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Directionality of Brain Microtubule Assembly *In Vitro*

(autoradiography/electron microscopy/viscometry)

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ABSTRACT **Chicks were injected intra-cerebrally with [^{3}H]leucine, and ^{3}H-labeled microtubules were assembled *in vitro* in the brain supernatants. Pieces of these ^{3}H-labeled microtubules were then incubated with unlabeled brain tubulin subunits under conditions where the subunits assembled onto the labeled pieces. Electron microscopic autoradiography of the negatively-stained microtubules showed all of the radioactivity at one end of the tubules as they increased in length. This clearly demonstrated that the microtubules of brain in this *in vitro* system were being assembled unidirectionally.**

For some time our laboratory has been interested in the directionality of assembly of microtubules during flagellar growth. In the initial studies, light microscopic autoradiography was used to show that material is added primarily to the distal end ("tip") of flagella during flagellar regeneration in *Ochromonas* and *Chlamydomonas* (1, 2). These early studies did not differentiate between the addition of material to the axonemal microtubules and the flagellar membranes, but Witman, using the *Chlamydomonas* flagella-regenerating system (2) coupled with techniques of flagellar fractionation (3, 10) and electron microscopic autoradiography, recently has been able to show that the *microtubules* of the flagella are assembled *primarily by distal addition* of subunits (4). However, it was unknown whether microtubules which do not arise from a basal body, as do the flagellar microtubules, also exhibit a polarity of growth, or if they instead elongate by addition of new subunits to *both* ends. Now, with the development of methods for the *in vitro* assembly of brain microtubules (5), it has been possible to determine the directionality of brain microtubule (neurotubule) assembly *in vitro*. The basic design of these experiments was to obtain highly-radioactive pieces of brain microtubules for use as "seeds" or nucleation centers for the assembly of unlabeled brain tubulin subunits. The site(s) of assembly of the unlabeled subunits onto the radioactive seeds would then be determined by electron microscopic autoradiography of negatively-stained preparations of the microtubules. If the radioactivity was observed on one end of the tubule, it would indicate uni-directional assembly; if the radioactive portion was localized in between two unlabeled segments, it would indicate bi-directional assembly. The results to be described below show that under the experimental conditions used, *assembly of microtubules in vitro occurs principally by uni-directional addition of subunits.*

Abbreviations: PM, polymerization solution; EGTA, ethyleneglycol bis(β aminoethylether)N,N'-tetraacetic acid; SDS, sodium dodecyl sulfate; HMW, high molecular weight.

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MATERIALS AND METHODS

Preparation of Pieces of ^{3}H-Labeled Microtubules. For the preparation of ^{3}H-labeled microtubules, 4mCi of [^{3}H]leucine (New England Nuclear, L-[4,5-^{3}H]leucine 41 Ci/mM) in 25 μl of phosphate-buffered saline [0.15 M NaCl–0.01 M sodium phosphate (pH 7.4)] was injected intracerebrally into a 2- to 3-day-old chick. Amino-acid incorporation into the chick-brain protein is linear for at least 30 min after such an injection (6). After 1 hr, the chick was decapitated, and the brain (about 0.9 g) was removed, suspended in a volume of polymerization solution (PM) [0.1 M Pipes, 1 mM GTP, 1 mM $MgCl_2$, 2 mM ethyleneglycol bis(β aminoethylether)N,N'-tetraacetic acid (EGTA) (pH 6.9)] (5, 17) equal to 1.5 times the weight of the brain, and homogenized by use of a glass homogenizer and motor-driven teflon pestle. The homogenate was centrifuged for 1 hr at 35,000 $\times$ *g* (Sorvall RC-2 centrifuge, SS-34 rotor, 17,000 rpm) at 4°. The supernatant was removed and incubated at 37° for 30 min to assemble the microtubules; the tubules were collected by centrifugation at room temperature for 10 min at 27,000 $\times$ *g* (Sorvall SS-1 centrifuge). These are called "1X-polymerized microtubules." The pellet of microtubules was washed by thorough resuspension in PM at room temperature and harvested by centrifugation at 27,000 $\times$ *g* for 15 min. The washed 1X-polymerized microtubules were resuspended in a volume of PM equal to half the volume of the original crude supernatant (about 0.5–1.0 ml per brain), broken up into pieces by homogenization with a glass-teflon homogenizer, and kept at room temperature until use. The pieces of microtubules had an average length of 3.5 μm and a specific activity of 1.5 to 2.0 $\times 10^7$ dpm/mg of protein.

