Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5759–5763, November 1979
Biochemistry

Dynein binds to and crossbridges cytoplasmic microtubules

(tubulin/periodicity/polarity/ATP dissociation)

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Communicated by Joseph G. Gall, August 23, 1979

ABSTRACT **Dynein isolated from *Chlamydomonas* flagellar axonemes binds to microtubules assembled *in vitro* from 6S brain tubulin dimers. The dynein arms bind periodically along the length of the microtubules with a center-to-center spacing of 24 nm, equal to the periodicity of dynein arms on intact axonemes. The arms project from the *in vitro* assembled microtubules at an angle of approximately 55°, thereby defining microtubule polarity. Dynein cosediments with microtubules through a sucrose gradient, as demonstrated by electron microscopy, gel electrophoresis, and ATPase analysis. In addition, dynein induces crossbridging between adjacent microtubules. Darkfield microscopy reveals that microtubules containing dynein are aggregated into large bundles; electron microscopy indicates that microtubules of the same polarity are crossbridged by a regular array of arms. Viewed by darkfield microscopy, addition of ATP to crossbridged microtubules causes their disaggregation; electron microscopy shows that the majority of these microtubules are no longer crossbridged. These observations are applicable to the determination of microtubule polarity and directionality of microtubule assembly *in situ* and suggest a role for dynein in cytoplasmic microtubule-based cellular movements.**

Microtubules are the major structural component of many different motile systems, where they play an important role in the generation of movement. Perhaps the best characterized microtubular organelle is the axoneme of cilia and flagella in which it has been established that the dynein arms cyclically crossbridge adjacent outer doublet microtubules and hydrolyze ATP to produce sliding (1–3). Recent data have suggested a role for a dynein-like ATPase in other microtubule-dependent cell movements. For example, anaphase chromosome movements in lysed mammalian cells require ATP and are blocked by vanadate, an inhibitor of dynein ATPase activity (4, 5). Antibodies directed against sea urchin sperm flagellar dynein inhibit mitosis when injected into fertilized sea urchin eggs (6) and bind to the mitotic apparatus, as shown by immunofluorescent staining (7). An association between dynein and cytoplasmic microtubules also has been suggested by reports that flagellar fractions containing dynein stimulate brain tubulin assembly (8) and that a protein with properties similar to those of flagellar dynein can be isolated from brain microtubules (D. B. Murphy and R. R. Hiebsch, personal communication).

We report here that dynein isolated from *Chlamydomonas* flagella binds to *in vitro* assembled brain microtubules and decorates them with periodic and angular projections. Moreover, dynein induces ATP-dissociable crossbridging between adjacent microtubules. These observations demonstrate that dynein can interact directly with cytoplasmic microtubules.

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MATERIALS AND METHODS

Axoneme Isolation and Dynein Preparation. Flagella were obtained from *Chlamydomonas reinhardii*, strain 21gr, by use of dibucaine as described (9). All subsequent operations were performed at 4°C unless otherwise noted. Isolated flagella were centrifuged at 27,000 × *g* for 10 min, resuspended in 10 mM Hepes/5 mM $MgSO_4$/1 mM dithiothreitol/0.5 mM EDTA/25 mM KCl, at pH 7.4 (buffer A), containing 0.04% Nonidet P-40, and centrifuged at 12,000 × *g* for 10 min. The pellet containing the axonemes was resuspended and washed twice with buffer A. To extract dynein, we resuspended axonemes in 2–3 ml of 0.6 M NaCl/10 mM Hepes/4 mM $MgSO_4$/0.2 mM EDTA/1 mM dithiothreitol, at pH 7.4. After 15 min the axonemes were centrifuged at 27,000 × *g* for 10 min, the axonemal pellet was reextracted as described above, and the two supernatants were combined and centrifuged at 27,000 × *g* for 15 min. The resulting dynein supernatant was then desalted by centrifugation through Sephadex G-25 (10) into 50 mM 1,4-piperazinediethanesulfonic acid (Pipes)/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)/0.5 mM $MgSO_4$, at pH 6.9, and concentrated to 3–6 mg/ml with Aquacide (Calbiochem). Dynein was subsequently clarified by centrifugation at 100,000 × *g* for 1 hr and stored at 4°C until use.

