The structure of microtubule ends during the elongation and shortening phases of dynamic instability examined by negative-stain electron microscopy

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Summary

Microtubules (MTs) are dynamic polymers that can exist in phases of elongation and rapid-shortening at steady-state. These phases have been observed *in vitro* and in living cells, and this property of MTs has been termed 'dynamic instability'. The purpose of this study was to use negative-stain electron microscopy (EM) to test if there are structural differences between the ends of MTs in the elongation and shortening phases, which could provide insight into the mechanisms of dynamic instability. MTs in the elongation phase were obtained by seeding either highly purified porcine brain tubulin (PC-tubulin) or tubulin containing microtubule-associated proteins (MTP), from isolated *Tetrahymena* axonemes. The results are that, in addition to intact cylindrical MTs, a significant fraction of the tubulin polymer in the elongation phase occurred as sheets of parallel protofilaments, as found in previous investigations with self-assembled MTs. Therefore, sheet formation is an intrinsic property of MT assembly that does not depend on the tubulin purity or the method of nucleation. Also, since sheets lack helical symmetry, at least a fraction of tubulin polymers seeded from axonemes did not assemble by helical addition of tubulin dimers to the ends, an assumption often made in mathematical models of dynamic instability. Sheets and intact MTs that were seeded from isolated axonemes, emanated both from the intact MT wall of the axoneme A-subfiber and from the incomplete wall of the B-subfiber. Therefore, axoneme seeds do not provide a homogeneous nucleation site for tubu-

lin growth, or produce a homogeneous population of tubulin polymers under our conditions.

Previous evidence has indicated that MT disassembly can occur by a segmental release of tubulin oligomers from the ends and at sites along the length of MTs. However, these studies were performed with MTP, and disassembly was induced by cold depolymerization. We examined MT shortening under conditions that closely represent shortening *via* dynamic instability, namely isothermal dilution at 37°C of self-assembled MTs. This was compared with the morphology of cold-disassembled MTs. The cold-depolymerization of MTs composed of MTP showed rings and protofilament curls as previously observed using similar methods. Surprisingly, cold-depolymerization of MTs assembled from PC-tubulin induced not only shortening, but also the opening of a large fraction of MTs into sheets, suggesting that the MT lattice contains a cold-labile seam. Under conditions that mimic stochastic shortening, MTs were intact, closed cylinders with ends that were approximately blunt. Therefore, rapid shortening occurs at the ends of the MT, without a long-range disruption of the MT wall. In conclusion, MTs in the elongation phase can have highly irregular ends and need not elongate by a helical assembly process. Conversely, MTs in the shortening phase can have relatively blunt, even ends and can depolymerize in a relatively uniform fashion.

Key words: microtubules, sheets, axonemes.

Introduction

Microtubules (MTs) are unusual polymers in that their ends can persist in either an elongation or a shortening phase under steady-state assembly conditions. MTs are composed of α - and β -tubulin protein dimer subunits assembled into parallel protofilaments arranged to form a hollow tubule. Cytoplasmic MTs typically contain 13 protofilaments, but cylindrical polymers of ~11–15 protofilaments and curved sheets have been assembled *in vitro* by varying assembly conditions (Dustin, 1984; Karecla *et al.* 1989). MT assembly consists of nucleation followed by an elongation phase, which occurs by the addition of

tubulin dimers to the MT end. The polymerization requires GTP to be bound to the β -subunit of the dimer (GTP-tubulin), and this GTP is hydrolyzed to GDP (GDP-tubulin) following the dimer addition. At random times, a MT can convert to a rapid-shortening phase in which depolymerization occurs at the ends until the MT disappears or changes its state back into the elongation phase (rescue). The frequency of conversion to the shortening phase (catastrophe) decreases at higher tubulin concentrations (Walker *et al.* 1988), while the shortening velocity is concentration-independent, but is sensitive to magnesium concentration (Gal *et al.* 1988; Caplow *et al.* 1988; O'Brien *et al.* 1990). The alternation between these phases

of MT elongation and rapid shortening have been termed 'dynamic instability' (Mitchison and Kirschner, 1984; Horio and Hotani, 1986; Walker *et al.* 1988). Recently, individual MTs undergoing dynamic instability have been directly observed using light microscopy *in vitro* (Horio and Hotani, 1986; Walker *et al.* 1988) and in living cells (Cassimeris *et al.* 1988; Sammak and Borisy, 1988; Schulze and Kirschner, 1988). At present, the mechanisms that cause dynamic instability are unknown but are thought to be related to changes in tubulin conformation induced by the hydrolysis of GTP bound to the β -subunit of the tubulin dimer (Kirschner and Mitchison, 1986; Walker *et al.* 1988; Caplow *et al.* 1988; O'Brien *et al.* 1987; Carlier *et al.* 1987; Mandelkow and Mandelkow, 1989; Melki *et al.* 1989). In these models, a cap of GTP-tubulin at the MT end is proposed to stabilize a labile core of GDP-tubulin.

The purpose of this study was to test if there are structural differences between MT ends in the phases of elongation *versus* shortening that might account for their behavior. We used the technique of negative-staining electron microscopy (EM) to visualize the morphology of MTs assembled from purified porcine brain tubulin. MTs in the elongation phase were obtained by seeding growth from isolated axonemes as described by Walker *et al.* (1988), while shortening phase MTs were produced by rapid dilution of steady-state, self-assembled MTs. A comparison was also made between MT disassembly induced by rapid dilution with that produced by cold-depolymerization, a commonly used method. The effect of tubulin purity on MT structure under all of the above conditions was tested by comparing the results for MTs assembled from highly purified tubulin (PC-tubulin) with those from tubulin containing microtubule-associated proteins (3-cycle tubulin; MTP). Preliminary results of these experiments have been reported in abstract form (Simon and Salmon, 1989).

