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Microtubule assembly nucleated by isolated centrosomes

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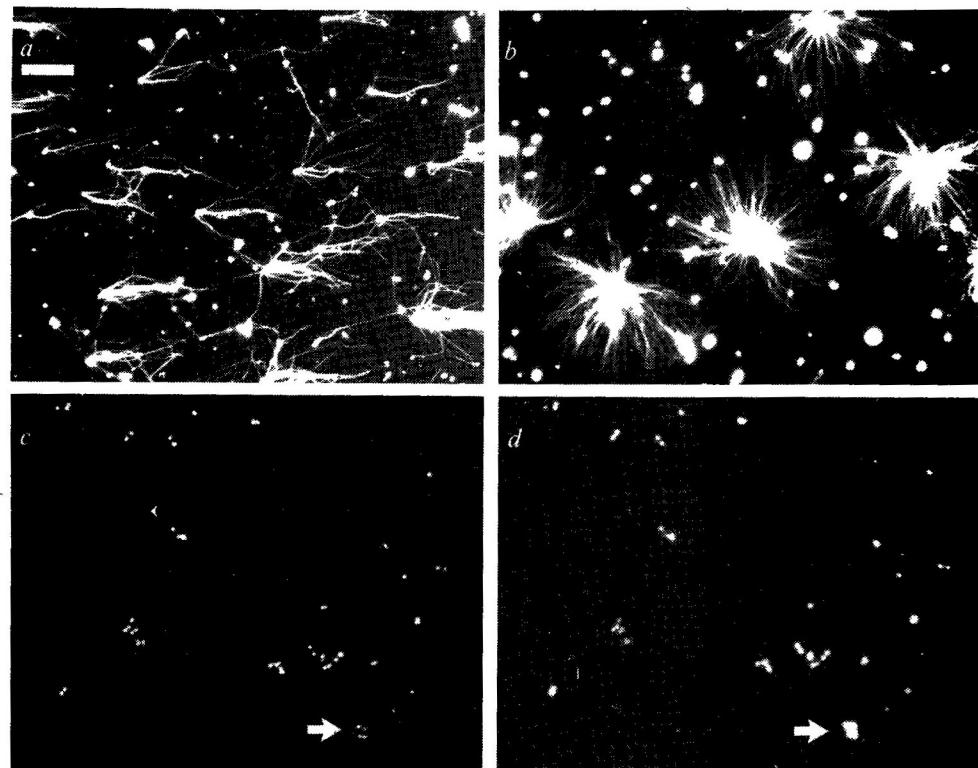
Microtubules are involved in the morphogenesis of most cells and are the structural basis of the mitotic spindle. We report here that purified centrosomes nucleate the assembly of microtubules with unusual dynamic properties. This may have important implications for the mechanism by which microtubule arrays are organized and stabilized in cells.

THE function of microtubules in cells is thought to depend on their specific spatial organization. In animal cells most microtubules are anchored at one end in a structure called the centrosome, which contains the centriole pair, and is surrounded by amorphous, osmophilic material^{1,2}. In cells lacking centrioles, but containing a focus of microtubule growth, similar material is found, as are antigenic determinants common to those found in the centrosome, suggesting that there are common structural components in all interphase microtubule organizing sites³.

A fundamental question about the centrosome is how it organizes the assembly of microtubules *in vivo* and thereby, at least in part, determines the spatial arrangement of microtubules in the cell. This question can be broken into two parts, one concerning the nucleation, and the other the anchoring and stabilization of microtubules. Nucleation is essentially a kinetic process,

involving the coalescence of microtubule subunits into a seed, which can subsequently elongate. A structure which promotes nucleation will shorten or remove the lag phase in polymerization, and thus give microtubules attached to the structure a kinetic advantage. Microtubule nucleation by centrosomes is demonstrated *in vivo* when microtubules regrow preferentially from the centrosomes after cells are released from drug-induced microtubule depolymerization⁴⁻⁶. This aspect of centrosome function has also been demonstrated *in vitro*, using permeabilized cells^{7,8}, in crude lysates⁹⁻¹¹ and centrosome-nucleus complexes¹²⁻¹⁴. As well as giving local microtubules a kinetic advantage by nucleation, the centrosome *in vivo* must be able to anchor and preferentially stabilize them for long times. If centrosome microtubules had the same thermodynamic or steady-state stability as free microtubules, the latter eventually would come to

