

Direct Observation of Microtubule Treadmilling by Electron Microscopy

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ABSTRACT Using an immunoelectron microscopic procedure, we directly observed the concurrent addition and loss of chicken brain tubulin subunits from the opposite ends of microtubules containing erythrocyte tubulin domains. The polarity of growth of the brain tubulin on the ends of erythrocyte microtubules was determined to be similar to growth off the ends of *Chlamydomonas* axonemes. The flux rate for brain tubulin subunits in vitro was low, $\sim 0.9 \mu\text{m/h}$. Tubulin subunit flux did not continue through the entire microtubule as expected, but ceased when erythrocyte tubulin domains became exposed, resulting in a metastable configuration that persisted for at least several hours. We attribute this to differences in the critical concentrations of erythrocyte and brain tubulin. The exchange of tubulin subunits into the walls of preformed microtubules other than at their ends was also determined to be insignificant, the exchange rate being less than the sensitivity of the assay, or $<0.2\%/h$.

Microtubule assembly is thought to be a steady state process involving the association and incorporation of GTP-tubulin during assembly and dissociation of GDP-tubulin during disassembly. Wegner, studying actin polymerization, predicted that actin filaments may exhibit a flux of subunits through the polymers at steady state due to the net addition and loss of subunits at opposite ends (1). This concept of treadmilling, adapted to microtubules, has been applied either implicitly (2, 3) or explicitly (4, 5) to several models of mitosis.

The conclusion from pulse-label (6–11), electron microscopic (12), and dark field (13) studies is that treadmilling, in vitro, probably does occur, but the variability and low magnitude of the values have made the very existence of the phenomenon the subject of recent debate. In no case up until now has it been possible to document fluxing by direct electron microscopic examination.

The goal of this study was to measure treadmilling rates at steady state in microtubule heteropolymers with distinct regions of erythrocyte and brain tubulin. Treadmilling was observed over time at steady state, after labeling the microtubules with an antibody specific for erythrocyte tubulin. The results from these measurements show that chicken brain microtubules exhibit a flux rate of 0.3 subunits/s ($0.9 \mu\text{m/h}$).

MATERIALS AND METHODS

Preparation of Chicken Brain and Erythrocyte Tubulin: Chicken brain tubulin was isolated by the method of Dentler et al. (14) in 0.1

M PIPES buffer containing 1 mM MgCl_2 , 2 mM EGTA, 1 mM GTP, and 4 M glycerol.

Chicken erythrocyte tubulin was prepared from chicken blood by the method of Murphy and Wallis (15). Tubulin was purified free of MAPs by ion exchange chromatography using Whatman P-11 phosphocellulose (PC)¹ (16) and cycled once before use. Unless stated otherwise, microtubule assembly buffer was 0.1 M Na-PIPES pH 6.94 containing 1 mM MgCl_2 , 1 mM GTP, and 5% glycerol.

Erythrocyte Tubulin Antibody: A rabbit polyclonal antibody was produced against the beta subunit of the erythrocyte tubulin dimer. The PC tubulin was dialyzed, reduced, and alkylated as described by Crestfield et al. (17), then further dialyzed against water and 2 mM mercaptoethanol and lyophilized. Beta subunits were produced by chromatography on hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) in the presence of SDS as described by Lu and Elzinga (18) and Little et al. (19). Antigen mixed with Freund's complete adjuvant was injected subcutaneously into three rabbits (420 μg each). The titer was increased by subsequent boosts (420 $\mu\text{g}/\text{boost}$) of the antigen in incomplete Freund's adjuvant. An affinity bed for the purification of specific antibody was prepared by conjugating erythrocyte beta tubulin to Sepharose 4B (20). Resin and antiserum were incubated together overnight. After washing the resin with PBS (10 mM phosphate pH 7.4 containing 137 mM NaCl and 14 mM KCl) and with PBS containing 0.5 M KCl, the antibody was eluted with 1 M acetic acid, neutralized to pH 4 with 1 M Tris, dialyzed against PBS, and frozen until use.