Materials and methods

Preparation of axonemes

Axonemes were prepared from *Tetrahymena thermophila*, strain SB255 (mucus-deficient), by a modification of Thompson *et al.* (1974) and Witman *et al.* (1978), as described by Drs Bruce R. Telzer and Leah T. Haimo (personal communication). Cells were grown in 2% Proteose Peptone (Difco Labs, Detroit, MI), 0.01 mM FeCl₃, plus two drops of Antifoam A (Sigma Chemical Co., St Louis, MO) at 32°C. Cells (density $\sim 5 \times 10^6$ cells ml⁻¹) were collected and washed twice in HC buffer (10 mM Hepes, 0.5 mM CaCl₂, pH 7.4) by centrifugation at 2000 *g*, 4°C, for 5 min. Cells were resuspended in 4× packed cell volume with HC buffer, then deciliated by adding dibucaine-HCl (Sigma; dissolved in ethanol) to 2 mM. After 1 min, the cell bodies were differentially removed by three cycles of centrifugation at 750 *g*, 4°C, for 5 min. The cilia were then collected at 12 000 *g*, 4°C, for 15 min, then washed and resedimented in HMDEK buffer (10 mM Hepes, 5 mM MgSO₄, 1 mM EDTA, 25 mM KCl, 1 mM dithiothreitol, pH 7.4). The pellet was resuspended in half the packed cell volume with HMDEK, mixed 1:1 (v/v) with 0.5% Nonidet P-40 (Sigma) in HMDEK, and set on ice for 20 min. The demembrated axonemes were pelleted and washed twice with HMDEK, and once in PM buffer (100 mM Pipes, 2 mM EGTA, 1 mM MgSO₄, 1 mM GTP, pH 6.9). The axonemes were stored at -20°C in 50% glycerol. Before use, axonemes were diluted >15-fold with PM buffer, sedimented at 7000 *g* for 15 min at 4°C, then resuspended with PM buffer.

Tubulin purification

Microtubule protein (MTP) consisting of tubulin and MT-associated proteins (MAPs), also named 3-cycled tubulin, was prepared from porcine brain according to Shelanski *et al.* (1973) and

Sloboda *et al.* (1976). Tubulin free from MAPs (PC-tubulin) was prepared by the further purification of MTP as described by Voter and Erickson (1984) using phosphocellulose chromatography followed by MT assembly/disassembly in 1 M sodium glutamate. Tubulin samples were frozen in liquid nitrogen and stored at -70°C.

Preparation of elongating and shortening MT samples for electron microscopy (EM)

Samples containing MTs in the elongation phase were prepared as follows. Mixtures of PC-tubulin or MTP at 1.4 mg ml⁻¹ and diluted axonemes in PM buffer were held on ice, then placed in a 37°C water bath to initiate polymerization. At specific time intervals, 10 μ l of the sample was placed on a 200 mesh, carbon-coated, 1% Formvar-coated (Shawinigan Resin Corp., Springfield, MA), glow-discharged copper grid. After 15 s, the grid was rinsed quickly with five drops of PM buffer without GTP at 37°C, stained with 5–10 drops of 1 or 2% uranyl acetate (Fisher Scientific Co., Fair Lawn, NJ), and then excess stain was blotted with filter paper. The time interval between the first rinse and stain drop was typically 4 s ($n=10$; s.d.=0.4). Shortening MTs were prepared by allowing 4 mg ml⁻¹ PC-tubulin or 3 mg ml⁻¹ MTP to self-assemble in PM buffer for ~ 30 min at 37°C. Depolymerization was induced either by dilution of samples to 0.5 mg ml⁻¹ with PM buffer at 37°C (isothermal dilution), which is at the extrapolated critical concentration for elongation as measured by Walker *et al.* (1988), or by cold-depolymerization by placing the undiluted sample into an ice-water bath. Portions were withdrawn from the samples at specific time points and prepared for EM as above, except that cold-depolymerized sample grids were rinsed in ice-cold PM buffer, less GTP. EM was performed on a Zeiss EM10CA at 80 kV acceleration typically at $\times 50\,000$, $\times 100\,000$, or $\times 200\,000$ magnification.

Results

MTs in the elongation phase

MTs in the elongation phase were obtained by seeding MT growth from the ends of isolated *Tetrahymena* axonemes. Under the conditions employed (PM buffer; 14 μ M tubulin; 37°C), the fraction of MTs in the elongation phase was >97%, based on the frequencies of catastrophe/rescue determined by Walker *et al.* (1988) for PC-tubulin and Pryer *et al.* (1990) for MTP. The MT ends were examined by negative-staining electron microscopy (EM) between 30 s to 10 min after warming to 37°C (Figs 1 and 2). At 30 s, MTs assembled from PC-tubulin had two to three MTs attached to each end of an axoneme, while MTP had 5–10 MTs per end. At this time point, tubulin polymer growth was short (~ 0.5 μ m). At 5 min, the PC-tubulin polymers had a predominant length between 3 and 6 μ m, while MTP polymers had reached that length by ~ 2 min. Fig. 2C shows a low-magnification image of assembly from an axoneme after 3 min. After longer time periods (>10 min), MTs were long (>10 μ m) and were sometimes detached from axonemes, probably due to breakage during handling. Therefore, time points longer than 10 min were not routinely examined.

The major fraction ($\sim 70\%$; see Table 1; Fig. 8A, below) of polymer assembled from PC-tubulin were MTs with ends that were approximately even or 'blunt', such that protrusions of protofilaments from the end usually did not exceed 10–15 nm ($\sim 1/2$ the MT width). The range in morphology of blunt MT ends as we define it is shown in Figs 1C,D, and 2A,B. Typically, blunt ends were not perfectly even but showed slight irregularity. Nonetheless, we consider these populations as blunt ends because of the uncertainty in the degree of structural preservation of the technique (see Discussion). MTs assembled from

Figs 1, 2. Electron micrographs of negatively stained porcine brain MTs in the elongation phase, nucleated from isolated axonemes. Fig. 1 (MTP) shows an irregular protrusion (A) and a protofilament sheet (B) extending from the distal end of an intact MT. Fig. 2 (PC-tubulin) shows a low-magnification view of MTs grown for 3 min from an isolated axoneme (C) with a distribution of MT lengths. In D, a spiral sheet (arrow) and several other sheets are emerging from an axoneme in addition to intact MTs. Also shown is a rare structure where a sheet emerged from the axoneme but is closed into an intact MT distally (arrowhead). Examples of what were considered blunt MT ends are shown in Fig. 1C,D and Fig. 2A,B. Bar, 50 nm.

Table 1. Quantitation of tubulin polymer morphology

Condition	% Blunt*	% Irregular†	% Sheets‡	n
PC elongation§				
1 min	68	1	30	92
5 min	67	5	28	123
10 min 15 s	69	0	31	152
MTP elongation§				
30 s	40	18	42	114
1 min 30 s	21	26	53	108
PC shortening				
37°C¶	96	2	2	170
Cold	82	0	18	320
MTP shortening¶				
37°C¶	92	8	0	181
Cold	73	24	3	148

PC, MTs assembled from highly purified tubulin. MTP, MTs assembled from tubulin containing MAPs (3-cycled tubulin).

* Blunt ends are considered protofilament protrusions that extend for less than ~15 nm from the end of intact MTs (e.g. Figs 4A–D, 5A–E).

† Irregular ends are protrusions that extend for at least several hundred nm (e.g. Fig. 4F) or have long edge curls (e.g. Fig. 6A,B) from the end of intact MTs.