Fig. 1 Assay of centrosomes. *a*, Regrowth using centrosomes on coverslips. N115 centrosomes (see Fig. 2 legend) were diluted into 5 ml of PE buffer (10 mM PIPES, 1 mM EDTA, pH 7.2 with KOH) and sedimented onto 11-mm round glass coverslips at 25,000g (15 min, 4 °C) in a swinging bucket rotor. Corex tubes (15 ml) were modified to contain a plexiglass plug which could be lifted out easily with the coverslip on top. A drop of 5% Triton X-100 was added to each tube and the coverslip removed. 100 µl tubulin (35 µM in PB) was added to each coverslip, which was incubated at 37 °C for 12 min in a humidified chamber. Microtubules were fixed by aspirating most of the tubulin and adding 1% glutaraldehyde in PB (80 mM PIPES, 1 mM EDTA, 1 mM MgCl₂, 1 mM GTP, pH 6.8 with KOH) at 37 °C for 3 min. The coverslip was rinsed in PB' (PB without GTP) and postfixed in methanol at -20 °C. Immunofluorescence was with DMIα monoclonal anti-tubulin³⁴ and rhodamine-conjugated goat anti-mouse antibody (Cappel). Antibodies were routinely diluted in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) + 0.1% Triton X-100 + 0.05% Na₃NO₂. Coverslips were washed in PBS + 0.1% Triton X-100 and mounted in 90% glycerol, 20 mM Tris, pH 7.8. Fluorescent microscopy was with a Zeiss Photomicroscope III, and photography used Kodak Technical Plan 2415 developed with HC110. *b*, Regrowth of centrosomes in solution. N115 centrosomes were mixed with tubulin at 0 °C in a final volume of 50 µl and a final concentration of 25 µM in PB. After 8 min at 37 °C, 200 µl of 1% glutaraldehyde in PB at 26 °C was added and then 3 min later, 1 ml of PB' at 0 °C. The regrown centrosomes were layered on top of a 5-ml cushion of 25% v/v glycerol in PB' in the modified corex tubes, and sedimented at 25,000g for 15 min onto a polylysine-coated coverslip made by dipping a glass coverslip in 1 mg ml⁻¹ polylysine, then aspirating dry. The supernatant was aspirated, and the interface above the cushion washed with 1% Triton X-100. The coverslip was then removed, postfixed and stained as above. *c, d*, Structural assay of centrosomes using immunofluorescence. N115 centrosomes were sedimented on a plain glass coverslip as in *a*. Coverslips were fixed directly in methanol at -20 °C and stained with a mixture of monoclonal anti-tubulin antibody and 5051 human antipericentriolar material³⁵. Mixed rhodamine-conjugated goat anti-mouse and fluorescein-labelled goat anti-human (Cappell) secondary antibodies were used. *c*, Tubulin; *d*, 5051. Scale bar corresponds to 6 µm in *a, b*; 3 µm in *c, d*.



Regrowth of centrosomes in solution. N115 centrosomes were mixed with tubulin at 0 °C in a final volume of 50 µl and a final concentration of 25 µM in PB. After 8 min at 37 °C, 200 µl of 1% glutaraldehyde in PB at 26 °C was added and then 3 min later, 1 ml of PB' at 0 °C. The regrown centrosomes were layered on top of a 5-ml cushion of 25% v/v glycerol in PB' in the modified corex tubes, and sedimented at 25,000g for 15 min onto a polylysine-coated coverslip made by dipping a glass coverslip in 1 mg ml⁻¹ polylysine, then aspirating dry. The supernatant was aspirated, and the interface above the cushion washed with 1% Triton X-100. The coverslip was then removed, postfixed and stained as above. *c, d*, Structural assay of centrosomes using immunofluorescence. N115 centrosomes were sedimented on a plain glass coverslip as in *a*. Coverslips were fixed directly in methanol at -20 °C and stained with a mixture of monoclonal anti-tubulin antibody and 5051 human antipericentriolar material³⁵. Mixed rhodamine-conjugated goat anti-mouse and fluorescein-labelled goat anti-human (Cappell) secondary antibodies were used. *c*, Tubulin; *d*, 5051. Scale bar corresponds to 6 µm in *a, b*; 3 µm in *c, d*.

predominate. Instead the reverse is found; free microtubules, if formed, are generally unstable with respect to centrosomal ones^{15,16}. This aspect of centrosome function has been discussed theoretically, and models have been proposed for centrosomal stabilization of microtubules by capping of a thermodynamically less stable end^{17,18} or by inducing a localized region in the cell which promotes microtubule assembly¹⁶. Experimental support for such models is at best indirect. To further study microtubule nucleation and stabilization, we have developed a method for the isolation of active centrosomes. By examining the assembly of pure tubulin onto isolated centrosomes, the kinetics of the nucleation process and the stability of nucleated microtubules was determined. The results of these studies have revealed important and unexpected features of microtubule polymerization, which bear on the role of the centrosomes in stabilizing and organizing microtubule arrays in cells.

Centrosomes and microtubule nucleation

To assay centrosomes during purification, we developed a functional assay in which microtubule assembly was dependent on centrosomes. To achieve this we needed a preparation of tubulin which polymerized well onto centrosomes but was deficient in spontaneous assembly. Tubulin purified by phosphocellulose chromatography in PIPES buffer without glycerol⁸ fulfilled these criteria and could be stored as aliquots at -70 °C without subsequent change in assembly properties. This preparation of tubulin appeared to consist of only α - and β -tubulin on overloaded polyacrylamide gels, although the more sensitive technique of immunoblotting revealed residual τ -protein (<0.1% of the total protein present). Very little spontaneous assembly of this tubulin occurred at concentrations as high as 25 μM (2.5 g l⁻¹) for 30 min, yet it readily assembled onto centrosomes.

