

# Evidence for Microtubule Subunit Addition to the Distal End of Mitotic Structures In Vitro

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**ABSTRACT** HeLa cells blocked in metaphase with 0.04  $\mu\text{g}/\text{ml}$  of the microtubule poison nocodazole were shown to contain large numbers of microtubules with typical mitotic organization but no centriole. Lysis of nocodazole-poisoned cells in a microtubule reassembly buffer containing 0.5 M PIPES, 2.5% dimethyl sulfoxide, 1 mM EDTA, 1 mM  $\text{MgCl}_2$ , 1 mM GTP, 1% Triton X-165, 0.5% sodium deoxycholate, 0.2% SDS, pH 6.9, preserved metaphase aster structures 5  $\mu\text{m}$  in diameter surrounded only by a thin, fibrous cell remnant. Inclusion of 2 mg/ml porcine brain microtubule protein in the lysis buffer produced asters up to 20  $\mu\text{m}$  in diameter with a birefringent retardation of 5–6 nm. In these large asters the central microtubules had normal morphology, but peripheral microtubules were clearly abnormal. Our interpretation is that in high PIPES lysis buffer, exogenous brain tubulin adds to the distal ends of preexisting aster microtubules to form abnormal microtubules. This observation supports the assumptions made by Borisy and by Summers and Kirschner in their interpretation of growth experiments to determine the microtubule polarity in mitotic structures.

Microtubules possess an intrinsic molecular polarity. This property has been determined for neurotubules and flagellar tubules by their behavior of growing faster *in vitro* at one end than at the other (1, 27). It has been confirmed for flagellar tubules by electron microscopy (2). The polarity of microtubules within the mitotic apparatus may play an important role in chromosome movement; several models for the mechanism of mitosis depend on such a property (3, 18, 20, 22, 31). The polarity of spindle microtubules has recently been investigated using isolated mitotic fragments to seed the growth of neurotubulin *in vitro* (4, 32). In these studies, the rate of growth of neurotubules attached to isolated kinetochores and centrosomes has been measured: the seeded neurotubules grow with a rate equal to that expected for subunit addition occurring only at the fast-growing end of a free tubule. The deduction of a polarity from these data requires a knowledge of the end of the tubule to which the subunits are adding. Both of the published studies make the assumption that subunit addition is occurring at the end distal to the microtubule-nucleating site (kinetochore or center). Indeed, some assumption was necessary because there was no information previously available concerning the zone of subunit addition.

We report here on the isolation of mitotic structures from cultured HeLa cells by means of a modification of the condi-

tions for the assembly of microtubule protein (MTP) reported by Himes et al. (14). This method has allowed us to mark the zone of neurotubule subunit addition to mitotic asters *in vitro*. We also report some observations on HeLa cells treated with the synthetic antimicrotubule drug, nocodazole (9), whose properties made possible our approach to the determination of the zone of astral growth.

## MATERIALS AND METHODS

### *Production of Mitotic HeLa Cells*

HeLa cells were cultured in roller bottles or culture flasks in Eagle's minimal essential medium buffered with 10 mM HEPES to pH 7.4 and supplemented with 7% calf serum. Cells were synchronized with 5 mM thymidine for 16 h (36). 8 h after removal of the thymidine, nocodazole (Aldrich Chemical Co., Inc., Milwaukee, Wis.) as a 1  $\mu\text{g}/\text{ml}$  stock in dimethylsulfoxide (DMSO) was added to the cultures to give a final concentration of 0.04  $\mu\text{g}/\text{ml}$ . Mitotic cells were shaken from the culture vessel 5–6 h after the addition of nocodazole. These dislodged cells were concentrated approximately fourfold by centrifugation and resuspension in  $\frac{1}{4}$  vol of the same nocodazole-poisoned medium. The poisoned cells were maintained as a suspension culture at 37°C during the course of experimentation.

### *MTP Purification and Assembly*

MTP was purified from porcine brain by the glycerol method of Shelanski et al. (28) in a buffer containing 0.1 M PIPES, 1 mM  $\text{MgCl}_2$ , 1 mM GTP, 1.0 mM

EGTA, pH 6.9. Protein was stored in the same buffer with 8 M glycerol at  $-20^{\circ}\text{C}$  and prepared for use fresh daily (34).

The MTP used in cell lysis experiments was depleted of ring oligomers by the method of Allen and Borisy (1). The use of this protein delayed the spontaneous initiation of microtubule assembly outside the lysed cell remnant, allowing the asters and cage to be purified by a discontinuous glycerol gradient without an adhering meshwork of microtubules.

### Preparation of Lysis Buffer

Nocodazole-poisoned HeLa cells were lysed in a buffer containing 0.5 M PIPES, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , 2.5% DMSO, 1% Triton X-165, 0.5% sodium deoxycholate, 0.2% SDS, pH 6.9. To avoid precipitation of the detergents, the lysis buffer was prepared as follows: The Triton,  $\text{MgCl}_2$ , DMSO, and EGTA were added to a volume of water approximately a third the final volume of lysis buffer. This solution was warmed to  $37^{\circ}\text{C}$ , the deoxycholate was added as a solid and dissolved. This solution was cooled on ice and the PIPES was added slowly as a 1.0-M stock, pH 7, with constant vortexing. SDS was added to the ice-cold solution from a 10% stock with vortexing. The solution was brought to its final volume by the addition of water. The complete lysis mixture was stable for  $\sim 1$  wk if refrigerated. Elevated temperatures for extended periods caused a slow precipitation of the detergents. The lysis buffer was used with or without added MTP. Just before use, GTP was added to the lysis buffer to a concentration of 1 mM.

