THE IN VITRO ASSEMBLY OF
FLAGELLAR OUTER DOUBLET TUBULIN

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ABSTRACT
Flagellar outer doublet microtubules were solubilized by use of sonication, and
the tubulin was reassembled in vitro into single microtubules containing 14 and
15 protofilaments. The tubulin assembly was dependent on both the KCl and
tubulin concentrations, exhibiting a critical concentration of 0.72 mg/ml at
optimum solvent conditions. Flagellar tubulin was purified by cycles of tempera-
ture-dependent assembly-disassembly and molecular sieve chromatography, and
characterized by two-dimensional gel electrophoresis. Although doublet microtu-
bules were not formed in vitro, outer doublet tubulin assembled onto intact A-
and B-subfibers of outer doublet microtubules and basal bodies of Chlamydo-
monas; the rate of assembly from the distal ends of these structures was greater
than that from the proximal ends. Microtubule-associated proteins (MAPs) from
mammalian brain stimulated outer doublet tubulin assembly, decorating the
microtubules with fine filamentous projections.

KEY WORDS cilia and flagella directionality of
assembly · flagellar tubulin · in vitro
assembly · microtubules · sea urchin sperm

Eukaryotic flagella have been well characterized
in terms of structure and function (38, 47) but
little is known about the assembly of their major
structural components, the flagellar microtubules.
Flagellar microtubules are found in a cylindrical
arrangement, the axoneme, which contains nine
outer doublet microtubules surrounding a central
pair of single microtubules. These flagellar microtu-
bules differ from the other cellular microtu-
bules—the cytoplasmic microtubules—not only in
their unique morphology and arrangement, but
also in their relative stability. Treatments which
rapidly disassemble cytoplasmic microtubules—
high pressure, low temperature, or drugs such as
colchicine—do not solubilize flagellar microtu-
bules (48). This resistance to solubilization, more
than anything else, has hampered attempts at
studying flagellar microtubule assembly in vitro.

Stephens successfully dissolved flagellar outer
doublet microtubules by the use of the ionic
detergent Sarkosyl. Reassociation of the resulting
tubulin subunits occurred after dilution in the
presence of magnesium and GTP, yielding single
microtubule-like polymers and ribbons of protofi-
laments (44, 46). However, this assembly oc-
curred in vitro at 0°C as well as in the presence of
colchicine which is known to inhibit flagellar mi-
crotubule assembly in vivo (33). Other methods
used to solubilize flagellar outer doublets have
included use of the organic mercurial, Salyrgan
(43), thermal melting (45, 52), and low ionic
strength dialysis after acetone extraction (32).
Tubulin prepared by these methods was not capa-
bale of assembly into microtubules. By contrast,
cytoplasmic tubulin from brain tissue has been
used extensively for in vitro assembly studies (8,
16, 21, 23, 25, 28, 37, 40, 50, 51). This tubulin
assembled onto flagellar axonemes and basal bod-
ies, suggesting that the assembly sites of flagellar
and brain tubulin were similar (1, 4, 6, 9, 34, 41).
It seemed, therefore, that to achieve flagellar microtubule assembly in vitro, a means for obtaining native, soluble flagellar tubulin from the highly stable flagellar axoneme was needed. Recently, this has been accomplished by Kuriyama, who showed that tubulin solubilized from sea urchin sperm flagellar outer doublets by extensive solubilization was isolated by sedimentation. The functional flagellar tubulin subunit was shown to be a dimer with a sedimentation coefficient of 6S. Moreover, tubulin prepared in this manner bound colchicine, and its assembly was cold reversible.

This report contains a further analysis of the in vitro assembly of flagellar tubulin subunits prepared by the method of Kuriyama (22). A preliminary description of this work has appeared (5).

MATERIALS AND METHODS

Isolation and Storage of Sperm

Sperm from the sea urchin Strongylocentrotus drobaschensis or Strongylocentrotus purpuratus were isolated by removing the gonads, mixing them in calcium-free artificial sea water (13), 10^-4 M EDTA, 10 mM Tris-HCl, pH 8.0 at 4°C (CET), and straining them through cheesecloth. The sperm were then sedimented in 40-ml graduated polycarbonate tubes at 5,000 g (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.; RC-5, HB-4 rotor) for 5 min at 4°C (45). After removal of the supernatant, the pellets of packed sperm were resuspended in 10 mM MgCl2, 0.3 M KCl, 20 mM Tris-thyroglycolate buffer, pH 8.2 at 4°C; this suspension was diluted 1:1 with 100% glycerol and stored at -20°C for up to 5 mo (54).