N115 neuroblastoma cells were initially chosen for centrosome isolation because they contain multiple centrioles and because the centrosomes seemed to be easily isolated in association with nuclei^{8,13,19-21} (Fig. 1). Centrosome preparations were exposed to tubulin and the extent of microtubule growth assayed by immunofluorescence. Spontaneous microtubule assembly was negligible. Centrosomes could be either regrown in solution or attached to a glass coverslip. The coverslip assay was more convenient in developing the purification scheme, as it was not limited either by centrosome concentration or by the buffer in which the centrosomes were placed. However, it was inferior for kinetic studies of centrosome-dependent microtubule growth, since the capacity of centrosomes was severely inhibited by adsorption to glass (Fig. 1a, b). For an accurate assessment of nucleation capacity, the solution assay was used. That the sites of microtubule regrowth were centrosomes was confirmed using antibody to tubulin, which recognizes the centriole cylinders (Fig. 1c), or with the human autoantibody against pericentriolar material (Fig. 1d)³. The staining pattern with these two antibodies gave similar distributions, showing the clustering of several centrioles into one N115 microtubule organizing centre. The pericentriolar material appears more irregular and diffuse, and sometimes connected adjacent centrioles (arrow in Fig. 1c, d). Immunofluorescence of naked centrosomes was used to determine their concentration during isolation, when conditions for preserving activity were already established. The antigenic determinants of both the centrioles and pericentriolar material were found to be much more robust than the nucleation capacity and are preserved by treatments which destroy nucleation capacity, such as 1 M NaCl or 2 M urea. Interestingly, treatment with 0.5 M KI or 2% sodium phosphotungstate at pH 7.0 appeared to solubilize the tubulin epitope (and destroy nucleation capacity) without perturbing the pericentriolar antigen staining. This suggests that the pericentriolar material may have a structural autonomy independent of the centriole cylinders.

We initially isolated complexes between centrosomes and nuclei from cells which were pretreated with nocodazole and cytochalasin B to depolymerize microtubules and weaken the actin meshwork¹³. However, there was a major difficulty in

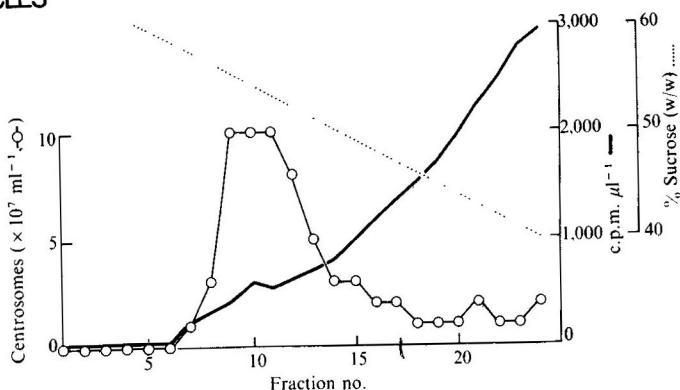


Fig. 2 Isolation of labelled N115 centrosomes by sucrose gradient sedimentation. Twenty confluent plates of N115 neuroblastoma cells were used. Cells were grown in DME H21 + 10% calf serum at 37 °C in 7.4% CO₂. For ³⁵S-methionine-labelled centrosomes, five plates of cells were labelled with 5 mCi of ³⁵S-methionine in medium containing 5% serum and 5% of the normal methionine complement for 18 h. These plates were combined with 15 unlabelled plates and processed as usual. Cells were pretreated with 10 $\mu\text{g ml}^{-1}$ nocodazole and 5 $\mu\text{g ml}^{-1}$ cytochalasin B in medium for 90–120 min at 37 °C. Cells were then collected in medium and transferred to 50-ml polypropylene tubes, 10⁸ cells per tube. All subsequent steps were at 0–4 °C. Cells were washed using a table centrifuge at full speed (1,500g). Washes were with PBS, then PBS/10+8% sucrose, then 8% sucrose. The final pellet was resuspended in 12.5 ml per tube of 1 mM Tris-HCl, 0.1% β ME (made using 2 M Tris-HCl stock, pH 8.0). To this was added 12.5 ml per tube of 1 mM Tris, 0.1% β ME, 1% Nonidet P-40 (NP40). The lysate was spun at 1,500g for 3 min. The supernatant was filtered through 37- μm nylon mesh and 1/50 volume of 50× PE (Fig. 1a) was added to the filtrate. This was transferred to 30-ml corex tubes and underlain with PE+20% w/w Ficoll+0.1% β ME+0.1% NP40, then spun at 25,000g for 15 min in a swinging bucket rotor at 2 °C. Most of the supernatant was aspirated and the Ficoll interface collected with a Pasteur pipette (2.5 ml per tube). Ficoll interfaces were pooled and layered onto a sucrose gradient. The gradient was poured to half fill an SW 28 tube (Beckman) and consisted of 20% (w/w)-62.5% (w/w) sucrose made up in PE+0.1% β ME+0.1% Triton X-100. The gradient was spun at 27,000 r.p.m. for 1 h at 2 °C in an SW28 rotor (100,000g); 30 drop fractions were collected from the bottom. To preserve nucleation capacity, the ionic strength was kept low throughout the preparation, but the activity was stable for several days at 0 °C or several months at -70 °C once in the final sucrose solution. Sucrose concentration was determined by refractometry, and centrosome concentration determined as for Fig. 1c, d. The three peak fractions were pooled, aliquoted, frozen on liquid nitrogen and stored at -70 °C. To prepare centrosomes from CHO cells, the following modifications were made: washes were done on the tissue culture plate, using an aspirator to remove wash solutions; an extra wash of 1 mM Tris-HCl, 0.1% β ME was used, then 10 ml per plate of the same buffer+0.5% NP40 was added. Plates were rotated gently at 4 °C for 10 min using a gyratory shaker (New Brunswick Scientific). The lysate containing centrosomes was collected and 50× PE was added before the low-spread spin to remove chromatin. The filtration step was omitted and the preparation proceeded as above.