Preparation of 6S Tubulin Dimers and Microtubule-Associated Proteins. Twice-cycled tubulin was prepared as described (11). To obtain 6S tubulin dimers and microtubule-associated proteins (MAPs), we loaded twice-cycled tubulin on a phosphocellulose column equilibrated in column buffer (50 mM Pipes/1 mM EGTA/0.5 mM $MgSO_4$/0.1 mM GTP, at pH 6.9) (11, 12). The void volume containing the purified 6S tubulin dimers was adjusted to 1.0 mM $MgSO_4$, concentrated to 20–25 mg/ml by ultrafiltration with an Amicon PM-30 membrane, desalted into column buffer, clarified by centrifugation at 100,000 × *g* for 1 hr, and stored as frozen droplets in liquid N_2. MAPs were eluted from the phosphocellulose column with 0.8 M NaCl in column buffer, concentrated to approximately 5 mg/ml, desalted into column buffer, clarified at 27,000 × *g* for 15 min, and stored as droplets in liquid N_2.

Microtubule Polymerization and Purification. A solution of 50 mM Pipes/2.5 mM $MgSO_4$/1.0 mM EGTA (buffer B) plus 1 mM GTP containing 6S tubulin, 6S tubulin and dynein, or 6S tubulin and MAPs was incubated at 30°C to polymerize microtubules. Assembled microtubules were obtained by centrifugation into a discontinuous sucrose gradient (0.5 ml of 40, 50, 60, and 70% and 2 ml of 80% sucrose in buffer B) at 35,000

Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MAP, microtubule-associated protein.

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× *g* for 30 min at 30°C (13). Microtubules polymerized from 6S tubulin alone banded at the 70–80% sucrose interface. Microtubules polymerized in the presence of dynein banded at the 60–70% sucrose interface, whereas those polymerized in the presence of MAPs were present throughout the 60% sucrose step. Microtubules removed from the sucrose gradient were visualized by negative staining or were diluted 1:1 with buffer B at 30°C and centrifuged at 27,000 × *g* for 20 min at 30°C prior to fixation for ultrastructural analysis or preparation for gel electrophoresis and ATPase assays.

To determine if dynein could bind to preassembled microtubules, we polymerized 12 mg of 6S tubulin per ml in buffer B containing 6 M glycerol. After 30 min, 10 μM colchicine was added to prevent additional microtubule assembly; 6 mg of dynein per ml in a volume half that of the microtubule preparation was added. The mixture was incubated at 30°C for 15–30 min prior to centrifugation into a discontinuous gradient as described.

To test the effect of ATP on dynein-crossbridged microtubules, we added 1 mM ATP, 16 mM creatine phosphate, and 0.1 mg of creatine phosphokinase per ml to a sample of polymerized microtubules.

Microscopy. Darkfield microscopy was performed as reported (13). Samples of assembled microtubules were mixed with an equal volume of 40% sucrose in buffer B at 30°C in order to decrease Brownian motion. Pellets of microtubules were prepared for thin sectioning as described (14). Material was fixed in 1% glutaraldehyde containing 1% tannic acid buffered at pH 7.0 with 10 mM sodium phosphate, followed by postfixation in 1% OsO_4 at pH 7.0. Samples to be negatively stained were fixed in 1% glutaraldehyde in buffer B and stained with neutralized 4% phosphotungstic acid in 4% sucrose (15).

Biochemical Assays. Turbidity measurements of microtubule assembly *in vitro* were performed as described (14) except that polymerizations were carried out at 30°C. ATPase activity of samples in 0.5 ml of buffer A at 20°C was measured by the one-step colorometric assay of Lin and Morales (16). Protein concentrations were determined with bovine serum albumin as a standard (17). Gel electrophoresis was performed as reported (14).