In some experiments (see *Discussion*) the preparation of 1X-polymerized microtubules obtained from the crude supernatant was further purified by depolymerization of the tubules at 4° for 30 min and centrifugation at 27,000 $\times$ *g* for 10 min at 4° to remove any remaining pieces of tubules, followed by re-polymerization of the subunits in the supernatant at 37° for 30 min to give "2X-polymerized microtubules." This polymerization–depolymerization could be repeated to obtain "3X-polymerized microtubules." With each successive polymerization, greater tubulin purity was achieved.

Preparation of Unlabeled Tubulin Subunits. Tubulin subunit preparations were obtained from chick brain homogenates prepared as described above except that the homogenate was centrifuged at 130,000 $\times$ *g* (Spinco, L2-65B, 40,000 rpm, 50 Ti rotor) for 1 hr at 4°. This crude, high-speed supernatant (S1) was used as a source of tubulin subunits (5, 17); the amount of tubulin was determined to be 20–30% of the total

protein by SDS acrylamide gel electrophoresis. The subunit preparation was made at the same time as the labeled microtubule pieces and was kept in the cold until used as described in the following section. In some experiments (see *Discussion*), 2X- or 3X-polymerized microtubules (see above) were depolymerized by cold treatment, and centrifuged at 40,000 rpm (50 Ti rotor) at 4° for 1 hr to obtain supernatants which contained 2X- or 3X-purified tubulin subunits.

Assembly of Unlabeled Tubulin Subunits onto ^{3}H-Labeled Microtubule Pieces. A volume of the microtubule pieces (2.2 mg of protein per ml) was added to an equal volume of the subunit preparation (S1, 15 mg of protein per ml, 3–4 mg of tubulin per ml) and the temperature raised to 37° to initiate polymerization. Aliquots were taken every 5 min for 30 min and fixed with an equal volume of 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0). The glutaraldehyde fixation was necessary to prevent breakage of the tubules while they were being prepared for electron microscopic autoradiography. After fixation, each aliquot was further diluted with either glutaraldehyde or PM so that an appropriate number of microtubules would be present on each electron microscope grid to make the analysis of tubule length and silver grain distribution on each tubule feasible.

Negative Staining and Electron Microscopic Autoradiography. A drop of the microtubule suspension was placed on a carbon-over-formvar-coated grid and the grid rinsed with four drops of 0.1 M KCl, followed by two drops of 2% cytochrome *c* in 1% amyl alcohol, and four drops of 1% uranyl acetate. The grid was then drained almost to dryness with filter paper. Preparation of the negatively-stained microtubules for electron microscopic autoradiography was carried out as described by Witman (4): Following staining, the grids were placed on a rotating platform in a vacuum evaporator and a second layer of carbon was evaporated onto the grids from an angle of 45° while continuously rotating the grids. The microtubules were thus sandwiched between two layers of carbon. Uranyl acetate-stained specimens prepared in this way are preserved throughout the autoradiographic processing. The grids were then coated with Ilford L-4 emulsion (7) and stored at 4° in light-tight Bakelite slide boxes containing a small pack of Drierite. After suitable intervals of time (usually 1–2 months), the grids were removed and processed at 20° as follows: Kodak Microdol-X, 5 min; 1% acetic acid, 30 sec; Kodak acid fixer, 5 min; and distilled water, 30 sec, followed by four 1-min rinses in distilled water. The grids were then dried and examined directly in a Philips 200 or 300 electron microscope without prior removal of the gelatin.

Viscometry. Viscometric analysis of tubulin assembly was carried out with Cannon-Manning semi-micro viscometers having outflow times for water at 37° of 47–50 sec.