### Tubulin Addition to *Tetrahymena* Pellicles

*Tetrahymena* were grown in 0.75% proteose peptone, 0.75% yeast extract (Difco Laboratories, Detroit, Mich.), 1.5% sucrose, 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{KH}_2\text{PO}_4$ , and 30  $\mu\text{g}/\text{ml}$  Sequestrine (Ciba-Geigy Corp., Ardsley, N. Y.). Cells were collected by centrifugation, lysed, and deciliated by resuspension in 0.1 M PIPES, pH 6.9, containing 1 mM EGTA, 2 mM  $\text{MgCl}_2$ , and 0.2% Nonidet P-40, and agitated on a vortex mixer at room temperature. The lysed, deciliated cells were collected by centrifugation and mixed with 1 mg/ml MTP in lysis buffer, incubated at  $37^{\circ}\text{C}$  for 6 min, then fixed by addition of an equal volume of 2% glutaraldehyde in 0.1 M PIPES, pH 6.9. Fixed pellicles were allowed to settle onto polylysine (1 mg/ml)-coated slides, then processed for electron microscopy as described below for HeLa cells.

### Lysis of HeLa Cells

A 0.7-ml aliquot of nocodazole-poisoned HeLa cells in suspension ( $10^5$  cells/ml) was pelleted by centrifugation at 300 g for 30 s at room temperature. The cell pellet was resuspended and incubated for 3 min at  $37^{\circ}\text{C}$  in 0.5 ml of a buffer containing 1 mM PIPES, 5% DMSO, 1 mM EGTA, 1 mM  $\text{MgCl}_2$  in heavy water ( $\text{D}_2\text{O}$ ). This buffer causes hypotonic swelling of the cells and maintains the microtubules within the cells. The swollen cells were pelleted as described above, lysed by the addition of 0.2 ml of lysis buffer with or without added MTP, and vortexed very briefly. Just before addition to the cell pellet, the lysis buffer was warmed for 20 s in a  $37^{\circ}\text{C}$  water bath. Cells were examined at room temperature on a Zeiss Photomicro II microscope.

### Electron Microscopy

HeLa cells and lysed cells were fixed for 15 min at  $37^{\circ}\text{C}$  by addition of 30% glutaraldehyde to a final concentration of 3%. Cells were rinsed twice in 10% sucrose in 0.1 M cacodylate buffer, pH 7.0, and then treated with 0.2% tannic acid in the same buffer for 3 min, all at room temperature. After tannic acid treatment, cells were rinsed three times as described above and osmicated in 1%  $\text{OsO}_4$  in 0.1 M cacodylate buffer for 5 min at room temperature. After three rinses with distilled water, the sample was carefully dehydrated in methoxyethanol (MetEt) at room temperature. We found that lysed cells were very sensitive to dehydration artifacts. The following procedure, however, proved effective: The sample was suspended in 2 ml of 30% MetEt, and 0.8 ml of 100% MetEt was added dropwise with mixing between each drop. Cells were pelleted from this 50% MetEt and resuspended in 2 ml of 50% MetEt to which was added 1.3 ml of 100% MetEt dropwise as above.

Cells were pelleted and resuspended in 1 ml of 70% MetEt; 2 ml of 100% was added as described above. Cells were again pelleted and resuspended in 1 ml of 90% MetEt and 1 ml of 100% MetEt twice. Cells were then rinsed twice with propylene oxide and flat-embedded in Spurr's resin by the method of Pickett-Heaps et al. (26). Individual cells were excised, serially sectioned, and stained with uranyl acetate and lead citrate. Microscopy was performed on a Philips 300 electron microscope.

Counts of microtubules in lysed cells were obtained from electron micrographs of lysed cells at various distances from the mitotic center. The position of a

section passing through the astral center was estimated by the symmetry and orientation of the microtubules rather than by finding the centriole pair which, as described below, was absent. Because the aster is a radially symmetric structure, the orientation of the microtubules is independent of the direction in which the aster is sectioned. This allowed a rather unambiguous identification of the astral center. The distance of a given section from the astral center was estimated by counting the number of serial sections that separated it from the center and assuming a section thickness of 90 nm. Such estimates are probably accurate to within 15% (19).

## RESULTS

### Nocodazole-poisoned HeLa Cells

Metaphase-arrested HeLa cells were obtained for this study by inhibiting cell cycle progression with nocodazole. Nocodazole is a synthetic drug that binds to tubulin at the colchicine site and microtubule polymerization *in vivo* and *in vitro* (8, 9, 16). HeLa cells poisoned with 0.04  $\mu\text{g}/\text{ml}$  nocodazole were very stably blocked in mitosis (37). Light microscope observation of poisoned mitotic cells indicated that one-third to one-half of the cells, depending on the culture, had condensed chromosomes clearly aligned on a metaphase plate. Often, tripolar and tetrapolar metaphase plates were seen in addition to the normal bipolar arrangement (Fig. 1).