Isolation of Flagellar Outer Doublets

Glycerol storage solution containing the equivalent of 100 ml of packed sperm was centrifuged at 35,000 g for 40 min (Sorvall RC-5, SS-34 rotor) at 4°C. All of the following steps were performed at 4°C. The sperm were resuspended in 320 ml of CET and centrifuged at 35,000 g for 20 min; the resultant pellet was brought up in a final volume of 250 ml of CET and 125-ml aliquots were placed in the 500-ml container of a Sorvall Omni-Mixer and sheared at full speed for 2 s (45). This suspension of sperm heads and tails was centrifuged at 1,000 g for 5 min (Sorvall RC-5 centrifuge, SS-34 rotor), the pellet of heads was discarded, and the centrifugation of the supernate was repeated. The supernate from this second 1,000-g centrifugation, containing mostly tails as well as some contaminating heads, was then centrifuged at 18,000 g for 10 min (Sorvall RC-5, SS-34 rotor), pelleting both heads and tails. After resuspension in 100 ml of CET, 25-ml aliquots were underlayed with 15 ml of 25% glycerol, 3 mM MgCl2, 10 mM Tris-HCl, pH 8.0 at 4°C, and centrifuged at 4,000 g for 5 min (Sorvall RC-5, HB-4 rotor). The contaminating sperm heads sedimented through the glycerol while most of the tails remained above the glycerol cushion. The supernate containing the sperm tails was removed and centrifuged at 27,000 g for 30 min (Sorvall RC-5, SS-34 rotor) to sediment the tails. The tails were demembranated with a solution containing 1% Triton-X 100 (Rohn & Haas Co., Philadelphia, Pa.), 3 mM MgCl2, 0.1 mM dithiothreitol (DTT), 30 mM Tris-HCl, pH 8.0 at 4°C (45), and the flagellar axonemes were dialyzed against 0.1 mM EDTA, 0.01% 2-mercaptoethanol, 1 mM Tris-HCl, pH 8.0 at 4°C for 18 h (17) removing the central pair microtubules and most of the dynein ATPase. Outer doublets, tubulin prepared in this manner were harvested by sedimentation at 40,000 g for 30 min (Sorvall RC-5, SS-34 rotor) and resuspended in 20 ml of polymerization mixture (PM) containing 5 mM 2-[N-morpholino]ethane sulfonic acid (Mes), 0.5 mM MgSO4, 1 mM ethylene glycol-bis-(β-aminoethyl ether)N,N' -tetraacetic acid (EGTA), 0.5 mM DTT, 1 mM guanosine-5'-triphosphate (GTP), pH 6.7 with KOH (22). This suspension was centrifuged at 40,000 g for 30 min and the pellet was resuspended in a volume of PM yielding a final protein concentration of 4–8 mg/ml. The suspension of outer doublets in PM was used in subsequent sonication experiments. 100 ml of packed sperm yielded ~150–200 mg of outer doublets.

Solubilization of Outer Doublets

Outer doublets in PM were sonicated in a 13 × 3.5 cm conical glass tube suspended in an ice water bath. Aliquots containing 6 ml of outer doublets at a concentration of 4–8 mg/ml in PM were sonicated continuously for 3 min using a Branson Model W140D Sonifier Cell Disrupter (Heat Systems-Ultrasoundics Inc., Plainview, N.Y.) equipped with the standard microtip tuned to a final output of 40–50 W. All subsequent steps were performed at 4°C. After sonication, the preparation was centrifuged at 40,000 g for 30 min (Sorvall RC-5, SS-34 rotor), and the supernate was removed and centrifuged again at 130,000 g for 1 h (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.; L2-65B, 50 Ti rotor). These sedimentations removed all of the particulate matter remaining after sonication as judged by electron microscopy of negatively stained preparations. The supernate was then concentrated by pressure dialysis on an Amicon PM 30 filter device (Amicon Corp., Lexington, Mass.) to a final concentration of 10–15 mg/ml; this solution of flagellar outer doublet tubulin (S-1) served as the starting material for all of the experiments described in Results.