Preparation of Microtubule Seeds and Subunits: Microtubule seeds were prepared by polymerizing PC erythrocyte tubulin at 37°C in 0.1 M PIPES pH 6.94 containing 10 mM MgCl_2 , 1 mM GTP, and 20% glycerol, and sedimenting and resuspending the polymers to 5–13 mg/ml in assembly buffer. Polymers were sheared by 10 passes through a 27-gauge needle (21). Brain PC tubulin subunits were diluted to the desired concentration in the same buffer at 5°C containing 0.25 U/ml acetate kinase and 50 mM acetyl phosphate as a GTP regenerating system (22). The stability of GTP (>95%) for periods up to

¹ Abbreviation used in this paper: PC, phosphocellulose.

3 h was confirmed by chromatography on polyethyleneimine plates (23) in LiCl/formic acid. The subunit preparations were lightly sonicated before use to disperse any oligomers (15).

Preparation of Heteropolymers for Measurement of Flux at Steady State: Two methods were used to prepare heteropolymers for the tubulin flux measurements. In method A, brain tubulin subunits (700 μ l, 1.25 mg/ml) were preincubated for 60 s in a 1-ml cuvette in a water-jacketed cuvette holder at 30°C. This time was determined to be sufficient for complete temperature equilibration. Erythrocyte microtubule seeds (25 μ l) were then added to obtain tubulin subunit addition at both ends of the microtubules. No brain microtubules formed by spontaneous self-assembly during this time. In method B, 250- μ l aliquots of elongating heteropolymers, produced by adding erythrocyte seeds to an equilibrated pool of brain tubulin (2.5 mg/ml), were removed and mixed with 1.0 ml prewarmed assembly buffer. This method rapidly brought the polymers close to steady state condition and produced short heteropolymers with a short length of brain tubulin polymer at each end.

For electron microscopic observations 20- μ l samples were taken at various times after dilution and fixed in assembly buffer containing 5% glutaraldehyde and labeled as described below.

Axonemes from the flagella of *Chlamydomonas reinhardtii* were prepared according to the method of Allen and Borisy (24) and used as seeds to establish the polarity of brain tubulin growth as described by Bergen and Borisy (12).

Labeling of Microtubule Heteropolymers with Tubulin Antibody—Protein A—Gold: Electron microscopic grids containing glutaraldehyde-fixed microtubules were rinsed successively with phosphate-buffered saline (PBS), 10 mM NaBH₄ to reduce the free aldehyde (25), PBS, and incubated with the erythrocyte tubulin antibody at 50 μ g/ml for 10 min. The grids were rinsed with PBS again and incubated with gold colloid coated with protein A (26–28). The preparations were rinsed with PBS, fixed with 5% glutaraldehyde in 0.1 M PIPES, pH 6.94, 1 mM MgCl₂, 1 mM GTP, and 5% glycerol, reduced with NaBH₄, and washed with PBS before negative staining with 1% uranyl acetate. The fixation after incubation with protein A–gold was required to prevent the redistribution of label.

Measurements of the decorated microtubules were made using a Zeiss EM 10A electron microscope (Carl Zeiss, Inc., Thornwood, NY). At least 25 microtubules from each grid were measured with respect to the length of the seed and the extent of polymerization at each end. This was chosen as the minimum number of microtubules to be counted because, to estimate accurately the average length of a microtubule seed population with a standard deviation of 1.15 μ m to within 0.5 μ m (maximum error of the estimate of 0.5 μ m at a 95% confidence level), a sample size of at least 24 microtubules was required.

Protein Concentration Determination: Total protein concentrations were determined by the Bradford protein assay (29). Bovine serum albumin (BSA) was used as a standard.

Biochemical Materials: PIPES, a sodium salt, was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Other chemicals and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Demonstration of Antibody Specificity for Erythrocyte Beta Tubulin in Microtubule Heteropolymers

The specificity of the erythrocyte beta tubulin antibody is demonstrated in Fig. 1 in an immunoblot of chicken brain and erythrocyte tubulin. Only the beta subunit of the erythrocyte tubulin was labeled. This antibody was also specific when used in an immunogold labeling procedure for electron microscopy. Erythrocyte microtubules were heavily stained compared with brain microtubules which allowed the two types of microtubules to be distinguished (Fig. 2).