Electron microscopy of the nocodazole-poisoned cells invariably revealed large numbers of microtubules. In all 10 cells examined, these microtubules had a metaphase-like organization (Figs. 1 and 2). That is, even in those cells without obvious metaphase plates, microtubules extended from a center located at the cell periphery toward the kinetochores of centrally located chromosomes. However, serial sectioning failed to reveal a centriole in four of five mitotic centers of nocodazole-treated cells. Fig. 3 shows a series of photographs taken from a complete series through one such mitotic center. In one nocodazole-poisoned cell we observed a centriole not associated with any microtubules at some distance from the centriole-free mitotic center (Fig. 4).

### Microtubule Assembly in Lysis Buffer

The conditions used for lysis of poisoned cells have enabled us to mark the zone of microtubule subunit addition in lysed cells. These conditions, described in Materials and Methods, are similar to those reported by Himes et al. (14, 15) and support the assembly of neurotubulin *in vitro*. We investigated these conditions with regard to their effect on microtubule assembly and largely confirmed the work of Himes and his colleagues, although some discrepancies exist. We confirmed that neurotubulin in 0.5 M PIPES buffer will assemble into protofilament ribbons (Fig. 5). We have also found that microtubule assembly occurs to the same extent in 0.5 M PIPES buffer in the presence of a strong detergent mixture, 1% Triton X-165, 0.5% deoxycholate and, 0.2% SDS, as measured by a sedimentation assay (17).

MTP assembly in lysis buffer which was nucleated by preexisting microtubules produced a polymer different than that observed with self-nucleated assembly. Isolated, deciliated *Tetrahymena* pellicles were used to nucleate the assembly of 1 mg/ml of MTP in lysis buffer as described in Materials and Methods. Pellicles fixed at  $0^{\circ}\text{C}$  showed no microtubule growth. The majority of the polymerization at  $37^{\circ}\text{C}$  occurred as elongation from the basal bodies. Grazing sections of such pellicles passed through the basal bodies in some areas, while in others they cut bundles of microtubules that grew outward from the basal bodies (Fig. 6). These elongated microtubules generally