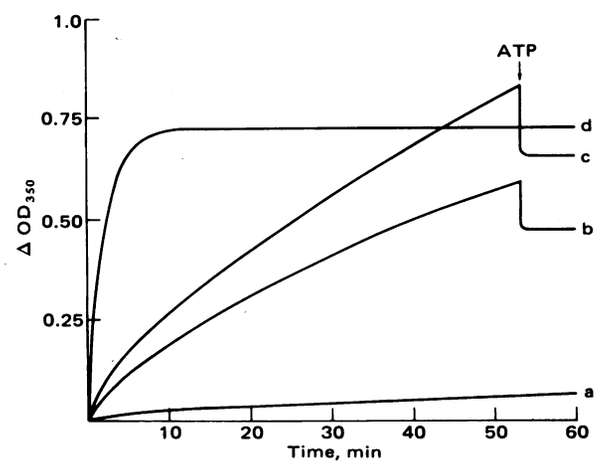

FIG. 1. Assembly of microtubules at 30°C from 5 mg of 6S tubulin per ml (a) plus 0.75 mg of dynein per ml (b), 1.5 mg of dynein per ml (c), or 0.4 mg of MAPs per ml (d). ATP (1 mM) was added to all samples at 55 min.

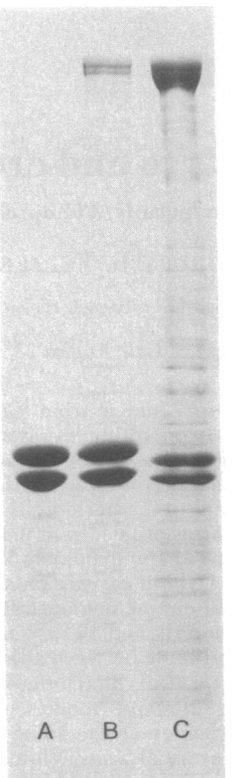

FIG. 2. Sodium dodecyl sulfate/urea gel showing cosedimentation of dynein with microtubules polymerized from 6S tubulin. Lane A, 40 μg of 6S tubulin; lane B, 40 μg of microtubules polymerized from 7 mg of 6S tubulin and 2.5 mg of dynein per ml and banded on a discontinuous sucrose gradient; lane C, 25 μg of dynein preparation.

RESULTS

The interaction of *Chlamydomonas* flagellar dynein with purified 6S brain tubulin was first studied turbidimetrically. When incubated at 30°C, 6S tubulin polymerized very slowly (Fig. 1). Addition of the dynein preparation to the 6S tubulin resulted in an increase not only of the rate (8), but also of the extent of assembly. Furthermore, dynein stimulated 6S tubulin assembly at concentrations of tubulin too low for self-assembly

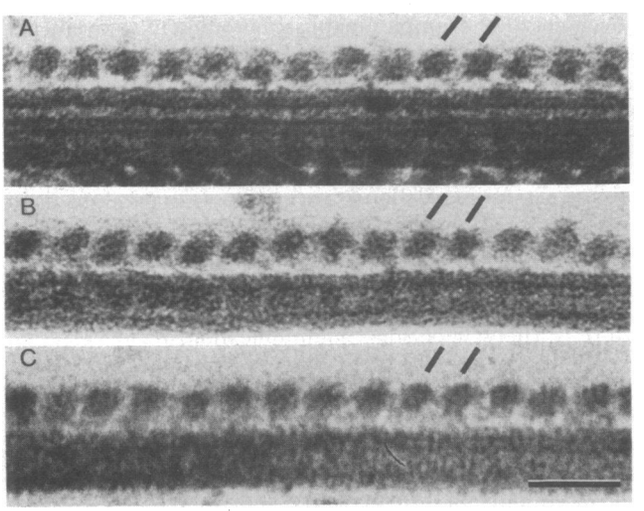

FIG. 3. Longitudinal sections of outer doublet of intact axoneme showing row of dynein arms (*A*) and of microtubules polymerized from 6S tubulin and dynein (*B* and *C*). Arms project from the microtubule at an angle of 55° and with a periodicity of 24.2 nm. Bar, 50 nm.