Electrophoresis. SDS–acrylamide gel electrophoresis was carried out as described by Fairbanks *et al.* (8). The two tubulins which comprise the microtubules (9, 10) migrate as one band on this gel system. After fixation and removal of excess SDS from the gel by thorough washing with 7% acetic acid, the gels were quantitatively-stained for protein with the dye Fast Green (11).

Thin-Sectioning of Microtubules. In vitro 3X-polymerized microtubules were centrifuged at 225,000 × *g* (Spinco L2-65B, 50 Ti rotor) for 30 min at 30° in 13-ml Oak Ridge tubes. The pellets were fixed *in situ* with 2.5% glutaraldehyde in PM, followed by 1% osmium tetroxide, dehydrated in ethanol, and then flat-embedded in Epon, all by standard procedures. The flat-embedded pellet was sectioned either perpendicular or parallel to its plane to obtain microtubule cross and longitudinal sections, respectively.

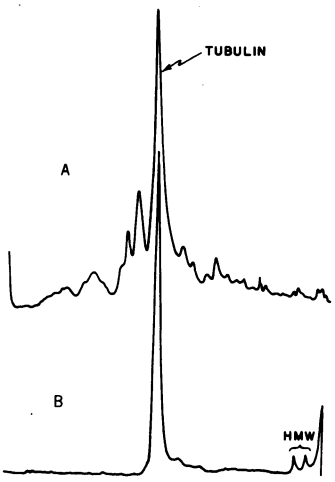

FIG. 1. Scans of Fast-green-stained SDS-acrylamide gels showing: (*A*) crude brain supernatant and (*B*) tubulin purified by *in vitro* assembly. HMW is high molecular weight, ATPase-containing protein which co-purifies with tubulin.

RESULTS

Purification of Tubulin. The purification of the radioactive microtubule pieces onto which unlabeled tubulin subunits were to be assembled is shown in the scans of Fast Green-stained SDS-acrylamide gels (Fig. 1). The crude brain supernatant (Fig. 1A) can be seen to contain two major proteins. The most prominent of these is tubulin with a relative molecular weight of 55,000 (9); the other is actin, with a relative molecular weight of 43,000 (E. Kuczmarski, unpublished results). After purification of the tubulin by two successive polymerizations and depolymerizations or after washing 1X-polymerized tubules with warm PM as described in *Materials and Methods*, SDS gel electrophoresis showed the presence of only one major protein with the electrophoretic mobility of tubulin (Fig. 1B). The remaining 10–15% of the protein which co-purified with tubulin was found primarily in two high-molecular-weight (HMW) proteins of about 350,000–450,000. The nature of these HWH proteins is not known, but we have determined that together they contain most of the ATPase activity of the purified microtubules. These HMW, ATPase-containing proteins have electrophoretic mobilities which are very close to but not identical with sea urchin flagellar dynein ATPase when they are compared on 3% SDS-acrylamide gels (R. Sloboda, S. Granett, and E. Kuczmarski, unpublished results).

Observation of Assembled Microtubules. The microtubules which had been assembled *in vitro* were observed in the electron microscope by both negative staining and thin-sectioning procedures (Fig. 2A, B, and C). Negative staining with uranyl acetate could be done quickly during the course of an experiment to assay the progress of *in vitro* assembly, and it was used to quantitate the increase in microtubule length with time (see below). Examination of thin-sectioned, purified microtubules showed that they were coated with an irregularly arranged filamentous material (Fig. 2A and B). The nature of this filamentous material is not known, but it appears similar to the filamentous material seen on microtubules of neurons *in situ* (13–16), and it might represent the only nontubulin protein(s) seen on SDS gels of purified micro-