subsequently detaching the centrosomes from the nucleus, to which they are tightly associated during interphase^{9,14}. This proved very difficult, although treatment with 2 M urea in 100% D₂O buffers was partially successful in detaching the centrosomes and preserving nucleation activity. The attachment, however, appeared to be more labile in living cells, and we found that detergent lysis of cells at very low ionic strength liberated centrosomes into solution, while the same treatment of isolated nuclei did not. The low ionic strength lysis appeared to solubilize most of the cellular actin, giving a lysate containing little particulate material apart from some chromatin. For this treatment to work, it was essential for the metal cation concentration in the lysate to be <0.5 mM. Centrosomes constituted the largest remaining particulate material after chromatin removal (Fig. 2).

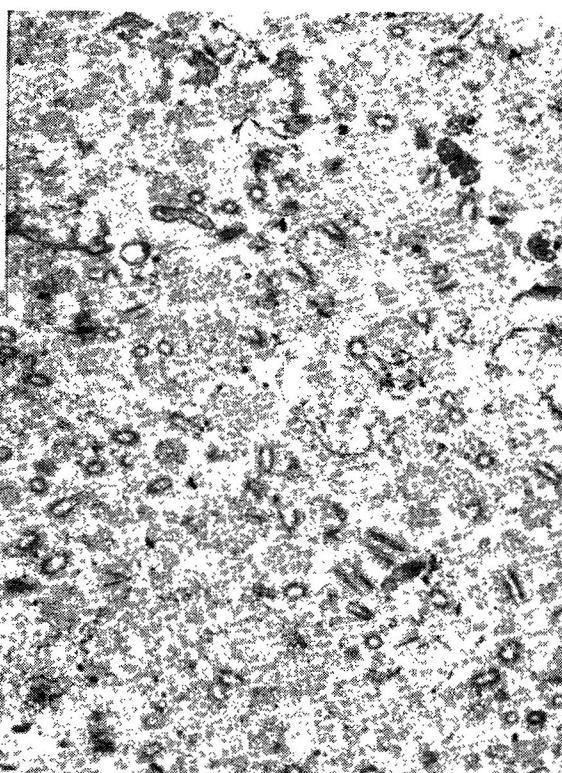


Fig. 3 Thin section of centrosomes. 10^8 N115 centrosomes, directly from the sucrose gradient, were diluted with 10 ml of 50 mM PIPES, 1 mM EDTA, pH 7.2 with KOH + 1% glutaraldehyde at 0°C and incubated for 30 min. The fixed centrosomes were then pelleted by spinning at 25,000g for 15 min. The pellet was washed twice in the same buffer without glutaraldehyde and resuspended in 2% OsO₄ in the same buffer. After 2 h at 0°C , the pellet was dehydrated through an ethanol series and propylene oxide, and embedded in Araldite. Sections (50 nm) were stained with uranyl acetate and lead citrate. Scale bar corresponds to 0.8 μm in the low-magnification field, and 0.1 μm in the inset.

Although N115 cells gave a good yield of centrosomes, they were heterogeneous in size, with one to ten centrioles per structure (Fig. 1c, d). As a source of more homogenous centrosomes, we used CHO cells which have a well characterized centriole cycle¹⁴. We modified the preparation slightly to take advantage of the adherent cell line and the more stable nucleus, but the effectiveness of a very similar protocol on two different lines suggests that it may have general use for centrosome purification (Fig. 2). The routine availability of a reproducible preparation of highly purified, stable centrosomes has opened the way for studies of centrosome function both *in vitro* and *in vivo*²².

Electron microscopy of a thin section of the N115 centrosome preparation reveals that the triplet structure of the centriole cylinder is well preserved and the pericentriolar material responsible for microtubule nucleation⁹ is visible (Fig. 3). We estimated that centriole cylinders constituted ~5% of the volume of the pellet. This is in agreement with quantitative immunoblotting and two-dimensional gel analysis, which indicated that 3–5% of the protein in the preparation was tubulin. When centrosomes were isolated from methionine-labelled cells (Fig. 2), the peak contained ~0.01% of the original counts; the overall yield obtained by counting centrioles was 30%, indicating a 3,000-fold purification. We estimate that the tubulin in a pair of centrioles should represent ~0.002% of the total cell protein, agreeing with our estimates that centrioles represent ~5% of the centrosome preparation. We cannot assess the functional importance of the remaining material, some of which must be the important pericentriolar components involved in microtubule nucleation.

Microtubule growth of centrosomes

Nucleation capacity of centrosomes was characterized by measuring the number and length of microtubules nucleated as a function of tubulin concentration. CHO centrosomes were regrown and fixed in solution, and then sedimented onto EM grids and visualized by rotary shadowing with platinum (Fig. 4b, c). Mean microtubule length increased at a constant rate at a given tubulin concentration, identical to the rate of increase for the plus ends of axonemes²³. This agrees with previous studies showing that *in vitro*, centrosomes nucleate microtubules with the plus ends distal¹⁰. We found considerable variation in microtubule number per centrosome, reflecting heterogeneity in

the population which may result from damage during isolation and the presence of different cell cycle stages. Mitotic centrosomes should be rare or absent in the preparation, since mitotic CHO cells would have been lost from the population during the plate washes. The number of microtubules nucleated per centrosome increased with tubulin concentration, starting at 4 μM and reaching a plateau above 20 μM (Fig. 4). This plateau may indicate saturation of nucleation sites, and the general shape of the curve is in agreement with earlier studies^{8,11,12}.