The addition of erythrocyte tubulin seeds to brain tubulin subunits at 30°C resulted in a rapid increase in the amount of light scattering which by electron microscopy was shown to be due to the polymerization of brain tubulin subunits on the ends of the exogenous seeds. We call these microtubules chimeric microtubules because they contain three distinct regions: a central region which contained the original eryth-

Stain Blot

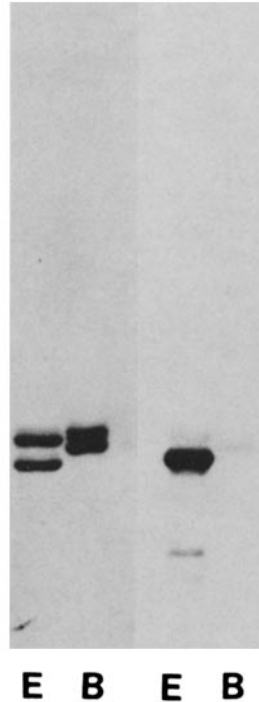


FIGURE 1 Demonstration of antibody specificity. Chicken erythrocyte tubule and chicken brain tubulin were fractionated by electrophoresis on a 10% polyacrylamide gel, pH 9.1 (30) and transferred to nitrocellulose paper (31). The strips were then incubated with anti-serum to beta erythrocyte tubulin diluted 1/500, followed by an incubation with ¹²⁵I-protein A. The left panel shows Amido black staining patterns of erythrocyte (E) and brain (B) tubulin. The right panel shows the corresponding autoradiogram after incubation with the anti-tubulin serum (1/500 dilution) and ¹²⁵I-protein A.

rocyte tubulin seed and two elongated ends which consisted of brain tubulin (Fig. 3).

Antibody labeling was required to distinguish the different segments of the chimeric microtubules since there were no apparent changes in the ultrastructure of the microtubule to demarcate the boundaries of each segment. This observation is consistent with the fact that erythrocyte and brain microtubules both contain similar numbers of protofilaments as determined by tannic acid staining (32) and thin sectioning: erythrocyte microtubule protein (13.6 \pm 0.7) or PC-tubulin (13.3 \pm 0.8), and brain microtubule protein (13.6 \pm 0.8) or PC-tubulin (13.6 \pm 0.7).

Evaluation of the Constancy of Polymer Mass and Microtubule Number Concentration at Steady State

The constancy of the microtubule number concentration was determined by counting microtubules on randomly selected grid squares (100 squares per time point; 400-mesh grids) of negatively stained microtubule samples. From these counts it was determined that there were no major changes in the microtubule number concentration for 4 h. The constancy of polymer mass was evaluated by monitoring the turbidity at 350 nm, by a sedimentation assay (21) and by quantitation of the lengths of brain tubulin polymer by electron microscopy. For the case in which seeds were added to brain tubulin subunits (1.25 mg/ml) without dilution, polymer mass increased by 75%, and steady state was not attained until after 100 min. When elongating polymers were diluted with buffer, polymer mass initially decreased during the first 2 min after dilution, and then increased by 30% over the next 20 min, after which the polymer mass remained constant (Fig. 4C).

