INITIATION AND GROWTH OF MICROTUBULES FROM MITOTIC CENTERS IN LYSED MAMMALIAN CELLS

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ABSTRACT

Metaphase PtK_1 cells, lysed into polymerization-competent microtubule protein, maintain a spindle which will gain or lose birefringence depending on the concentration of disassembled tubulin subunits used in the lysis medium. Concentrations of tubulin subunits greater than the equilibrium monomer value promote a rate and extent of birefringence increase that is proportional to the subunit concentration. Increase in spindle birefringence can be correlated with an increase in tubule number, though the relationship is not strictly linear. Increase in spindle tubule number is due to an in vivo-like initiation of tubules at the mitotic centers, as well as tubulin addition onto pre-existing spindle fragments. Colcemid-treated prometaphase cells lysed into polymerization-competent tubulin develop large asters in the region of the centrioles and short tubules at kinetochores, making it unlikely that all microtubule formation in lysed cell preparations is dependent on tubulin addition to short tubule fragments. Asters can also form in colcemid-treated prometaphase cells lysed in tubulin that is incapable of spontaneous tubule initiation, suggesting that the centriolar region serves a tubule-initiator function in our lysed cell preparations. The ability of the centriole to initiate microtubule assembly is a time-dependent process—a ripening effect takes place between prophase and late prometaphase. Ripening is expressed by an increase in the number and length of tubules found associated with the centriolar region.

Formation of the mitotic spindle is dependent on the assembly of microtubules (MT) from tubulin subunits. This assembly is generally controlled with respect to time in the cell cycle, location within the cell, and orientation of the microtubules. Most, if not all, of the proteins required for the formation of the mitotic spindle are present before the onset of mitosis (9, 12, 29, 34, 38). Weisenberg (43) has shown that in eggs of the surf clam *Spisula* tubulin concentration in unactivated eggs is comparable to that during metaphase in activated eggs. These observations suggest that tubule assembly for spindle formation is not initiated by an immediate increase in the concentration of tubulin; a change must occur in the cytoplasm that shifts the tubulin-assembly reaction to favor polymer formation. There are studies which strongly suggest that the formed spindle is in equilibrium with an unassembled state of its subunits (16, 35, 36, 37). Many factors have been identified that affect this equilibrium, but little is known at present of the ways in which the cell regulates spindle formation.

Understanding the mechanism(s) by which the cell shifts its equilibrium toward MT formation will not necessarily explain the spatial control of

the organization process. Aster or spindle formation occurs in part as a result of MT initiation at specific regions of the cell called mitotic centers (reviewed by 22, 28, 47); Picketts-Heaps (27) has coined the term "microtubule organizing centers" (MTOCs)[1] for all cellular regions that serve as initiating sites for MT growth. Weisenberg and Rosenfeld (46) have obtained direct evidence that it is a change in the mitotic center, not the tubulin, that triggers MT formation: they found that an activated organizing center from *Spisula* eggs is capable of forming an aster with tubulin derived from unactivated eggs, while unactivated centers will not. MOTCs may control microtubule assembly by spatial positioning of subunits, by activating tubulin in some way, by nucleating assembly, or by some combination of these and other mechanisms (39, 40, 45, 46).

Spindle formation probably depends on MT initiation at the kinetochores as well as at the mitotic centers (spindle poles). Independent tubule-organizing activity of the kinetochores has often been suggested (5, 20, 21); in some instances a complete spindle forms in association with each kinetochore with no apparent microtubule contribution from the centriolar region (8, 14, 15). Recently B. Telzer of Yale University (personal communication) has reported that kinetochores on chromosomes isolated from HeLa cells can initiate tubule formation in vitro in the presence of exogenous tubulin.

With the discovery of conditions that promote efficient MT polymerization in vitro (44), it has become possible to make cell-free preparations of mitotic spindles that appear to contain active mitotic centers (7, 17, 30). Mammalian spindle structure after lysis is dependent on the concentration of disassembled tubulin subunits in the buffered, lysis medium over a rather broad range of tubulin concentrations (25). When the concentration of unassembled subunits is greater than the in vitro equilibrium value, there is a net gain in metaphase spindle birefringence (BR) and tubule number. When the concentration of subunits in the lysis medium approximates the equilibrium monomer value, the BR of the spindle is maintained for several minutes at a level similar to that in vivo. When the unassembled subunit concentration falls below the equilibrium value, the BR of the spindle fades within seconds; the rate of fading depends on the tubulin concentration.

In this paper, we give a detailed structural comparison of normal mammalian spindles with spindles augmented after lysis with exogenous tubulin. We also address the problem that some of this augmentation of BR and tubule number may come from addition of tubule subunits onto preexisting tubule fragments in the spindle. A system is described in which colcemid is used in vivo to obtain tubule-free cells. We present evidence that the mitotic centers and kinetochores become competent to initiate tubule growth during the colcemid block and will do so after lysis when the colcemid is washed out and polymerization-competent tubulin is provided. This sytem is used to show that mitotic centers improve in their capacity to initiate tubules at the time the spindle normally forms. At that time, they become able to initiate assembly of tubulin which would not otherwise polymerize.

