the initiation or regulation of actin filament assembly (see reviews by Pollard and Cooper, 1986, Stossel et al., 1985) or, possibly, cytoplasmic microtubule assembly (see Khawaja et al., 1988). Taken together, these studies show that capping structures are important components of a variety of cytoskeletal structures. Careful study of their functions may reveal more about the regulation of cytoskeletal filament assembly by a variety of cell types.

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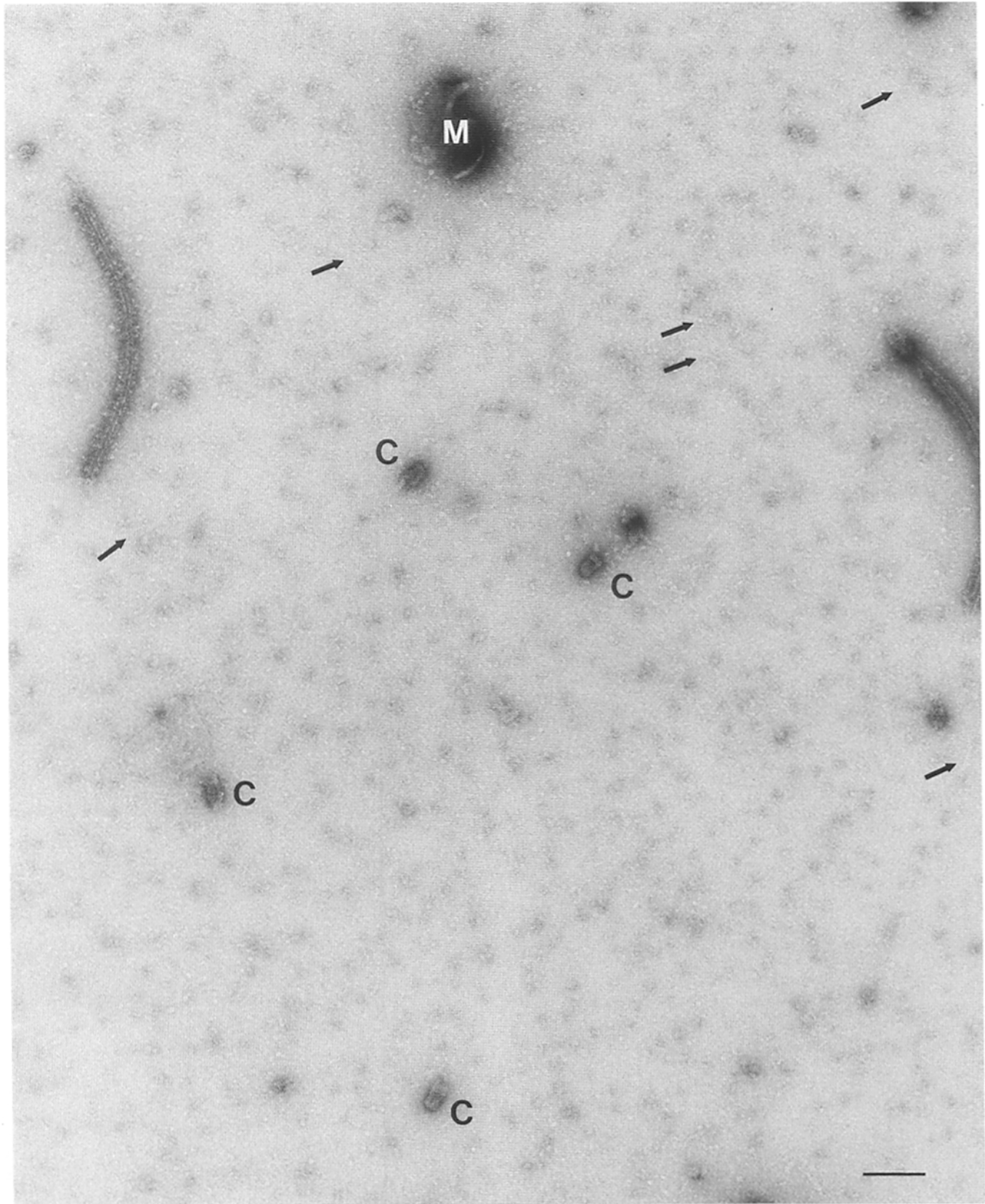
Figure 11. Composition of the MgCl₂ supernatants after release of capping structures as described in Fig. 10. Supernatants contain both central microtubule caps (*C*) and distal filaments (*arrows*) as well as fragments of central microtubules, membrane vesicles (*M*), and particulate structures presumably released from the axonemes. Bar, 0.1 μm.

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