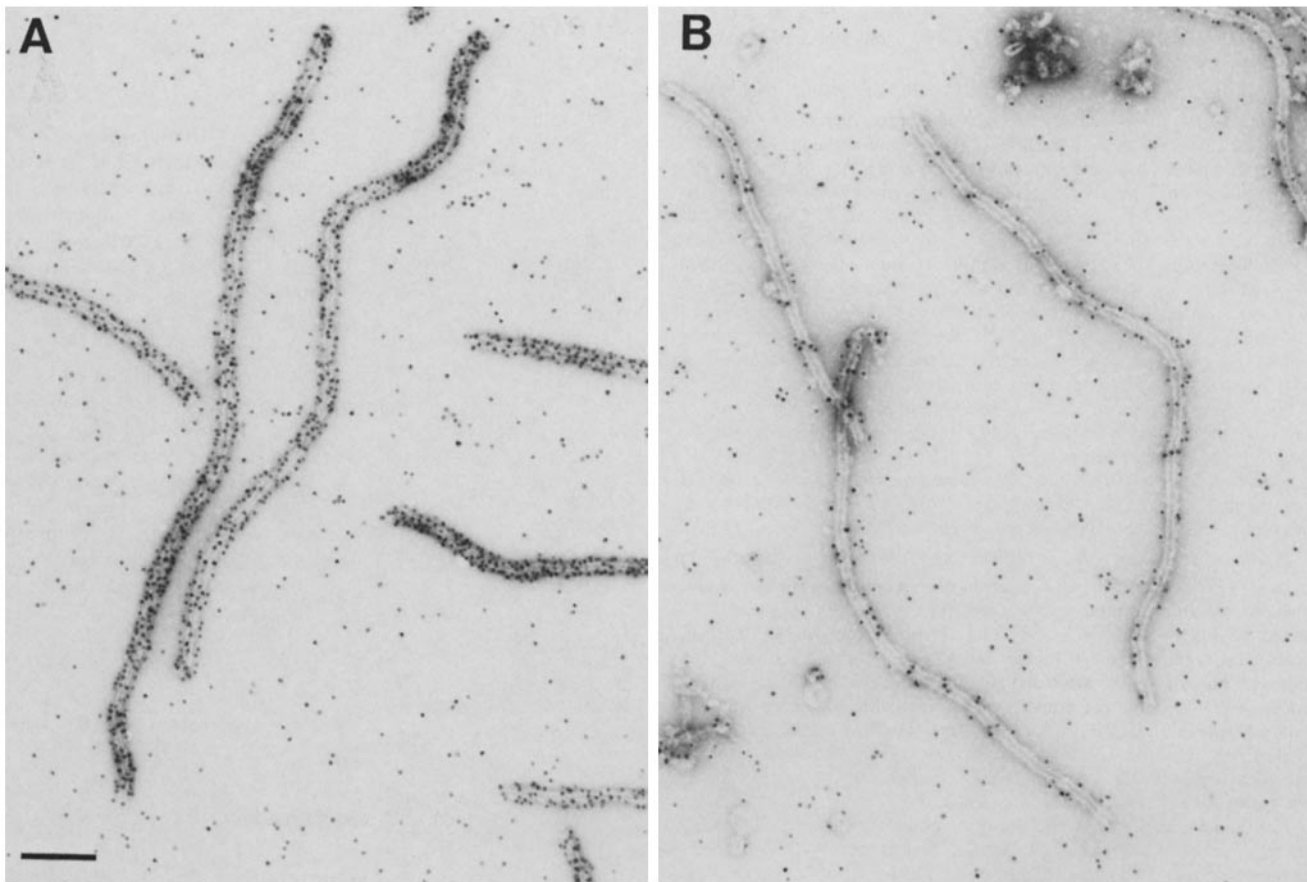


FIGURE 2 Labeling of microtubules by the erythrocyte beta tubulin antibody. Microtubules were polymerized from erythrocyte tubulin (A) or brain tubulin (B) and fixed in 5% glutaraldehyde in assembly buffer. The two types of polymers were incubated with 50 $\mu\text{g}/\text{ml}$ affinity-purified beta tubulin antibody and protein A-gold. Bar, 0.2 μm . $\times 50,000$.

Polarity of Microtubule Growth

Chlamydomonas axonemes were used as alternate seeds to compare the polarity of growth of erythrocyte and brain tubulin. As in the case of porcine brain tubulin (12), the growth of both chicken brain and erythrocyte tubulin from the distal (+) end was faster than that from the proximal (-) end.