MATERIALS AND METHODS

Preparation of Tubulin

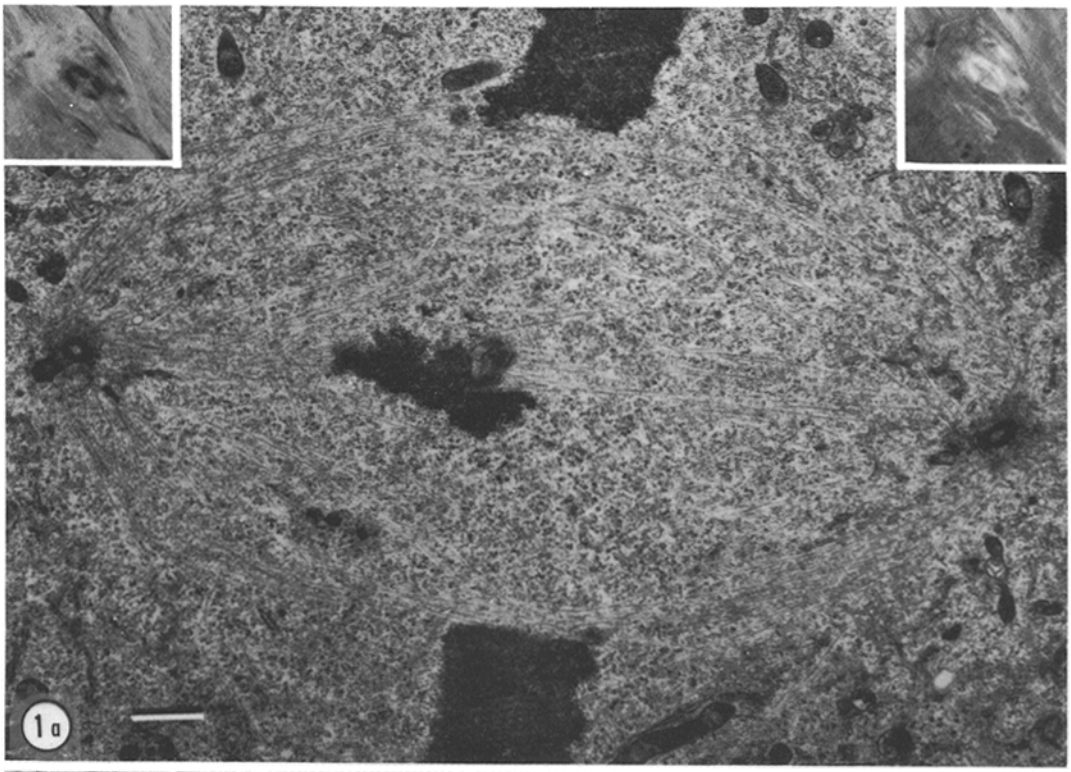
MT protein was prepared following the method of Weisenberg (44) and later modifications by Borisy et al. (3) and Shelanski et al. (33). One or two cycles of a reversible temperature-dependent assembly procedure were used for purification of either chick or porcine neurotubulin. Tubulin pellets were stored at −70°C for as long as 4 wk before use. The tubulin was resuspended in 100 mM piperazine-*N*-*N*'-bis(2-ethane sulfonic acid) (PIPES, pH 6.94), 1 mM $MgSO_4$ and 1 mM guanosine triphosphate (GTP). Triton X-100 was used in all lysis experiments at 0.1%, a concentration which does not affect tubulin polymerization. Our once-washed cycle I tubulin resulted in approximately 80% tubulin and 15% high molecular weight components, as measured by densitometric tracing on SDS acrylamide gels stained with Coomassie blue.

High-speed supernatant preparations were made following the method of Allen and Borisy (1). The capacity of tubulin solutions to form MTs in vitro at 37°C was monitored by negative staining and electron microscopy or light scattering at 350 nm as described elsewhere (11). Protein concentration was determined by the method of Lowry et al. (19).

Tissue Culture Cells

PtK_1 cells were used in all experiments and maintained as described previously (7). In preparation for light

[1] ***Abbreviations used in this paper:*** **BR**, birefringence; GTP, guanosine triphosphate; kMT, kinetochore microtubule; MT, microtubule; nkMT, nonkinetochore microtubule; MTOC, microtubule organizing center; **PIPES, piperazine-*N*-*N*'-bis (2-ethane sulfonic acid).**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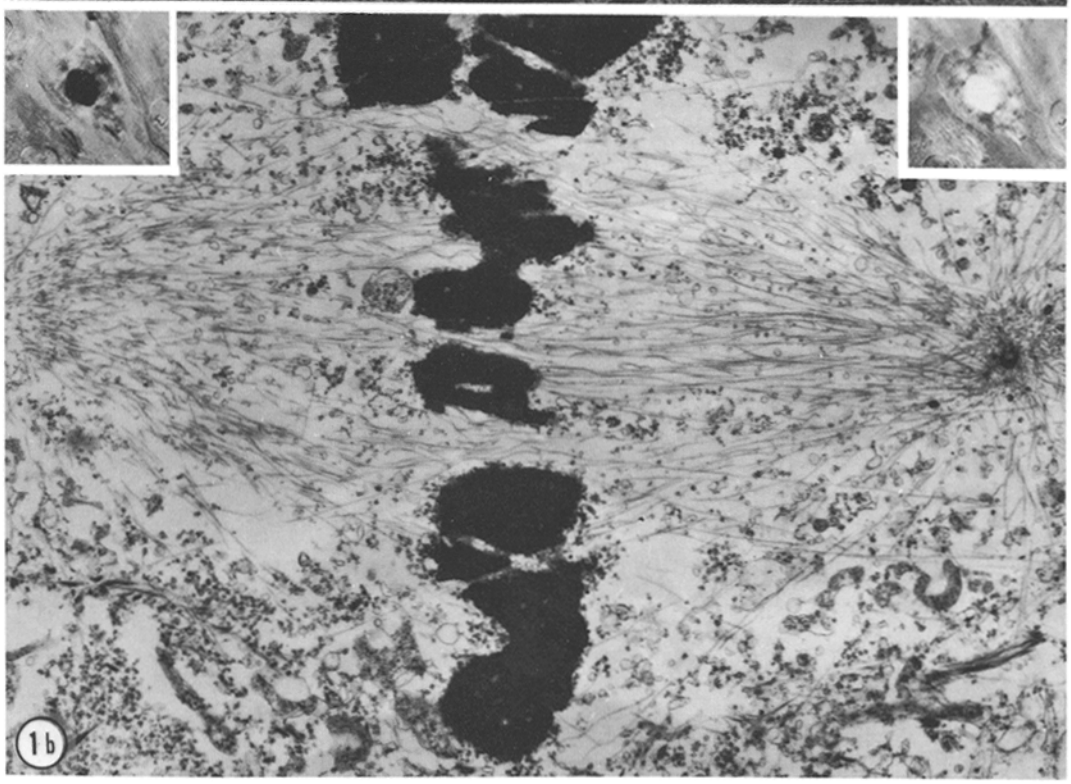
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microscopy, the cover slips were mounted on slides with cover slip fragments as spacers and waxed down with a 1:1:1 mixture of vasoline, lanolin, and paraffin. The preparations were maintained at 35°C on the microscope stage with an air-curtain incubator. Temperature-shift experiments were performed by passing water at either 0° or 37°C through a thin perfusion chamber used as a supporting slide on the microscope stage. When changes in the cell medium were to be made, new solutions were flushed under the cover slip by using filter paper to draw out the old medium.