Assay for Tubulin Assembly

Flagellar tubulin assembly was measured by the absorbance change at 350 nm in a temperature-controlled cuvette chamber of a Gilford recording spectrophotom-
Two-dimensional electrophoresis was performed by making S-1 0.15 M in KCl and warming the temperature from 0°C to 35°C. The presence or absence of microtubules after turbidity measurements was confirmed by electron microscopy of negatively stained samples.

**SDS-Urea Polyacrylamide Gel Electrophoresis**

Proteins prepared for electrophoresis by the method of Laemmli (24) were loaded onto slab gels (20 × 15 × 0.15 cm) of the Laemmli formulation with the following alterations: (a) sodium dodecyl sulfate (SDS) was deleted from both the stacking and separation gels and, (b) the separation gel was composed of both a 4-16% linear acrylamide gradient and a 1-8 M linear urea gradient. After electrophoresis, slab gels were fixed in 12.5% trichloroacetic acid, and stained with Coomassie Blue (ICL United States, Inc., Wilmington, Del.). Alternatively, cylindrical gels (10 × 0.6 cm) were run and stained quantitatively with Fast Green (18) as described by Sloboda et al. (39). The gels were scanned at 650 nm with a Gilford Model 240 spectrophotometer with a linear transport, and the areas under the peaks were determined by planimetry. All tubulin concentrations given in the text were determined by quantitative electrophoresis.

**Protein Determination**

Protein determinations were performed with the method of Lowry et al. (27) as modified by Schacterle and Pollack (36), using bovine serum albumin as a standard.

**Two-Dimensional Electrophoresis**

Two-dimensional electrophoresis was performed by the method of O'Farrell (30), with the exception that the second dimension separation gel was composed of a urea-acrylamide gradient as described above. The pI's of the polypeptides were determined by slicing the isoelectric focusing gels every 0.5 cm, eluting the ampholines with NaOH (10, 31, 48). The pellets were fixed over-night (12-18 h) and, after a brief wash in 0.15 M phosphate buffer, pH 6.7, they were fixed at 0°C in 2% osmium tetroxide, 6% tannic acid, 0.15 M NaH2PO4, pH 6.7 with NaOH (10, 31, 48). The pellets were fixed overnight (12-18 h) and, after a brief wash in 0.15 M phosphate buffer, pH 6.7, they were fixed at 0°C in 2% OsO4, in 0.15 M phosphate buffer, pH 6.7 for 2 h. After rapid dehydration through a graded series of acetone solutions at room temperature, the pellets were embedded in the Epon formulation of Spurr (42). Thin sections, cut on a Porter-Blum MT2 ultramicrotome (Dupont Instruments-Sorvall, Dupont Co., Wilmington, Del.), were routinely stained with uranyl acetate and lead citrate before examination at 60 kV in a Philips 201 electron microscope.

**Negarve Staining of Microtubules**

Routine negative staining was performed by placing a drop of the microtubule-containing preparation on a
carbon-over-Formvar-coated copper grid, removing most of the liquid with filter paper, and inverting the grid onto a drop of 5% glutaraldehyde in PM for \( \frac{1}{2} \) to 1 h. The grids were then negatively stained in neutralized 4% phosphotungstic acid in 0.4% sucrose (34).

To assay flagellar tubulin assembly onto basal bodies or axonemes, a drop of tubulin in PM, containing 0.15 M KCl, was placed on a silicone-coated glass slide at 0°C. The axonemes or basal bodies in PM were applied to carbon-over-Formvar-coated copper grids and, after 5-10 s, the grids were drained to near-dryness with filter paper. The grids with the attached axonemes or basal bodies were inverted onto the tubulin drops and the glass slide was moved to a warming tray at 35°C. At various times during assembly, the grids were transferred to a drop of 5% glutaraldehyde in PM and fixed for \( \frac{1}{2} \) to 1 h before negative staining with phosphotungstic acid (34). The negatively stained preparations were viewed at 80 kV in a Philips 201 electron microscope. Microtubule length measurements were made from tracings of enlarged negatives, and at least 50 microtubules were measured at each time point. The electron microscope was calibrated with a carbon replica of an optical diffraction grating.

**Isolation of Microtubule-Associated Proteins (MAPs)**

The MAPs (39) were isolated from in vitro-assembled calf brain microtubules by the phosphocellulose chromatographic method of Weingarten et al. (50), as modified by Sloboda et al. (40). The MAPs were desalted into PM on a Sephadex G-25 column before their incubation with soluble flagellar tubulin (S-I). The MAP fraction was composed principally of the high molecular weight proteins (14, 28, 39, 40).