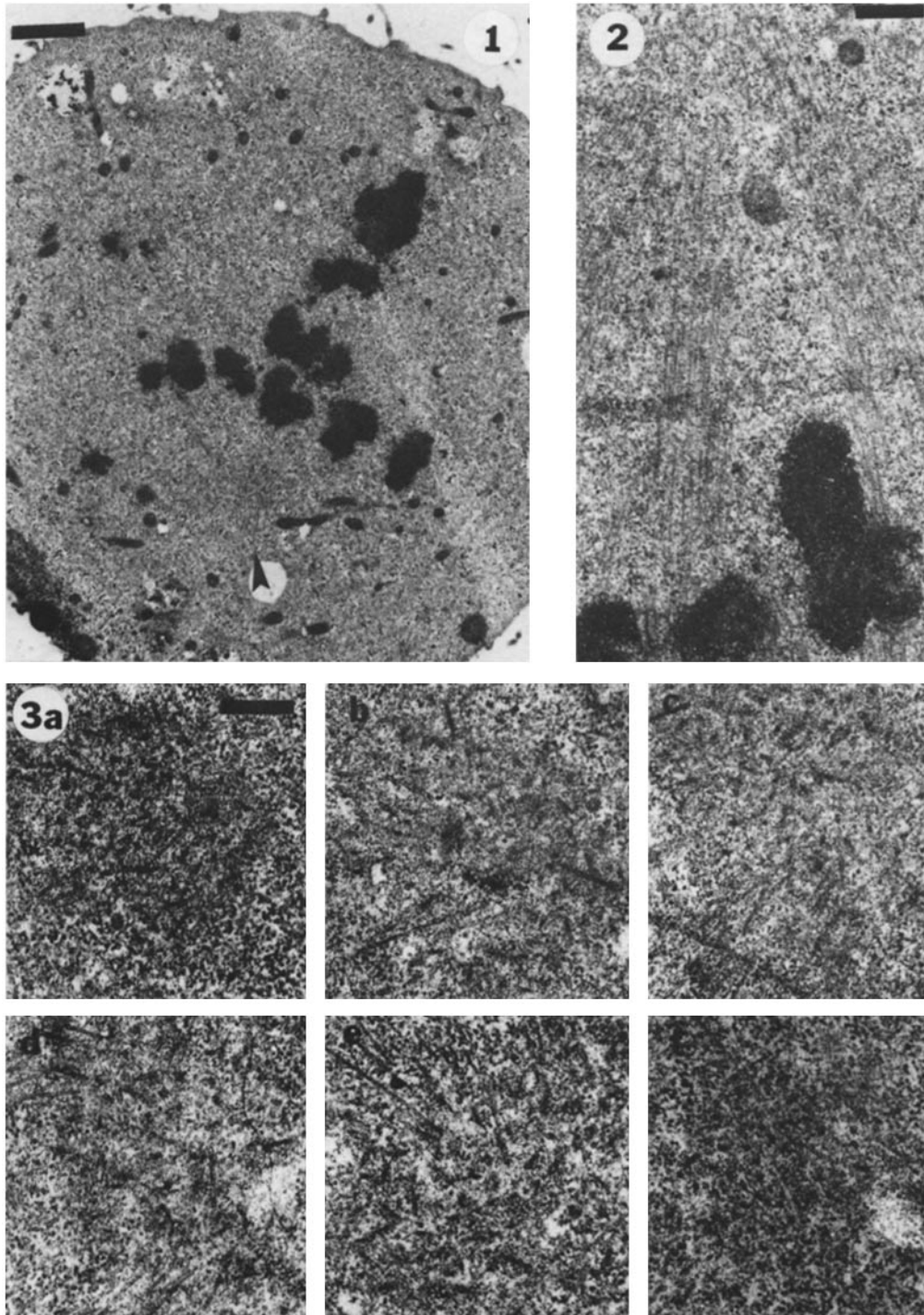


FIGURE 1 A thin section through a nocodazole-poisoned, metaphase-arrested HeLa cell. This cell shows a clear, tripolar, metaphase-plate arrangement of chromosomes. One of the three mitotic centers is apparent in this section and is marked by an arrow. Bar,  $1.6 \mu\text{m}$ .  $\times 6,200$ .

FIGURE 2 A higher magnification image of the spindle region in a metaphase-arrested, nocodazole-poisoned HeLa cell. Pole-to-kinetochore tubules are abundant. Bar,  $0.6 \mu\text{m}$ .  $\times 16,400$ .

FIGURE 3 (a-f) Six electron micrographs from a complete serial of sixteen sections through a mitotic center in a nocodazole-poisoned cell. In this case, as in three others, no centriole was found in the centrospheric, osmiophilic cloud. Bar,  $0.4 \mu\text{m}$ .  $\times 27,700$ .

had lateral arms associated with them. Often, cross sections of these tubules looked like pinwheels with curved lateral arms decorating the hollow tubule. In contrast, self-nucleated assembly of MTP in lysis buffer produced very few tubules showing patent lumens.

#### Isolation of Asters

Nocodazole-poisoned, metaphase-arrested cells were selected from culture bottles by mechanical agitation. Cells were harvested by centrifugation at  $600 \text{ g}$  for  $1 \text{ min}$ . The cell pellet

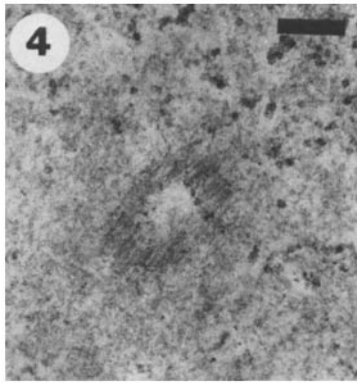


FIGURE 4 An electron micrograph of a section through a centriole in a nocodazole-poisoned, metaphase-plate cell. The mitotic center is many sections away. Clearly, this centriole was not acting as a mitotic center. Bar, 0.2  $\mu\text{m}$ .  $\times 45,500$ .

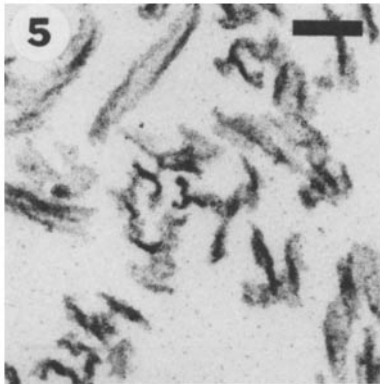


FIGURE 5 An electron micrograph of a section through a pellet of porcine brain microtubule protein polymerized in 0.5 M PIPES, 2.5% DMSO, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM GTP, 1% Triton X-165, 0.5% sodium deoxycholate, 0.2% SDS, pH 6.9. Bar, 0.1  $\mu\text{m}$ .  $\times 90,000$ .

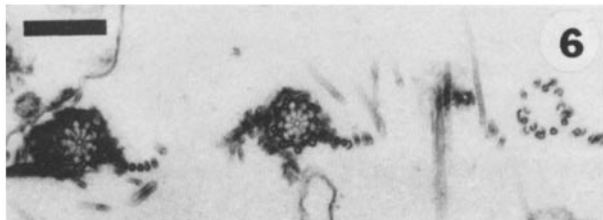


FIGURE 6 A grazing section of a deciliated *Tetrahymena* pellicle which has nucleated microtubule elongation in lysis buffer. On the left, the section contains cross sections of basal bodies at two different levels. On the right is a tuft of neurotubulin polymer extending from a basal body. Note that tubules with evident lumens and hooked decoration form in lysis buffer when nucleated by existing microtubules. Compare to Fig. 5. Bar, 0.3  $\mu\text{m}$ .  $\times 36,000$ .

was resuspended in 1 mM PIPES, 1 mM EGTA, 5% DMSO in heavy water ( $\text{D}_2\text{O}$ ), pH 6.9, for 3 min at 37°C. This caused hypotonic swelling of the cells while stabilizing the existing microtubule structures. Swollen cells were centrifuged as described above and resuspended in lysis buffer with or without porcine brain MTP. The only observed effect of swelling was to reduce the amount of cytoplasmic debris adhering to the isolate; the isolated asters were unchanged if cells were lysed without previous hypotonic swelling.

