

Microtubule Dynamics and Microtubule Caps: A Time-resolved Cryo-Electron Microscopy Study

Eva-Maria Mandelkow, Eckhard Mandelkow, and Ronald A. Milligan*

Max-Planck-Unit for Structural Molecular Biology, c/o DESY, D-2000 Hamburg 52, Germany; and *Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. Microtubules display the unique property of dynamic instability characterized by phase changes between growth and shrinkage, even in constant environmental conditions. The phases can be synchronized, leading to bulk oscillations of microtubules. To study the structural basis of dynamic instability we have examined growing, shrinking, and oscillating microtubules by time-resolved cryo-EM. In particular we have addressed three questions which are currently a matter of debate: (a) What is the relationship between microtubules, tubulin subunits, and tubulin oligomers in microtubule dynamics?; (b) How do microtubules shrink? By release of subunits or via oligomers?; and (c) Is

there a conformational change at microtubule ends during the transitions from growth to shrinkage and vice versa? The results show that (a) oscillating microtubules coexist with a substantial fraction of oligomers, even at a maximum of microtubule assembly; (b) microtubules disassemble primarily into oligomers; and (c) the ends of growing microtubules have straight protofilaments, shrinking microtubules have protofilaments coiled inside out. This is interpreted as a transition from a tense to a relaxed conformation which could be used to perform work, as suggested by some models of poleward chromosome movement during anaphase.

UNLIKE other self-assembling biopolymers, microtubules are not simply static structures but show a behavior termed dynamic instability (Mitchison and Kirschner, 1984); that is, they can switch spontaneously between phases of growth and shrinkage. This is observed not only with microtubules assembled *in vitro*, but also in living cells (Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Cassimeris et al., 1988). The possible physiological functions of this behavior, e.g., in mitosis, have recently been reviewed by Mitchison (1988).

The phase transitions in a population of microtubules may be uncorrelated so that the ensemble shows an apparent steady state. They can also be synchronized, leading to oscillations of assembly and disassembly which can be observed by light scattering, X-ray scattering, and EM (Pirolet et al., 1987; Carlier et al., 1987; Mandelkow et al., 1988; Lange et al., 1988; Melki et al., 1988; Wade et al., 1989; Obermann et al., 1990). Oscillations require GTP which is hydrolyzed during microtubule assembly. Depending on how GTP is supplied one can distinguish two types of oscillations: the first is maintained by excess GTP in the solution; it has typical periodicities ~ 1 –3 min. The second type of oscillation is achieved by a GTP regenerating system with low initial guanosine 5'-diphosphate (GDP)¹; it has longer periodicities (~ 10 min; Pirolet et al., 1987).

Several models have been proposed to explain the oscillations. Their common feature is that at the end of the assembly phase microtubules convert from growth to shrinkage, and that the disassembly products containing bound GDP are only slowly regenerated to the assembly-competent tubulin-GTP subunits. Differences between the models are concerned with the nature of the breakdown products which could be either tubulin-GTP subunits or tubulin oligomers with bound GDP. We have previously proposed that microtubules disassemble by the release of oligomers, both in standard assembly buffer and in oscillation conditions. This was based on cryo-EM observations of cold-disassembled microtubules (Mandelkow and Mandelkow, 1985) and on time-resolved X-ray studies of oscillations (Mandelkow et al., 1988; Lange et al., 1988). The involvement of oligomers was apparent both from our earlier X-ray experiments and negative stain EM of oscillations (of the first type) and from cryo-EM experiments (second type of oscillations; Wade et al., 1989). By contrast, light scattering does not discriminate between the possibilities, and thus alternative models not involving oligomers were proposed (Carlier et al., 1987; Chen and Hill, 1987). We therefore decided to investigate the distribution of tubulin assembly forms during microtubule assembly, disassembly, and oscillations by cryo-EM. The advantages over other imaging methods are that it circumvents some of the artifacts of negative staining, and it combines high structural and high temporal resolution. This allows one to take "snapshots" of an ongoing reaction.

1. *Abbreviations used in this paper:* GDP, guanosine 5'-diphosphate; MAP, microtubule-associated protein; PC-tubulin, phosphocellulose-purified tubulin.

A second motivation for this study was the question of the conformational state of microtubule ends. It has been known for some time that tubulin protofilaments can assume two principal conformations, straight (as in a microtubule) or coiled (as in the cold-stable tubulin rings). The coiled conformation was observed mainly in conditions where microtubules are not assembled, for example low temperature, high Ca^{2+} , GDP, etc. Because of this the role of the coiled conformation remained ambiguous; it was considered either as a precursor of assembly or as an accidental by-product. However, there were also indications that coiling of protofilaments was an intermediate state in microtubule disassembly (Mandelkow and Mandelkow, 1985). The question received renewed attention when it was suggested that chromosome-to-pole movement during anaphase could be generated by the disassembly of microtubules at the kinetochore without requiring ATP hydrolysis (Koshland et al., 1988; Coue et al., 1991). This would imply that the energy was stored in the microtubule structure and could be released by disassembly of microtubules at the kinetochores. This in turn suggested a conformational change, e.g., in the form of a straight-to-coiled transition (see review by Mitchison, 1988). One aim of this study was therefore to find out if disassembling microtubule ends had a different conformation from assembling ones in conditions other than cold disassembly. Extending our previous findings we now show for a variety of conditions that the coiled state of a protofilament is characteristic of disassembling microtubule ends, while assembling ones have straight protofilament ends, some of which may protrude beyond the rest. This implies that neither growth nor shrinkage follows a helical mechanism, that microtubules can shrink by direct release of coiled protofilament fragments, and that the phase transition between growth and shrinkage is accompanied by a pronounced conformational change which in principle could be used to generate a force.

Materials and Methods

Protein Preparation

Microtubule protein and phosphocellulose-purified tubulin (PC-tubulin) was prepared from pig brain as described previously (Mandelkow et al., 1985). Aliquots of the protein were stored in liquid nitrogen until use. For standard microtubule assembly the buffer was 0.1 M Na-Pipes (pH 6.9 at 20°C), with 1 mM each of MgSO_4 , EGTA, DTT, and GTP. For oscillations, this buffer was supplemented with 20 mM MgSO_4 , 60 mM NaCl, and 4 mM GTP. The kinetics of assembly or oscillation was checked by light or X-ray scattering. All data shown here were derived from aliquots of the same protein preparation whose behavior was highly reproducible. Since the protein concentrations were rather high, especially in the oscillation experiments (up to 25 mg/ml), the phases of assembly and disassembly could also be monitored by observing the light scattering by eye.

EM

Vitrified samples were obtained by the blotting procedure as described (Dubochet et al., 1982; Milligan et al., 1984; Mandelkow and Mandelkow, 1985). Briefly, 5- μl aliquots of assembling, disassembling, or oscillating solutions (concentrations up to 25 mg/ml) were withdrawn at various time points, placed on a holey, carbon-coated grid, blotted with filter paper, and plunged into liquid ethane by means of a guillotine device. The vitrified samples were transferred to a cold stage (model 626; Gatan, Inc., Warrendale, PA) and observed in an electron microscope (model CM 12; Philips Electronic Instruments, Inc., Mahway, NJ) equipped with an anticontaminator (model 651N; Gatan Inc.). Specimens were surveyed at low dose and

low magnification ($\times 2,650$). Images of selected areas over holes in the carbon were taken at 35,000 magnification using the low-dose facility. The electron image film (SO-163; Eastman Kodak Co., Rochester, NY) was developed in full-strength developer (D19; Eastman Kodak Co.) for 12 min. For each condition we made typically 3-10 experiments (a total of ~ 100) from which $\sim 1,500$ images were obtained.

Temperature Control

Since microtubules are very sensitive to temperature it was important to control it accurately. Before an experiment the microtubule solution was kept in a water bath at 37°C. At first we prepared the grids in a warm room at 37°C and normal humidity. However, under these conditions the temperature of a drop of solution placed on the grid decreased by 6-10°C within a few seconds because of evaporative cooling. A change of this magnitude already induces partial microtubule disassembly. The temperature drop could only be avoided by keeping the humidity in the room at 95-100% by boiling water. The time taken for blotting and plunging the sample was 1-2 s; vitrification in the cryogen itself takes ~ 0.1 ms (Cyrklaff et al., 1990).

Results

Structures of Dynamic Microtubules

We have used time-resolved cryo-EM to study microtubule assembly, disassembly, and oscillations; examples are shown in Figs. 1-6. Before dealing with the dynamic aspects we describe some of the structural features. Vitrified protein samples have a low intrinsic contrast (because of the small difference in scattering power between the protein and the solvent). The contrast was optimized in three ways: (a) a large value of underfocus ($\sim 2.7 \mu\text{m}$); (b) making the layer of solution very thin (~ 100 nm); and (c) selecting areas over holes in the carbon. In these conditions microtubules and tubulin oligomers are clearly resolved, although individual tubulin subunits cannot be discerned reliably.

The structural features of microtubules are similar to those described in previous reports (Mandelkow and Mandelkow, 1985; Wade et al., 1990). The edges appear as dark lines which correspond to several protofilaments seen in projection (usually 4-5, depending on protofilament number and angle of view). On the inside one can discern two or three dark striations which arise from pairs of superimposed protofilaments from the front and back of the tubule. Some microtubule show an alternation between areas of two and three internal striations, separated by "fuzzy" regions. This is explained by the fact that microtubules reassembled in vitro often contain 14 protofilaments, and that there is a gradual twist of the protofilaments around the microtubule axis which generates different views in projection. The separation between successive fuzzy regions is typically ~ 200 -300 nm (corresponding to a protofilament pitch 28 times larger, i.e., ~ 5.6 -8.4 μm), but it can also vary over a wide range (e.g., between 250 and 660 nm in a single microtubule). The superposition of front and back, in combination with the supertwist, generates a chevron pattern indicating the structural polarity of microtubules (Fig. 5 *b*, top). Microtubules with only two internal striations, unequal edges, and very little or no supertwist probably contain 13 protofilaments (Fig. 5 *b*, bottom; see Wade et al., 1990).