**RESULTS**

**Solubilization and Characterization of Outer Doublet Tubulin**

The sonication procedure of Kuriyama (22) was effective in solubilizing outer doublet microtubules, extracting an average of 40% of the outer doublet protein (Table 1). SDS-urea polyacrylamide gel electrophoresis indicated that the major components of the solubilized fraction (S-I) were \( \alpha \)- and \( \beta \)-tubulin (Fig. 1c). Although other polypeptides were present in the S-I fraction, most of these had molecular weights less than 94,000 in contrast to the intact axonemes (Fig. 1a) and purified outer doublets (Fig. 1b). Quantitative analysis of cylindrical gels showed that tubulin comprised 82 ± 4% by weight of the total S-I protein. This tubulin enriched fraction was used in all of the experiments described below unless otherwise specified.

**Table 1**

| Axonemes | Outer doubles | Outer doubles solubilized | Outer doubles solubilized | Soluble tubulin |
|----------|---------------|---------------------------|---------------------------|-----------------|
| mg       | mg            | %                         | mg                        | mg              |
| 205      | 95            | 36                        | 38                        | 30              |
| 319      | 168           | 90                        | 54                        | 74              |
| 402      | 230           | 92                        | 40                        | 75              |

* In each experiment, sonication of outer doublets was performed as described in Materials and Methods.
† The average solubilization from seven experiments was 40% by weight.
§ The solubilized protein was determined to be 82 ± 4% tubulin.

**Self-Assembly of Solubilized Outer Doublet Tubulin**

Solubilized outer doublet tubulin (S-I) in PM at 0°C was assembled into microtubules by adding KCl and raising the temperature to 35°C. As the KCl concentration was raised from 0 to 0.1 M, at constant tubulin concentration, increasing amounts of assembly were observed (Fig. 2). Raising the KCl concentration above 0.1 M did not increase the final amount of microtubules formed, and so all subsequent in vitro experiments were carried out at a “saturating” level of KCl, 0.15 M.

The in vitro-assembled flagellar microtubule preparation, when negatively stained and observed in the electron microscope, was seen to consist of tangled networks of single microtubules (Fig. 3). In thin sections, the microtubules also appeared single (Fig. 4c). However, unlike any of the microtubules in the native, intact axonemes (Fig. 4a and b), these microtubules contained 14 and sometimes 15 protofilaments (Fig. 4d). Therefore, the in vitro-assembled microtubules differed in at least two basic structural features from the outer doublet microtubules from which their tubulin was derived: only single microtubules were formed in vitro, and none of these contained 13 protofilaments (Fig. 4c and d) like the A-subfiber of the outer doublet (Fig. 4b).

The assembly of S-I tubulin into microtubules in vitro was concentration dependent (Fig. 5). When the final amount of assembly (\( \Delta A_{350} \)) was plotted against tubulin concentration, a critical concentration of 0.72 mg/ml was obtained (Fig. 6) corresponding to an apparent equilibrium constant, \( K_e = 1.5 \times 10^5 \text{ M}^{-1} \). To determine whether kinetic factors capable of lowering this critical concentration were present in the axonemes but
FIGURE 1 SDS-urea polyacrylamide gel electrophoresis showing steps in the preparation of soluble outer doublet tubulin. (a) Whole axonemes before low ionic strength dialysis; (b) outer doublets, after low ionic strength dialysis; (c) protein solubilized (S-1) by sonication of the outer doublets. α and β represent the two non-identical tubulin monomers. The migration positions of various molecular weight markers are indicated at the left. 60 μg of protein was loaded onto each slot.

were removed during outer doublet isolation, whole axonemes (see Fig. 1 a) in PM were also sonicated, yielding a preparation which was 77 ± 2% tubulin. The tubulin solubilized from axonemes or purified outer doublets assembled with the same critical concentration (Fig. 6), indicating that factors which might affect the final amount of assembly were not released from the intact axonemes by sonication.

Purification of Flagellar Outer Doublet Tubulin

Since the in vitro reassembly of flagellar tubulin seemed comparable to the in vitro reassembly of brain tubulin, the assembly-disassembly procedure (37), useful in purifying brain tubulin, was used to purify flagellar tubulin from the S-1 supernate. As a result of cycles of assembly-disassembly (see Materials and Methods), the percentage of tubulin in the preparation increased from 82 ± 4% to 94 ± 1% (Fig. 7a and b). The only other proteins which remained after cycling the tubulin twice were of a lower molecular weight than β-tubulin (L MWs; Fig. 7b); no proteins of a molecular weight higher than α-tubulin were observed.