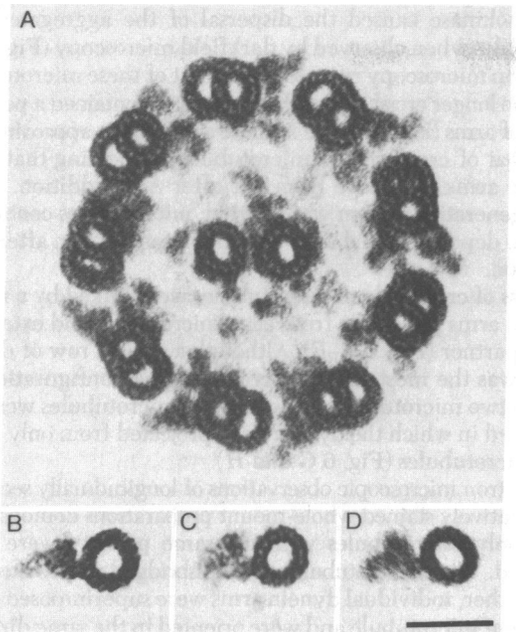

FIG. 4. Cross sections of *Chlamydomonas* axoneme (*A*) and of microtubules polymerized from 6S tubulin and dynein (*B–D*). Arms on assembled microtubules have a morphology similar to the outer row of arms on axonemes. Bar, 50 nm.

(data not shown). The stimulation of assembly was less than that observed when the MAP fraction was added, and the turbidity did not plateau during the course of the experiment. Addition of 1 mM ATP to samples of polymerized microtubules caused a rapid decrease of about 15% in the optical density of preparations containing dynein but had little effect on either microtubules polymerized from 6S tubulin alone or those polymerized in the presence of MAPs.

Polymerized microtubules containing bound dynein were separated from unbound dynein by centrifugation into a sucrose gradient. Gel electrophoresis indicated that the high molecular weight bands comprising dynein, as well as several minor bands in the dynein preparation, cosedimented with the microtubules (Fig. 2). Dynein also cosedimented with microtubules assembled prior to dynein addition, indicating that dynein could bind to intact microtubules and suggesting that tubulin present in the dynein preparation was not incorporated into these microtubules. The microtubules had an ATPase activity of 0.17 $\pm$ 0.03 μmol of P_i per min per mg, whereas microtubules assembled from 6S tubulin or 6S tubulin and MAPs had no measurable ATPase activity. For comparison, *Chlamydomonas* axonemes had an ATPase activity of 0.25 $\pm$ 0.06 μmol of P_i per min per mg.

Electron microscopic observations of microtubules polymerized in the presence of dynein revealed that dynein arms projected periodically in a linear array along the microtubules (Fig. 3 *B* and *C*). Similar observations were made when dynein was added to a preparation of preassembled microtubules. The center-to-center spacing of dynein arms of 24.2 $\pm$ 1.2 nm ($\pm$SD, $n = 255$) is equal to the periodicity of dynein arms along outer doublet microtubules of *Chlamydomonas* axonemes, as illustrated in Fig. 3*A*. Dynein arms projected from the wall of *in vitro* assembled microtubules at an angle of 54.6° $\pm$ 5° ($n = 97$), similar to the angle of arms projecting from intact axonemes. Moreover, dynein arms on a given microtubule were all oriented in the same direction, thus defining microtubule polarity. In cross sections of microtubules containing dynein, the distinctive ultrastructure of the arms could be discerned (Fig. 4 *B–D*) and resembled that of dynein present in the outer row of arms on axonemes (Fig. 4*A*). In addition, when a cross section of a microtubule contained more than one dynein arm, the arms were always pointed in the same direction (i.e., the hooks on the arms on a given microtubule pointed either clockwise or counterclockwise) (see Fig. 7 *C* and *D*). Microtubule polarity could, therefore, be determined in cross sections.