‡ Sheets are parallel arrays of protofilaments extending the entire polymer length (e.g. Figs 2D, 3A) or as a protrusion from an intact MT (e.g. Figs 1B, 7A,B).

§ MTs in the elongation phase were seeded from isolated axonemes for the times given (PM buffer at 37°C).

¶ MTs in the shortening phase were induced by isothermal dilution using PM buffer at 37°C for 1 min.

|| Cold-disassembled MTs.

MTP exhibited a range of ends that were much more irregular than those assembled from PC-tubulin, often occurring as protrusions several hundreds of nm in length that contained less than a full complement of protofilaments (Fig. 1A). The apparent width of the intact MTs varied between samples from 22–40 nm due to the degree of MT flattening on the grid due to adsorption, as previously discussed by McEwen and Edelstein (1980) and Erickson (1974a).

Surprisingly, both MTP and PC-tubulin assembly from axonemes frequently appeared on the grid surface as sheets of parallel protofilaments rather than intact cylindrical MTs. The tubulin sheets could be categorized into two distinct populations: (1) sheets that were short extensions (<1 µm) from the ends of intact MTs distal to the axoneme seed (Fig. 1B); and (2) sheets that emanated directly from the axoneme ends and extended for the entire polymer length (Figs 2D, 3A). We term these sheets 'distal ends' and 'entire length', respectively. Infrequently, tubulin sheets were observed proximal to the axonemes with intact MTs distally, as shown in Fig. 2D (arrowhead). At 5–10 min after initiation of assembly, sheets were seen that extended several µm (3–6 µm) or approximately as long as the intact MTs. The sheets sometimes appeared in twisted, helical shapes (Fig. 2D, arrow). The number of protofilaments in both types of sheets (distal ends *versus* the entire length) ranged from 9 to 16, but was typically ~13. Frequently, the number of protofilaments decreased gradually from approximately 13 to 9 as the distance from the axoneme or from the end of an intact MT increased, giving the appearance of a slight taper. The sheet ends had a range of morphology including flat, tapered protrusion and arc-shaped (Fig. 3B,C,D, arrowheads). The frequency of both distal ends and entire length sheets when grouped together relative to intact, closed MTs are given in Table 1

Table 2. Occurrence of tubulin polymer sheets at the ends of intact MTs versus the entire polymer length during elongation from axonemes

Elongation time (min)	Protofilament sheet occurrence		n
	% Distal ends*	% Entire length†	
1	21	79	96
5	22	78	18
10.25	19	81	57

PC-tubulin in the elongation phase was seeded from axonemes, then samples were prepared for EM at the times given (see Materials and methods).

* Distal ends are protofilament sheets extending only from the ends of intact MTs distal to the axoneme.

† Entire length means sheets emanating directly from the axoneme and persisting for the entire polymer length.

and Fig. 8A (below), and was approximately 30 % for PC-tubulin and 50 % for MTP. Within the population of PC-tubulin sheets, the proportion occurring as sheets only on the distal ends of growing MTs was 20 % while that for sheets for the entire polymer length was 80 % (see Table 2). The fraction of sheets *versus* intact MTs, and also the type of sheet, did not shift in population over the 10 min in which it was examined for MTs assembled from PC-tubulin (Tables 1,2). The morphologies for MTP were not tabulated. It is important to note that the negative staining procedures do not preserve the three-dimensional structure of the tubulin polymers. The sheets observed by our methods probably are 'C-shaped' polymers of similar curvature to MTs in solution that have become flattened onto the grid by adsorption (see Discussion).

Nucleation from the incomplete wall (11 protofilaments) of the axoneme B-subfiber could potentially explain the presence of sheets that extend the entire polymer length, while intact MTs could emanate exclusively from the complete A-subfiber (13 protofilaments). This possibility was tested by scoring the polymer morphology from the subfibers between 1 and 10 min of growth of 14 µM PC-tubulin. The axoneme subfibers can be discerned because the A-subfiber has a greater width than the adjacent B-subfiber (Fig. 3A). Cylindrical, intact MTs assembled from PC-tubulin had 85 % (*n*=85) emanating from the A-subfiber and 15 % from B-subfiber, consistent with results of Scheele *et al.* (1982) and Binder *et al.* (1975), who found that under low tubulin concentrations MTs preferentially grew from the A-subfibers. Sheets, either at the distal ends of MTs or occurring for the entire polymer length, had 44 % (*n*=36) from the A-subfiber and 56 % from the B-subfiber. Thus, intact MTs and sheets emanated from both the A- and B-subfibers. Sometimes sheets emanated from both A- and B-subfibers of the same outer doublet, and others emanated from adjacent outer doublets forming sheets of >13 protofilaments; however, these were not scored. Therefore, the various types of polymer morphology are not strictly associated with the site of nucleation from the axonemes.

The polarity of the ends of isolated axonemes used in these experiments (cell-body proximal *versus* distal) could not be morphologically distinguished from each other. It was determined, however, that 32 % of axonemes had sheets on both ends (*n*=44) at 1, 5 and 10 min after growth initiation of PC-tubulin. Conversely, 68 % of axonemes had one end with only intact MTs, with the other end having sheets and intact MTs. Therefore, sheets and intact MTs were present on both the proximal and distal axon-

Fig. 3. Electron micrographs of tubulin protofilament sheets assembled from PC-tubulin. The conditions are the same as for Fig. 2. Tubulin assembly frequently occurred as sheets (see Figs 1,2). In A, sheets are seen emanating from both the A- and B-subfibers of the axoneme outer doublets (labeled *a*, *b*, respectively). The photograph has been dodged at the axoneme level (open arrowhead) to allow visualization of the subfibers (compare with Fig. 2D). The ends of sheets (arrowheads in B,C,D) showed a range of morphology from irregular (B,C), to almost even (D). Bar, 50 nm.

eme ends, although the exact distributions were not measured in these experiments.

MTs in the shortening phase

Isothermal dilution. To capture MTs in the rapid shortening phase, it was necessary initially to form long MTs (~ 50 – $200\ \mu\text{m}$) because of the fast MT disassembly rates of 20 – $30\ \mu\text{m min}^{-1}$ per free end (Walker *et al.* 1988; Pryer *et al.* 1990) and the lag time required to manipulate the preparation for negative staining. Since there was a limit on the length to which MTs could be grown from axonemes before breakage occurred (see above), long MTs were prepared by self-assembly (not nucleated from axonemes) of 3 or $4\ \text{mg ml}^{-1}$ PC-tubulin or MTP for 30 min at 37°C . These conditions favored the formation of long, intact MTs (Kirschner *et al.* 1975; Voter and Erickson, 1984). Shortening was then induced by diluting the sample isothermally to a concentration at which net disassembly occurs ($5\ \mu\text{M}$; see Materials and methods). MTs assembled from PC-tubulin formed a dense, entangled mass on the grid in the EM samples examined at the earliest time points (15–20 s). Over time, the density of MTs on the grid decreased until by 2 min only one or two MTs per grid hole (200 mesh; $\sim 100\ \mu\text{m}$ width) could be observed. The density of MTs in the MTP samples was similar but not as sparse as PC-tubulin samples at 2 min.