The ends of microtubules can appear in different shapes. The basic distinction is that the protofilaments can be either straight (Fig. 1) or coiled (Figs. 2 and 3), with a curvature

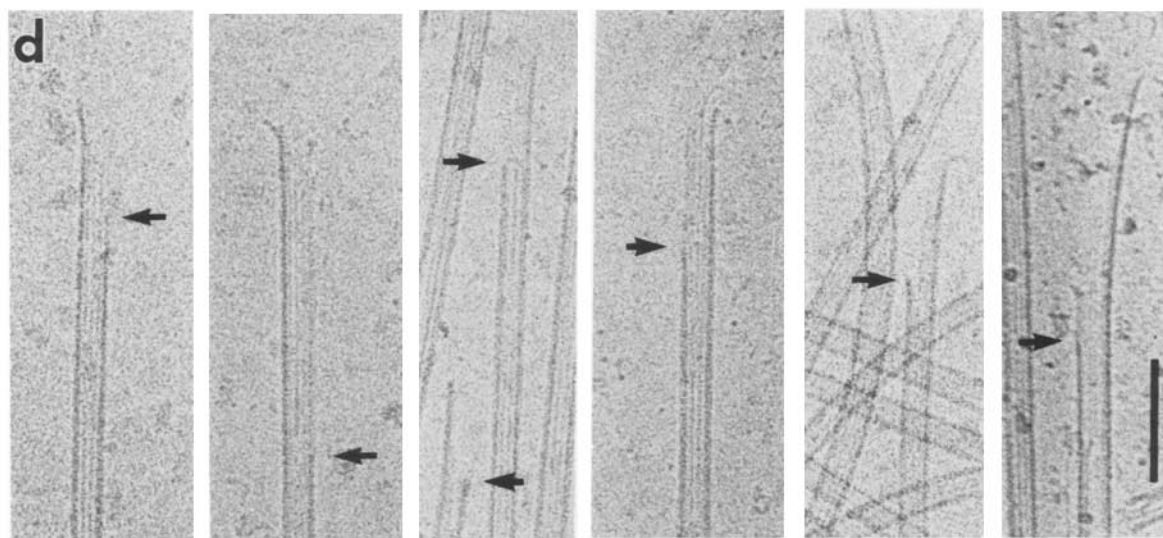
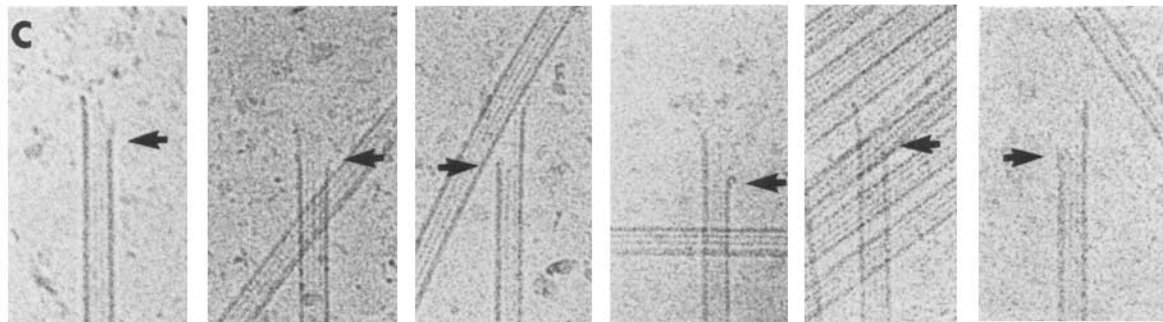
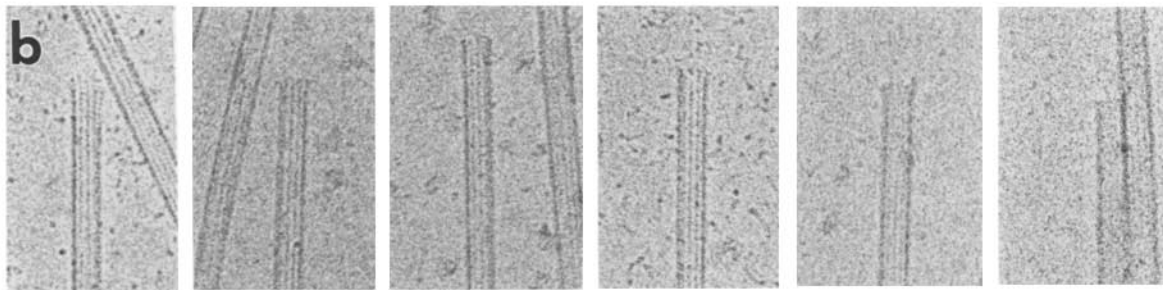
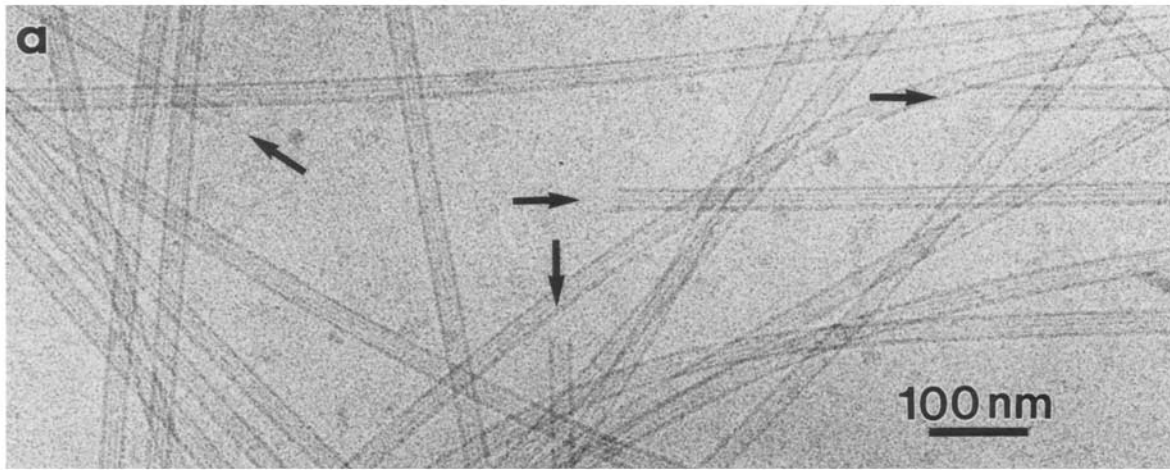


Figure 1. Growing microtubules in standard assembly conditions (PC-tubulin, 5 mg/ml, time = 2:25 min). (a) Survey with several blunt microtubule ends (arrows). (b–d) Higher magnification with different protrusions of protofilaments. (b) Blunt ends, all protofilaments terminate almost simultaneously. (c) Short protrusions of protofilaments, up to about 50-nm long. (d) Long protrusions (>50 nm). Bar, 100 nm.

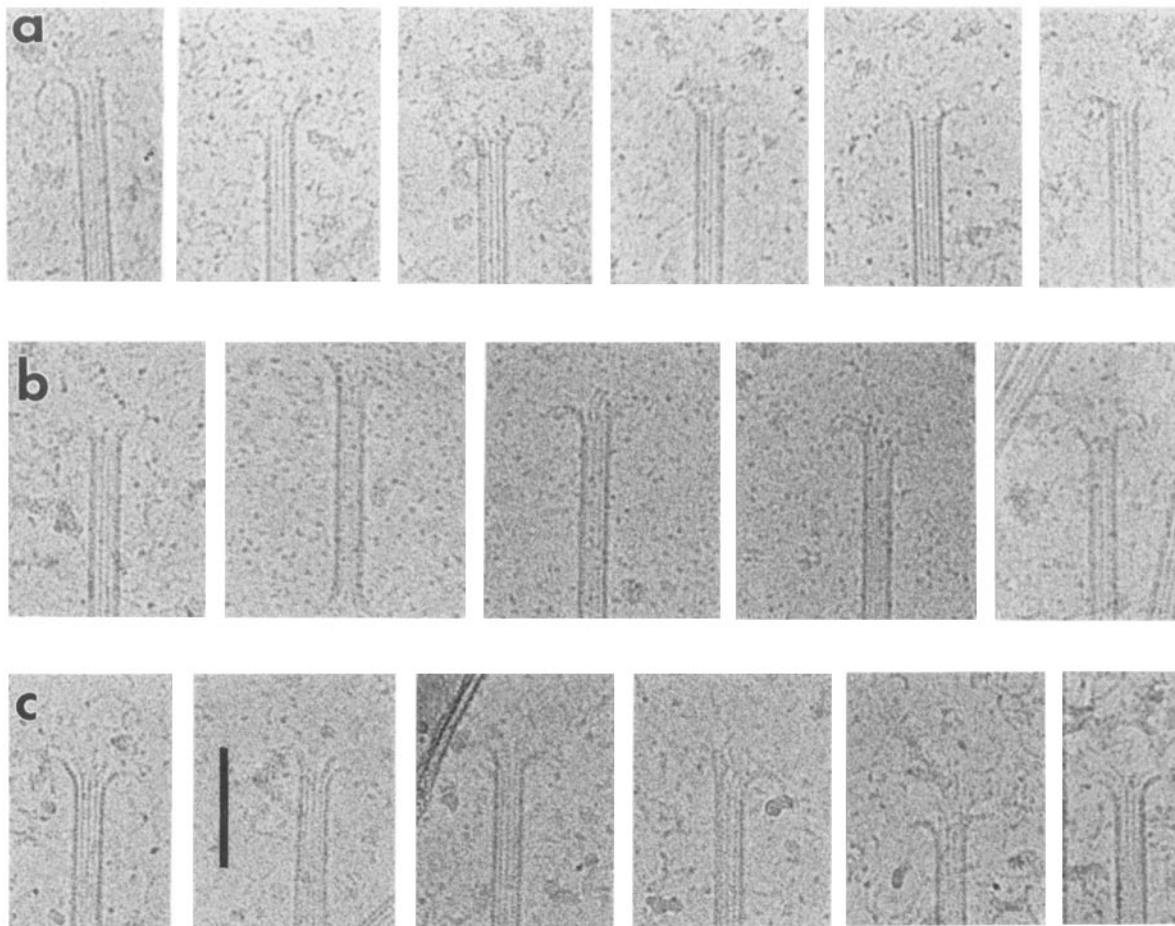


Figure 2. Microtubules during shrinkage in different conditions. Note the coiled protofilaments at microtubule ends. (a) Shrinkage in standard assembly buffer 8 s after isothermal dilution from 22 to 7.3 mg/ml. (b) Shrinkage in standard assembly buffer 14 s after cooling the solution to 4°C. (c) Shrinking microtubules (11 mg/ml), 12 s after the addition of 4 mM Ca²⁺. Bar, 100 nm.