Cells that were lysed in buffer without added MTP produced small asters or spindles 4–6 nm in diameter with a birefringence

of 0.7–1.2 nm retardation (Fig. 7). These asters were surrounded by a thin, fibrous cell remnant we have called the cage. These “endogenous asters” were composed of normal microtubules arranged radially about mitotic centers (Fig. 8). No centrioles were observed in serial section through these endogenous asters. Very little material other than microtubules and the aster-encompassing cage was observed in sections through the lysed cells.

Swollen cells that were lysed in buffer containing 2 mg/ml porcine brain microtubule protein produced structures as seen in Figs. 9 and 10. 85% of these lysed cells contained one or more large asters up to 25  $\mu\text{m}$  in diameter surrounded by a fibrous cage. We have referred to these asters as exogenous asters, as they were produced in the presence of exogenous porcine brain tubulin. The birefringent retardation by these asters, measured in a region halfway between the astral center

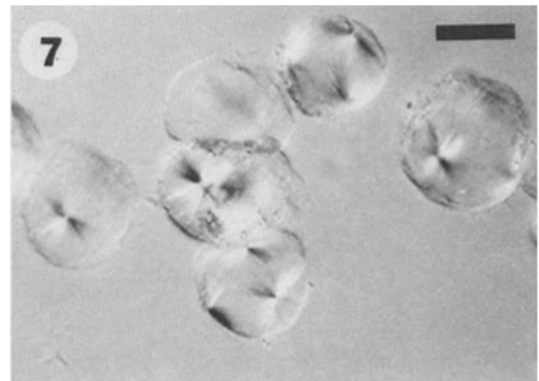


FIGURE 7 A polarization light micrograph of endogenous asters and the spindle-encompassing cage (see text). Bar, 15  $\mu\text{m}$ .  $\times 680$ .

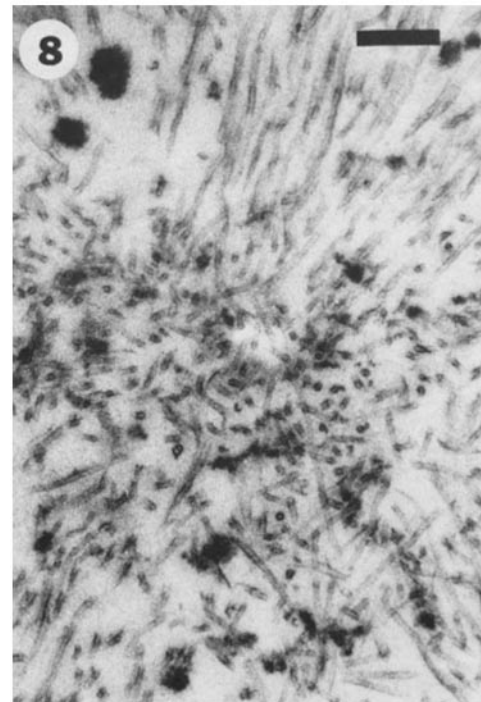


FIGURE 8 An electron micrograph of a thin section through the center of an endogenous aster. The black splotches are believed to be material from the cage. A concentration of such material forms a perimeter around all the asters seen in thin section just as the cage surrounds asters seen in the light microscope. Bar, 0.3  $\mu\text{m}$ .  $\times 36,000$ .

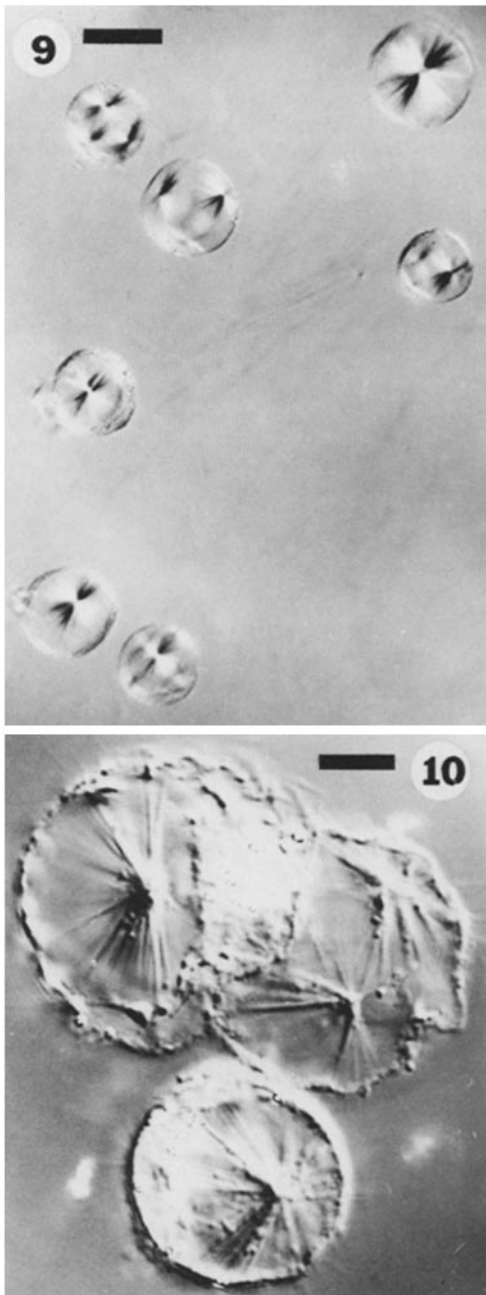


FIGURE 9 A polarization light micrograph of exogenous asters and surrounding cage (see text). Bar, 18  $\mu\text{m}$ .  $\times 560$ .