Colcemid blocks were performed by adding colcemid (Ciba Corp., Summit, N.J.) to cover slip preparations in conditioned Ham's F-12 medium at a final concentration of 1×10^{-5} M. After 10 min at 37°C and 5% CO_2, the cover slip and medium containing colcemid were mounted on a slide and maintained at 37°C on the microscope stage with an air-curtain incubator. Colcemid was removed by drawing out the medium and replacing it with tubulin lysis solution.

Microscopy

Measurements of the BR of the mitotic apparatus were made with a Zeiss polarization microscope equipped with a $\lambda/30$ Brace-Kohler compensator. The compensator angle was measured by mechanically coupling a potentiometer to the compensator (S. Inoué, Marine Biological Lab, Woods Hole, Mass., personal communication). Spindle retardation, here called birefringence, was measured by determining the compensator setting necessary to bring the central portion of the half spindle to extinction. All light micrographs were recorded on Kodak Tri-X 35 mm film (Eastman Kodak Co., Rochester, N.Y.) and developed in Diafine. Prophase motions of the chromosomes were monitored with differential interference contrast optics.

Cells observed in light microscopy were marked on the glass cover slip by an objective scribe (Leitz). The preparation was fixed with 3% glutaraldehyde buffered in 100 mM PIPES, pH 6.9, and then osmicated, stained, dehydrated, and embedded by standard techniques. Cover slips were placed cell-side down on Epon-filled bottle caps made of polyethylene. The cells previously monitored with light optics were excised, sectioned, picked up on plastic and carbon-coated slot grids, stained with uranyl acetate and lead citrate, and examined in a Philips 300 microscope.

RESULTS

Metaphase

Spindle structure after lysis is dependent on the concentration of polymerizable MT subunits in the buffered lysis medium. Fig. 1 compares a longitudinal thin section of a metaphase spindle fixed in vivo with a similar section of a spindle prepared by lysis in a high concentration of tubulin subunits. The polarization insets of similarly treated cells compare the relative BR of a normal PtK_1 metaphase spindle with that of a lysed spindle preparation 15 min after lysis. Quantitative measurements on these cells by polarization optics show a fourfold increase in BR in the region of the spindle after lysis. The electron micrographs of the lysed cell show the spindle remains intact and the cytoplasm is well extracted with only fragments of membranous material remaining. The chromosomes remain intact and appear slightly more condensed than in vivo. Kinetochore structure is not noticeably altered, and MTs can be seen ending in these regions. Some tubules in both lysed and unlysed cells pass through the chromosomes, making slender channels in the chromatin. A few tubules protrude beyond the domain of the spindle. There is an increase in both the total number and apparent length of spindle tubules with lysis.

A more quantitative comparison of the cells shown in Fig. 1 *a*, *b* was obtained by laying a ruler on high-magnification prints parallel to the metaphase plate and counting the number of tubules that intersected one edge of the ruler. We then averaged the maximum number of tubules thus determined from each of five comparable sections on either side of the metaphase plate near the spindle axes. The mean and standard deviation for the unlysed cell was 30.4 ± 6.9 tubules. This factor

FIGURE 1 (*a*) Ptk_1 metaphase spindle fixed in vivo. The prominent polar regions each contain a pair of centrioles. The chromosomes in the center of the spindle show sister kinetochores where MTs terminate. × 9,700. Bar is 1 μm. Inserts are polarization optical light micrographs of a living cell similar to the cell shown in thin section. × 440. (*b*) PtK_1 metaphase cell lysed in 7 mg/ml tubulin warmed for 30 s at 37°C before lysis and fixed 15 min after lysis. These lysis conditions result in a threefold increase in tubule number compared to the spindle in (*a*). The chromosomes appear more condensed and the kinetochores remain intact. Clear zones in the chromosome structure are formed by MTs. The cytoplasm is well extracted, only membranous fragments remaining. × 9,700. Inserts are polarization optical light micrographs of a cell lysed under comparable conditions to that shown in thin section. × 440.

of about 1.72 in one dimension of the spindle cross section suggests that the spindles differ by the square of this ratio, i.e. by about 3 for the total number of tubules per spindle cross section. Note, however, that the distance between the poles in these cells does not change much under the conditions that promote an increase in tubule number, and the spindle retains the general pattern and appearance of the spindle in vivo.

Fig. 2 *a–d* demonstrates the relationship between the rate and extent of in vitro polymerization of the tubulin used in these experiments. Light scattering has been shown to be a reliable method to monitor MT polymerization (11). Fig. 2 *a* shows the development of light scattering when varying concentrations of tubulin were warmed from 0° to 37°C and monitored for 24 min. The higher the tubulin concentration, the greater the rate and extent of polymerization as measured by light scattering. Fig. 2 *b* shows that the greater the initial concentration of unassembled tubulin, the greater the rate and extent of BR increase in metaphase spindles. Fig. 2 *c* is a different expression of light-scattering data, plotting the rate of increase in scattering and the maximum scattering obtained vs. the concentration of total protein.