The average number of microtubules per centrosome increased gradually with tubulin concentration which is surprising as phase transitions generally show a step concentration dependence. This finding could be due to heterogeneity in the kinetics of nucleation, or to inherent differences in stability of microtubules grown at different concentrations. To examine this question, we measured the stability of free microtubules as a function of tubulin concentration.

To initiate the bulk polymerization of purified tubulin, we seeded its assembly with a small amount of microtubule fragments generated by spontaneous polymerization of the same tubulin in an assembly-promoting buffer containing 30% glycerol and 10 mM MgCl₂²⁴. Below 14 μM total tubulin, the microtubules depolymerize; above this concentration there is a linear increase of polymer with total tubulin, indicating a sharp phase transition (Fig. 5). The slope of this line is 0.68, probably indicating that two-thirds of the tubulin (stored as frozen aliquots) is competent for assembly. The concentration below which microtubules polymerize has previously been called the 'critical concentration'^{25,26}, by analogy with other phase transitions. We prefer the term 'steady state concentration' to indicate that this is the monomer concentration that exists when polymer is assembled to steady state¹⁸. When corrected for inactive tubulin, the true steady-state concentration is ~10 μM . However, we use here 14 μM , because this is the effective value for our preparation. This value may be compared with typical values found for tubulin and microtubule-associated proteins of 1–3 μM ^{27,28}. A 10-fold higher steady-state concentration for pure tubulin in aqueous PIPES buffer is in reasonable agreement with earlier estimates^{29,30}; pure tubulin may be incapable of polymerizing in less favourable aqueous buffers³¹.

There is a striking difference between the minimum concentration for microtubule assembly off centrosomes (3–4 μM) and

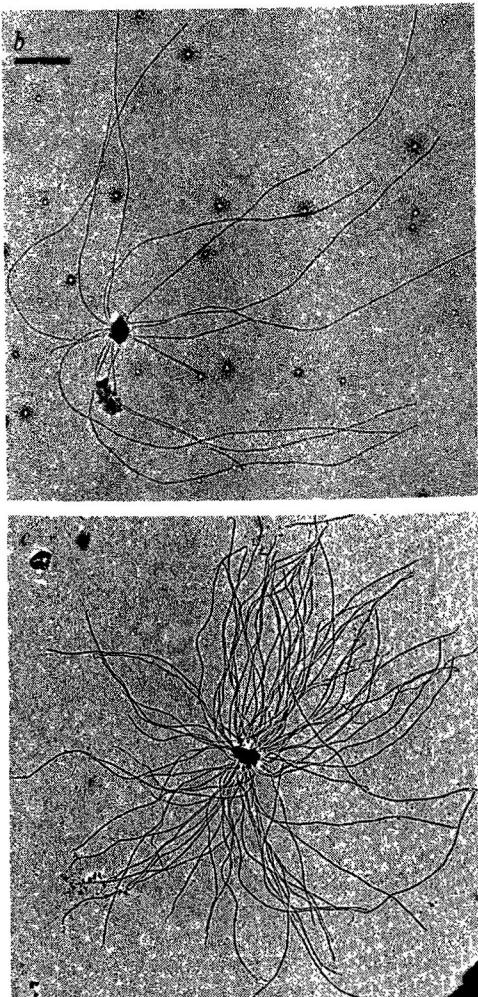
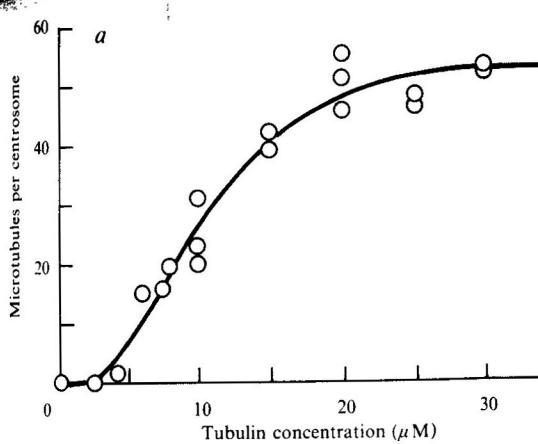


Fig. 4 Microtubule nucleation as a function of tubulin concentration. *a*, Number of microtubules nucleated per centrosome; *b*, *c*, typical images at 7.5 μM and 25 μM , respectively. Scale bar = 1.4 μm . Tubulin at appropriate concentration in PB was prewarmed for 2 min. 5×10^5 CHO centrosomes were added, and the mixture incubated in a final volume of 100 μl for 3–20 min at 37 °C (depending on tubulin concentration). The mixture was fixed by adding 300 μl of 1% glutaraldehyde in PB' at 26 °C and 3 min later, 600 μl of PB' at 0 °C. In all operations involving pipetting or mixing regrown centrosomes, great care was taken to avoid shear by growing microtubules to equal length (10–15 μm) at different tubulin concentrations. 50 μl of the mixture were sedimented onto a 150-mesh Parlodion-coated grid using the EM90 rotor in the airfuge (Beckman) at 90,000g for 10 min. The grids were removed from the rotor, washed in 0.01% Triton X-100 and air-dried; they were then rotary-shadowed with platinum at an angle of 8°. Microtubules per centrosome were counted directly in the electron microscope at a magnification of $\times 5,000$. 100 centrosomes were counted per point.