Microtubules polymerized from 6S tubulin and observed by darkfield or electron microscopy appeared as disperse, single microtubules (Fig. 5 *A* and *E*). On the other hand, microtubules polymerized in the presence of dynein appeared aggregated and regularly beaded when observed by darkfield illumination (Fig. 5 *B* and *C*), and negatively stained preparations indicated

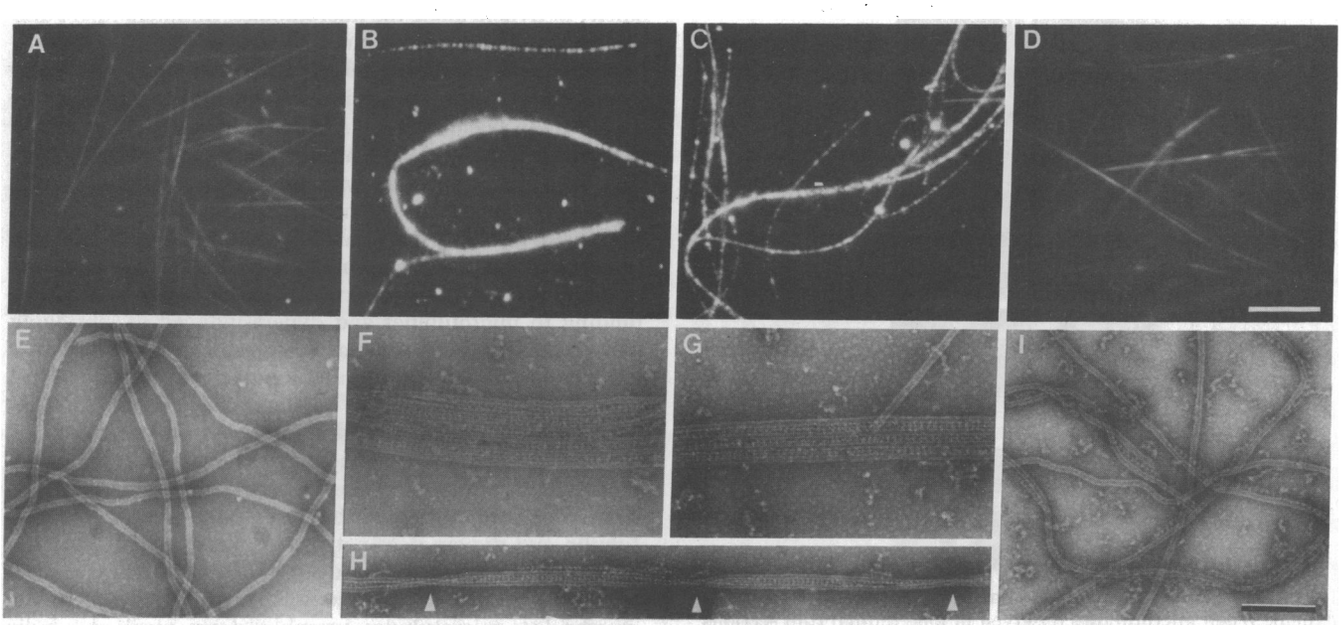

FIG. 5. Darkfield (bar, 5 μm) (*A–D*) and electron micrographs (bar, 0.25 μm) (*E–I*) of assembled microtubules. (*A* and *E*) Microtubules assembled from 6S tubulin. (*B, C,* and *F–H*) Microtubules assembled from 6S tubulin and dynein; crossbridged microtubules twist over each other (arrows, *H*). (*D* and *I*) ATP (1 mM), creatine phosphate, and creatine phosphokinase added to microtubules polymerized from 6S tubulin and dynein. Note dynein arms on microtubules in *I*.