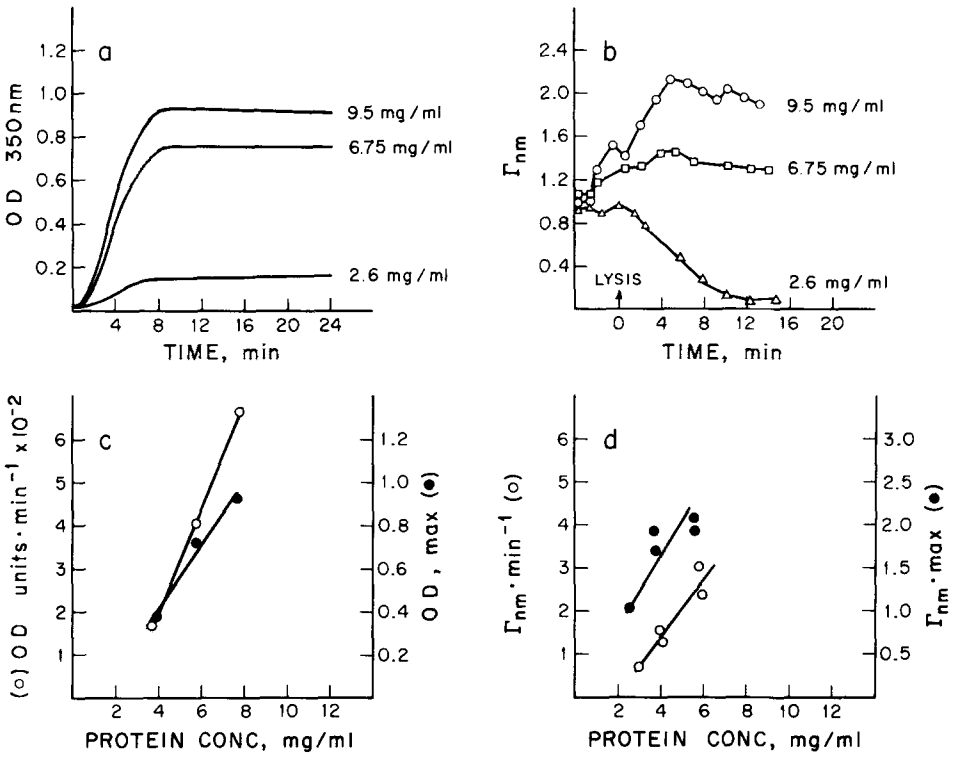


FIGURE 2 (*a*) Development of light scattering of tubulin solutions after warming from 0°C to 37°C. The greater the concentration of protein, the greater the rate and extent of light scattering development. (*b*) Comparison of changes in BR of metaphase cells lysed in varying concentrations of cycle-II tubulin brought to 37°C for 30 s before lysis. The rate of BR increase as well as the maximum levels achieved are dependent on total protein concentration. Cells lysed in low concentrations of tubulin may show an initial increase in BR followed by a decrease that coincides with the time that the tubulin solution reaches equilibrium. (*c*) Relationships between protein concentration and rate of development of light scattering (○) and maximum levels achieved (●) measured in experiments similar to that performed in Fig. 2 *a*. Both show an apparent linear relationship, the greater the protein concentration the greater the rate of light scattering development and maximum levels achieved. (*d*) Relationship of protein concentration used in lysis solution on the net rate of BR increase (○) and maximum values achieved (●) in metaphase spindles. Data is from a series of experiments performed in a fashion similar to that seen in Fig. 2 *b*. The rate and extent of BR increase in metaphase cells is to a large extent proportional to the protein concentration in the lysis medium.

Both the rate and the extent of polymerization are approximately proportional to the protein concentration. Fig. 2 *d* shows a similar expression of BR data. Here the case is less clear, probably as a result of the difficulty in obtaining measurements and of the natural variability of the cells. Nonetheless, the higher the concentration of tubulin in the lysis mixture, the greater the rate and net gain in spindle BR. We have noted, however, that as tubulin concentration in the lysis medium exceeds 8–10 mg/ml, the increase in BR and tubule number in metaphase spindles does not increase in proportion to tubulin concentration. Tubulin polymerization measured by development of light scattering is so rapid at high tubulin concentrations that the subunit pool is depleted almost to its equilibrium concentration within 4 min. We presume that by the time the lysis mixture enters the cell, the disassembled tubulin concentration is consequently reduced and its ability to augment spindle BR and tubule number has declined.

Table I shows the resulting change in tubule number and BR when different metaphase spindles are lysed in varying concentrations of unassembled tubulin. At tubulin concentrations of 3.0 mg/ml warmed at 37°C for 5 min before lysis, the BR and maximum tubule number are similar to those of a normal metaphase cell fixed in vivo. Lysis of spindles into tubulin solutions above this concentration results in a net gain in BR and tubule number, although the two are not related in a strictly linear fashion. Concentrations of tubulin below 3.0 mg/ml warmed for 5 min will not preserve spindle integrity—a loss of BR is coupled with a decrease in tubule number. Loss of spindle BR and tubule number in some cells can also occur from lysis of spindles in tubulin solutions that have already come to equilibrium. Several factors could account for this, for instance hydrolytic enzyme activity in the lysed cell or an inability of the tubulin solution truly to equilibrate across what is left of the cell cortex.

A different quantitative comparison of spindle structure for lysed and unlysed cells can be obtained by counting the number of tubules in serial cross sections and displaying these data with reference to position on the spindle axis (6, 23). Fig. 3 shows such a MT distribution for a normal metaphase spindle, a metaphase spindle lysed in a high concentration of tubulin, and another lysed in a concentration of tubulin below the equilibrium value. The normal microtubule distribution of a cell fixed in vivo shows an increase in tubule number proceeding from the centriole toward the metaphase plate. The drop in tubule number at the metaphase plate is in part the result of microtubules ending on kinetochores (kMT). The bottom profile represents a spindle lysed in concentrations of tubulin that will not maintain spindle structure. This results in a decrease in total tubule number, especially in the region of the metaphase plate. The number of MTs associated with the centriolar region is one-third that of a normal metaphase spindle, while the number at the plate has dropped to one-sixth its normal value.

TABLE I

Effect of Unpolymerized Tubulin Concentration on Spindle Tubule Number and Birefringence

BR_{onm}	BR_{fnm}	Max. tubule no.	Tubulin concn.
			mg/ml
0.50	2.65	6,930	8
0.52	0.48	1,885	3.0
0.51	0.15	607	0.75

Birefringence was measured on metaphase spindles before lysis, and again 20 min after lysis in various tubulin concentrations shown. The same metaphase cell in each case was prepared for electron microscopy, and the maximum number of tubules counted from serial cross sections.

Tracking tubules through serial sections of this cell shows that most of the kinetochore fibers are retained; most of the tubule loss is in the nonkinetochore class of MTs. The retention of kMT and not nonkinetochore microtubules (nkMT) increases the relative depth of the well in the region of the metaphase plate. This is in agreement with previous observations which suggest that nkMT are more labile that kMT (4, 7).