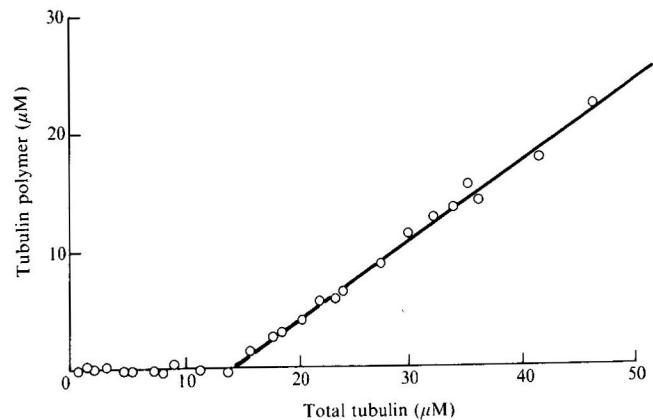


Fig. 5 Purification of tubulin and determination of steady-state concentration. 2-cycle beef brain microtubules²⁴ were resuspended and Dounce-homogenized in CB (50 mM PIPES, 1 mM EGTA, 0.2 mM MgCl₂, pH 6.8 with KOH) and 1 mM GTP. After 20 min at 0 °C they were sedimented at 150,000g for 30 min at 2 °C. 30 ml of the 10 mg ml⁻¹ supernatant were applied to a 200-ml phosphocellulose column (Whatman P11) equilibrated in CB at 4 °C. The column was eluted at 100 ml h⁻¹ and the peak flow through fractions pooled; GTP was added to a concentration of 1 mM, and the tubulin was frozen in aliquots on liquid nitrogen and stored at -70 °C. To induce spontaneous polymerization, tubulin was placed in a final buffer of 30% v/v glycerol, 80 mM PIPES, 1 mM EGTA, 10 mM MgCl₂, 1 mM GTP pH 6.8 with KOH, at a tubulin concentration of 40 μM . After 40 min at 37 °C, microtubules were sheared by six rapid passes through a 1.5-inch 22 gauge needle to produce uniform seeds. Tubulin was brought to a final buffer of PB (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, 1 mM GTP, pH 6.8 with KOH), and a GTP regenerating system (RS; final concentration 10 mM Na-acetyl phosphate + 1 U ml⁻¹ of acetate kinase) was added³⁵. This solution containing 59 μM tubulin was prewarmed to 37 °C, then 1/100 volume of seeds was added. After 30 min at 37 °C, the solution was sheared by two passes as above. After 5 min at 37 °C, the microtubule-containing solution was diluted by varying amounts, using warm PB + RS. Aliquots (200 μl) of diluted microtubules were allowed to equilibrate for 40 min at 37 °C. Half of each aliquot was transferred to 0 °C, and half was immediately sedimented at 90,000g for 4 min in the airfuge at 37 °C. After 30 min at 0 °C, the remaining half-aliquots were subjected to an identical spin at 4 °C. Protein concentrations were determined by Coomassie blue binding assay using a bovine serum albumin standard and a molecular weight of 10⁵ daltons for tubulin dimer. Polymer concentration was calculated as cold supernatant minus warm supernatant, and cold supernatant differed from total protein by <5%. The experiment was repeated on three tubulin preparations with similar results.

the steady-state concentration for assembly of pure tubulin (14 μM) (Figs 4, 5). Centrosomes seem able to nucleate microtubules well below the steady-state concentration, with microtubules first seen at 3–4 μM , despite the fact that free microtubules are unstable below 14 μM . Although this could possibly be due to differences in critical concentration between the two ends of the microtubule, we consider it unlikely as we have not found such differences in studying polymerization from axonemes²³. Furthermore, the amount of nucleation by centrosomes varies over a wide concentration range, whereas free microtubules show a sharp discontinuity in stability at the steady-state concentration and thus the expected shape of a phase transition (Figs 4, 5).

To study the effects of tubulin concentration on microtubule stability and avoid effects due to the kinetics of nucleation, intermediate tubulin concentrations were approached by diluting centrosomes presaturated with microtubules at high concentrations. Microtubule polymerization was initiated well above the steady-state concentration, resulting in centrosomes saturated with about 50 microtubules each. We found that if the tubulin was diluted to below the steady-state concentration, microtubules were lost from the centrosomes in a time- and concentration-dependent manner (Fig. 6). Surprisingly, the