Another method useful in purifying brain tubulin is molecular sieve chromatography (14, 40). To determine whether this procedure would also be useful in purifying solubilized flagellar tubulin, S-1 tubulin was chromatographed on a Bio-Gel A-1.5 m column and two peaks were resolved: one chromatographing in the void volume (Peak I) and another chromatographing ahead of the included volume (Peak II) (Fig. 8). Peak I (Fig. 9b) contained tubulin and nearly all of the nontubulin proteins. When compared to the S-1 column load, Peak I was enriched for proteins with molecular weights higher than α-tubulin. Peak II (Fig. 8) contained few proteins; the major protein was a lower molecular weight protein which chromatographed ahead of the included volume.

FIGURE 2 Effect of KCI concentration on S-1, outer doublet tubulin assembly (see Fig. 1c). The tubulin concentration was constant at 2.8 mg/ml; incubation was at 35°C.
Figure 3 In vitro-assembled flagellar microtubules, glutaraldehyde-fixed and negatively stained with phosphotungstic acid. Bar, 1 μm.

9c), on the other hand, contained 87 ± 2% tubulin and polypeptides of a lower molecular weight than β-tubulin. There was a greater number of low molecular weight polypeptides in Peak II tubulin (Fig. 9c) than in 2X tubulin (Fig. 9d), and this was the reason for the difference in purity (87 vs. 94% tubulin, respectively). Peak II tubulin was capable of self-assembly in PM containing 0.15 M KCl, but the kinetics and efficiency of its polymerization were not tested.
FIGURE 4 Thin sections of flagellar microtubules fixed in the presence of tannic acid as they appear in situ and after assembly of S-1 outer doublet tubulin in vitro. (a) Intact axoneme showing nine outer doublets surrounding two central pair microtubules; bar, 0.2 μm; (b) outer doublet, demonstrating 13 protofilaments in the intact A-subfiber and 10 protofilaments in the C-shaped B-subfiber; bar, 20 nm; (c) single microtubules assembled from outer doublet, S-1 tubulin in vitro; bar, 0.2 μm; (d) higher magnification of the in vitro-assembled microtubules in Fig. 4c, showing 14 and 15 protofilaments in the in vitro-assembled microtubules; bar, 20 nm.
The purity of both 2X and Peak II tubulin was further analyzed by two-dimensional electrophoresis (30) (Fig. 10a and b). The tubulin could be resolved into two major spots: an α-spot with a pI of \(5.48 \pm 0.04\), as well as a β-spot with a pI of \(5.26 \pm 0.02\). In the Peak II preparation, another spot with a molecular weight identical to β-tubulin, but focusing at a higher pH, also was visualized (Fig. 10b, arrow).

Neither the LMW proteins of the 2X preparation nor the more numerous low molecular weight proteins from Peak II of the Bio-Gel column were present in the second dimension gel; presumably, they did not enter the first dimension isoelectric focusing gel.

**Directionality of Assembly of Flagellar Tubulin In Vitro**

Previously, it was shown that brain tubulin would assemble in vitro onto flagellar axonemes...
and basal bodies (1, 4, 6, 9, 34, 41), and that this heterologous assembly was directional (1, 6, 34, 41). To determine whether flagellar tubulin grew directionally from flagellar seeds, axonemes or basal bodies from *Chlamydomonas* were incubated in the presence of outer doublet tubulin as described in Materials and Methods.

Flagellar microtubules assembled onto both the distal and proximal ends of axonemes and basal bodies, although those on the distal ends were longer than those on the proximal ends (Fig. 11a and b). The rate of flagellar tubulin assembly onto the axonemes was quantitated by incubating the axonemes at 35°C in the presence of two concentrations of Peak II flagellar tubulin in PM containing 0.15 M KCl (Figs. 8 and 9c), and stopping the reaction at various times during assembly with glutaraldehyde (see Materials and Methods). After negative staining, microtubule lengths were measured. These experiments demonstrated that the rate of assembly from the distal end was much greater than the rate of assembly from the proximal end (Fig. 12).