The MT profile of metaphase spindles lysed in high concentrations of tubulin subunits shows an overall increase in MT number (25). The regions of the spindle that show the greatest percentage increase are the areas around the centrioles and the metaphase plate. There is a fivefold increase in tubule number in the area immediately adjacent to the centriole. Tracking of kMT bundles through EM serial sections of a spindle lysed in high concentrations of tubulin shows that the number of tubules ending on kinetochores remains similar to that found in vivo at 30 ± 5. Thus, the kMT number has stayed relatively constant, whereas the total tubule number has increased by as much as five times. We conclude that the increase in spindle

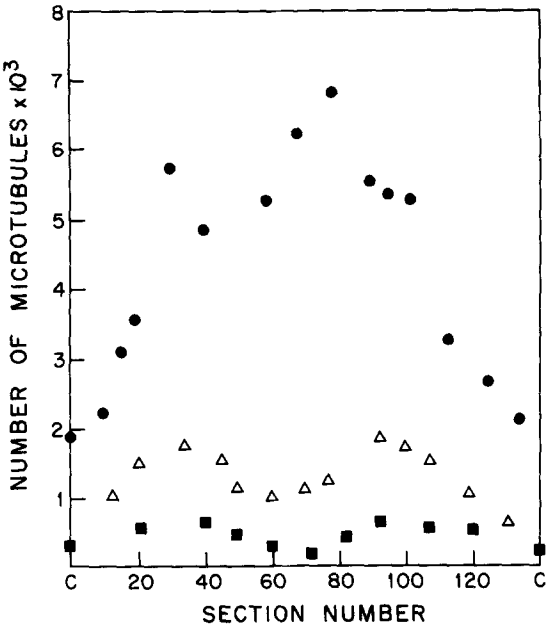


FIGURE 3 Microtubule number vs. position along the spindle axis measured in numbers of sections. Each section is about 90-nm thick. C represents the position of the centrioles. MT profile of a metaphase cell fixed in vivo (Δ) shows an increase in MT number from the centriole towards the body of the spindle. The drop in tubule number at the metaphase plate is in part due to kMTs terminating. (●) is an MT profile of a metaphase cell lysed in 8 mg/ml tubulin brought to 37°C for 30 s before lysis and fixed after 15 min. There is approximately a fivefold increase in tubule number thoughout the domain of the spindle with the largest percentage increase occurring in the regions of the centrioles and metaphase plate (■) is a profile of a metaphase spindle lysed in 1.0 mg/ml tubulin brought to 37°C for 1 min before lysis and fixed after 15 min. There is a net loss of tubule number, especially in the region of the metaphase plate.

number is accounted for almost exclusively by an increase in nkMTs, even in the region of the metaphase plate, although the possibility that the normally heterodisperse lengths of kMTs is increased to form a more homogenous length distribution cannot be ruled out.

It is possible that the number of kMTs does not increase during lysis of spindles in high concentrations of tubulin because the tubule sites at the kinetochore are both limited in number and saturated in a normal metaphase cell. We therefore tried to remove some kinetochore fibers and then subject the spindle to conditions in which MT number would be greatly augmented. Cells in metaphase were chilled for 6–8 min at 0°C on a microscope stage until BR levels as measured by polarization optics were reduced to levels below 0.20 nm. This treatment gave almost complete depolymerization of nkMT and a loss of approximately 50% of the kMTs as measured by tracking of MTs in cold-treated unlysed cells (data not shown). Cells were then lysed in tubulin at 0°C and warmed to 37°C on the microscope stage. After 10 min at 37°C there was a two- to threefold increase in BR over the initial in vivo level; however, increase in BR could not be attributed to nkMTs. Fig. 4 shows six electron micrographs of serial cross-sections from a cell treated in this fashion containing a kinetochore bundle as it approaches and then terminates at a kinetochore. The number of tubules that ends on this kinetochore is about 16 (there are two tubules which are difficult to track with confidence), about 15 less than usually found in vivo.

The substantial increase in spindle MT number resulting from lysis of metaphase cells in high concentrations of tubule subunits may derive both from initiation of new tubules and from elongation of existing ones. Elongation of MT fragments known to be present in the spindle (10, 13, 24, and unpublished results) would clearly increase the number of tubules seen at any one spindle cross section. However, it would seem difficult to account for a fivefold increase in tubule number, especially in the astral region outside the main body of the spindle, if addition of tubulin onto pre-exising fragments were the only mechanism involved in tubule number increase.

It therefore seems likely that some of the increased numbers of tubules come from tubules initiated during or after lysis. We believe that few, if any, of these new tubules form as a result of spontaneous nucleation of tubules similar to that occurring in vitro. The region inside the remainder of the cell cortex after lysis, but outside the spindle, is devoid of tubules, but many can be seen outside the remaining cell cortex. We tentatively infer that spontaneous nucleation occurs only rarely within the lysed cells and that new spindle-associated tubules are initiated by the remnants of the physiological MTOCs.

To obtain direct evidence on whether mitotic centers can nucleate tubules after lysis, we have investigated the effect of exogenous tubulin on the centriolar and kinetochore regions of lysed prometaphase cells that are devoid of MT fragments. We have taken advantage of the potent MT inhibitor,

colcemid, to treat prophase cells in a way that prevents the formation of the spindle and disassembles all tubule fragments observable with the electron microscope. In the next section, we describe assembly of tubules after lysis of colcemid-treated cells.

Prometaphase

Prometaphase cells were chosen to examine the role of the centriolar region and the kinetochores in MT assembly. Cells at this point in the normal mitotic cycle show formation of MTs at the centriolar regions and the first association of MTs at kinetochores. Cells entering mitosis in the presence of colcemid do not form MTs, although chromosome condensation and nuclear-envelope breakdown proceed in the presence of the drug. There is substantial literature on the effects of colcemid in blocking mitosis and altering fine structure (4, 5).