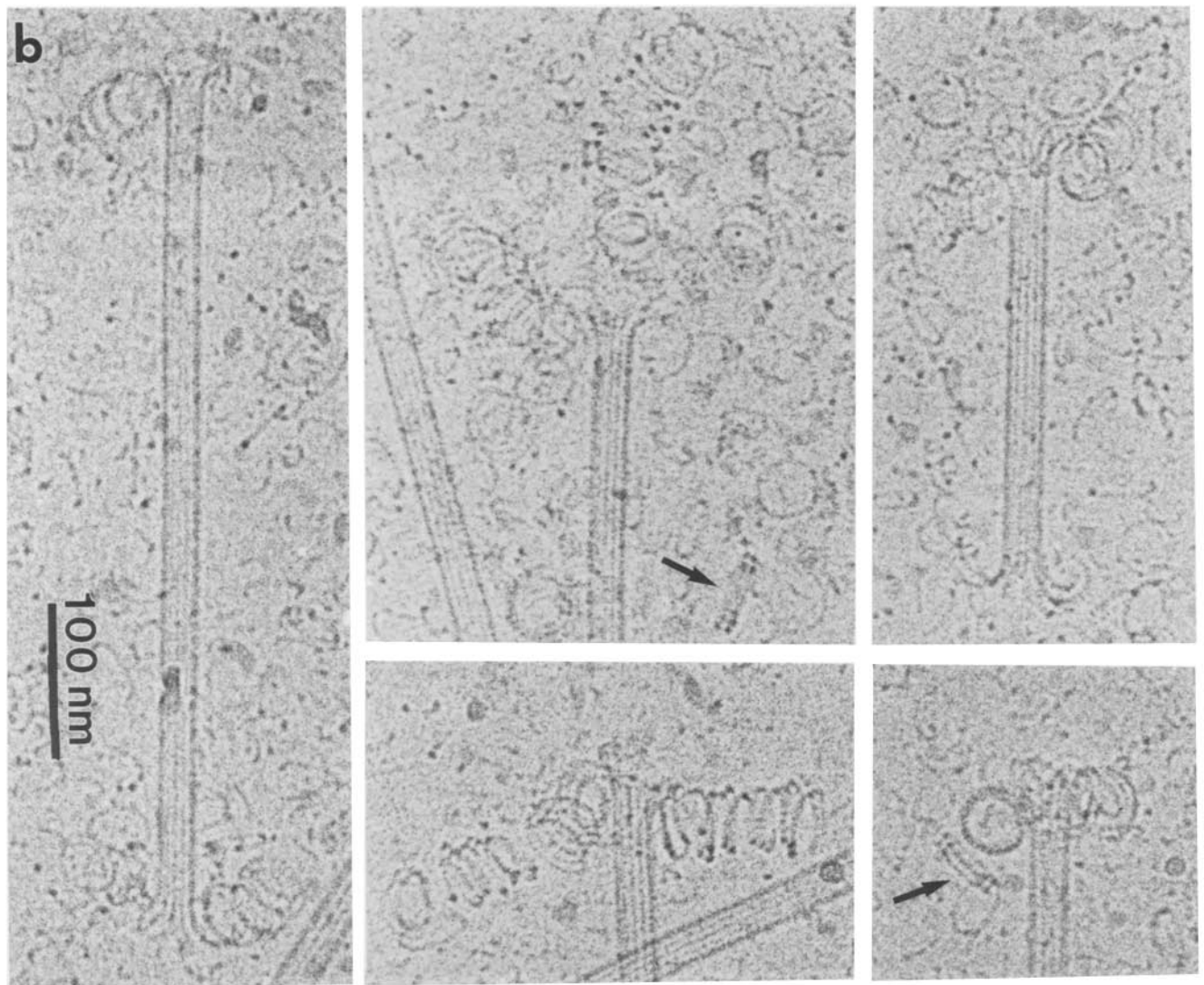
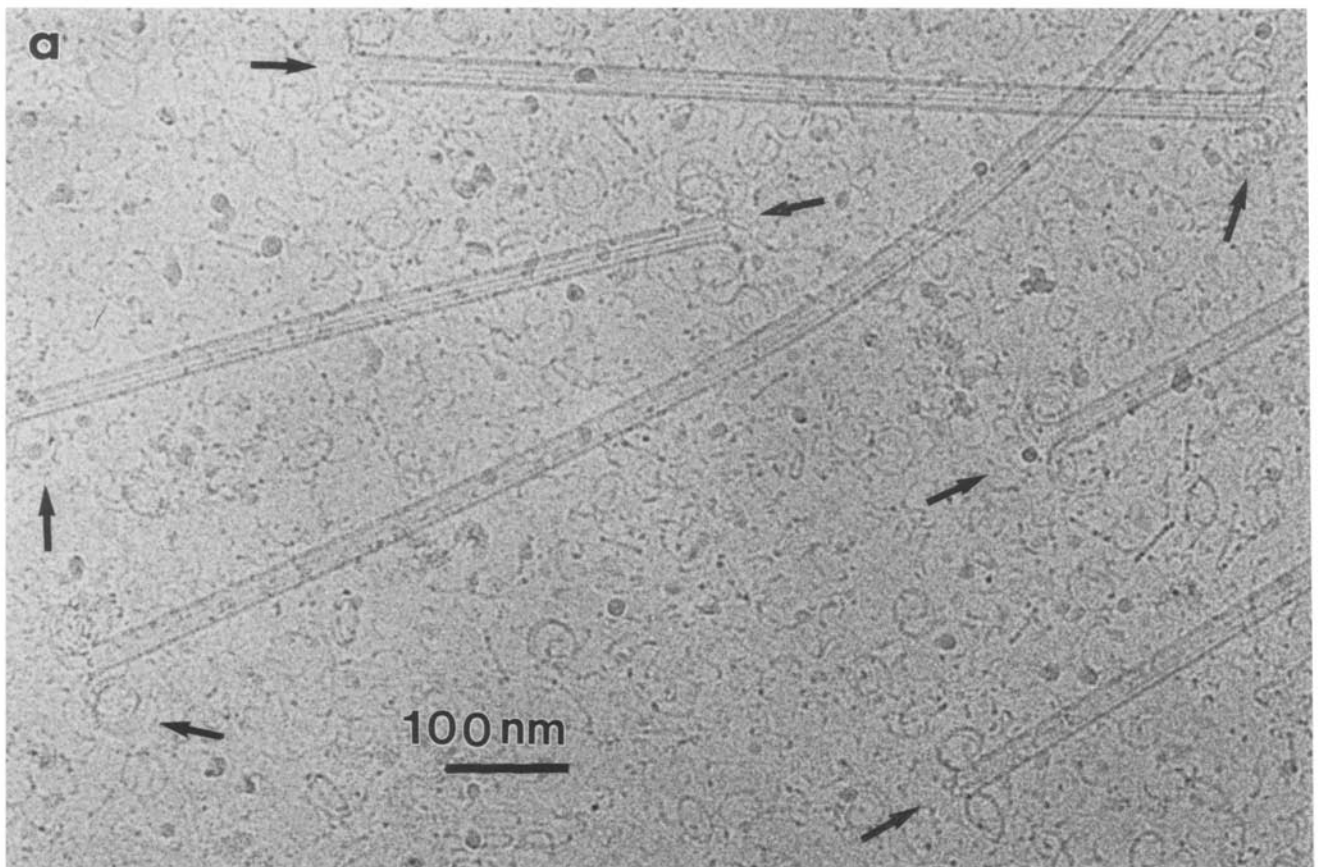
similar to that of tubulin rings (see below). The difference is related to the state of assembly or disassembly, as discussed later. In either case some of the protofilaments can extend beyond the rest. If they are straight, this gives the microtubules a “tapered” or “jagged” appearance (Fig. 1, *c* and *d*), reminiscent of the interzone microtubules in mitosis (McIntosh et al., 1985). In cross-section these regions would be C shaped like an incomplete microtubule wall. The protofilament protrusions can be 100–200-nm long. If they are too short to be detected we call the microtubule end “blunt” (Fig. 1, *a* and *b*; note that even a blunt end must be slightly jagged because of the microtubule surface lattice, but this is difficult to detect at our resolution). On the other hand, when the protofilaments at a microtubule end are coiled they can appear either as short curved stubs (Fig. 2), or, if they are longer, as rings or even spirals (Fig. 3). The direction of coiling is roughly inside out, i.e., protofilaments tend to coil away from the microtubule axis. Thus in projection the coiling is most apparent at the two edges where the coils are seen face on.

Besides microtubules the most prominent structures are the tubulin oligomers; they are particularly prominent in os-

cillation conditions (Fig. 4; and compare the background in Fig. 1 and Fig. 5). Oligomers consist of coiled protofilaments, often closed into rings whose mean diameter varies between 36 and 40 nm. Most of the rings have a single turn, but cases of two concentric turns are seen as well. Rings viewed face-on appear as circles; side views are recognized as straight bars with darker knobs at their ends, corresponding to several protein subunits in projection (Fig. 5, *b* and *d*, arrows). Double bars (Fig. 3 *b*, arrows) indicate that a pair of rings can be stacked axially. Along the ring perimeter one frequently finds indications of a substructure with a 4-nm spacing, consistent with the spacing of tubulin monomers along protofilaments (Mandelkow et al., 1983). These rings are similar to those commonly observed with brain microtubule protein at low temperature.

Not all oligomers form closed rings; many are smaller and are recognized as curved lines, equivalent in contrast to one or two protofilaments, with lengths frequently on the order of 40 to 60 nm. A striking feature is the frequent occurrence of dark dots, ~4–5-nm wide (e.g., Figs. 5 and 6). They are mostly seen in the ring-containing background areas outside the microtubules. We interpret them as short protofilaments

Figure 3. Shrinking microtubules (11 mg/ml), 12 s after the addition of 20 mM Mg²⁺. (a) Survey, (b) details at higher magnification. Note the pronounced coiling at microtubule ends and the increased concentration of oligomers in the background. Bar, 100 nm.