It is interesting to note that the number of microtubules which assembled onto the distal ends of basal bodies and axonemes was routinely greater than the 11 microtubules present in these seed structures (nine A-subfibers of the outer doublets + two central pair microtubules). The reason for this was that both the complete A-subfibers and the partially circular B-subfibers of the outer doublets were capable of nucleating single microtubules (Fig. 11b and c).

**Stimulation of Flagellar Tubulin Assembly by Brain MAPs**

Similarities in the in vitro assembly of brain and flagellar tubulins have been described (22). Furthermore, both brain and flagellar tubulins have similar critical concentrations for assembly (reference 25 and Fig. 6); single microtubules form from either brain or flagellar tubulin in vitro (reference 31 and Fig. 4), and the directionality of assembly of brain and flagellar tubulins onto axonemes or basal bodies appears to be identical.

**Figure 8** Fractionation of S-1 outer doublet tubulin on Bio-Gel A-1.5m. Peak I chromatographs at the void volume of the column while Peak II is partially included; 1-ml fractions were collected; see Fig. 9b and c for the composition of these peaks.

**Figure 9** SDS-urea polyacrylamide gel electrophoresis of the fractionation of S-1 outer doublet tubulin (See Fig. 8). (a) S-1 outer doublet tubulin which was loaded onto the column; (b) protein that eluted at the void volume (Peak I); (c) the protein partially included in the column (Peak II); (d) tubulin purified by cycling shown for the sake of comparison with Fig. 9c. Note that fewer minor components are present in Fig. 9d than Fig. 9c. 60 µg of protein was loaded onto each slot.
Brain microtubules purified by temperature-dependent cycles of assembly and disassembly copurify with a group of nontubulin proteins known collectively as microtubule-associated proteins or MAPs (39). MAPs have been shown to stimulate brain tubulin assembly (28, 40, 50), decorating the microtubules with fine, filamentous projections (14, 28). In view of the similarities between brain and flagellar tubulin polymerization in vitro, experiments were performed to determine whether brain MAPs would stimulate the assembly of flagellar tubulin. Since flagellar tubulin self-assembled without added factors in PM with 0.15 M KCl (Figs. 2-4), it was necessary to partially inhibit this self-assembly by performing these experiments in 0.05 M KCl (see Fig. 2 and reference 22) to determine whether there was a stimulatory effect of the MAPs. Under these conditions, MAP addition stimulated both the initial rate and final amount of flagellar tubulin (S-1) assembly (Fig. 13). Furthermore, when the flagellar microtubules which were assembled in vitro in the absence and presence of brain MAPs were analyzed by electron microscopy, those formed in the absence of the MAPs were smooth-walled (Fig. 14a and b) while those formed in the presence of the MAPs were decorated with a fine filamentous material (Fig. 14c and d).

DISCUSSION

This report demonstrates that tubulin from sonicated flagellar outer doublet microtubules of sea urchin sperm is capable of self-assembly into single microtubules. These microtubules contain 14 and 15 protofilaments rather than the 13 protofilaments characteristic of most microtubules in vivo (49). The reason for this increase of one to two protofilaments is not known, but a similar phenomenon has been reported for in vitro-assembled brain microtubules and was attributed to the type of buffer used in the polymerization mixture (31). The formation of single microtubules from outer doublet tubulin in vitro is not peculiar to sea urchin sperm tubulin but also occurs when *Chlamydomonas* flagellar tubulin (solubilized in the same manner) is reassembled in vitro.²

² L. I. Binder, and J. L. Rosenbaum. Unpublished results.
Figure 11. Assembly of outer doublet tubulin onto Chlamydomonas basal bodies and axonemes as visualized in phosphotungstic acid negatively stained preparations of glutaraldehyde-fixed material. (a) Outer doublet tubulin assembled onto both distal and proximal ends of a basal body; the distal end of the basal body is demarcated by the collar region; bar, 1 μm; (b) outer doublet tubulin assembled onto both distal and proximal ends of an axoneme; the distal end of the axoneme is splayed, while the proximal end remains evenly bundled together; arrow indicates an outer doublet with microtubules emanating from both A- and B-subfibers; bar, 1 μm; (c) higher magnification of an outer doublet showing assembly of outer doublet tubulin from both the A- and B-subfibers; bar, 0.5 μm.