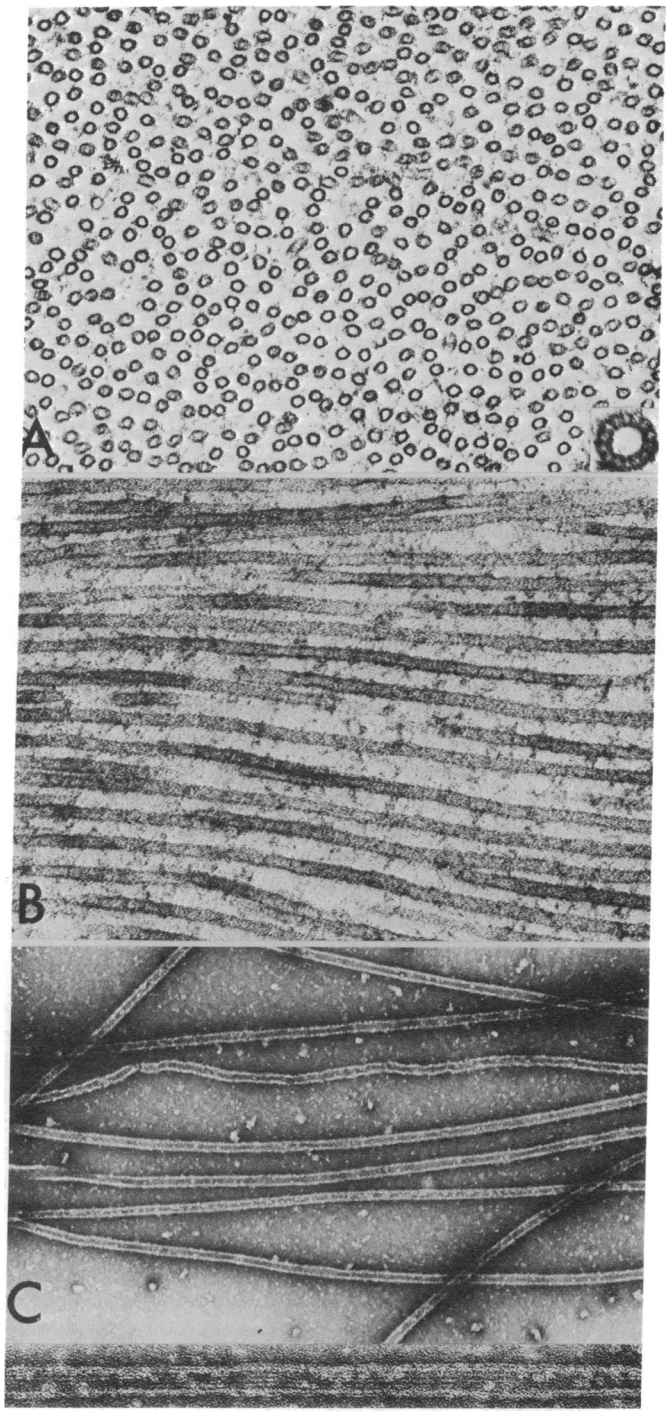

FIG. 2. Electron micrographs of *in vitro*-assembled brain tubules. (*A*) Cross sections of microtubules; inset shows tubule from preparation stained with tannic acid (26), to show subunits; (*B*) longitudinal section showing filamentous material associated with *in vitro*-assembled microtubules; (*C*) low- and high-power (*inset*) micrographs of glutaraldehyde-fixed, negatively-stained microtubules.

tubules, i.e., the high molecular weight, ATPase-containing protein(s) (12).

Demonstration of Tubulin Subunit Assembly onto Pieces of Microtubules. To determine the directionality of subunit assembly onto pieces of microtubules, it was desirable that the subunits assemble primarily onto the pieces of ^{3}H-labeled microtubules and that little assembly occur in the subunit preparation by itself. This requirement was met by centrifuging the subunit preparation (S1) at 130,000 $\times$ *g* for 60 min to remove any possible microtubule initiating centers which may have been present in the supernatant (17). Viscometric analysis of the subunit preparation in PM at 37° showed that there was almost no increase in viscosity for at least 35 min (Fig. 3), and no microtubules were observed in the suspension by electron microscopy. Likewise, the preparation of washed microtubule pieces showed little change in viscosity when incubated by itself in PM at 37° (Fig. 3). However, when the subunits were mixed with the microtubule pieces, there was an immediate and rapid increase in viscosity, indicating the assembly of microtubules (Fig. 3).