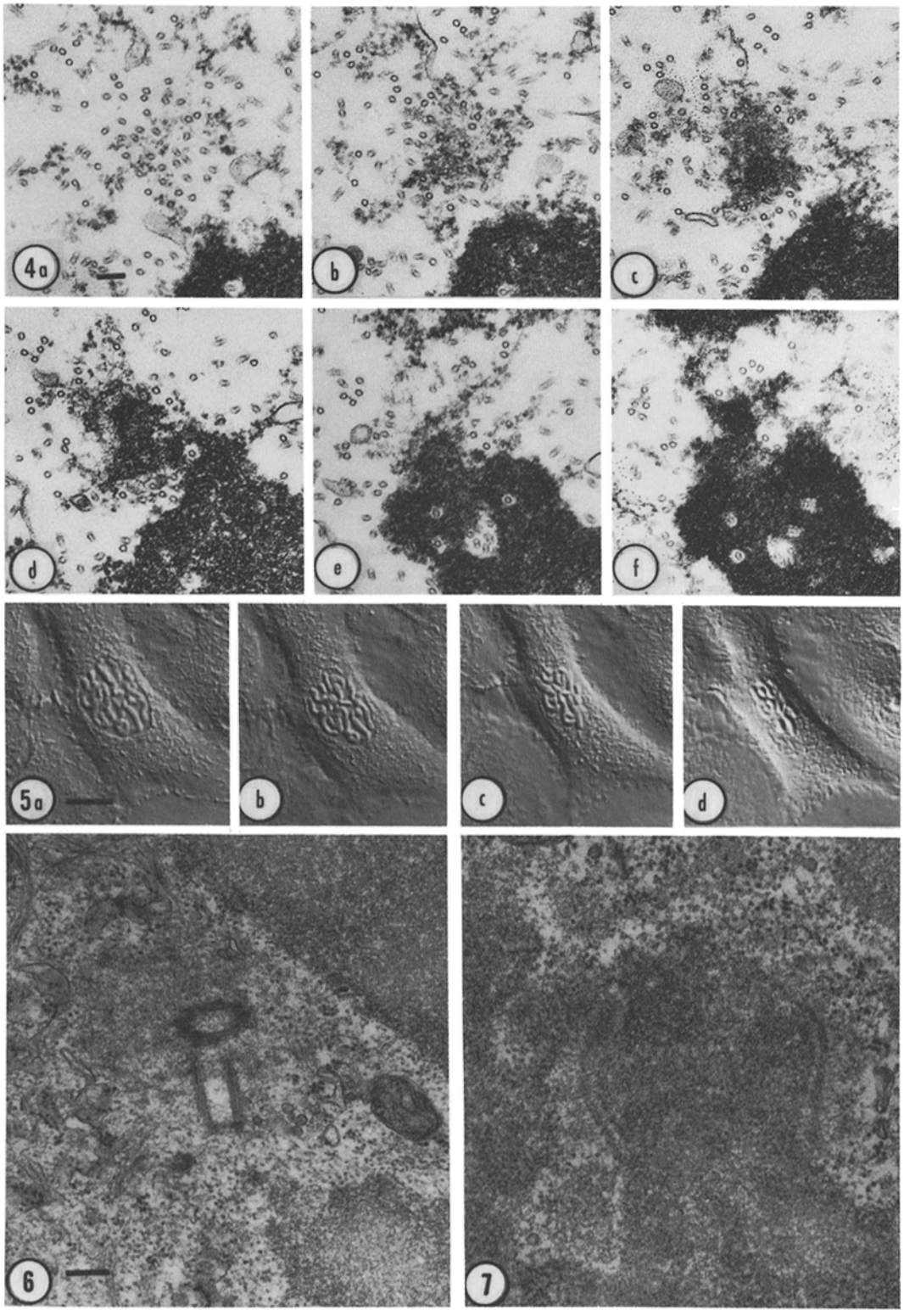
Characterization of the Colcemid Block

Fig. 5 *a–d* shows a series of differential interference contrast images of a cell going from prophase through prometaphase in the presence of 1×10^{-5} M colcemid. Chromosome condensation is not inhibited, and chromosome position can be seen to change before nuclear envelope breakdown (Fig. 5 *b*). After nuclear-envelope breakdown and in the absence of spindle formation, the chromosomes coalesce to the center of the cell (Fig. 5 *c*, *d*). Chromosome condensation, prophase chromosome motions, nuclear-envelope breakdown, and rounding-up of the cell occur normally in the presence of colcemid. It appears that only the formation of MTs is inhibited. Fig. 6 is an electron micrograph of the centriolar region of a colcemid-blocked cell 5 min after nuclear-envelope breakdown. There are no apparent tubules or tubule fragments, although a dense felt-work of loosely organized electron-dense material is seen on the periphery of the centrioles. In the presence of this concentration of colcemid, pairs of centrioles do not separate to their normal bipolar position. Fig. 7 shows a kinetochore region in the same cell, with sister kinetochores at opposite sides of the chromosome. The outer layers are associated with a dense matrix extending outward from the chromosome, similar to that found at normal prometaphase (32). The middle layer of the trilamellar kinetochore structure in other colcemid-treated chromosomes has been noted in the literature (4, 5, 32), but since the kinetochore is not completely differentiated in PtK_1 by late prometaphase, we cannot be sure if the absence of the middle layer is the result of colcemid or incomplete differentiation.

Close examination of the outer layer of the kinetochore shows a hint of what may be tubule fragments, though their structures do not appear complete. We have included this micrograph to demonstrate that even cells blocked by a high concentration of colcemid may show incomplete depolymerization of tubule-like structures at the kinetochore. In several other colcemid-treated cells examined at an earlier stage of prometaphase and showing less well differentiated kinetochore structure, we have not observed any structure that could be construed as tubules.

Colcemid-blocked prophase cells were individually followed with the light microscope through nuclear envelope breakdown; 5 min thereafter they were lysed into buffers containing polymerization-competent tubulin. Before lysis, there is no evidence of astral BR (Fig. 8 *a*), and the differential interference contrast image (Fig. 8 *b*) shows the typical arrangement of chromosomes in a colcemid-blocked mitotic cell after nuclear envelope breakdown. After lysis in high concentrations of unpolymerized tubulin, asters form, and the BR increases for up to 10 min, at which time it seems to achieve a plateau. Fig. 8 *c* shows the polarization optical image of the asters 15 min after lysis. The apparent radial symmetry of MT density around the centriole gives both positive and negative contrast when a few nanometers of compensation are added. The differential interference contrast image in Fig. 8 *d* shows the effects of lysis on the visibility of the chromosomes and the pairs of mitotic centers after extraction of the cytoplasm. Colcemid-treated prometaphase cells lysed in assembly buffer without tubulin do not show any tubules when examined with electron microscopy.

As the BR of the asters increases after lysis, there is no evidence for an increased separation of the centrioles. In an effort to separate the mitotic centers by tubule growth, asters formed by lysis of colcemid-blocked, prometaphase cells in high concentrations of tubulin have been augmented further by the addition of more unpolymerized tubulin. In this case, there is again no evidence for mitotic-center separation as BR increases. Fig. 9 is an electron micrograph of the asters from the same cell shown in Fig. 8 *a–d*. MTs can be seen radiating from the centriolar region. One centriole was cross-sectioned, and the triplets forming the blades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can be seen. The other centriole was sectioned longitudinally, thus showing the length of the centriole. Tubules are in close association with the centrioles, even inside them, but they do not appear to emanate directly from the structure. There is no specific MT orientation to suggest that the centriole is directly involved as a nucleator. The centriole in longitudinal section shows some electron-dense material present in its immediate vicinity, even though the cytoplasm is well extracted by the detergent treatment.