Examination of Microtubule Flux

Two procedures were used to prepare chimeric microtubules for the examination of tubulin subunit flux at steady state. The results of both methods are described separately since each procedure provided observations not seen with the other technique. As in the case for growth off axonemes, brain tubulin elongation off erythrocyte microtubule seeds was consistently longer at one end than the other. Therefore, we have called the long end the plus end and the short end the minus end. A graphic display of the results of one experiment produced by method A is shown in Fig. 4A, in which each microtubule is represented as a tripartite line. The center segment represents the original erythrocyte tubulin seed, and the segments to either side represent regions of brain tubulin elongation. The tubules are oriented so their short ends are all on one side and the long ends are on the other.

In case A, there was initially growth at both ends of the erythrocyte seed with the plus end growing much faster than the minus end (Fig. 4B). 10 min after initiation the minus

ends began to depolymerize and continued to depolymerize for the next 10 min at a rate of 0.7 $\mu\text{m}/\text{h}$ (0.3 subunits/s) until all of the brain tubulin polymer was gone from the minus ends of most of the microtubules. Once the erythrocyte seeds were exposed, no further shortening occurred. During the time of minus end depolymerization the plus end continued to grow at a much faster rate (5.3 $\mu\text{m}/\text{h}$). This experiment demonstrated that depolymerization at the minus end and polymerization at the plus end could occur simultaneously as predicted by treadmilling. However, we were not able to obtain a true flux rate, because the system was not at steady state as indicated by the imbalance in the elongation rates at plus and minus ends, and by the observation that the polymer mass increased slowly with time.

In case B, initial elongation was produced using a higher concentration of the brain subunits, resulting in longer minus ends. These chimeric microtubules were diluted into buffer, causing some depolymerization followed by a phase of polymerization which continued at both ends for 20 min after which the minus ends began to depolymerize. As judged by constancy of turbidity, a constant sum of the mean lengths of the plus and minus end, and an unchanging microtubule number, steady state was attained at this time. For the next hour the minus end shortened at a rate of 0.8 $\mu\text{m}/\text{h}$ (0.4 subunits/s) that nearly matched the rate of 1.1 $\mu\text{m}/\text{h}$ (0.5 subunits/s) at which the plus end lengthened (Fig. 4C). Therefore, in the second case we produced a system in which true flux could be measured.