Shortening MTs assembled from PC-tubulin displayed MT ends that were virtually always blunt (as defined above) with no curls, splays or sheets (Fig. 5A–E; Table 1; Fig. 8B). MTs assembled from MTP also had predominantly blunt ends (Fig. 4A–D) with no sheets, but also showed splayed ends of $\sim 100\ \text{nm}$ in length, short protofilament curls (Fig. 4E) and irregular tapered ends (Fig. 4F) in a small percentage of samples (see Table 1 and Fig. 8B). The data listed in Table 1 were compiled after 1 or 2 min post-dilution for PC-tubulin and MTP, respectively, a time that allowed all MT ends to enter the shortening phase (see Discussion).

Cold-depolymerization. Cold temperature (cold-depolymerization) has been used previously in EM morphology studies (see Discussion) as a method for inducing MT disassembly. Since it is possible that cooling induces shortening by a different mechanism than occurs in MTs undergoing dynamic instability, we compared the results of cooling with those produced by isothermal dilution. Cold-depolymerization was induced by placing self-assembled MTs, at a concentration of 3 or $4\ \text{mg ml}^{-1}$, on an ice bath (see Materials and methods). Grids prepared from MTs cooled for less than 2 min were too dense with long MTs to be useful, while those prepared 10–20 s later did not have any MTs (although MTP concentrations showed a wider range than PC-tubulin). Therefore, samples were normally observed 2 min after cold treatment. Overall, the morphology of cold-depolymerized MTP was strikingly dissimilar to that of PC-tubulin MTs as described below.

At 2 min post-cooling, MTs assembled from PC-tubulin had lengths that ranged from 1 to $6\ \mu\text{m}$. The polymers usually were not straight but had twists and kinks. Long sheets were frequently observed, and extended from half to the entire length of the polymer in the form of twisted and curled sheets (Fig. 7A,B, arrowheads). Sometimes the sheets extended from the end of an intact MT, and other sheets had intact MTs on either side. The sheets often contained a full complement of ~ 13 protofilaments (Fig. 7A,B, arrowhead), which sometimes split apart (Fig. 7B, arrows). The ends of intact MTs were blunt

except that short ($\sim 15\ \text{nm}$) protofilament curls were often observed (Table 1 and Fig. 8C).

Cold-depolymerized MTs assembled from MTP had ends that frequently consisted of protofilament rings (Fig. 6A, arrowhead) and curls, often appearing as ‘rams’ horns’ (Fig. 6B, arrowhead). Irregular ends also occurred in the shape of tapered protrusions, which were composed of protofilaments that extended to different lengths from the end (Fig. 6A, arrow). In addition, rings of protofilaments were found scattered in the background (Fig. 6A,B). Sheets of any type were only rarely observed (see Table 1 and Fig. 8C), unlike the sheets seen in the cooled PC-tubulin preparations.

Discussion

Microtubule elongation

Limitations on the interpretation of the EM results must take into account how the sample preparation may have affected the MT morphology. MTs in the elongation phase were prepared by adsorbing the sample to the grid, washing quickly with a few drops of isothermal buffer, then quickly applying uranyl acetate. The washing step before uranyl acetate fixation was essential, because without it the fixed sample contained an unacceptable amount of precipitated protein that obscured visualization of the MTs. An important question is whether shortening could have occurred on the grid during the washing step, which was $\sim 4\ \text{s}$ (see Materials and methods). This is the same time scale as is measured for the onset of rapid shortening following infinite dilution using a flow chamber and video-enhanced differential interference contrast (video-DIC) microscopy (Walker *et al.* 1989). However, the MT ends probably did not convert to the rapid-shortening phase because, under the three conditions tested (elongation, shortening, cold-depolymerization), each displayed MTs of different morphology. In addition, sheets were not an anomaly of the sample preparation, since they were found specifically in the elongation-phase samples and cold-depolymerized PC-tubulin, and not in the isothermal dilution grids. The results were consistent between three preparations of axonemes, two preparations of PC-tubulin, and multiple EM grids. We also examined tubulin growth seeded from sea urchin sperm axonemes and sheets were again observed (data not shown). We tested glutaraldehyde as a fixing agent to allow the sample grid to be rinsed without the possibility of MT disassembly. However, 1% glutaraldehyde produced unusual types of MT morphology such as long, single protofilament strands connecting MTs together and the loss of protofilament structure with time in fixation (data not shown), and was therefore not used in these studies. Glutaraldehyde anomalies of MT fixation have been previously reported by Himes *et al.* (1982).

A surprising result from our studies is that seeded polymerization of MTP or PC-tubulin from isolated *Tetrahymena* axonemes often consisted of sheets of parallel protofilaments in addition to intact MTs. This result is similar to previous observations made for MT elongation under conditions of self-assembly (no added seeds) as discussed below. Erickson (1974a) and Kirschner *et al.* (1975) observed sheets in the form of helical and planar ribbons during self-assembly of brain MTP. These structures consisted of 5–13 protofilaments, and predominated during the initial 30 s to 2 min of polymer elongation. After 8–10 min, complete, closed MT cylinders predomi-

Figs 4, 5. Electron micrographs of MTs in the shortening phase induced by isothermal dilution. MTs were self-assembled at 37°C for 30 min, then diluted isothermally below the critical concentration for assembly with PM buffer. Samples were prepared for EM at 2 or 1 min post-dilution for MTP or PC-tubulin MTs, respectively. Fig. 4 (MTP) shows a range of MT ends with varying degrees of evenness that were considered blunt ends (A–D). MTs assembled from MTP also displayed a population with protofilament protrusions extending from an intact MT as shown in F (arrowhead). In E, an end with a protofilament curl is shown. Fig. 5 (PC-tubulin) shows an array of blunt MT ends (A–E). Bar, 50 nm.