FIGURE 10 A Nomarski image of exogenous asters and surrounding cage (see text). Bar, 6.6  $\mu\text{m}$ .  $\times 1,500$ .

and the distal extremity, varied among esters between 4.3 and 5.9 nm. The birefringence of exogenous and endogenous asters, as well as any aster structure seen in Nomarski optics, disappeared completely upon cooling to 0°C or upon addition of 5 mM  $\text{CaCl}_2$ . The cage, on the other hand, was stable under these conditions (Fig. 11). In certain giant, multinucleate cells, lysis produced a large number of asters within a single cell remnant (Fig. 12). A small yield, ~10%, of asters free of the surrounding cage (Fig. 13) was obtained by lysing metaphase-arrested cells that had been treated with cytochalasin B (20  $\mu\text{g}/\text{ml}$ ) added to the nocodazole-containing medium for 20 min at 37°C before lysis. Asters and/or asters and shells were separated from the soluble cell lysate by centrifugation at 1,000 g

for 10 min at room temperature on a 10–50% discontinuous gradient of glycerol in 0.5 M PIPES buffer without detergent. Asters and/or asters and cages were located at the 10–50% interface.

The exogenous asters were composed of microtubules with radial arrangement (Fig. 14). Within 2.5  $\mu\text{m}$  of the aster center 87% of the microtubules had a normal morphology, while 82% of the microtubules distal to this radius had the lateral arms (Table I). Generally, these decorated tubules had a uniform orientation consistent with their all having arisen from a center (Fig. 15).

### Isolation of Spindles

The aster isolation method can be used to isolate spindle structures surrounded by a fibrous cage from cells allowed to recover from nocodazole poisoning.

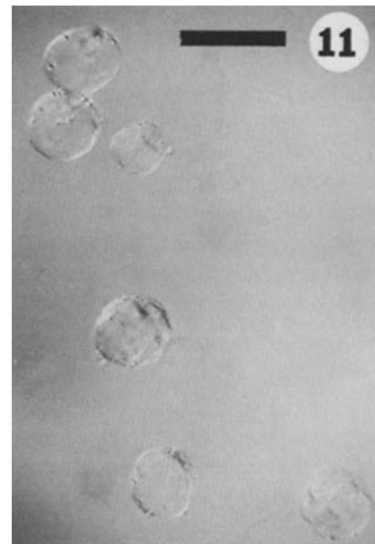


FIGURE 11 A polarization light micrograph of HeLa cells after lysis as for exogenous asters with addition of 5 mM  $\text{CaCl}_2$ . A 2-min incubation on ice of a preparation of exogenous asters produces an identical image. Asters but not cages are cold and calcium labile. Bar, 36  $\mu\text{m}$ .  $\times 400$ .

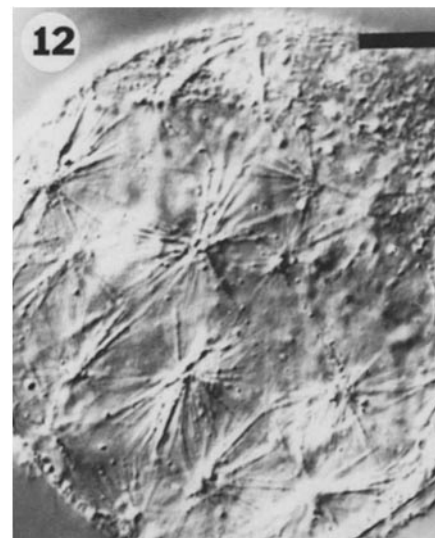


FIGURE 12 A Nomarski image of a giant, multinucleate HeLa cell lysed to produce exogenous asters. Some such cells produced more than 50 asters per cell remnant. Bar, 9  $\mu\text{m}$ .  $\times 1,100$ .

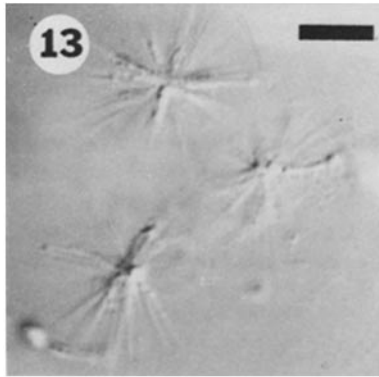


FIGURE 13 A Nomarski image of three exogenous asters without a surrounding cage produced by treatment of cells with cytochalasin B before lysis. Bar, 6.6  $\mu\text{m}$ .  $\times 1,500$ .