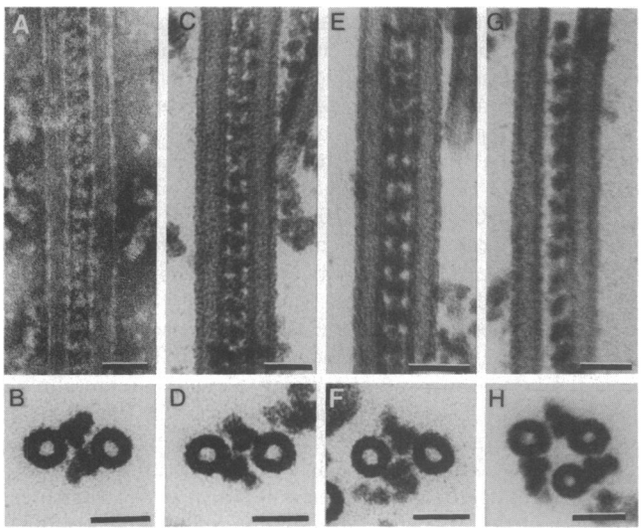

FIG. 6. Microtubules crossbridged by dynein. Negative stain (*A*) and thin sections (*B–F*) of microtubules crossbridged by arms from each microtubule. (*G* and *H*) Microtubules crossbridged by arms from only one microtubule. In *G*, the row of dynein arms originates from the microtubule on the left and crossbridges the microtubules on the right. Bar, 50 nm.

that these microtubules were bridged together in bundles of two or more microtubules (Fig. 5 *F–H*) which often twisted over each other (Fig. 5*H*). Addition of 1 mM ATP and an ATP-generating system containing creatine phosphate and creatine phosphokinase caused the dispersal of the aggregated microtubules when observed by darkfield microscopy (Fig. 5*D*). Electron microscopy revealed that most of these microtubules were no longer crossbridged, but many still contained a periodic array of arms (Fig. 5*I*). The ATPase activity was approximately 75% that of crossbridged microtubules, indicating that some dynein arms may have been lost after ATP addition. If the ATP-generating system was omitted, microtubules containing dynein depolymerized as in approximately 10 min after ATP addition.

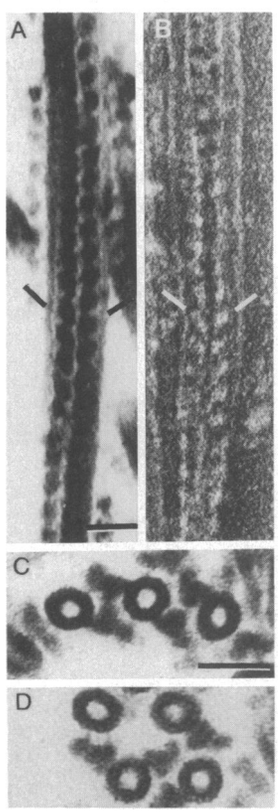

FIG. 7. Microtubules of the same polarity are crossbridged by dynein. When microtubules observed in longitudinal thin sections (*A*) and negative stains (*B*) cross over each other, the dynein arms appear oriented in the same direction. In cross sections, the dynein arms within groups of crossbridged microtubules are all oriented counterclockwise (*C*) or clockwise (*D*). Bar, 50 nm.

Pairs of crossbridged microtubules were linked by a row of dynein arms projecting from each microtubule and extending to its partner (Fig. 6 *A–F*). Although a double row of dynein arms was the most common crossbridging configuration between two microtubules, crossbridged microtubules were also observed in which the dynein arms projected from only one of the microtubules (Fig. 6 *G* and *H*).

Electron microscopic observations of longitudinally sectioned or negatively stained whole-mount preparations demonstrated that only microtubules with the same polarity were crossbridged. When microtubules of a crossbridged pair twisted over each other, individual dynein arms were superimposed on the adjacent microtubule and were oriented in the same direction (Fig. 7 *A* and *B*). That crossbridged microtubules were of the same polarity was also determined in cross sections. Dynein arms on a given group of microtubules were all oriented in the same direction (Fig. 7 *C* and *D*; also see Fig. 6 *B*, *D*, *F* and *H*).