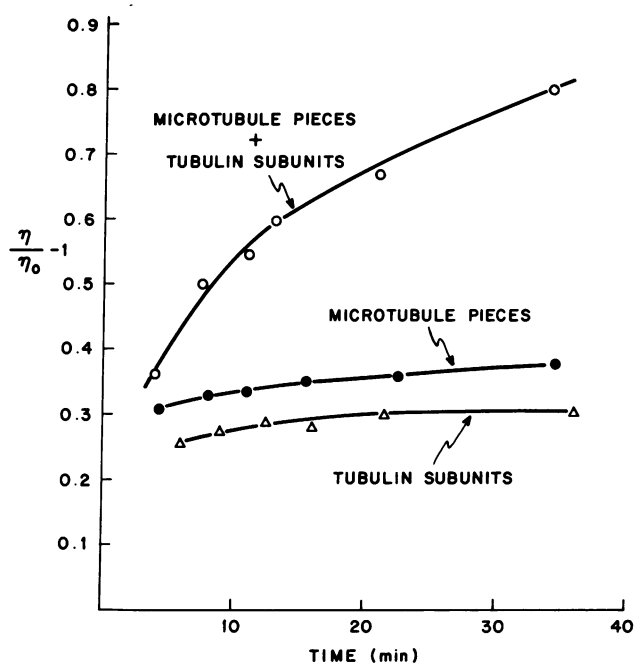

FIG. 3. Viscometric analysis of *in vitro* microtubule assembly showing tubulin subunits and microtubule pieces separately and mixed (see *text*).

To show definitively that the tubulin subunits were assembling onto the added pieces of microtubules, measurement of the lengths of fixed, negatively-stained microtubules were made at intervals after the addition of the subunits to the pieces. As can be seen in Fig. 4, the short pieces of microtubules of average length 3.5 μm which were present at time zero disappeared as the incubation at 37° progressed, while longer pieces appeared. The viscosity of the preparation increased simultaneously with the formation of the longer tubules.

The experimental requirements were therefore met during the first 35 min of the incubation when it could be shown by both viscometric analysis and microtubule length measurements that tubulin subunits were assembling primarily onto the pieces of microtubules.

Directionality of Tubulin Subunit Assembly onto Microtubule Pieces. Pieces of ^{3}H-labeled microtubules were incubated with the unlabeled subunits as described above. At intervals following the initiation of assembly by raising the temperature to 37°, aliquots were fixed, negatively-stained, and prepared for autoradiography. Following development of the autoradiographs, the distance of the silver grain(s) from the nearest end of a tubule at each time interval was determined. Microtubules typical of each time interval are illustrated in Fig. 6; the results of the silver grain distribution and

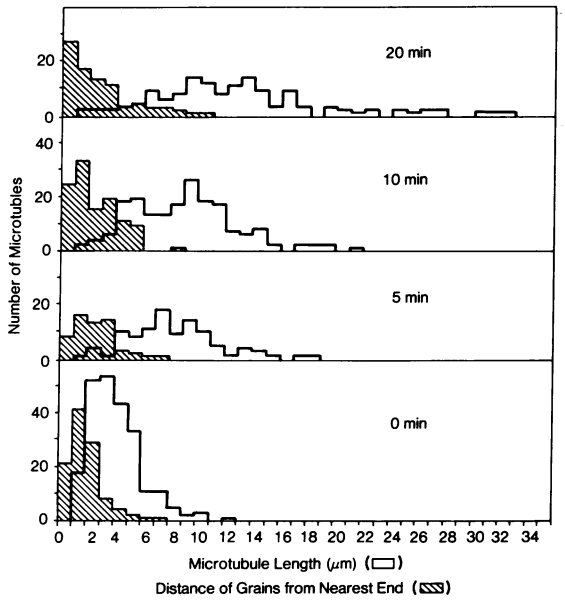

FIG. 4. Analysis of microtubule lengths (*empty bars*) and silver grain distance from nearest end of tubule (*cross-hatched bars*) during assembly of unlabeled tubulin subunits onto ^{3}H-labeled microtubule pieces. Most tubules had one silver grain. As the tubule lengths increase with time, the grains remain distributed near the ends of the tubules.