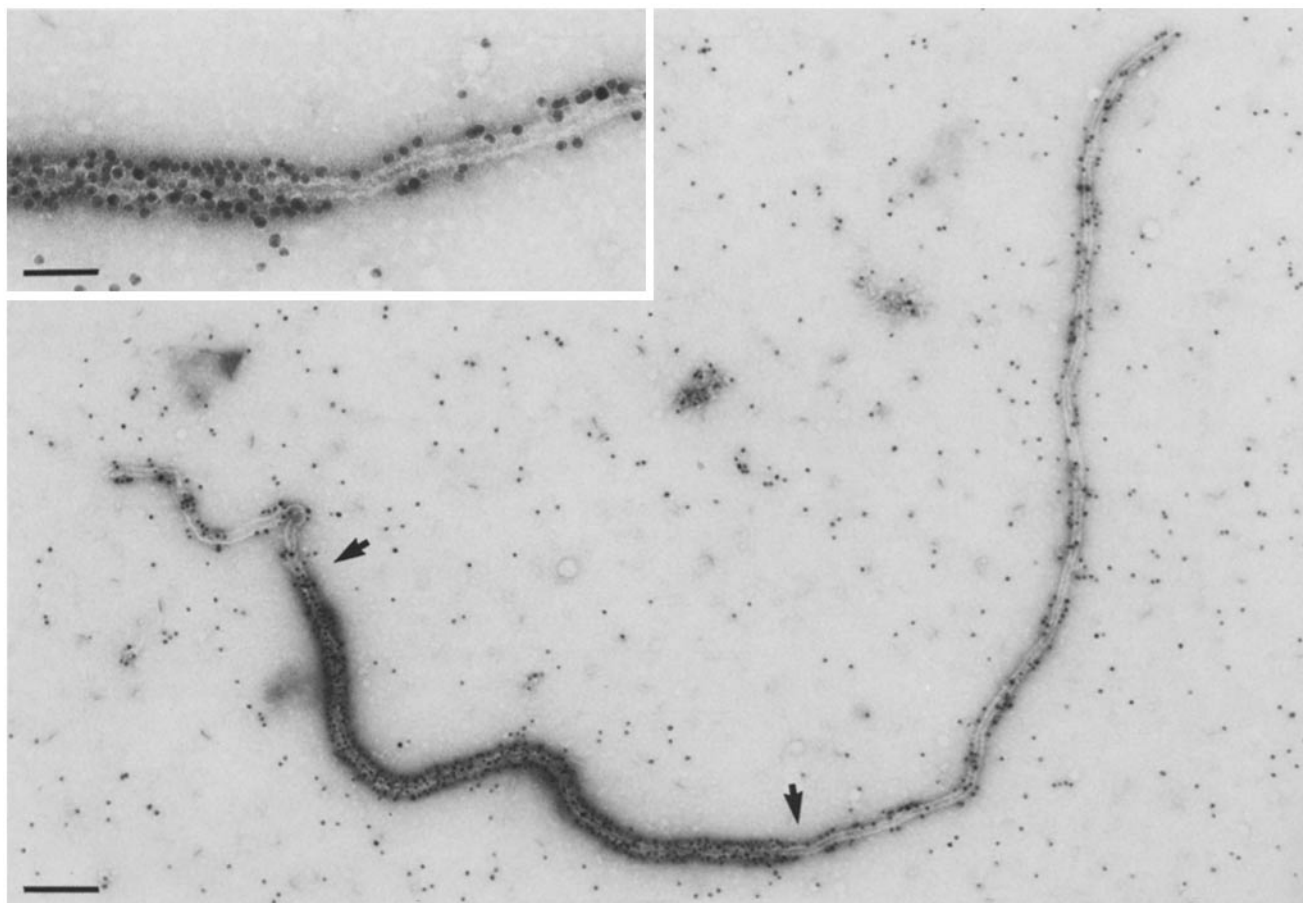


FIGURE 3 A chimeric microtubule composed of brain and erythrocyte tubulin. Erythrocyte tubulin seeds were added to brain tubulin, and the resulting microtubules were stained with erythrocyte tubulin antibody and protein A-gold. The original seed, delineated by arrows, is heavily labeled by the gold particles. Bar, 0.2 μm . $\times 52,000$. Inset shows an enlarged view of the junction between the erythrocyte tubulin seed and the brain tubulin polymer. Bar, 0.08 μm . $\times 125,000$.

We expected that disassembly at the minus (–) ends would continue through the erythrocyte tubulin seed until the distinct regions of antibody labeling disappeared. However, as seen in Fig. 4A the minus ends shortened until the erythrocyte tubulin seeds were exposed (the microtubules contained brain tubulin at only one end) after which the polymers remained remarkably stable for several hours. The mean seed length remained unchanged for the duration of the experiment, being 3.4 ± 1.1 and 3.3 ± 2.6 μm at 5 min and 3 h, respectively. A significant number of microtubules (~30%) retained short brain tubulin caps at their minus ends even after several hours.

Absence of Subunit Exchange into Polymer Walls

To examine the possibility that tubulin dimers may exchange with subunits along the walls of preformed microtubules (33), we examined the composition of brain tubulin polymers in the presence of erythrocyte tubulin subunits. Based on the density of gold labeling of polymers assembled from varying ratios of erythrocyte and brain tubulin we found that a concentration of erythrocyte tubulin greater than 5% of the total (~80 erythrocyte subunits/ μm brain microtubule polymer) was sufficient to elevate the labeling above the baseline level of brain microtubule labeling (Rothwell, S. W., W. A. Grasser, and D. B. Murphy, manuscript in preparation). At this level of resolution we saw no evidence for insertion of

erythrocyte subunits into the walls of the brain microtubule seeds even after 5 h at 37°C.