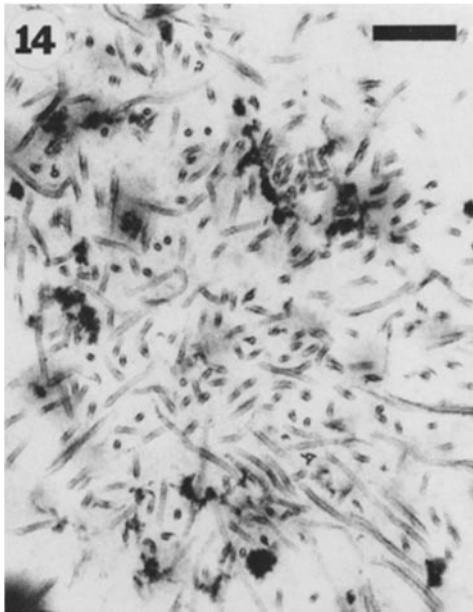


FIGURE 14 An electron micrograph of a section through an exogenous aster  $\sim 1\mu\text{m}$  from the center. The majority of microtubule cross sections are of normal morphology. Bar, 0.3  $\mu\text{m}$ .  $\times 36,000$ .

Metaphase-arrested cells that were rinsed free of nocodazole will reenter the mitotic cycle. Cells that had recovered from nocodazole poisoning for 30 min were swollen and lysed in 0.5 M PIPES buffer containing 2 mg/ml MTP as outlined above. Light microscope observation of such lysed cells revealed spindle-shaped fiber arrays devoid of chromosomes and any astral structure. The retardation of these spindles varied from 5.3 to 8.6 nm. Spindles with chromosomes intact were obtained by lysing cells in a lysis mixture in which the SDS concentration had been lowered to 0.02% (Fig. 16). Spindles of stages later than metaphase were obtained by lysing cells after longer periods of recovery from the nocodazole block.

## DISCUSSION

The true metaphase arrest of HeLa cells caused by low doses of nocodazole allowed us to obtain the large number of mitotic spindle isolates used in these experiments. Our observations argue that low doses of nocodazole do not have the same effect on cultured cells as do low doses of colcemid and colchicine, though all appear to bind to the same site on tubulin (16).

Although low doses of colchicine (24) and colcemid (7) do allow some microtubule assembly, nocodazole-treated cells show relatively normal mitotic organization of these tubules. Colchicine and colcemid arrest are colloquially referred to as metaphase arrest, however, they generally cause a ring-shaped configuration of condensed chromosomes called a C-mitosis (10). Despite the largely normal array of microtubules in nocodazole-poisoned cells, such cells are very stably arrested in mitosis (37). Low doses of colchicine, in contrast, allow some cells to “creep” past mitosis into interphase (24). Low doses of nocodazole allow microtubule assembly to metaphase, but block further progress completely. Two interpretations come readily to mind. Some particularly nocodazole-sensitive microtubules are required for anaphase movements or nocodazole inhibits the breaking of the kinetochore link that joins the bivalent chromosomes. Because the majority of reported effects of nocodazole are on microtubule assembly (8, 9, 16), the former interpretation seems more likely.

The existence of multiple initiating sites in cultured interphase cells, and the role of the centriole, if any, in organizing the mitotic apparatus are both topics surrounded by controversy (6, 13, 25, 30). Our finding of multiple, centriole-free mitotic centers in nocodazole-treated cells suggests that the tubule-initiating material of the pole matures normally without association with centrioles. In the absence of centrioles, this tubule-initiating material divides into a variable number of aggregates, each serving as a mitotic center.

The lysis of nocodazole-poisoned, metaphase-arrested HeLa cells in the lysis buffer without neurotubulin produced relatively small aster structures that were shown to be composed of normal microtubules. We believe that these endogenous asters are the remnant of the microtubule array that existed in the drug-blocked cell. The lysis of metaphase cells with our conditions preserves the microtubules and cage which existed in the arrested cells but little else.

The conditions we used for lysis of poisoned cells support the addition of characteristic, abnormal tubulin polymers to the ends of preexisting microtubules. The neurotubulin polymer nucleated by *Tetrahymena* basal bodies showed lateral arms extending from the wall of the newly grown microtubule (Fig. 6). We have used this unusual morphology as a marker for the site of tubulin addition to a mitotic structure. We believe that the zone of tubulin subunit addition to mitotic microtubules is at the end distal to the mitotic center. This interpretation is supported by the following observations: The absence of ribbon structures within the cage, the profusion of tubules with an evident lumen and lateral arms, and the uniform orientation of microtubules (Fig. 15) argue that the decorated tubules were the result of elongation. If these decorated tubules

TABLE I  
Microtubule Cross Sections Seen in Endogenous and Exogenous Asters

	Endogenous asters		Exogenous asters	
	Normal	Deco-rated	Normal	Deco-rated
Microtubule cross sections within $\sim 2.5\text{-}\mu\text{m}$ radius of the aster center	1,156	39	1,134	178
Microtubule cross sections outside $\sim 2.5\text{-}\mu\text{m}$ radius	—	—	339	1,504