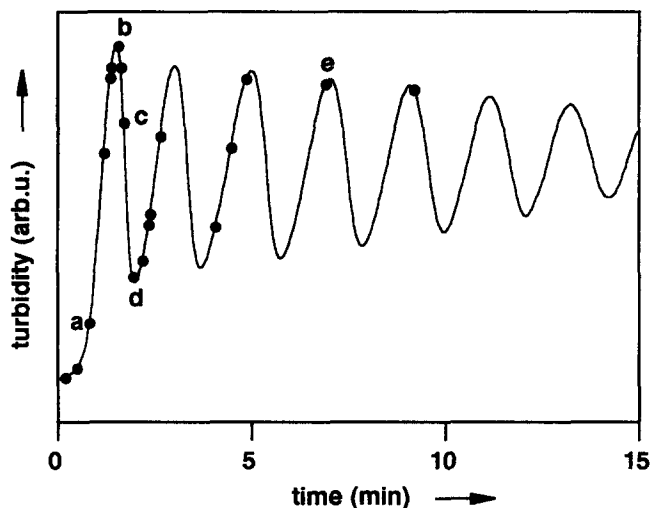


Figure 4. Oscillations of a solution of PC-tubulin (23 mg/ml, oscillation buffer) monitored by turbidity. The initial temperature jump from 4 to 37°C is at time 0. (●) Show the time points at which samples were rapidly frozen for cryo-EM.

seen end-on, arising from several tubulin subunits in projection. The images show that the oligomers are heterogeneous; when they are long enough, ~110–120 nm, they form rings because of their intrinsic curvature, but shorter ones only show up as coiled protofilament fragments.

In this paper, we use the term oligomer to denote the protofilament fragments and rings just described. However, there is also another type of oligomeric structure which is seen as a globular particle of roughly 20–30-nm diam (Fig. 7) which appears to be a transition between the inactive (GDP containing) oligomers and microtubules. This point will be taken up later.

Conformation of Microtubule Ends during Assembly and Disassembly

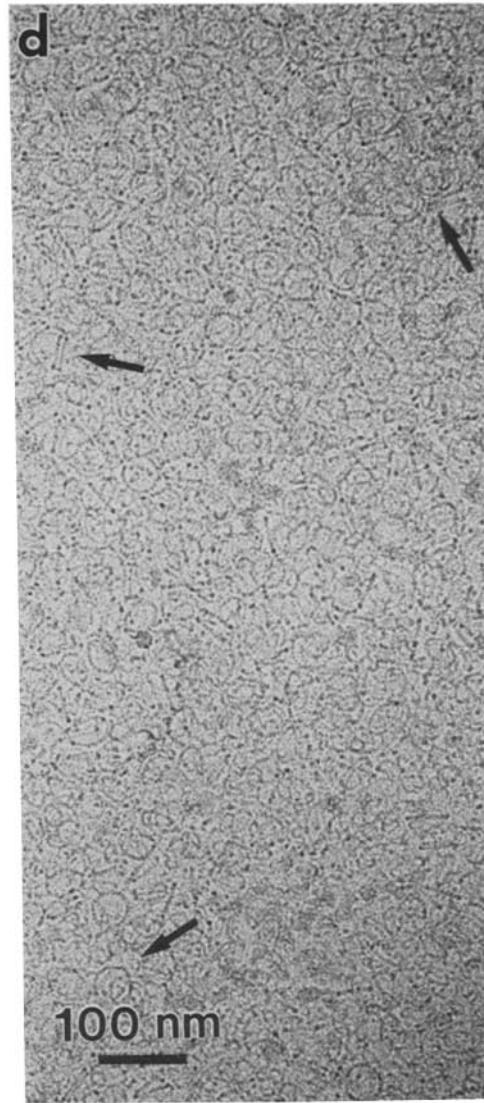
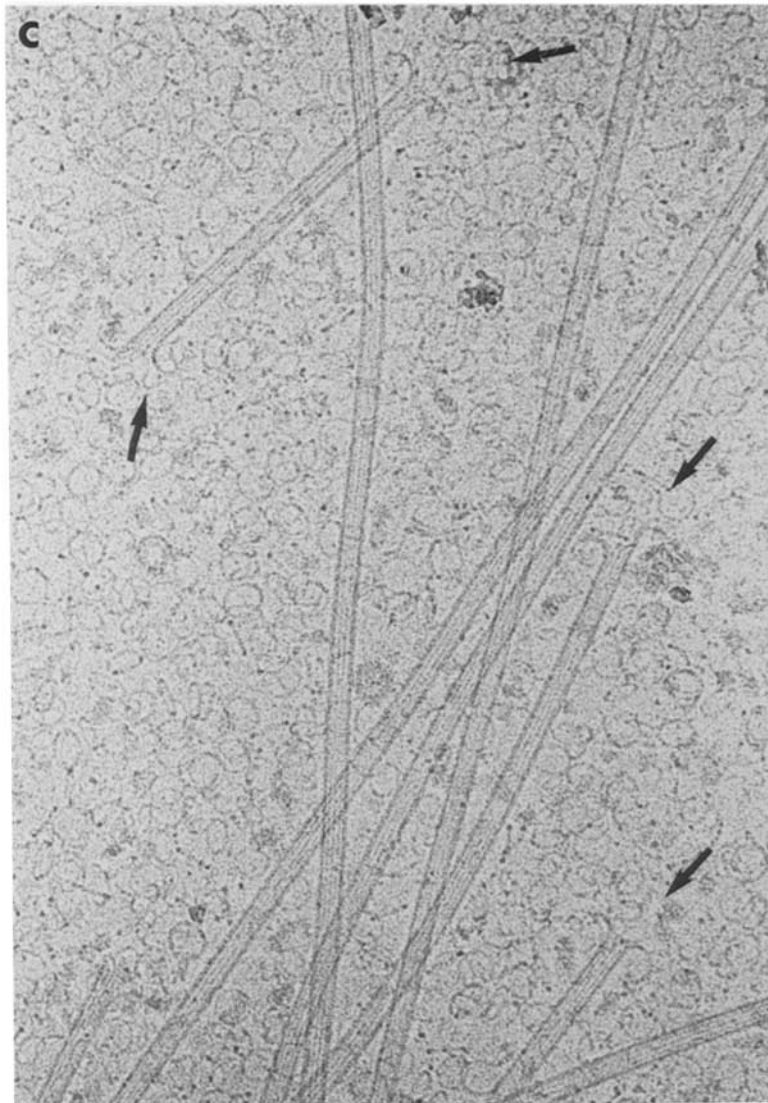
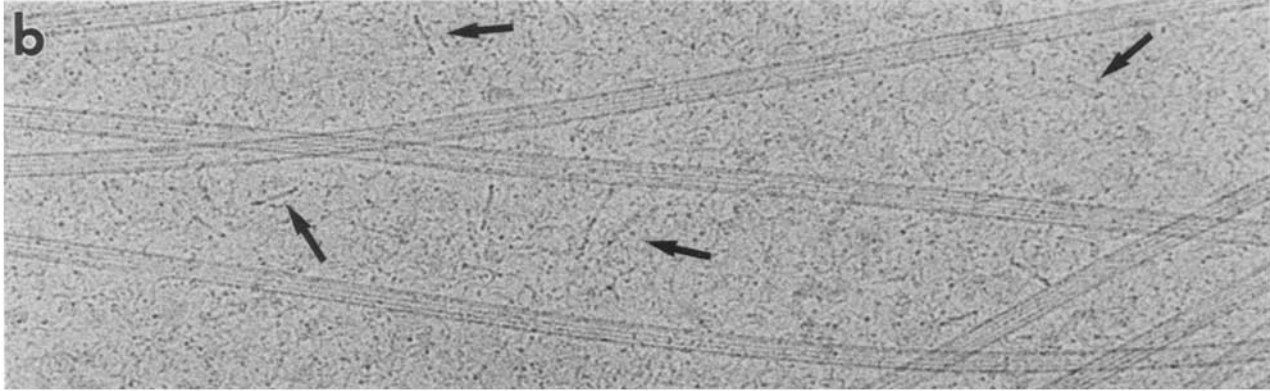
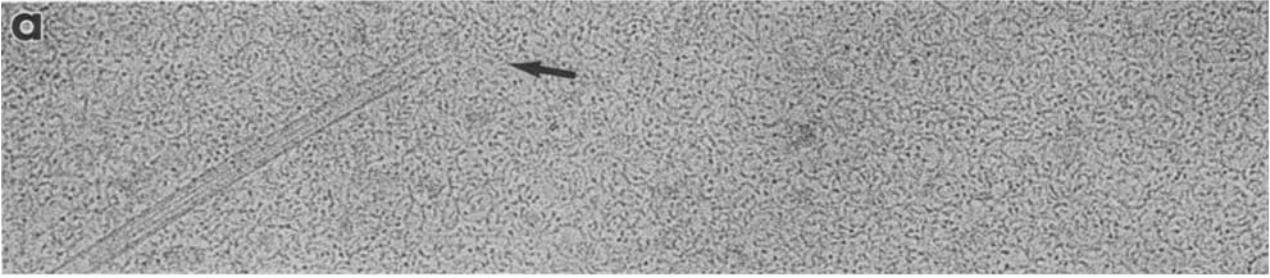
A central point to understanding microtubule dynamics is the question of what determines the transition from growth to shrinkage and vice versa. A number of studies have attempted to define the nature of the phase change at microtubule ends in terms of its biochemical properties (e.g., nucleotide binding) and related theoretical modeling (Hill and Chen, 1984; Bayley et al., 1989), but the structural rearrangements at microtubule ends have remained uncertain. EM would seem the method of choice for studying these conformational changes. However, the traditional approaches (e.g., negative staining) make it difficult to distinguish be-

tween real and artefactual observations (see Discussion). This prompted us to use cryo-EM of solutions of synchronous microtubules. Earlier observations on microtubule-associated protein (MAP)-containing microtubules had already suggested that cold-induced disassembly generated fraying microtubule ends and coiled protofilament fragments, rather than just endwise dissociation of subunits (Kirschner et al., 1974; Mandelkow and Mandelkow, 1985). We have now extended these studies to microtubules without MAPs and to a variety of well-defined conditions of synchronous growth or shrinkage.