The self-assembly of outer doublet tubulin occurs above a relatively low critical concentration (Fig. 6) and, after purification by assembly-disassembly, the only polypeptides present other than α- and β-tubulins are the LMW proteins, with molecular weights lower than β-tubulin (Figs. 7b and 9d). Quantitative electrophoresis indicates that, at most, they comprise 5-6% of the total protein after two cycles of polymerization and depolymerization. Nagle et al (29) have shown that, in cultured cells, a low molecular weight protein of 49,000 daltons co-purifies with tubulin during cycling and is involved in microtubule assembly; however, in the flagellar assembly system, it is not known whether the LMW proteins have any assembly-promoting activity or whether they co-purify stoichiometrically with flagellar tubulin. In the analogous brain tubulin assembly systems, high molecular weight proteins which co-purify with tubulin through cycles of assembly-disassembly (39) have been shown to stimulate assembly in vitro (28, 40).
Previously, Bloodgood and Rosenbaum (7) demonstrated that a soluble extract from flagellar axonemes increased the initial rate of purified brain tubulin assembly in vitro without affecting the final equilibrium amount of polymer formed. Attempts to test the effect of this flagellar factor on flagellar tubulin assembly, however, were hampered by the factor's insolubility under the buffer conditions employed in outer doublet tubulin reassembly. Additional research in this area is warranted to determine whether the flagellar factor (7) is active in promoting flagellar tubulin assembly in vitro.

The possibility that nontubulin factors can affect flagellar tubulin assembly is indicated by the results which show that the MAPs from brain microtubules influence both the initial rate and final, apparent equilibrium amount of outer doublet tubulin reassembly in vitro (Fig. 13). This, and the fact that the MAPs decorate the in vitro-assembled flagellar microtubules (Fig. 14c and d) suggests that a common binding site or sites for the MAPs may exist on both brain and flagellar tubulin molecules. This suggestion is consistent with the results of a recent report which demonstrated that brain MAPs bind to native Chlamydomonas outer doublets and prevent the binding of the dynein-ATPase (19).

Flagellar tubulin self-assembly in vitro may be dependent only on tubulin, even though the LMW proteins are still present after two cycles of assembly-disassembly. This possibility is reinforced by the fact that extensively purified brain tubulin can be polymerized in vitro without accessory factors (20, 25). Specifically, Lee and Timasheff (25) reported that purified brain tubulin assembly occurred above a critical concentration of 0.5 mg/ml ($K_r = 2.2 \times 10^5$ M$^{-1}$; $\Delta G^\circ = -7.6$ kcal/mol); similarly, flagellar tubulin assembly occurs above a critical concentration of 0.72 mg/ml ($K_r = 1.5 \times 10^5$ M$^{-1}$; $\Delta G^\circ = -7.3$ kcal/mol) (Fig. 6). Given that the brain tubulin molecule itself contains all the information necessary to form a microtubule, it seems quite possible that the same is true for the flagellar tubulin molecule.

Flagellar tubulin assembly in vitro is a rapid event whether the tubulin is self-assembled in 0.15 M KCl (Fig. 5) or stimulated to assemble by brain MAPs at 0.05 M KCl (Fig. 13). Similarly, brain tubulin assembly in vitro is also very rapid, and these kinetics can be attributed, at least in part, to the presence of ring-shaped aggregates thought to be nucleation intermediates (8). It is important to note that similar ring-shaped aggregates were neither observed in flagellar tubulin

![Figure 12](image-url) Rates of assembly of Peak II outer doublet tubulin onto both distal (Δ and ○) and proximal (▲ and ●) ends of Chlamydomonas axonemes at two tubulin concentrations. At least 50 microtubules were measured at each time point. Incubation was at 35°C in PM containing 0.15 M KCl. Error bars denote + and − one standard error of the mean.

![Figure 13](image-url) Stimulation of S-1 tubulin assembly with brain MAPs. Incubation was at 35°C in PM containing 0.05 M KCl. The MAP and tubulin concentrations were constant at 0.73 mg/ml and 2.8 mg/ml, respectively.

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Figure 14 Longitudinal and cross sections of in vitro-assembled flagellar microtubules (a and b) in the absence of brain MAPs and, (c and d) in the presence of brain MAPs; bar, 0.2 μm.
preparations in 0.15 M KCl nor in flagellar tubulin preparations in 0.05 M KCl in the presence of brain MAPs. Furthermore, when either of these mixtures was cold-dissociated after in vitro assembly, ring-shaped aggregates were still not seen (data not shown). Obviously, flagellar tubulin assembly is preceded by some form of nucleation event; however, the definition of this process and possible identification of a nucleation structure awaits further investigation.