Figs 6, 7. Electron micrographs of MTs in the shortening phase induced by cold-depolymerization. MTs were assembled as in Figs 4 and 5, then disassembly was induced by placing the sample into an ice bath for 2 min. Fig. 6 (MTP) shows protofilament protrusions (arrow in A), edge curls, protofilament rings (arrowhead in A), and rams' horns (arrowhead in B). Fig. 7 (PC-tubulin) shows long, twisted sheets of parallel protofilaments, which often extended for a large fraction of the polymer length (arrowhead in A,B). Sometimes the sheets appeared to split into individual protofilaments (arrows in B). Note the absence of rings in Fig. 7 compared to Fig. 6. Bar, 75 nm.

nated. Voter and Erickson (1984) observed similar results with self-assembled brain PC-tubulin in glycerol-containing buffers. At 0.78 mg ml^{-1} tubulin, sheets of a few micrometers in length were observed at 12 min. As time increased to 26 min, the number of sheets increased and many intact MTs were also formed. At 135 min, the polymers were mostly in the form of intact MTs with 10–20% by number as sheets. Detrich *et al.* (1985) and Detrich and Jordan (1986) found that purified sea urchin

Fig. 8. Bar graphs represent the distribution of MT blunt ends, sheets and irregular ends under the conditions of elongation, shortening and cold depolymerization as observed by EM. The data and terminology were taken from Table 1. A. MTs in the elongation phase seeded from axonemes. Data for MTP were taken at 1.5 min, and PC-tubulin was averaged for 1–10.25 min post-warming. B. MTs (self-assembled) in the shortening phase induced by isothermal dilution at 37°C . C. MTs were cold-depolymerized by placing self-assembled MTs on an ice bath. MTP are MTs assembled from tubulin containing MAPs (3-cycled) and PC-tubulin is highly purified tubulin. This figure illustrates the marked distribution in morphology between the three conditions of assembly/disassembly.

egg tubulin formed sheets during the initial stages of self-assembly, which elongated up to μm lengths at time periods of 45 s to 10 min. Eventually, most of the polymer formed intact MTs after an overshoot of turbidity, which the authors partly attributed to polymer sheets that formed during the initial polymerization. Sheets were also found during the initial stages of self-assembly of sea urchin sperm axoneme outer doublet B tubulin (Linck and Langevin, 1981). We did not measure a shift in population from sheets to intact tubules (Table 1), or from sheets that extended the entire polymer length to those only at the distal ends (Table 2). This discrepancy may be due to the relatively short time periods in which our samples were observed (<10 min).

Erickson (1974a,b) suggested that small arrays of parallel protofilaments are the initial nucleation sites for self-assembled tubulin, which elongate two-dimensionally until a full complement of protofilaments is formed, thereupon sheets close or 'zipper' to form intact MTs (see Kirschner, 1978). Therefore, sheets were suggested to be a phenomenon specific to self-assembled tubulin resulting from the lack of pre-existing nucleation sites. However, we obtained similar results with seeded polymerization of MTP or PC-tubulin from isolated axonemes. This is in agreement with Dentler and Rosenbaum (1977), who examined the regeneration of *Chlamydomonas* flagella that had been resorbed using a high salt treatment. They found that 70% of the ends of regenerating flagella A-subfiber MTs had sheets at their distal ends. The above results indicate that sheet formation during tubulin polymer elongation is an intrinsic property of MT assembly that does not depend on the source and purity of the tubulin, or on whether MTs are polymerized by self-assembly or nucleated from axonemes.

The occurrence of tubulin sheets seeded from axonemes may be explained in several ways. First, it can be argued that we used the heterologous system of porcine brain tubulin and *Tetrahymena* axonemes, therefore MTs with aberrant lattices were formed. This is unlikely because sheets were found with regenerating flagella (Dentler and Rosenbaum, 1977), a homogeneous system. Second, sheets that extend the entire polymer length could represent initial polymer that eventually would close or zipper to form an intact MT (Erickson, 1974a). A sheet completing the zippering process could be MTs with sheets on the distal ends, which comprised 6% by number of total polymer assembled from PC-tubulin (calculated from Tables 1,2). Third, the ends of the axoneme seeds could have been damaged during the isolation procedures. Thus, a combination of intact and frayed axoneme subfiber ends could have served as the tubulin initiation sites. It is not possible to discriminate between the above possibilities, but indeed it may be a combination. This is supported by the heterogeneous structures and polymer initiation sites observed for the tubulin polymer in the elongation phase (see Results).

The spatial arrangements of tubulin dimers in axoneme outer doublet A- and B-subfibers, determined from EM diffraction (Amos and Klug, 1974), have been termed the A- and B-lattices, respectively. The arrangement of dimers in MTs having an A-lattice was deduced to have perfect helical symmetry, and elongation was by sequential, helical addition of tubulin dimers to the MT ends. In contrast, a B-lattice MT has a discontinuity or 'seam' in the lattice where the dimers in the protofilaments are out of register after completing a helical turn. These lattices were reconcilable with the fact that the A-subfiber is an

intact MT (13 protofilaments), while the B-subfiber (11 protofilaments) is an incomplete MT wall (reviewed by Mandelkow and Mandelkow, 1989).

Theoretical models to explain MT dynamic instability have assumed that MTs are in an A-lattice, and therefore have perfect helical symmetry (Bayley *et al.* 1990; Chen and Hill, 1985). However, this assumption may not be correct. Structural analysis has shown that self-assembled MTs (Mandelkow *et al.* 1986; McEwen and Edelstein, 1980; Crepeau *et al.* 1978) and isolated axoneme central-pair MTs (Linck *et al.* 1981) have B-lattice structures, while the A-lattice has been reported only for the A-subfiber of intact axonemes (Amos and Klug, 1974). B-lattice structures, or at least MTs containing a seam, are probably also present in at least a fraction of the polymer seeded from axonemes in our preparations. This is supported by the presence of distal sheets on intact MTs, irregular ends that suggest individual protofilament elongation, and the cold-labile seam of MTs assembled from PC-tubulin. Erickson and Voter (1986) noted that long, open sheets formed from self-assembled tubulin could apparently be stable over long time periods. They postulated that MT closure is not a crucial step in elongation, and does not confer higher stability for elongation. Thus, tubulin polymer growth need not occur by helical dimer addition, since sheets lack helical structure, but instead could elongate by individual protofilament extension. This concept has been applied to B-lattice MT growth because of the discontinuity or seam in the structure, which also precludes helical symmetry (Mandelkow *et al.* 1986; Linck and Langevin, 1981). Although it has not been ascertained whether tubulin sheets (or C-shaped structures) can undergo dynamic instability, dynamic instability has been directly observed with self-assembled MTs (Horio and Hotani, 1986), which have a B-lattice (Mandelkow *et al.* 1986; McEwen and Edelstein, 1980). Therefore, any proposed model of dynamic instability must account for the presence of a discontinuity in the lattice structure, and must not presume strictly helical assembly/disassembly.