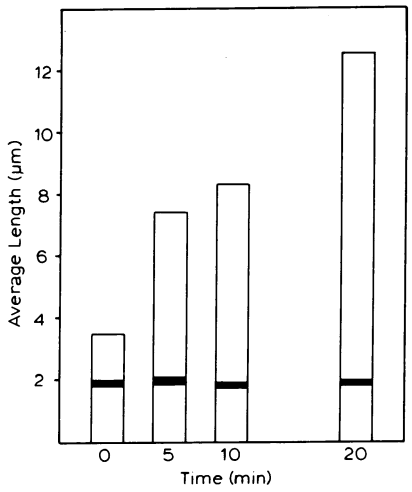

FIG. 5. *Average* lengths of microtubules (*open bars*) with time after mixing tubulin subunits with ^{3}H-labeled microtubule pieces. The *dark bar* on each open bar shows the *average* distance of the silver grains from one end of the tubules at each time.

microtubule length analyses are shown in Figs. 4 and 5. It can be clearly seen that as the microtubules increased in length, the silver grains consistently appeared near one end of the tubule, in the same position as in the original preparation of ^{3}H-labeled microtubule pieces. The results therefore show that *the unlabeled subunits were being added onto one end of the ^{3}H-labeled pieces of microtubules and that microtubule assembly in this system under the experimental conditions employed is principally uni-directional.*

DISCUSSION

These results show that when pieces of brain microtubules are used as initiation centers for the *in vitro* assembly of brain tubulin subunits, assembly is principally uni-directional. That subunit assembly is occurring onto a pre-determined end of the microtubule piece seems likely, but this cannot be proven since there is no way of distinguishing one end of a piece from another. However, we and others have recently been able to demonstrate that pieces of axonemal microtubules from *Chlamydomonas* flagella or sea urchin sperm can act as initiation centers for the *in vitro* assembly of chick brain tubulin subunits (18–20). In these *heterologous* systems (flagellar microtubules + brain subunits) one can determine onto which end of the microtubule the brain subunits are assembling since it is relatively easy to distinguish the proximal from the distal end of isolated flagellar microtubules (4, 18). In these experiments we were able to show that assembly was entirely onto the distal end of the flagellar microtubules at low brain subunit concentrations (1 mg of tubulin per ml), and onto both the distal and proximal ends at high subunit concentrations (5 mg of tubulin per ml); in the latter case, however, the *rate* of assembly was at least 5–8 times greater onto the distal in comparison to the proximal end (ref. 20; L. Binder, W. L. Dentler, and J. Rosenbaum, in preparation). The reason for this concentration-dependent assembly of brain tubulin subunits onto *both* ends of flagellar microtubules is not known but it indicated that it was important to determine the directionality of assembly using different concentrations of subunits in the *homologous* neurotubule assembly system described here. Since a small amount of assembly of brain tubulin onto a nonfavored end of a piece of brain microtubule (as with the *proximal* addition of subunits onto flagellar microtubules) would be difficult to detect by autoradiographic procedures, these experiments were carried out using the microtubule "decoration" procedure recently described by Borisy (27).