As a reference we describe microtubules growing in standard assembly buffer after raising the temperature from 4 to 37°C (Fig. 1). The ends of these microtubules are characterized by straight protofilaments which usually terminate almost simultaneously; these ends appear blunt (Fig. 1, *a* and *b*, arrows). In other cases some protofilaments protrude beyond the rest in a nearly straight fashion (up to 100 nm), giving the microtubule end a tapered appearance (Fig. 1, *c* and *d*). This shows that microtubules grow along protofilaments rather than in a helical manner, consistent with their nonhelical lattice (as discussed previously, Mandelkow et al., 1986). A rough classification showed that 60% of the growing microtubules had blunt ends (meaning that protofilament protrusions, if present, were too short to be detected with certainty), 26% were clearly tapered, with protrusions up to 50 nm, and 10% had even longer protrusions (Table I). A minor fraction (4%) had coiled ends, even during overall growth, which is indicative of shrinkage as discussed below. This could be explained by several factors: (*a*) In principle the microtubules are dynamically unstable so that a fraction would be expected to shrink even in conditions favorable for assembly. (*b*) The transfer of the microtubules to the grid and the blotting could lead to some breakage and disassembly. This effect is particularly noticeable with very thin layers (<~50 nm), where the surface roughness leads to kinks and breakages (e.g., at the edges of the holes in the carbon film, or where two microtubules superimpose on one another; see Fig. 7 *b* for an example).

In disassembly conditions the microtubules have almost invariably coiled ends, as if they were peeled apart (Figs. 2 and 3; and Table I). The length of the peeling protofilaments depends on buffer conditions, whereas the curvature is usually similar to that of rings (allowing for some floppiness and variations because of the effective viewing angle). Fig. 2 shows examples of moderate peeling where the coiled protofilament ends are shorter than a ring circumference (dilution, low temperature). When depolymerization is induced by adding several mM Mg²⁺ the coiling is most pronounced (spiralling protofilaments up to several gyres, Fig. 3). This

Figure 5. Survey of cryo-EM images during different stages of the oscillations. (*a*) Solution at beginning of first cycle (time = 0:50 min after T-jump; point *a* in Fig. 4), showing part of a growing microtubule (note the straight end) and numerous oligomers in background (rings and smaller ones). (*b*) First assembly maximum (time = 1:32 min; point *b* in Fig. 4). The short dark bars are rings seen edge on (arrows). The microtubule coming from the top right corner illustrates the supertwist for a 14 protofilament particle. It shows an alternation between two and three internal striations, and when viewing along the length at a shallow angle one can see a chevron pattern pointing to the left, indicating polarity. The microtubule starting in the lower half on the left has only two continuous internal striations and probably contains only 13 protofilaments. (*c*) During disassembly following the first maximum (time = 1:43 min; point *c* in Fig. 4). Note microtubules predominantly with coiled ends (arrows). (*d*) Minimum between first and second cycle (time = 1:56 min; point *d* in Fig. 4). Note enhanced concentration of oligomers (compare *a* and *b*) and globular aggregates in the lower and upper right (compare Fig. 7). Bar, 100 nm.



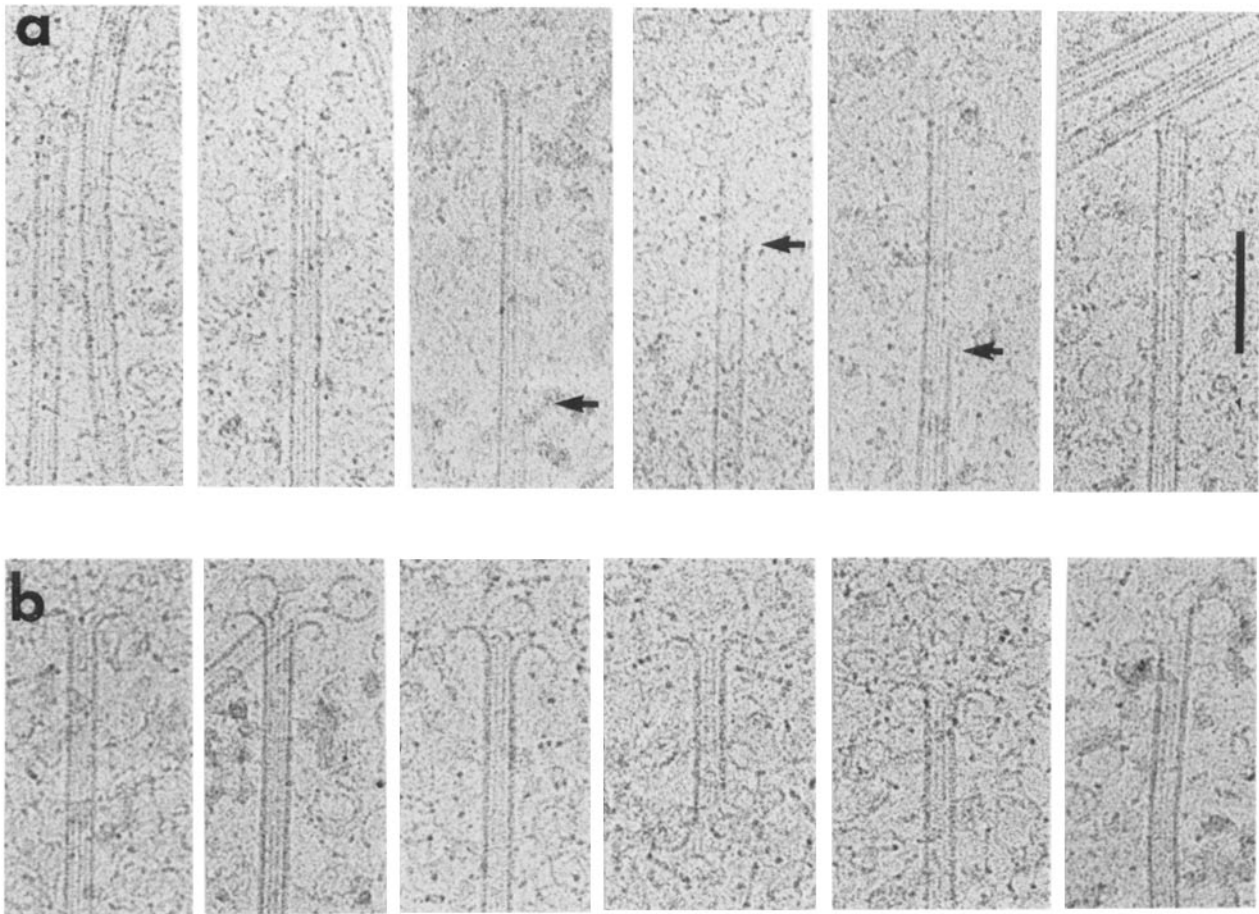


Figure 6. Higher magnification views of microtubules during oscillations. (a) Growing microtubules during first assembly phase, showing straight ends (blunt or tapered). The arrows show the start of tapering. (b) Shrinking microtubules during first disassembly phase, showing coiled ends. Conditions as in Fig. 5. Bar, 100 nm.

generates a “ram’s horn” appearance, especially when the vitrified layer is very thin so that the gyres are forced to take up to a face-on-view. Fig. 3 *b* is a striking example of Mg^{2+} -induced disassembly, showing how protofilaments peel off in extended coils and finally break away (*arrow*). These data show that coiled oligomers are an intermediate stage of microtubule disassembly, even in conditions where rings are not stable by themselves (such as dilution).

Microtubules have two ends (plus and minus) that are functionally distinct (fast and slow growth), and the question arises whether the above features occur only at one or at both ends. This can be answered by using the chevron pattern as a marker of polarity, or by studying microtubules short enough so that both ends are visible simultaneously. We did not detect a significant difference in the structure of the two

ends, i.e., growing ends are straight in both cases, shrinking ends are both coiled (e.g., Fig. 3 *b*).

Dynamic Instability and Microtubule Oscillations

The above experiments set the scene for observing dynamic instability. We were especially interested in oscillations where dynamically unstable microtubules are synchronized. We followed the distribution of microtubules and oligomers by taking snapshots during oscillations for up to five cycles (Fig. 4). Microtubules were numerous at the maxima, but rare at the minima. This means that during oscillations individual microtubules can disappear completely and that nucleation of new ones takes place not only at the beginning, but also at later times. The determination of microtubule and oligomer concentrations by cryo-EM is semi-quantitative

Figure 7. Special globular structures. (a) PC tubulin (5 mg/ml) in normal assembly buffer, frozen in the early stages of assembly (2:25 min). Note globular structures in background. (b) Higher magnification of (a), showing internal structure reminiscent of laterally associated short protofilaments (*arrow*). (c) PC tubulin (23 mg/ml) in oscillation buffer during disassembly after first maximum (1:43 min; compare Fig. 2). The globular structures are more numerous and often merge into one another (*lower right*). (d) Higher magnification from the same specimen. (e–g) Oligomers and globular structures during oscillations in negative stain. (e) Disassembly just before second oscillation minimum (note oligomers equivalent to short protofilaments, and darkly stained globular aggregates). (f) 10 s after *e*, transition between rodlike oligomers and globular structures. (g) 25 s after *e*, during assembly. Note many short microtubules and globular structures, linked like chains on a bead. Bars, 100 nm.