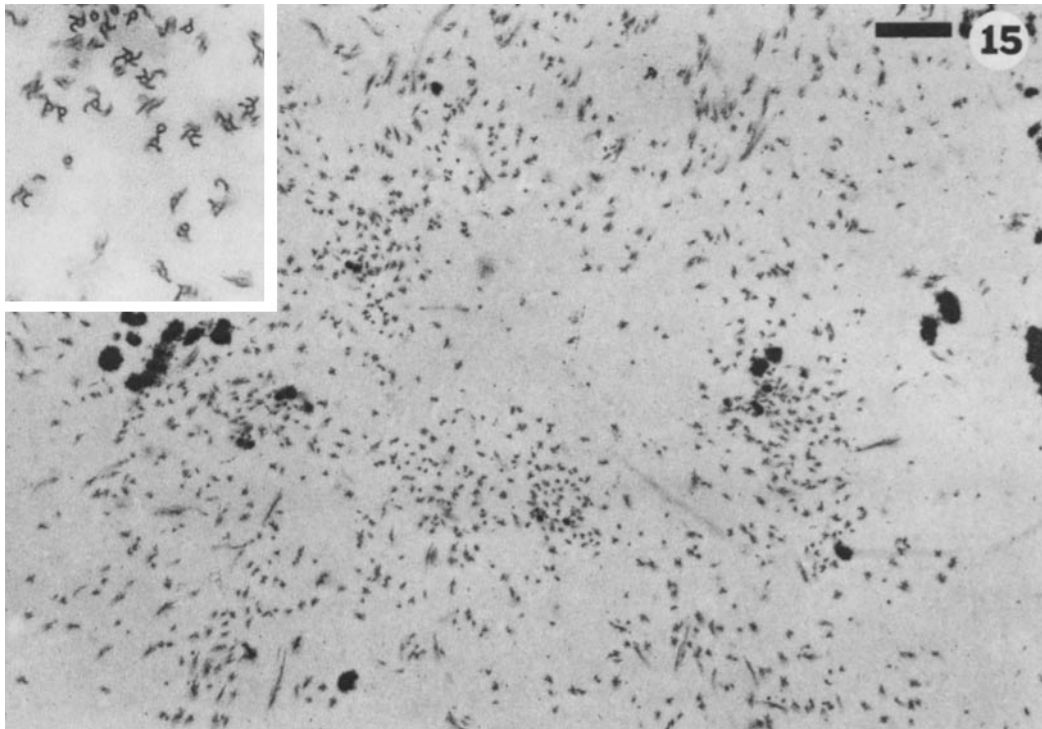


FIGURE 15 An electron micrograph of a section through an exogenous aster  $\sim 6 \mu\text{m}$  from the cell center. Note that the great majority of microtubules have curved lateral arms extending from the wall of the microtubule. This is seen in higher magnification in the *inset*. Note also the uniform orientation of microtubule cross sections. Bar,  $0.7 \mu\text{m}$ .  $\times 15,000$ . *Inset*:  $\times 53,000$ .

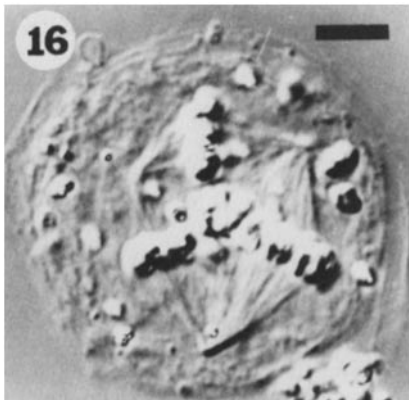


FIGURE 16 A Nomarski image of a HeLa cell that had been allowed to recover from nocodazole treatment for 30 min before lysis. The cell was lysed at  $37^\circ\text{C}$  in  $0.5 \text{ M}$  PIPES,  $2.5\%$  DMSO,  $1 \text{ mM}$  EGTA,  $1 \text{ mM}$   $\text{MgCl}_2$ ,  $1 \text{ mM}$  GTP,  $1\%$  Triton X-165,  $0.5\%$  sodium deoxycholate,  $0.02\%$  SDS, pH 6.9 containing  $2 \text{ mg/ml}$  porcine brain MTP. The 10-fold lower concentration of SDS preserves the chromosomes. Bar,  $3.6 \mu\text{m}$ .  $\times 2,800$ .

had arisen *de novo*, one would expect to find ribbons or random orientations of tubules. The high concentration of normal microtubules near the center of the aster (presumably the endogenous aster) and the high concentration of abnormal polymers in the periphery argue that the elongation occurred at the distal end of the microtubules. The experiments support the validity of the assumption made in the growth rate studies of Borisy (4) and Summers and Kirschner (32) that exogenous tubulin adds at the distal end of mitotic microtubules. It must be noted, however, that the conditions we have used for microtubule assembly are hardly physiological. For that matter,  $0.1 \text{ M}$  PIPES buffer may depart substantially from conditions

in the cytoplasm. It is clearly of interest to try to carry experiments on the zone of growth of spindle tubules into the cell and determine the zone of growth *in vivo*.

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