Growth of MTs at Kinetochores

Structural differentiation of the kinetochores is not inhibited by the presence of colcemid, even though the structure is partially extracted by colcemid treatment at stages later than prometaphase (32). Lysis of a colcemid-blocked cell in early prometaphase in high concentrations of tubulin results in a few tubule-like structures at the kinetochore (Fig. 10 *a*). Short tubule fragments less than 0.05 μm long are found oriented perpendicularly to the main axis of the kinetochore structure, as seen in Fig. 10 *a*, *b*. Lysis of a late prometaphase or early metaphase cell (Fig. 10 *b*) in high concentrations of tubulin shows an increase in the number and length of tubules associated with the kinetochore. The tubules appear to be in direct contact with the kinetochore. Since no tubules of any kind have been seen to grow on chromosomes of lysed prophase cells, the differentiation of the kinetochore may control to some extent the apparent ability of the chromosomes to initiate tubules or tubule fragments after the prophase to prometaphase transition. In no case, however, is the length of tubules associated with kinetochores in our material comparable to the length of those present in the centriolar region under the same lysis conditions.

Ability of the Centrioles to Assemble Initiation-Incompetent Tubulin

We have demonstrated that the centriolar region, and to a smaller extent the kinetochore, can initiate the assembly of tubulin that can spontaneously initiate its own assembly in vitro. Allen and Borisy (1) have shown that fragments of doublet MTs from flagella can serve as seeds to nucleate the in vitro assembly of tubulin that will not spontaneously initiate polymerization. Following their methods, we have tried to determine whether the mitotic centers can serve a similar nucleating function in lysed cells.

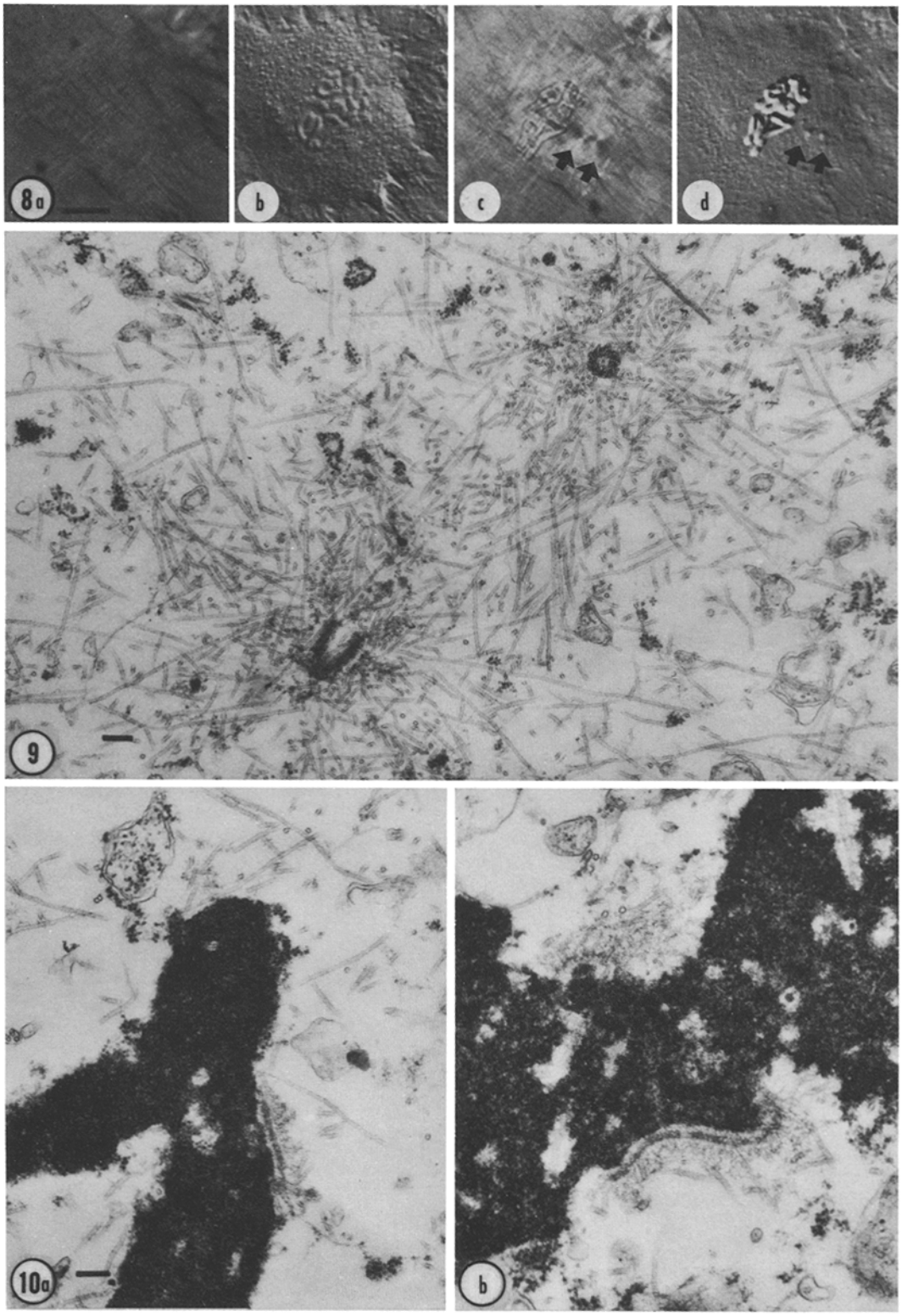
Tubulin capable of polymerization, but not of spontaneous nucleation, was prepared by centrifugation at such high speeds that all presumptive nucleating structures were pelleted and polymerization-competent, initiation-incompetent tubulin remained in the supernate (2). Tubulin prepared in this fashion eventually polymerize after a lag period that is largely dependent on tubulin concentration. Colcemid-blocked prometaphase cells were lysed in tubulin from a high-speed supernate which shows a minimum lag period of 15 min in light-scattering development after warming. Development of light scattering was due to tubule polymerization and not to the formation of nontubule aggregates. Within 3–5 min after lysis at

FIGURE 4 Serial cross-sections of a bundle of MTs that end on a kinetochore. A metaphase cell was chilled to 0°C for 6 min in vivo, then lysed into 5 mg/ml tubulin at 0°C, and warmed on a microscope stage for 15 min before fixation. Cross-sections revealed a net gain in total spindle MTs. However, only 16 MTs appear to end on this kinetochore, compared with 30 ± 5 found in vivo. The kinetochore is distinguished from chromatin by a more diffuse appearance (*b*, *c*,). Bar is 0.1 μm. × 38,500.