DISCUSSION

These experiments document the first direct electron microscopic observation of tubulin flux through microtubules *in vitro*. Preparations of chimeric microtubules, sampled over time, clearly show simultaneous loss of tubulin subunits at one end and the gain of subunits at the other end, while the microtubule number and polymer mass remain constant. Although our estimate of the flux rate of brain tubulin subunits through microtubules is low (~0.9 $\mu\text{m}/\text{h}$), it is consistent with both the low rates calculated by other investigators (3, 7, 10–13, 34) and the low efficiency of treadmilling ($s = 0.13$) previously measured by us (35).

Continuous treadmilling at these rates should result in the disappearance of the original seed, but, in our system, this was not the case. Instead, a metastable condition was observed which we attribute to the different kinetic properties of the two tubulin types. When the critical concentration values determined for erythrocyte tubulin (plus end = 1.6 μM ; minus end = 1.8 μM) are compared with brain tubulin (plus end = 2.7 μM ; minus end = 4.7 μM) (35), it is apparent that the erythrocyte moiety has a greater potential for polymerization than does the brain species. One would therefore predict that as soon as the brain tubulin depolymerizes, exposing the

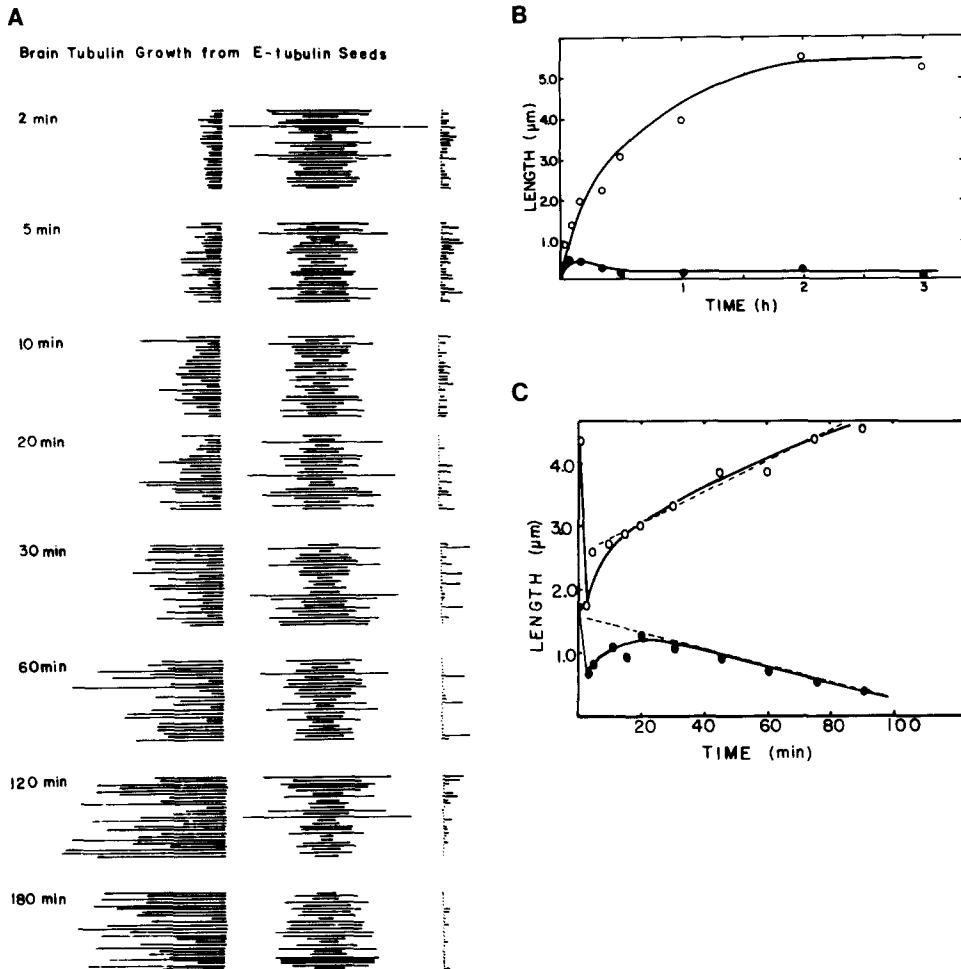


FIGURE 4 Changes in microtubule length with time. (A) Graphic display of the behavior of the hybrid microtubules over time. Each three-part line represents one microtubule that was measured on the electron microscope. The middle segment represents the erythrocyte tubulin seed, and the segments to either side represent brain tubulin polymer. T_0 equals the initiation of brain tubulin from the erythrocyte seeds. No further dilutions were performed on the reaction mixture (method A). (B) The lengths of elongated brain tubulin polymer at plus (\circ) and minus (\bullet) ends from A were plotted versus time. (C) In a second experiment (method B) the mean lengths of elongated brain tubulin polymer at each end of the seed were plotted versus time after dilution into warm assembly buffer (t_0 equals time of dilution). Calculated rates of depolymerization from the minus end (\bullet) and polymerization at the plus ends (\circ) were 0.84 and 1.14 $\mu\text{m}/\text{h}$, respectively.

erythrocyte seed at the minus end to the subunit pool, new polymerization should occur. However, as the bulk of the subunits are brain tubulin, the growth segment will once again terminate in a protein type with a high critical concentration and should rapidly depolymerize. Cycles of alternating growth and depolymerization could account for the metastable configuration we observed and explain the fact that a few microtubules with short brain tubulin segments at their minus ends were present even after 3 h (Fig. 4A). Thus, when domains of erythrocyte and brain tubulin are coupled together in a single microtubule, it is possible that a polymer is created which never reaches the conventional steady state condition of continuous unidirectional flux.