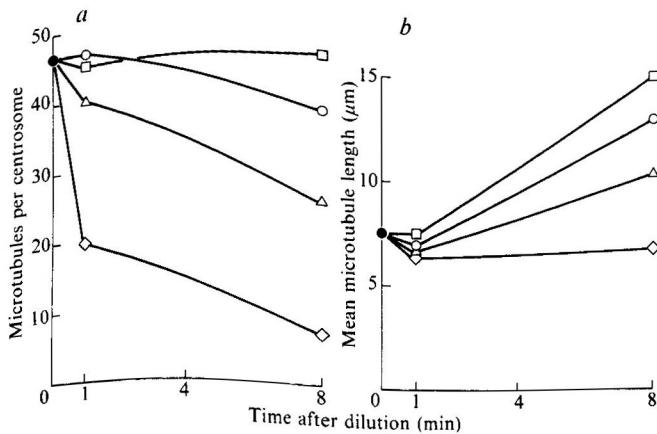


Fig. 6 Dilution of regrown centrosomes. *a*, Microtubules per centrosome; *b*, mean microtubule length. Microtubule nucleation was initiated at 25 μM tubulin as for Fig. 4. Regrown centrosomes were either fixed immediately, or diluted with warm PB, incubated further at 37 °C and fixed at the appropriate time (1–8 min). The final concentration of tubulin was 15 (\square), 10 (\circ), 7.5 (\triangle) or 5 μM (\diamond). Fixation and grid preparation were as described in Fig. 4 legend, except that the dilution was varied to keep the number of centrosomes per grid constant. To determine microtubule length, random centrosomes were photographed at a final magnification of $\times 3,000$. The negatives were digitized directly at $\sim \times 5$ magnification using a Numonics model 1224 digitizer interfaced to an HP85 computer. Absolute length was calibrated by photographing a calibration grid. 100 regrown centrosomes were counted and averaged for each point on *a* and 100 microtubule lengths averaged for each point on *b*. For numbers, s.d. was typically $\sim 30\%$ of the mean and for lengths at 8 min, s.d. was 2–3 μm . Thus, for sample sizes of 100, the number of microtubules at the lowest concentration and the length at the highest concentration differ from the initial values to a highly significant extent after 8 min.

remaining microtubules continued to grow at a concentration-dependent rate. For example, when the centrosomes were diluted to 7.5 μM tubulin, the mean length increased by 40% ($P < 0.001$) but the mean number decreased by 40% ($P < 0.001$) in 8 min. This did not occur by the preferential loss of short microtubules but by a general shift in the entire distribution. At the lowest concentration measured, the mean length decreased slightly at 8 min, although in this population there were some microtubules longer than in the original population (Fig. 6).

This loss of microtubules following dilution was quite unexpected, as was the continued growth of remaining microtubules. The microtubules could have dropped off, depolymerized, or fallen apart in some catastrophic manner. These hypotheses were tested in an experiment in which regrown centrosomes were diluted to 4.5 μM tubulin, where loss in microtubule number is very rapid. By fixing 15 and 30 s after dilution, it was possible to trap the intermediates in microtubule loss as large numbers of short microtubules. Thus, the microtubule loss following dilution appeared to result from endwise depolymerization—presumably from the distal end, although proximal loss remained a formal possibility. We conclude that at tubulin concentrations below the steady-state level, microtubules on centrosomes exist as two populations, some growing and some shrinking; the fraction in the two populations depends on the free tubulin concentration.

One possible explanation for this behaviour would be structural differences between growing and shrinking microtubules. Conceivably, the centrosome could nucleate microtubules with different stabilities by imposing some structural constraint on the microtubule lattice. The existence of such different microtubule classes could explain the heterogeneous data of Figs 4 and 6. If this were the case, the microtubules nucleated at a low tubulin concentration should be more stable and have a lower critical concentration than those nucleated at higher concentration. We tested this hypothesis by initiating polymerization on centrosomes at a tubulin concentration giving about half the saturating number of microtubules. After microtubules nucleated at this concentration had grown to $\sim 6 \mu\text{m}$, more tubulin was added to reinitiate microtubule assembly, which resulted in a bimodal initial length distribution (Fig. 7). The length distribution on any given centrosome was also bimodal. After dilution, the populations remained bimodal, with equal fractions in each population, despite loss of over half the microtubules (Fig. 7). This is clearly inconsistent with the hypothesis. The peaks even appear to move up slightly in length during this short time, illustrating again that the remaining microtubules continued to grow. It thus seemed that microtubules depolymerize at random following dilution, and furthermore that depolymerizing microtubules had a significant probability of reverting to polymerizing behaviour, the upper and lower peaks would have merged. This is consistent with the data of Fig. 6, which also suggests that the shrinking and growing microtubules are not readily interconvertible. If they were, the mean length of the remaining microtubules would be much lower than found.