Isolated axonemes, which have been used in numerous previous studies to seed tubulin growth, did not provide homogeneous initiation sites in our samples. MTs and sheets emanated from either the A- or the B-subfiber, or a combination of the two. The MTs that emanated from the A-subfibers could be a direct template extension of the axoneme, and therefore be continuous with the subfiber. However, this cannot be the case for MTs on the B-subfiber, since it has an incomplete MT wall. Scheele *et al.* (1982) determined the number of protofilaments in MTs grown from *Chlamydomonas* axonemes. When brain tubulin was seeded under conditions where growth occurs only on the A subfiber, the protofilament number was virtually always 13 (the A subfiber also contains 13). At higher tubulin concentrations, nucleation was shown to occur from both the A- and B-subfiber. Under these conditions, both 13 and 14 protofilaments per MT predominated. Since the B-subfiber has only 11 protofilaments, then 'nucleation' rather than strict template extension occurred. The authors suggested that the conformation of the B-subfiber may impose lattice constraints on the nucleating MTs that dictate the final protofilament number and subunit arrangement. Another example of nucleation rather than template addition has been observed for MTs seeded from centrosomes (Evans *et al.* 1985), which produced MTs with 13 protofilaments, even under buffer conditions that favored 14 protofilaments for self-assembled MTs. Therefore, individual MTs and sheets

seeded from axonemes probably elongate by a variety of mechanisms and could potentially have heterogeneous lattice structures.

Images of elongating MTs analysed by video-enhanced differential interference contrast light microscopy (video-DIC microscopy) have not discerned protofilament sheets (Walker *et al.* 1988). This is not surprising, since both a MT and a sheet are detectable, but not resolvable by this technique. In addition, tubulin sheets in solution probably have a curvature similar to an intact MT, so that the tubulin polymer is in the form of a tubule but with incomplete lateral associations between the edge protofilaments. In fact, the natural curvature of tubulin sheets has been used to identify the intrinsic structural polarity of MTs (Heidemann and McIntosh, 1980). This possibility is supported by cross-sections of assembling MTs, which sometimes appear 'C-shaped' (Erickson, 1974a; Mandelkow *et al.* 1986). Thus, when a sheet is adsorbed to an EM grid, the forces of adsorption cause the structure to open and lie flat.

Shortening microtubules

A possible fixation artifact of MTs in the shortening phase could be that one side of the MT may be adsorbed to the EM grid and stabilized from further shortening while the opposite side could continue to shorten. The end result would be a protrusion of the side of the MT proximal to the grid. An increase in irregularities could also be due to fixation occurring at unequal rates between protofilaments, thereby some protofilaments would be stabilized before others. Since it is difficult to reconcile an artifact of fixation that would create more even ends, then the blunt ends as we define them, which often have protofilaments that end at slightly different lengths, could be an underestimate of the actual evenness.

MT shortening induced by isothermal dilution (see Materials and methods) closely represents the rapid-shortening phase of dynamic instability. Walker *et al.* (1988), using video-DIC microscopy, found that the frequency of conversion from elongation to rapid-shortening was increased at lower tubulin concentrations, while the shortening velocity was independent of tubulin concentration. Therefore, isothermal dilution is equivalent to increasing the probability of converting to the shortening phase without affecting the velocity or mechanism of shortening. Shortening MTs produced by this method, assembled from either MTP or PC-tubulin, displayed predominantly even, blunt ends. This suggests that rapid shortening does not occur by a gross change in morphology of the MT end. Since we have not shown how rapidly and accurately the ends are fixed (see above), it is possible that fraying may have occurred over short distances of several dimer lengths. We can eliminate the possibility that depolymerization induced by isothermal dilution occurs at sites along the length of the polymer, since MTs remain intact. This is consistent with video-DIC microscopy, which shows that MTs shorten at the ends without a change in the optical density of the MTs along their lengths (Walker *et al.* 1988). In addition, the rate of bulk MT depolymerization depends on the number of filament ends (Johnson and Borisy, 1977). Therefore, any proposed mechanism of MT shortening must be consistent with relatively uniform shortening (within several dimer lengths) of all protofilaments from the MT ends.

It has been postulated that MT rapid-shortening may involve a long-range steric change in the MT wall (Kirschner and Mitchison, 1986; Walker *et al.* 1988).

Indeed, previous observations using cryoelectron microscopy of cold-depolymerized MTs, assembled from MTP, indicated that MT disassembly occurred by a segmental release of protofilaments from the ends and all along the length of MTs (Mandelkow and Mandelkow, 1985; Mandelkow *et al.* 1985). Similar morphological observations were made by Kirschner *et al.* (1974) using conventional negative-stain EM. They examined cold-depolymerized MTP MTs and observed rings or spirals depending on how long the preparation was cooled. If the samples were diluted at room temperature, a 'rams'-horn' appearance was observed, similar to our Fig. 6B. The conclusion stated by these authors was that MT disassembly proceeds by release of segments of protofilaments that coil into spirals, or at least the protofilaments of disintegrating MTs have a tendency to coil into the spiral or circular form. The authors also suggested that lowering the temperature weakens lateral bonds, but not the longitudinal ones. These observations have been made in other laboratories (Kirschner and Williams, 1974; Erickson, 1974b; Weisenberg, 1974; Mandelkow *et al.* 1985) and are confirmed by our MTP results. More recently, it was determined that the ring structures are composed and induced by MAPs in the preparations (Erickson and Voter, 1986; Voter and Erickson, 1984). However, these results are specific to cold-depolymerization and may not be representative of MTs exhibiting shortening *via* dynamic instability. This is based on our observation that cold-depolymerized MTs have distinctly different morphology than MTs in the shortening phase induced by isothermal dilution, a method that closely mimics stochastic shortening (see above).

Cold-disassembled MTs composed of PC-tubulin often exhibited sheets of protofilaments (Fig. 7A,B) that are strikingly different than those assembled from MTP, which displayed ends that formed irregular protrusions, curls, and rings but no sheets (see Results). The formation of sheets with PC-tubulin may indicate that there is a cold-induced conformational strain in the MT lattice forcing it open into sheets. This is evidence for the discontinuity or seam in the MT wall parallel to the MT longitudinal axis (Mandelkow and Mandelkow, 1989). If lateral associations are not intrinsically weakened except at the seam, then the splaying observed with MTP may be due to a MAP-induced 'peeling' force parallel to the long axis of the MT, which can overcome the lateral protofilament association forces. This is consistent with the observation that rings are formed directly from MTs during cold-disassembly, measured by radiolabeled nucleotide exchange (Zeeberg *et al.* 1980). Therefore, cold-induced splaying of MTP assembled MTs may be due to an active force attributed by the MAPs, as opposed to a passive weakening of protofilament lateral interactions. An additional effect due to MAPs could be to increase the MT lateral bond strength at the seam and thereby prevent cold-induced sheet formation.