Brain and flagellar tubulin molecules share a number of physical and chemical properties. In Kuriyama’s initial report (22) on outer doublet tubulin assembly, the functional tubulin subunit was shown to behave as a dimer with a sedimentation coefficient of 6S, and its in vitro assembly was shown to be inhibited by sulfhydryl blocking reagents. Brain microtubule protein exhibits both of these properties (21, 23). More recently, it was demonstrated that α- and β-brain tubulin subunits have isoelectric points of 5.4 and 5.3, respectively (3), similar to the pI values of 5.48 and 5.26 for flagellar α- and β-tubulins. Furthermore, the α-subunit of brain tubulin was shown to contain a small amount of heterogeneity, exhibiting one minor and one major spot on two-dimensional gels (3). Flagellar α-tubulin also shows isoelectric heterogeneity on occasion; however, the number of α-spots seems to depend on how the sample is stored before isoelectric focusing (data not shown).

The directionality of assembly of outer doublet tubulin onto axonemes and basal bodies closely resembles that previously observed for brain tubulin. In both instances, the preferred end for assembly from these structures is the distal end (references 1, 4, 6, 34, 41 and Fig. 11). Moreover, assembly from the distal end is faster than assembly from the proximal end (references 6, 34 and Fig. 12), indicating that, even after the initiation of assembly onto axonemes or basal bodies, the kinetics of assembly are governed by the inherent growth polarity of the nucleating structure.

Doublet microtubules are not formed in vitro even when the soluble flagellar outer doublet tubulin is provided with doublet templates (axonemes and basal bodies; Fig. 11). Instead, single microtubules assemble from both A- and B-subfibers of axonemes and basal bodies. Optical diffraction studies have shown that the surface lattices of the A- and B-subfibers are different, and that the surface-lattice of the B-subfiber precludes the formation of an intact, closed, tubular structure (2, 46, 47). In fact, microtubules formed from the B-subfiber in vitro often appear as flattened sheets whether the tubulin subunits employed are from brain (6, 34) or flagella (data not shown). Furthermore, Stephens demonstrated that heat-solubilized B-subfiber tubulin was only capable of forming flattened sheets in vitro (46).

In light of these observations, experiments should be performed to determine: (a) the surface-lattice of the microtubules assembled from both A- and B-subfibers in vitro, and (b) the shape of the tubulin structures (i.e., intact single microtubules or C-shaped tubulin sheets) assembled onto the B-subfibers in vitro.

The reason why doublet microtubules are not formed in vitro is not known. Although calculations based on the data in Table I show that only 46% of the outer doublet tubulin is rendered soluble, there was no indication of a selective solubilization of either A- or B-subfiber as a result of sonication. In addition, others have observed a progressive shortening of both subfibers of intact doublet microtubules during sonication (15). These results suggest that the tubulin used in these experiments was a mixture of both A- and B-subfiber tubulins.

When outer doublets were solubilized by means other than sonication (26, 53), the whole structure was not dissolved. A ribbon of three protofilaments was left which was thought to compose part of the partition region between the A- and B-subfibers (26, 53). This ribbon consisted of tubulin and four or five other polypeptides (26). It is possible that the incomplete solubilization of the ribbon tubulin or one or all of the polypeptides associated with it accounts for our failure to form doublet microtubules from the outer doublet tubulin. When preparations of intact ribbons were added to flagellar tubulin, however, outer doublets still were not formed (data not shown).

Recently, doublet-like microtubules were formed by assembly of brain tubulin in the presence of high MgCl₂ (12). However, it is not known whether or not these were “true doublets” composed of a 13-protofilament tubule in lateral continuity with a 10- or 11-protofilament C-shaped tubule (See Fig. 4b). In fact, others have shown that doublet-like microtubules formed in vitro from brain tubulin when viewed in thin section after fixation in the presence of tannic acid are not doublets in the conventional sense, but instead are either 8-protofilament sheets tenuously...
attached to 14-protofilament microtubules, or interlocking rings formed by two microtubules (11). In the case of flagellar tubulin assembled in vitro, Kuriyama reported that doublet-like structures were visible in negatively stained preparations (22). Although we observed similar phenomena in nonfixed negatively stained preparations, these were clearly seen to be two single microtubules lying in close apposition. Furthermore, our thin-section electron microscopic analysis confirmed that no doublet microtubules were formed in vitro (Fig. 4c).

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