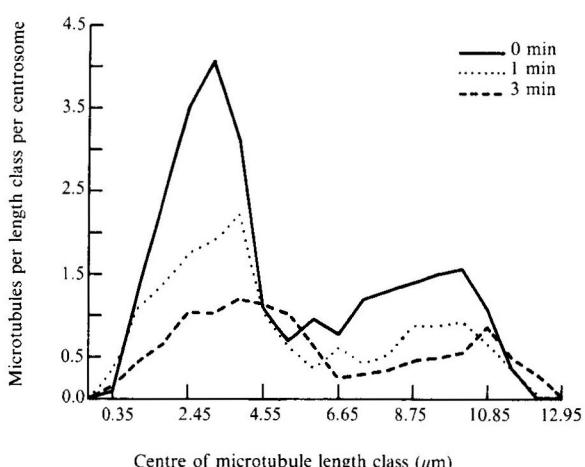


Fig. 7 Bimodal length experiment. Microtubule nucleation was initiated on centrosomes at 7.5 μM tubulin in PB. After 13 min at 37 °C, sufficient prewarmed tubulin at 40 μM in PB was added to bring the solution to 25 μM . After 1.5 min at this concentration, an aliquot of the mixture was fixed and the remainder was diluted with warm PB to a final concentration of 5 μM . Aliquots were fixed 1 and 3 min after this final dilution. Fixed, regrown centrosomes were sedimented onto grids, photographed and measured as in Figs 5, 6. 100 centrosomes were counted to obtain the number per centrosome. For length distributions 700–1,000 microtubules were measured. The length distributions at each time were plotted as a histogram with 20 bars, then the ordinates of the histograms were scaled so that the y axis reflects the number of microtubules per centrosome in each size class. The lines connect the centre points of the top of each scaled histogram bar. The mean number of microtubules per centrosome was 27 (0 min), 16 (1 min) and 11 (3 min).

Conclusions

The data described here indicate that the centrosome is capable of microtubule nucleation at tubulin concentrations where individual microtubules are unstable and can only exist transiently. A newly nucleated microtubule can grow for a while but will eventually start to depolymerize with a probability which increases with decreasing concentration of tubulin. Once initiated, the microtubule will generally disappear completely, leaving an unoccupied nucleation site. This site can then initiate a new microtubule. Because the centrosome can continuously renucleate, it will always be at the centre of an astral microtubule array, which will thus appear stable. Each individual microtubule is unstable, but a stable amount of polymer is found despite the overall tubulin concentration being below that at which free microtubules are stable.

If such properties are manifest in the cell, they have profound consequences for the structure and dynamics of the microtubule cytoskeleton; they could explain why most of the microtubules in many cell types originate from the centrosome, and why free microtubules, formed for example by removal of depolymerizing drugs, are unstable with respect to centrosomal microtubules¹⁵.

The *in vitro* experiments demonstrate not only that microtubule assembly off the centrosome is kinetically preferred, but also that centrosomes will be the source of microtubules in the cell in the long term. Although differences in the affinity of the ends of microtubules for tubulin could accentuate the stability of centrosomal microtubules near the steady-state concentration¹⁷, the properties observed here could operate well below

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the steady-state concentration for either end of a microtubule and in these conditions would predominate.

Further consequences of this dynamic behaviour are considered in the following paper²³, which also considers the effects of the microtubule having two ends. We also propose a model making use of the concept of a cap of GTP-containing subunits at the ends of growing microtubules^{32,33} to account for the coexistence of shrinking and growing microtubules in a single population.

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Dynamic instability of microtubule growth

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We report here that microtubules *in vitro* coexist in growing and shrinking populations which interconvert rather infrequently. This dynamic instability is a general property of microtubules and may be fundamental in explaining cellular microtubule organization.

MICROTUBULES are structural filaments in the cytoplasm which are spatially organized and extremely dynamic^{1,2}. Recently, considerable effort has been directed towards understanding what produces and stabilizes specific arrangements of microtubules in cells and by what means microtubules can completely reorganize their spatial distribution. In the accompanying paper³, we suggest that microtubules nucleated by centrosomes can grow transiently at tubulin concentrations below those at which free microtubules are stable, and that nucleated microtubules coexist as shrinking and growing populations which rarely interconvert. This behaviour is clear only when individual microtubules rather than bulk populations are studied. Here we generalize the results from microtubules nucleated by centrosomes to free microtubules. We examine the detailed kinetics of microtubule assembly to try to account for these unusual dynamic properties.

Microtubule dilution

The crucial experiment demonstrating unusual dynamics in the preceding paper was that in which the microtubule number and length distributions were measured after centrosomes were regrown initially at a high tubulin concentration, then diluted³. The conclusion we drew from that experiment was that some microtubules continued to grow at the same time as others were lost by depolymerization from their distal ends. It seemed likely that this was a general property of microtubules. We therefore

describe here a similar experiment with free microtubules (Fig. 1). Microtubules were first made by spontaneous polymerization in an assembly-promoting buffer. These were then used as seeds and diluted extensively into a tubulin solution well above the steady-state concentration and allowed to elongate for 4 min. The seeds, which were initially 1-2 μm long, elongated to form a sharp distribution with a mean length of 18.3 μm (Fig. 1b). This actively growing population was either fixed immediately or first diluted with warm buffer to just above (15 μM) or below (7.5 μM) the steady-state concentration. To assess both the length and number concentration, fixed microtubules were quantitatively sedimented onto grids for electron microscopy. This procedure gave a highly reproducible number concentration (Fig. 1) and when a known polymer mass was used it gave the expected mean length (see, for example, Fig. 4).

The result of this experiment was very similar to that found using centrosome nucleated microtubules³, that is, below the steady-state concentration microtubules were found to both grow and shrink (Fig. 1). Above the steady-state concentration, the number of microtubules remained approximately constant (Fig. 1a) and their length increased from 18.3 to 40.2 μm in 10 min, retaining a fairly sharp distribution (Fig. 1d). Below the steady-state concentration, however, the number concentration decreased with time (Fig. 1a), but the mean length still increased from 18.3 to 21.5 μm in 10 min (Fig. 1c). The rate of microtubule