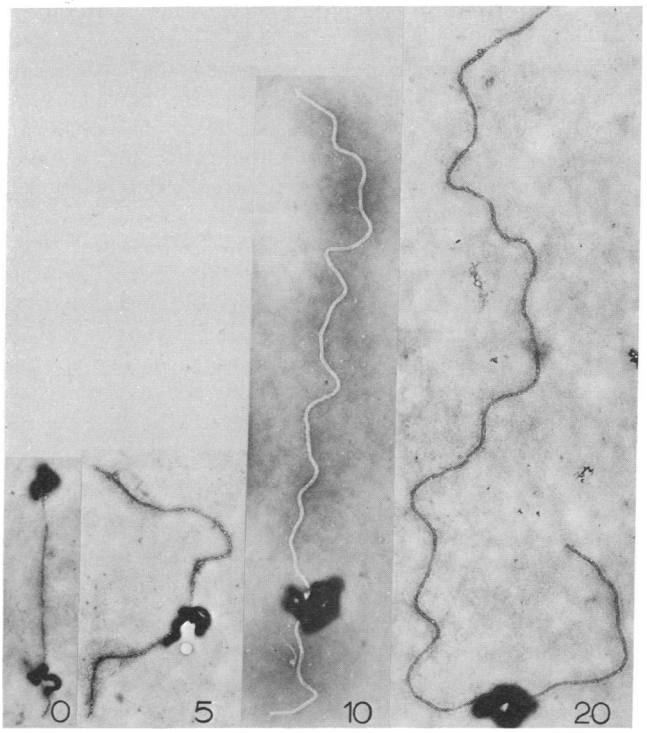

FIG. 6. Electron microscopic autoradiographs of negatively-stained microtubules showing tubule elongation and silver grain localization with time after mixing ^{3}H-labeled microtubule pieces with unlabeled tubulin subunits. The wavy appearance of the microtubules was due to fixation with glutaraldehyde prior to autoradiography. Time in min in lower right of each micrograph.

TABLE 1. *Tubulin assembly onto DEAE-Dextran-labelled microtubule pieces* (▨)

Experiment	Subunit concentration (mg/ml)	Number unidirectional	Number partially bidirectional	Number bidirectional
A. 3× PIECES* + 3× SUBUNITS*	7.5	38 (93%)	3	0
B. 3× PIECES + 3× SUBUNITS	5.0	50 (93%)	3	1
C. 3× PIECES + 3× SUBUNITS	2.5	55 (89%)	5	2
D. 1× PIECES† + 3× SUBUNITS	5.0	50 (91%)	3	2
E. 1× PIECES + 2× SUBUNITS‡	9.5	63 (81%)	10	5
F. 1× PIECES + 2× SUBUNITS	17.5	29 (81%)	4	3
G. 1× PIECES + SUBUNITS§	4.5	19 (70%)	7	1

Microtubule pieces were labeled with DEAE-Dextran and were incubated with subunits at 37°. Samples were negatively stained for electron microscopy at 1-min intervals up to 11 min. In highly concentrated subunit preparations (e.g., A.E.F), DEAF-Dextran label was not clearly distinguishable after 5 min but sufficient assembly had occurred to allow the determination of directionality of assembly.

* Carried through three polymerization/depolymerization cycles (*Materials and Methods*).

† Washed 1× microtubule pieces (*Materials and Methods*).

‡ Carried through two polymerization/depolymerization cycles (*Materials and Methods*).

§ High-speed supernatant (S1) of chick-brain homogenate (*Materials and Methods*).

In this method, microtubule pieces which are completely decorated with DEAE-Dextran (Pharmacia) are incubated with tubulin subunits under conditions where the subunits assemble onto the decorated pieces. The decorated pieces of the microtubules can then be distinguished within the newly assembled microtubules by negative staining, and the directionality of tubulin assembly onto the pieces can be determined. The results of these experiments are shown in Table 1. Regardless of whether one uses low or high concentrations of tubulin subunits, purified or unpurified subunit preparations, or microtubule pieces obtained from purified or unpurified tubulin, assembly is principally uni-directional. The reason for the low percentage of bi-directional assembly (10–20%) which is routinely observed with this technique is under investigation. In contrast, the heterologous system of flagellar tubules with brain tubulin subunits differs in that high percentages (80–100%) of axonemes showing bi-directional assembly can be obtained using concentrations of subunits (purified or unpurified) which give principally uni-directional assembly with pieces of neurotubules (20).

The finding that *in vitro* neurotubule assembly is uni-directional is of obvious importance with respect to the mechanism by which microtubules assemble during neurite outgrowth and axoplasmic flow. The finding is also relevant to those theories of mitotic movements which depend on the directionality and localization of assembly and disassembly of the microtubules of the mitotic apparatus (21). It is, therefore, important to determine where the microtubules of the mitotic apparatus are assembled and if their assembly is also uni-directional. Recently, it has become possible to assemble brain tubulin subunits onto the isolated, microtubule-depleted mitotic apparatus of tissue culture cells and various marine invertebrate eggs (22–25). By use of highly-radioactive brain tubulin subunits and autoradiographic procedures such as were used to determine directionality of microtubule assembly in these studies, it should also be possible to determine the directionality and site(s) of assembly of brain microtubules onto the tubule-depleted mitotic apparatus.

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