DISCUSSION

Results of this study indicate that *Chlamydomonas* flagellar dynein specifically binds to microtubules assembled *in vitro* from brain tubulin. The periodicity of the bound dynein is 24 nm, a multiple of the 8-nm tubulin dimer and equal to the spacing of dynein along outer doublet microtubules of axonemes (9, 18–20). Because microtubules or regions along a single microtubule are either completely decorated with or devoid of dynein, it is possible that one dynein arm bound to a microtubule enhances the binding of dynein at an adjacent site. This cooperative effect may thus establish the observed periodicity of dynein along the microtubules. Although MAPs also bind to assembled microtubules, they do so with a periodicity of 32 nm (14, 21) and their helical superlattice appears different from the linear array of dynein arms observed on microtubules in this study. The fact that MAPs can bind to dynein-extracted axonemes and prevent the reattachment of dynein (22) suggests that MAPs may interfere with the cooperativity of dynein binding at adjacent sites along the microtubule.

Dynein, like MAPs, appears to stimulate both the rate and extent of microtubule assembly by lowering the concentration of tubulin required for polymerization. Because both dynein binding to microtubules and dynein-induced crossbridging between microtubules may each cause an increase in turbidity unrelated to the process of microtubule assembly, the critical concentration for assembly of tubulin in the presence of dynein cannot be determined by turbidimetric measurements.

Electron microscopy demonstrates that microtubules are crossbridged to each other along their length by rows of dynein arms. The fact that the turbidity of suspensions containing tubulin and dynein does not plateau may indicate that microtubules are being crossbridged into large bundles, as is observed by darkfield microscopy. That dynein binds to and crossbridges microtubules assembled from purified tubulin suggests that the dynein binding sites on both the A and B subfibers of axonemes may themselves be composed of tubulin rather than of accessory proteins.

Microtubules possess an inherent polarity determined by their directionality of assembly (15, 18, 23). All dynein arms on crossbridged groups of microtubules are oriented in the same direction, thereby indicating that crossbridged microtubules possess the same polarity. It is not surprising that only microtubules with the same polarity can be crossbridged by flagellar dynein because both the A and B subfibers within the axoneme possess the same polarity.

After ATP addition, the turbidity of a preparation of crossbridged microtubules decreases and aggregated microtubules disperse, as observed by both darkfield and electron microscopy. Similar results were reported with trypsin-treated *Tetrahymena* outer doublet microtubules, which aggregated after ATP depletion and dispersed upon ATP readdition (24). ATP causes some dynein arms to be lost from the *Tetrahymena* outer doublets as well as from the assembled microtubules in this study, suggesting that ATP may cause a conformational change in the dynein arm, thereby making its attachment to the microtubule less stable. It is possible, therefore, that some crossbridged microtubules separate from each other as a result of a loss of dynein arms. However, because the majority of ATP-treated microtubules still contain dynein arms but are no longer crossbridged, it is probable that ATP also causes a relaxation of dynein crossbridges. The facts that cytoplasmic microtubules can be bound and crossbridged by dynein and that the crossbridges appear to be dissociated by ATP suggest that movements, including the procession of chromosomes and pole separation during mitosis, the translocation of organelles in the axon, and the migration of melanosomes, may be mediated by a dynein-like ATPase interacting with microtubules.

By comparing the angle of dynein arms bound to microtubules in longitudinal sections or the orientation of dynein arms in cross sections to that of dynein bound to native axonemes, the polarity of microtubules can now be defined. Dynein can, therefore, be used to determine the polarity of microtubules within the cell or those assembled *in vitro*, a technique analogous to that using heavy meromyosin to decorate actin filaments and determine their polarity (25).

We thank Drs. Timothy McKeithan and Ted Clark for helpful discussions and Nina Pierpont and Diethild Holub for expert assistance. This research was supported by U.S. Public Health Service Grant GM 14642-12 and American Cancer Society Grant IN-31-S.

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