It is also interesting that two of the features of dynamic instability as described by Mitchison and Kirschner (36) were not observed: (a) Microtubule number concentration remained constant for 4 h (1 mg/ml microtubule polymer), and (b) the lengths of the erythrocyte microtubule seeds in the heteropolymers remained constant despite exposure of their minus ends for up to 3 h.

While the *in vitro* flux rate we observed is consistent with other measured *in vitro* rates, they are considerably lower than the *in vivo* rates of chromosome movement during anaphase. A comparison of our mean rate of 0.9 $\mu\text{m}/\text{h}$ to *in vivo* rates in various organisms such as *Haemanthus katherinae* (47 $\mu\text{m}/\text{h}$) (37), PtK₁ cells (126 $\mu\text{m}/\text{h}$) (38), or *Chaetopterus* (198 $\mu\text{m}/\text{h}$) (39), shows that the *in vitro* rate is 50–100 times less. If treadmilling plays a significant role in mitosis,

the cellular environment must contain factors that dramatically increase the flux rates. Most of the microtubule associated proteins that have been identified up until now stabilize microtubules in both their phosphorylated or nonphosphorylated forms, and cause a decrease in the flux rate (10, 40–43). Therefore, other cytoplasmic factors such as the concentrations of calcium (44) or nucleotide (45) may be important for regulating microtubule stability *in vivo*. However, using fluorescence photobleach recovery methods after microinjection of fluorescent tubulin into living cells (46, 47) or examination of the dynamics of derivatized tubulin *in vivo* (48), there still has been no convincing demonstration of tubulin flux in microtubules *in vivo*. Therefore, the significance, for cellular processes, of the microtubule treadmilling observed *in vitro* remains to be established.

Finally, the discovery of chimeric microtubules locked in a metastable state suggests that a cell could produce microtubule heteropolymers of altered stability by changing the rate of synthesis of different tubulin variants. Recent work by Thompson et al. (49) and Gunderson et al. (50) demonstrates that biochemically distinguishable subsets of microtubules may be contained in the same microtubular array. Therefore, the actual tubulin content of the polymer and the distribution of the isoforms within the microtubule, either condensed into distinct domains or dispersed in a homogeneous manner, could greatly affect the flux rates. However, confirmation of this theory will require more investigation to define the composition and regulation of various microtubular organelles.

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