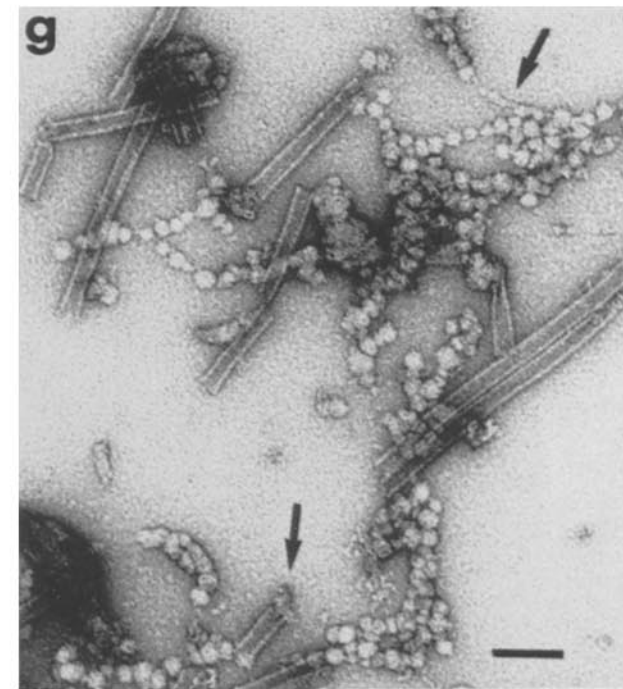
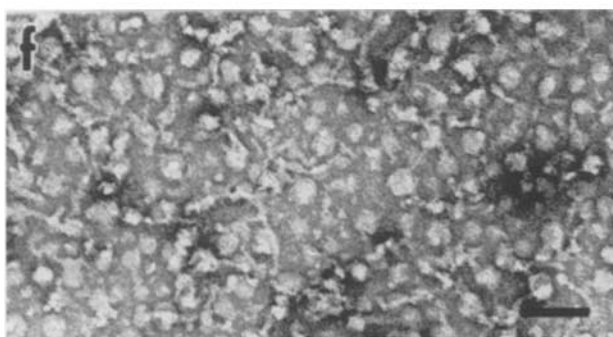
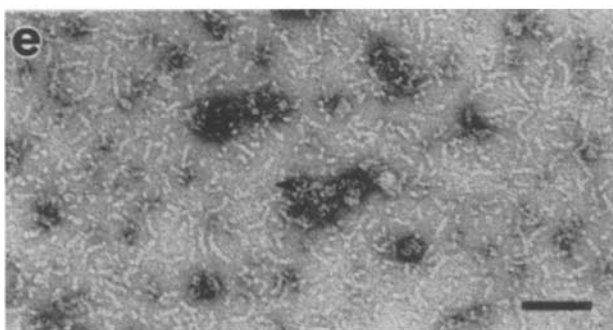
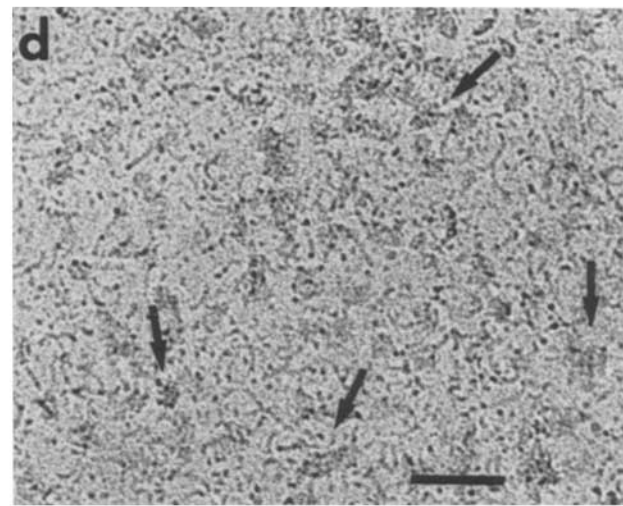
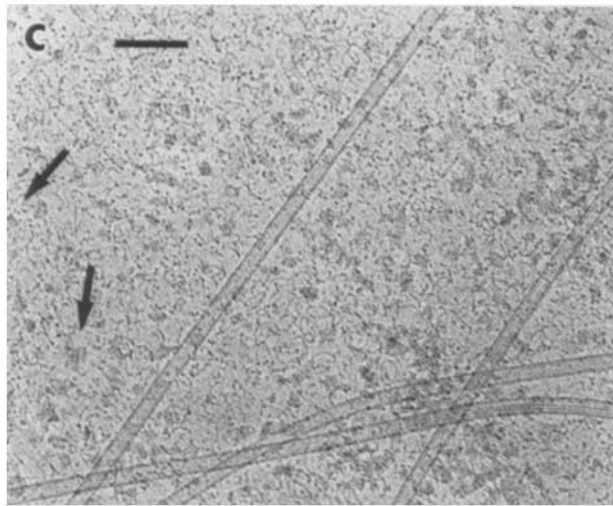
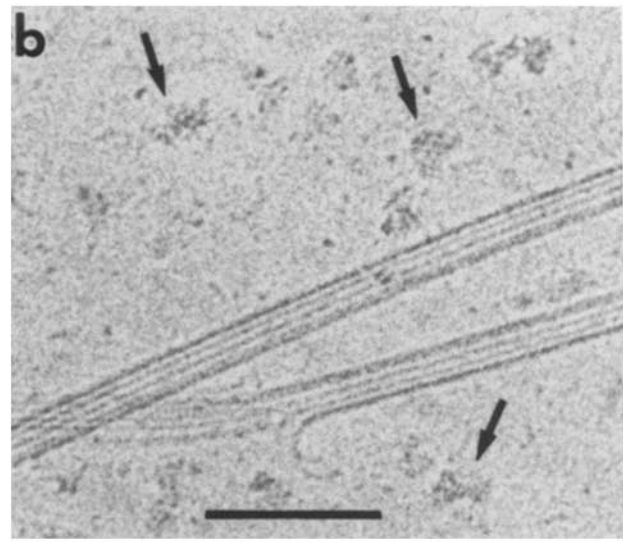
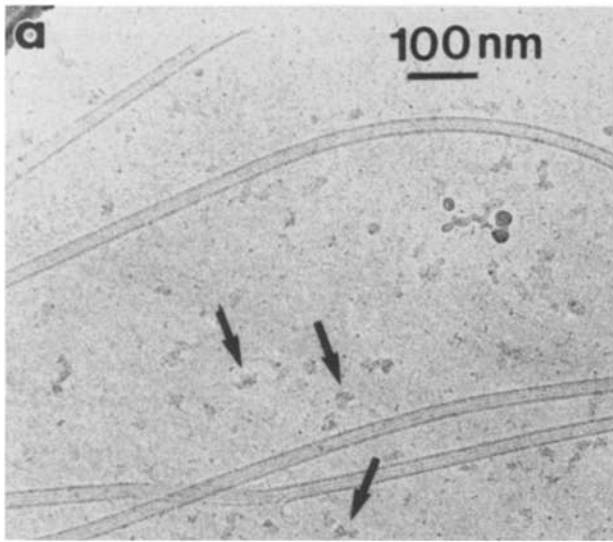


Table I. Quantitation of Conformations at Microtubule Ends in Different Phases of Growth, Shrinkage, and Oscillations

	Percent straight	Percent coiled	<i>n</i>
A. Growth (standard assembly):	96	4	400
Blunt ends	60		
Tapered ends, <50 nm	26		
Tapered ends, >50 nm	10		
B. Shrinkage (37°C):			
Dilution	17	83	100
4 mM Ca ²⁺	10	90	100
20 mM Mg ²⁺	0	100	100
C. Oscillations:			
First peak (1:33 min)	48	52	200
Shrinking phase (1:43 min)	4	96	200
First valley (1:56 min)	37	63	40
Growing phase (2:12 min)	82	18	70
Growing phase (4:29 min)	71	29	180

The correlation between "pure" assembly and disassembly (A and B) with straight or coiled ends, respectively, is very high; it is lower in the case of oscillations (C), probably because of limited amplitude, imperfect synchrony, and other factors. *n*, the number of microtubule ends examined.

since high-contrast images could be obtained only in selected areas over holes in the carbon film. Moreover, it is not known to what extent the different species were lost on the filter paper during blotting. However, the earlier X-ray data showed that in an assembly maximum the microtubule fraction typically contains 70% of the protein, the rest being oligomers and dimers, while during an assembly minimum the microtubule fraction decreases to 20% or less, depending on the amplitude of the oscillation. This is in qualitative agreement with the results presented here. We note in particular that a substantial fraction of rings is always present even at 37°C (compare Figs. 5 and 1).

During the oscillations there is a correlation of assembly and disassembly phases with straight and coiled microtubule ends, respectively, but it is less perfect than in the "pure" cases discussed previously (Table I). In other words, the net assembly phase was dominated by straight microtubule ends, the net disassembly phase by coiled ones, but there was a significant fraction showing the opposite behavior. This suggests that the synchrony is not complete; only part of the protein contributes to the net oscillations (see Fig. 4), and even the contributing fraction must be partly desynchronized to account for the shape of the oscillation curve (according to computer modeling discussed elsewhere; A. Marx, personal communication).

Globular Particles

During the time-resolved cryo-EM studies we also observed a different type of oligomeric structure. It has a roughly globular shape (~20–30 nm), and an ill-defined substructure (Fig. 7). Such globular structures would tend to be disregarded or be considered as an artifact of little significance. However, careful inspection of images from oscillating solutions show that these structures also oscillate with the same frequency as microtubules and oligomers. They are most noticeable around the assembly minima. In favorable cases one observes a substructure reminiscent of juxtaposed protofilaments (Fig. 7 *b*, arrows). Attempts to reveal the sub-

structure with more contrast by negative staining were not successful; in this case the structures appear to collapse into stain-excluding blobs (Fig. 7, *e–g*). During the early stages of the assembly phase the blobs have a striking tendency to coalesce into chains of beads (Fig. 7 *g*).

Although the globular particles are most pronounced during oscillations, they are found in the early stages of normal assembly as well (Fig. 7, *c* and *d*). This suggests that they somehow reflect the activation of tubulin by GTP and may be related to microtubule nucleation. The substructure seen in Fig. 7 *b* can be interpreted as a very short opened up microtubule wall; this would be compatible with the two-stage nucleation mechanism proposed by Voter and Erickson (1984). These observations point out the need for distinguishing different types and functions of small tubulin assemblies. One type (called oligomers in this paper) is a product of microtubule disassembly; it is readily observed and has a fairly well-defined structure (coiled protofilaments), and is inactive with respect to microtubule assembly. On the other hand, the initial stages of microtubule formation must correspond to distinct and assembly-competent types of oligomer, and the globular particles being likely examples.