The morphological analysis of shortening MT ends in this study does not address the events that occur at the onset and initial stages of shortening. For example, the transition period from elongation to rapid shortening could exhibit a transient structural transition at the polymer ends (see Kirschner and Mitchison, 1986) that would not have been observed by our methods. A technique to address the initial stages of shortening must incorporate rapid fixation, perhaps by rapid-freezing (Mandelkow *et al.* 1985; Wade *et al.* 1989), during the first several seconds after dilution. The technique of cryoelec-

tron microscopy has the advantage of extremely rapid sample fixation and can eliminate the need for exogenous staining (Mandelkow and Mandelkow, 1985).

In conclusion, our data suggest the following: (1) seeded tubulin polymer growth *in vitro* does not occur by simple template extension of the axoneme MT subfibers because polymorphic structures are formed. (2) Dynamic instability can occur by non-helical pathways of elongation/shortening because MTs can have an intrinsic seam in their lattice structure. (3) MT shortening occurs at the ends of MTs, with no long-range change in morphology. (4) Cold-depolymerized MTs have an altered mechanism of shortening when compared to stochastic shortening *via* dynamic instability. Overall, MTs in the elongation phase can have highly irregular ends and need not elongate by a helical assembly process. Conversely, shortening phase MTs can depolymerize in a relatively uniform fashion, indicating that a gross disruption of the MT wall is not required for disassembly to take place.

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References

- AMOS, L. A. AND KLUG, A. (1974). Arrangement of subunits in flagellar microtubules. *J. Cell Sci.* 14, 523-549.
- BAYLEY, P. M., SCHILSTRA, M. J. AND MARTIN, S. R. (1989). A simple formulation of microtubule dynamics: Quantitative implications of the dynamic instability of microtubule populations *in vivo* and *in vitro*. *J. Cell Sci.* 93, 241-254.
- BAYLEY, P. M., SCHILSTRA, M. J. AND MARTIN, S. R. (1990). Microtubule dynamic instability: numerical simulation of microtubule transition properties using a lateral cap model. *J. Cell Sci.* 95, 33-48.
- BINDER, L. I., DENTLER, W. L. AND ROSENBAUM, J. L. (1975). Assembly of chick brain tubulin onto flagellar microtubules from *Chlamydomonas* and sea urchin sperm. *Proc. natn. Acad. Sci. U.S.A.* 72, 1122-1126.
- CAPLOW, M., SHANKS, J., BREIDENBACH, S. AND RUHLEN, R. L. (1988). Kinetics and mechanism of MT length changes by dynamic instability. *J. biol. Chem.* 263, 10943-10951.
- CARLIER, M.-F., DIDRY, D. AND PANTALONI, D. (1987). MT elongation and guanosine 5'-triphosphate hydrolysis. Role of guanine nucleotides in MT dynamics. *Biochemistry* 26, 4428-4437.
- CASSIMERIS, L., PRYER, N. K. AND SALMON, E. D. (1988). Real-time observations of microtubule dynamic instability in living cells. *J. Cell Biol.* 107, 2223-2231.
- CHEN, Y.-D. AND HILL, T. L. (1985). Monte Carlo study of the GTP cap in a five-start helix model of a microtubule. *Proc. natn. Acad. Sci. U.S.A.* 82, 1131-1135.
- CREPEAU, R. H., MCEWEN, B. AND EDELSTEIN, S. J. (1978). Differences in α and β polypeptide chains of tubulin resolved by electron microscopy with image reconstruction. *Proc. natn. Acad. Sci. U.S.A.* 75, 5006-5010.
- DENTLER, W. L. AND ROSENBAUM, J. L. (1977). Flagellar elongation and shortening in *Chlamydomonas* III. Structures attached to the tips of flagellar MTs and their relationship to the directionality of flagellar MT assembly. *J. Cell Biol.* 74, 747-759.
- DETRICH, H. W. III AND JORDAN, M. A. (1986). Mechanism of assembly of sea urchin egg tubulin. *Ann. N.Y. Acad. Sci.* 466, 529-543.
- DETRICH, H. W. III, JORDAN, M. A., WILSON, L. AND WILLIAMS R. C. JR (1985). Mechanism of microtubule assembly. *J. biol. Chem.* 260, 9479-9490.
- DUSTIN, P. (1984). *Microtubules*. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag.
- ERICKSON, H. P. (1974a). Microtubule surface lattice and subunit structure and observations on reassembly. *J. Cell Biol.* 60, 153-167.
- ERICKSON, H. P. (1974b). Assembly of microtubules from preformed, ring-shaped protofilaments and 6-S tubulin. *J. supramolec. Struct.* 2, 393-411.
- ERICKSON, H. P. AND VOTER, W. A. (1986). Nucleation of MT assembly. *Ann. N.Y. Acad. Sci.* 466, 552-565.