Discussion

Microtubules are dynamically unstable, they switch spontaneously between phases of assembly and disassembly (Mitchison and Kirschner, 1984). This property makes microtubules unique among polymers, and it plays an important role in the morphogenesis of the cytoskeleton (Mitchison, 1988). Although the length changes of microtubules can be observed directly by video microscopy (Horio and Hotani, 1986), the underlying mechanism has remained elusive. Most discussions are centered around a biochemically distinct "cap" of subunits at microtubule ends (e.g., consisting of tubulin-GTP; Carlier et al., 1984). However, the nature of this cap has remained controversial (for recent discussions see Stewart et al., 1990; Caplow and Shanks, 1990*b*). We have therefore taken a different approach and asked if one can detect structural changes at microtubule ends related to microtubule dynamics. This question is closely linked to that of microtubule oscillations which can be regarded as a synchronized form of dynamic instability, and to the role of tubulin oligomers proposed earlier (Mandelkow et al., 1988). In particular we have addressed the following questions: (a) can we verify by direct imaging that oligomers are involved in the dynamics of microtubules?; (b) how do microtubules disassemble? Does this occur by release of tubulin subunits, or by release of oligomers?; (c) can we detect a conformational difference between growing and shrinking microtubule ends?; (d) Is there a difference between the structural transitions at the plus and minus ends?

Oligomers Are Released from Shrinking Microtubules and Oscillate in Antiphase

Since oligomers are difficult to detect by methods such as light scattering or optical microscopy there has been a debate concerning their presence during oscillations. The experimental data of Carlier et al. (1987) and Pirollet et al. (1987) were initially interpreted without assuming oligomers. In modeling a reaction mechanism for the oscillations one has to assume a slow rate-limiting step between microtubule

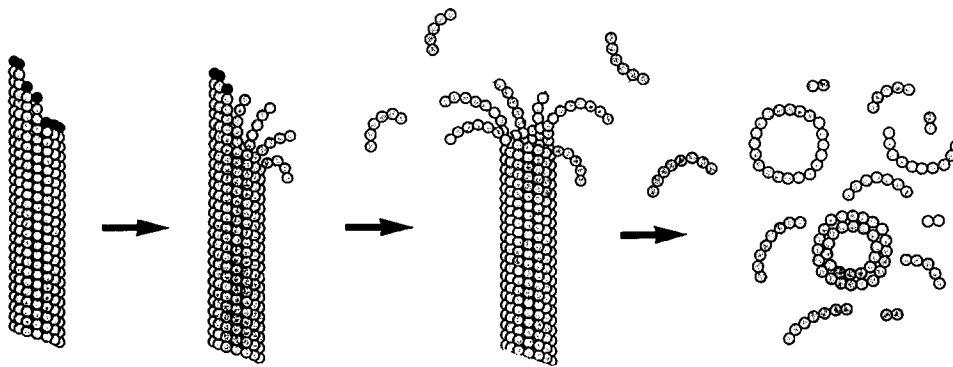


Figure 8. Diagrams summarizing the structural transitions at microtubule ends (drawn at one end only for simplicity). Each circle represents a tubulin monomer, no distinction is made between α and β subunits. From left to right: (a) Growing microtubule with straight protofilaments, sometimes protruding in a straight manner. The terminal subunits (black) are possibly in an assembly-promoting chemical state, e.g., tubulin-GTP; (b) microtubule in the transition to shrinkage. Some protofilament ends are beginning to coil inside-out; (c) shrinking microtubule with pronounced protofilament coiling; and (d) oligomeric disassembly products.

disassembly and the regeneration of assembly-competent tubulin-GTP, on the order of 0.02 s^{-1} . This was initially assumed to be in the nucleotide exchange on tubulin (Chen and Hill, 1987) which lead to an apparent discrepancy with the measured exchange rate because this is 10 times faster, $\sim 0.2 \text{ s}^{-1}$. The difficulty disappeared when oligomers were introduced as an intermediate stage, as suggested by X-ray scattering experiments (Mandelkow et al., 1988). Nevertheless, since the interpretation of scattering data is indirect it was important to verify the interplay between microtubules and oligomers by time-resolved EM.

Inspection of images obtained at different time points during the oscillations shows two main features (Figs. 4–6). Firstly, there is always a substantial fraction of oligomers, even at the maximum of microtubule assembly, and even at 37°C where oligomers disappear in standard assembly conditions. Secondly, the concentration of oligomers is minimal when microtubules are maximal, and vice versa, i.e., the two species fluctuate in antiphase. Since the oligomers contain nonexchangeable GDP (Zeeberg et al., 1980) they represent a nonpolymerizable storage form of tubulin, i.e., this protein is transiently inactivated with regard to assembly. This is an essential requirement for obtaining synchronized dynamic instability.

When disassembly is induced in conditions other than oscillatory ones (e.g., dilution, cold, divalent cations; see Figs. 2 and 3), the pathway of shrinkage is structurally similar to that during oscillations. There are variations in the extent of coiling which depend on buffer conditions: the oligomers peeling off from microtubule ends may be long, e.g., several gyres in the presence of high Mg^{2+} (Fig. 3) or shorter, typically a quarter of a gyre after isothermal dilution or cold shock in standard buffer (Fig. 2). The second variation concerns the concentration of oligomers remaining in solution after disassembly (high with several mM divalent cations, low after dilution or cold treatment in standard buffer). In fact the conditions generating extended coils at microtubule ends are similar to those known to stabilize oligomers (e.g., MgH). We also note that the coiling of microtubule ends is characteristic of the disassembly process itself, irrespective of whether microtubules disappear as a

whole. For example, microtubules that disassemble after a dilution have coiled ends, even when the final protein concentration is still above the critical concentration. Conversely, steady-state microtubules that are not dynamically unstable (because of taxol or MAPs, say) have straight ends (not shown).

These observations are explained by the scheme in Fig. 8. The first step in microtubule disassembly is coiling of protofilament ends so that the lateral bonds between them are sliced open; after that they peel off and subsequently break apart. The size of the “ram’s horns” depends on how stable the coiled conformation is in the buffer conditions chosen. The oligomeric breakdown products could either disassemble further (down to the size of a dimer), or reassociate, e.g., into rings, the equilibrium again being determined by buffer conditions, temperature, etc. These data confirm and extend our previous oscillation model, an updated version of which is shown in Fig. 9. The data also provides a connection between the observations that Mg^{2+} favors the formation of coiled oligomers (Frigon and Timasheff, 1975), and at the same time increases microtubule disassembly rates (Gal et al., 1988; O’Brien et al., 1990). The link is that protofilament coiling weakens the lateral bonds in a microtubule, increases the rate of protofilament “peeling” (which is faster than simple subunit dissociation), and thereby leads to the “ram’s horns” observed here.

One way to distinguish between oligomers and dimers is the rate of nucleotide exchange; it is slow with ring oligomers but fast with dimers (Zeeberg et al., 1980). Thus, if oligomers were the breakdown products of microtubules one would expect a slow nucleotide exchange. However, Caplow and Shanks (1990a) found that after isothermal dilution the exchange occurred at the fast rate ($t_{1/2} = 5 \text{ s}$); they concluded that microtubules first disassemble into tubulin subunits which then reassociate into oligomers. This seems to contradict our conclusions on disassembly via the oligomer pathway, but the two sets of observations could probably be reconciled by a more detailed kinetic study. For example oligomers are heterogeneous in size and structure, and they might equally well differ in nucleotide exchange rates. Thus, an initial population of smaller oligomers might exchange

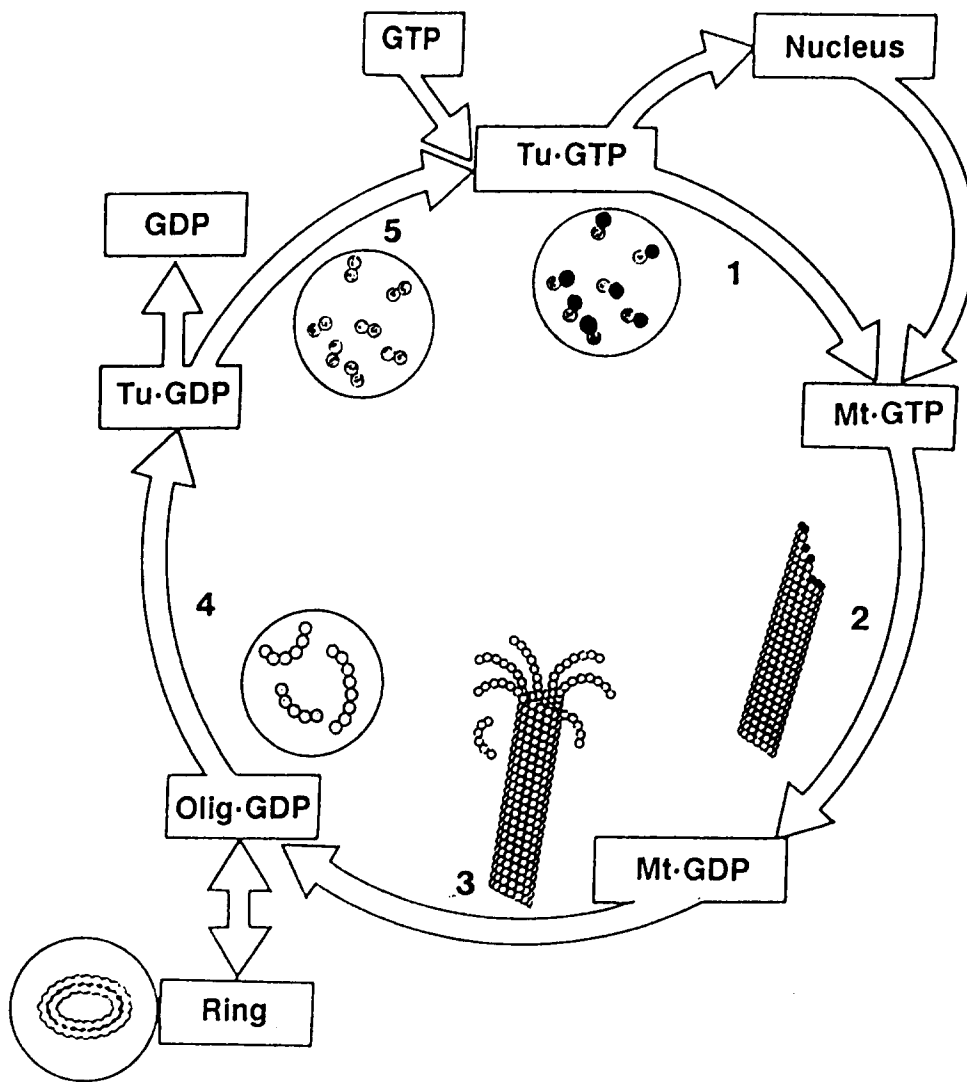


Figure 9. Diagram of the oscillation cycle (adapted from Mandelkow et al., 1988) showing the relationship between tubulin, microtubules, and oligomers, and the structural transitions at microtubule ends. The terminal subunits at growing ends are shown in black to indicate an altered conformation.

faster than the later population where annealing into rings or other structures has taken place (as indicated by the last step in Fig. 8 or by the transition between oligomers and rings in Fig. 9). Alternatively, disassembling oligomers might be short lived, especially after the strong dilution used in the Caplow and Shanks study (1990a), so that their transient existence would not show up in nucleotide exchange studies. In fact, from a kinetic point of view this case would be difficult to distinguish from endwise disassembly of subunits.

Details of the *in vitro* studies aside, the question remains of how the mechanism of oscillations *in vitro* relates to that of microtubule dynamics in living cells. Examples of microtubule-related oscillations have been reported, e.g., during mitosis (Bajer, 1982), but the more general case is that of irregular dynamic instability (Mitchison and Kirschner, 1984; Horio and Hotani, 1986). In other words, cellular microtubules are generally not as synchronized as they are during oscillations *in vitro*. However, even when one assumes that the length fluctuations in cells are stochastic one has to explain the reaction pathway. Here, too, microtubule disassembly via oligomers offers the attractive possibility that oligomeric tubulin is temporarily prevented from reassembly, thus allowing extended shortening. The state of the

subunit pool in cells is not well defined since there is no way to distinguish small oligomers from subunits. We expect that MAPs play a major role in determining the relative stability of tubulin polymers and oligomers inside cells; this hypothesis is currently being tested by studying the effect of different MAPs and their state of phosphorylation on the pathway of microtubule growth and shrinkage.

Conformational States of Microtubule Ends

Protofilaments are observed in two main structural states, either straight (as in microtubules), or curled inside out (as in rings). Rings and other types of coiled structures have been observed by many authors, but their relationship to assembly has been interpreted in different ways. The prevailing view was that rings were involved in microtubule assembly (various models reviewed by Kirschner, 1978); an alternative was that coiling was a sign of microtubule disassembly induced by low temperature (Kirschner et al., 1974; Mandelkow and Mandelkow, 1985). This view remained controversial, partly because of the possible artefacts of cold disassembly and negative stain EM. This was one reason for addressing the problem using the high temporal and structural resolution of cryo-EM.

The question of the conformation of microtubule ends gained additional significance with the concept of dynamic instability. To explain phase changes at microtubule ends one had to assume at least two different states of the terminal subunits which could be distinct, say, in terms of their bound nucleotide (GTP cap hypothesis; Carlier et al., 1984; Hill and Chen, 1984) or some other parameter. Finally, it was found that minus-end directed movement of chromosomes (analogous to anaphase) and the concomitant microtubule disassembly did not require exogenous energy, raising the possibility that the energy was stored in the polymer and released via a conformational change (Koshland et al., 1988; Mitchison, 1988; Coue et al., 1991).

We show here that straight protofilaments are typical of growing microtubules. Their ends are usually smooth, but some protofilaments may also protrude for up to 100 nm or more in an essentially straight manner. This means that microtubules grow essentially by elongation of protofilaments (and not in a helical fashion), and that there is a cooperative interaction between neighboring protofilaments which prevents one from growing too long without the others. Similar observations have been made with growing microtubules in living cells; examples are the "jagged ends" of interzone microtubules in mitosis (McIntosh et al., 1985), or the C-shaped ends of microtubules assembling in platelets (Behnke, 1967).

On the other hand, coiled protofilaments are typical of shrinking microtubules; it occurs in a variety of conditions and thus appears to be an intermediate stage in disassembly. As with growth, the pathway of shrinkage is also not compatible with a helical mechanism. The curvature of coiled protofilaments is similar to that of rings, suggesting a close relationship between the underlying tubulin conformations, as diagrammed in Fig. 8.

In principle one could link the structural observations to the GTP cap hypothesis as follows: tubulin-GTP enforces a straight conformation of protofilaments which allows a microtubule to grow. Tubulin-GDP prefers a conformation that would lead to coiling of protofilaments (Howard and Timasheff, 1986); this breaks the microtubule lattice and causes shrinking. This would mean that the tubulin-GDP subunits in the interior of a microtubule would be in an unfavorable, tense conformation, ready to relax into the coiled conformation. In the interior the protofilaments remain straight, in spite of their tense conformation, because of the many lateral bonds between them. However, the ends are exposed, less stabilized by interprotofilament bonds, and thus the relaxation into the coiled conformation takes place most readily at the ends (breakage and coiling of the interior takes place only in rare cases, see Fig. 7 *b*). According to this view, the energy of GTP hydrolysis would be stored in the microtubule lattice in the form of a tense state and could be liberated by disassembly, as required by the model of Koshland et al. (1988; and see below). However, a problem with the interpretation is that the chemical nature of the cap is still controversial (for recent reports see Stewart et al., 1990; Caplow and Shanks, 1990*b*). Thus it would be premature to equate the observed straight or coiled conformations with a given nucleotide status of the protein.

Microtubules are polar structures; the two ends differ in terms of assembly rates and dynamic instability (Horio and Hotani, 1986; Cassimeris et al., 1988). One therefore could

have expected some differences in the structural transitions at the two ends; this was however not observed. Both ends were coiled during disassembly and straight during assembly. This means that tubulin undergoes similar conformational transitions at both ends, even though their rates of growth or shrinkage differ.

In a study related to ours, Simon and Salmon (1990) used negative stain EM to observe growing and shrinkage microtubules. Depending on conditions, they frequently observed long incomplete microtubule walls (seen as "sheets" in negative stain) at the ends of growing microtubules, while disassembly produced blunt ends. The authors concluded that growth takes place in an irregular fashion (i.e., involving extended sheets), while shrinkage is rather uniform, without gross disruption of the microtubule wall. Our results differ in several respects. Firstly, most of our growing microtubules have blunt ends (Fig. 1, *a* and *b*). Secondly, extended open microtubule walls (corresponding to sheets) were not observed during growth, although short protrusions do occur in 1/3 of the cases (Fig. 1, *c* and *d*). Thirdly, our shrinking microtubules have coiled ends (Figs. 2 and 3); this is similar to earlier observations of Kirschner et al. (1974) and our previous cryo-EM experiments on MAP-containing microtubules (Mandelkow and Mandelkow, 1985). The data imply that our growing ends are compatible with the microtubule lattice, but the shrinking ones are not; formally this is the opposite of Simon and Salmon's (1990) conclusions. These differences are probably explained by staining and drying artefacts, as well as by the limited temporal resolution of negative staining. In any case, the cryo-EM data confirm that neither growth nor shrinkage is helical, in agreement with Simon and Salmon (1990).

Role of Globular Structures

In this paper we have emphasized the role of tubulin oligomers in microtubule disassembly. This is however somewhat unsatisfactory because microtubules must first be created before they can fall apart, and the nuclei of assembly must also be oligomers of some kind. In contrast to the inactive disassembly products these nuclei must be active, i.e., assembly competent, and presumably have a different structure. At first sight it might seem fruitless to search for such nuclei because their concentration would be extremely low (in the nM range; Obermann et al., 1990), and because they might not be identified reliably by EM. However, during the studies on oscillations we noticed consistently another oligomeric structure whose appearance seemed to correlate with the onset of microtubule assembly. We have termed them "globular particles" to distinguish them from the coiled, rod-, or ringlike oligomers generated during disassembly (Fig. 7). We then noted similar structures during assembly in standard conditions as well. These particles are probably comparable to the "agglomerates" seen by Wade et al. (1989) in their oscillation conditions, especially when GTP is added to a solution of oligomers (their Fig. 5 *b*). Superficially the particles also resemble the "plugs" described by Azhar and Murphy (1990). In certain cases these particles showed a remarkable tendency to associate with one another, sometimes like beads on a chain, and with microtubule emanating from them (Fig. 7 *g*, *arrows*). Negative stain images did not reveal a defined substructure, but the cryo-EM images could be interpreted as very small sections of microtubule walls,

with short protofilaments lying side by side (Fig. 7 b, arrows).

It is not certain whether these structures are microtubule nuclei in the strict sense (which in any case is a matter of definition). However, we suggest that the particles are aggregates of GTP-activated tubulin that could be related to the process of nucleation and therefore tell us what the pathway might be. If the interpretation is correct we can draw two conclusions: (a) microtubules are not generated from a helical nucleus. This is in line with the other observations (mentioned before) showing that microtubules do not behave as helices, even when their structure is described in terms of a helical aggregate; and (b) nuclei are two-dimensional assemblies of tubulin. This would agree with the model of Voter and Erickson (1984) derived from assembly kinetics and model calculations. Further investigations will show whether the globular particles can be analyzed in sufficient detail to allow a comparison with the existing models.

Implications for Cellular Transport Processes

For the present discussion one important result is that there is a conformational change on disassembling microtubule ends in all conditions studied. Building a microtubule with straight protofilaments is accompanied by GTP binding and hydrolysis; after that, the protofilaments tend to relax back into their coiled conformation which has the effect of breaking the microtubule lattice up, starting preferentially at their weakest points, the ends, and thereby releasing the energy stored in the polymer. This could be exploited by a living cell to produce movement without additional chemical energy. One example mentioned above is that of chromosome movement during anaphase, as proposed by Koshland et al. (1988) and recently confirmed by Coue et al. (1991). A second example may be that of the microtubule dynamics in the amoeba *Reticulomyxa* which appears to be based on a rapid interconversion between microtubules and coiled protofilaments (Chen and Schliwa, 1990). These transitions probably involve not only tubulin, but specialized MAPs as well, emphasizing the need to characterize the role of MAPs on conformational states of microtubules.

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