- EVANS, L., MITCHISON, T. AND KIRSCHNER, M. (1985). Influence of the centrosome on the structure of nucleated MTs. *J. Cell Biol.* **100**, 1185–1191.
- GAL, V., MARTIN, S. AND BAYLEY, P. (1988). Fast disassembly of MTs induced by Mg^{2+} or Ca^{2+} . *Biochem. biophys. Res. Commun.* **55**, 1464–1470.
- HEIDEMANN, S. R. AND MCINTOSH, J. R. (1980). Visualization of the structural polarity of microtubules. *Nature* **286**, 517–519.
- HIMES, R. H., JORDAN, M. A. AND WILSON, L. (1982). The effects of glutaraldehyde on the bound nucleotide content and morphology of MTs *in vitro*. *Cell Biol. Int. Rep.* **6**, 697–704.
- HORIO, T. AND HOTANI, H. (1986). Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature* **321**, 605–607.
- JOHNSON, K. A. AND BORISY, G. G. (1977). Kinetic analysis of microtubule self-assembly *in vitro*. *J. molec. Biol.* **117**, 1–31.
- KARECLA, P., HIRST, E. AND BAYLEY, P. (1989). Polymorphism of tubulin assembly *in vitro*. *J. Cell Sci.* **94**, 479–488.
- KIRSCHNER, M. W. (1978). MT assembly and nucleation. *Int. Rev. Cytol.* **54**, 1–71.
- KIRSCHNER, M. W., HONIG, L. S. AND WILLIAMS, R. C. (1975). Quantitative electron microscopy of MT assembly *in vitro*. *J. molec. Biol.* **99**, 263–276.
- KIRSCHNER, M. W. AND MITCHISON, T. (1986). MT dynamics. *Nature* **324**, 621.
- KIRSCHNER, M. W. AND WILLIAMS, R. C. (1974). The mechanism of MT assembly *in vitro*. *J. supramolec. Struct.* **2**, 412–428.
- KIRSCHNER, M. W., WILLIAMS, R. C., WEINGARTEN, M. AND GERHART, J. C. (1974). MTs from mammalian brain: Some properties of their depolymerization products and a proposed mechanism of assembly and disassembly. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1159–1163.
- LINCK, R. W. AND LANGEVIN, G. L. (1981). Reassembly of flagellar B($\alpha\beta$) tubulin into singlet microtubules: Consequences for cytoplasmic microtubule structure and assembly. *J. Cell Biol.* **89**, 323–337.
- LINCK, R. W., OLSON, G. E. AND LANGEVIN, G. L. (1981). Arrangement of tubulin subunits and microtubule-associated proteins in the central-pair microtubule apparatus of squid (*Loligo pealei*) sperm flagella. *J. Cell Biol.* **89**, 309–322.
- MANDELKOW, E. AND MANDELKOW, E.-M. (1989). Tubulin, microtubules, and oligomers: Molecular structure and implications for assembly. In *Cell Movement*, vol. 2: *Kinesin, Dynein, and Microtubules Dynamics* (ed. F. D. Warner and J. R. McIntosh), pp. 23–45. New York: Alan R. Liss, Inc.
- MANDELKOW, E.-M. AND MANDELKOW, E. (1985). Unstained MTs studied by cryo-electron microscopy substructure, supertwist and disassembly. *J. molec. Biol.* **181**, 123–135.
- MANDELKOW, E.-M., RAPP, R. AND MANDELKOW, E. (1985). Cryo-electron microscopy and freeze fracture studies of microtubules. In *Microtubules and Microtubule Inhibitors* (ed. M. DeBrabander and J. DeMey), pp. 51–60. Amsterdam: Elsevier Science Publishers.
- MANDELKOW, E.-M., SCHULTHEISS, R., RAPP, R., MULLER, M. AND MANDELKOW, E. (1986). On the surface lattice of MTs: Helix starts, protofilament number, seam, and handedness. *J. Cell Biol.* **102**, 1067–1073.
- MC EWEN, B. AND EDELSTEIN, S. J. (1980). Evidence for a mixed lattice in microtubules reassembled *in vitro*. *J. molec. Biol.* **139**, 123–145.
- MELKI, R., CARLIER, M.-F., PANTALONI, D. AND TIMASHEFF, S. N. (1989). Cold depolymerization of microtubules to double rings: Geometric stabilization of assemblies. *Biochemistry* **28**, 9143–9152.
- MITCHISON, T. AND KIRSCHNER, M. (1984). Dynamic instability of MT growth. *Nature* **312**, 237–242.
- O'BRIEN, E. T., SALMON, E. D., WALKER, R. A. AND ERICKSON, H. P. (1990). Effects of magnesium on the dynamic instability of individual microtubules. *Biochemistry* (in press).
- O'BRIEN, E. T., VOTER, W. A. AND ERICKSON, H. P. (1987). GTP hydrolysis during MT assembly. *Biochemistry* **26**, 4148–4156.
- PRYER, N. K., WALKER, R. A., BOURNS, B. D., SOBOEIRO, M. F. AND SALMON, E. D. (1990). Microtubule-associated proteins modulate microtubule dynamic instability *in vitro*: Real-time observations using video microscopy. *J. Cell Biol.* (in press).
- SAMMAK, P. J. AND BORISY, G. G. (1988). Direct observation of microtubule dynamics in living cells. *Nature* **332**, 724–726.
- SCHEELE, R. B., BERGEN, L. G. AND BORISY, G. G. (1982). Control of the structural fidelity of MTs by initiation sites. *J. molec. Biol.* **154**, 485–500.
- SCHULZE, E. AND KIRSCHNER, M. (1988). New features of microtubule behavior observed *in vivo*. *Nature* **334**, 356–359.
- SHELANSKI, M. L., GASKIN, F. AND CANTOR, C. R. (1973). MT assembly in the absence of added nucleotides. *Proc. natn. Acad. Sci. U.S.A.* **70**, 765–768.
- SIMON, J. R. AND SALMON, E. D. (1989). The structure of microtubule ends during the elongation and shortening phases of dynamic instability observed by negative-stain electron microscopy. *J. Cell Biol.* **109**, 341a.
- SLOBODA, R. D., DENTLER, W. L. AND ROSENBAUM, J. L. (1976). MT associated proteins and the stimulation of tubulin assembly *in vitro*. *Biochemistry* **15**, 4497–4505.
- THOMPSON, G. A., BAUGH, L. C. AND WALKER, L. F. (1974). Nonlethal deciliation of *Tetrahymena* by a local anesthetic and its utility as a tool for studying cilia regeneration. *J. Cell Biol.* **61**, 253–257.
- VOTER, W. A. AND ERICKSON, H. P. (1984). The kinetics of MT assembly. *J. biol. Chem.* **259**, 10 430–10 438.
- WADE, R. H., PIROLLET, F., MARGOLIS, R. L., GAREL, J.-R. AND JOB, D. (1989). Monotonic versus oscillating MT assembly: a cryo-electron microscope study. *Biol. Cell* **65**, 37–44.
- WALKER, R. A., O'BRIEN, E. T., PRYER, N. K., SOBOEIRO, M. F., VOTER, W. A., ERICKSON, H. P. AND SALMON, E. D. (1988). Dynamic instability of individual MTs analyzed by video light microscopy: Rate constants and transition frequencies. *J. Cell Biol.* **107**, 1437–1448.
- WALKER, R. A., PRYER, N. K. AND SALMON, E. D. (1989). Dilution of individual microtubules observed in real time *in vitro*. Determination of the delay preceding the onset of rapid shortening. *J. Cell Biol.* **109**, 342a.
- WEISENBERG, R. C. (1974). The role of ring aggregates and other structures in the assembly of MTs. *J. supramolec. Struct.* **2**, 451–465.
- WITMAN, G. B., PLUMMER, J. AND SANDER, G. (1978). *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. *J. Cell Biol.* **76**, 729–747.
- ZEEBERG, B., CHEEK, J. AND CAPLOW, M. (1980). Exchange of tubulin dimer into rings in MT assembly-disassembly. *Biochemistry* **19**, 5078–5086.

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