FIGURE 5 Differential interface contrast images of a prophase cell (*a*, *b*) passing through prometaphase (*c*, *d*) in the presence of 1×10^{-5} M colcemid. Colcemid does not seem to affect chromosome condensation (*a*), nuclear envelope breakdown (*c*), or rounding up of the cell (*d*). In the absence of spindle formation, chromosomes coalesce to center of cell (*d*). Bar is 10 μm. × 680.

FIGURE 6 Centriolar region of a prometaphase cell incubated in 1×10^{-5} M colcemid for 25 min before fixation. There is a dense felt-work of material in the region immediately surrounding the pair of centrioles and no tubule-like structures can be identified. Bar is 0.2 μm. × 32,400.

FIGURE 7 Sister kinetochores from the same cell as in Fig. 6. There are structures which could be construed as tubule fragments in the region immediately adjacent to the outer layer of the kinetochore. × 37,400.


8a
b
c
d
9
10a
b

37°C, faint, radially symmetrical patterns of BR could be seen with polarization optics. After 10 min, a definite aster could be identified. When the preparation was fixed and examined by electron microscopy, the centriolar regions were shown to have MTs associated with them (Fig. 11). The arrangement of MTs here is similar to that seen in cells lysed with initiation-competent tubulin. Since the tubulin used in the lysis medium showed no polymerization as measured by light scattering for at least 15 min, initiation-incompetent tubulin will form MTs in the presence of the centriolar region in lysed cell preparations. It seems highly probable that the centriolar region is acting as an MTOC to initiate MT formation in vitro.

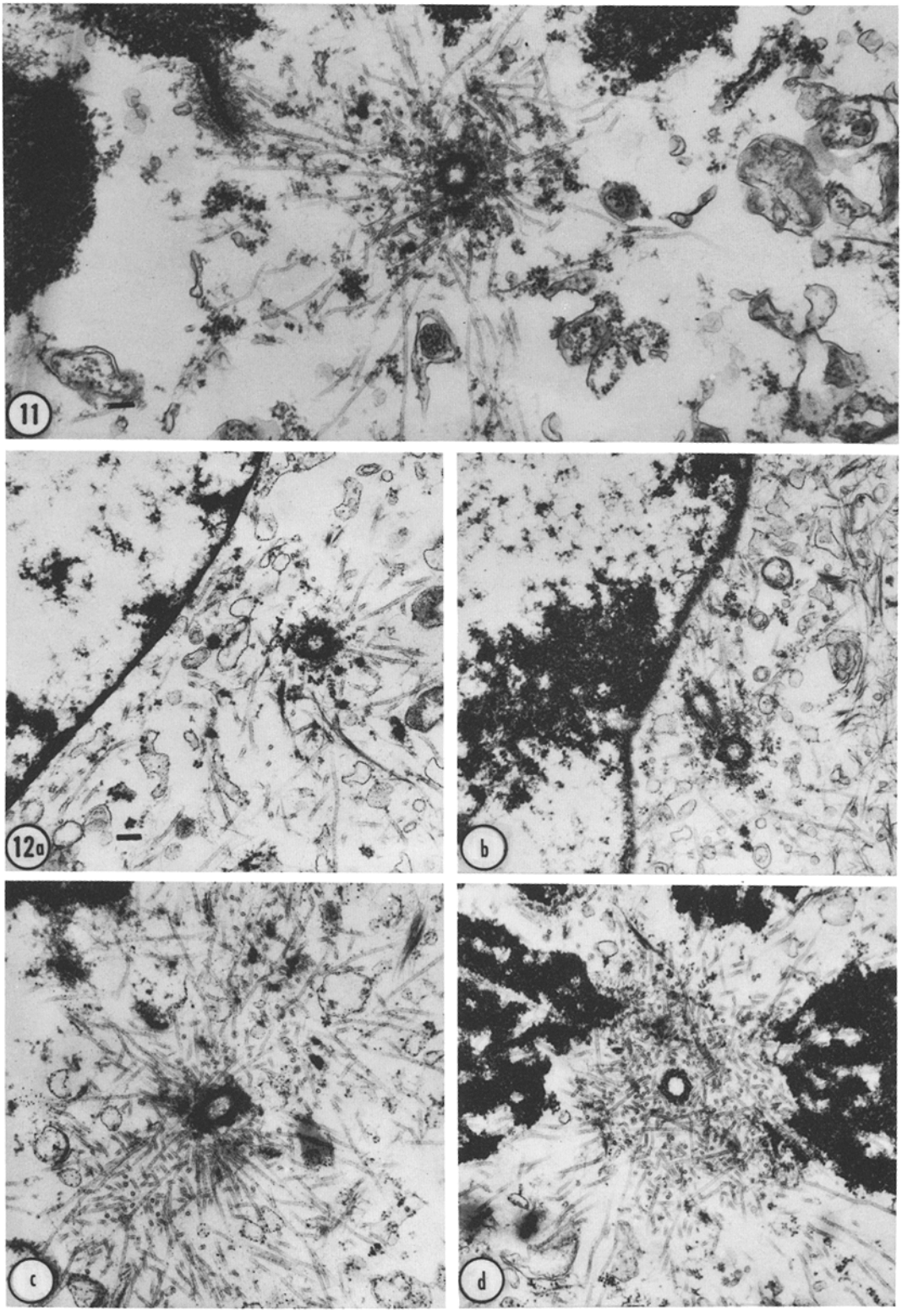
Time-Dependent Maturation of the MT-Initiation Capacity of the Mitotic Center

The number of tubules forming at the centriolar region of a lysed, colcemid-treated cell is dependent on the concentration of exogenous tubulin subunits, as one would expect, but a comparison of cells lysed under similar conditions show that factors within the lysed cell as well can affect the ability of the centriolar region to initiate MTs. Fig. 12 *a–d* is a series of electron micrographs showing MT assembly at centriolar regions, ranging from interphase (Fig. 12 *a*) to late prometaphase (Fig. 12 *d*). Fig. 12 *a*, *c* is from the same cover slip where lysis was done in 6.5 mg/ml tubulin. Fig. 12 *b*, *d* is from a cover slip lysed in 7.0 mg/ml of tubulin. The centriolar region of interphase cells can apparently nucleate a small number of MTs, and there are few MTs associated with the centriole at this stage in vivo. The nuclear envelope remains intact under these lysis conditions. Fig. 12 *b* shows a cell in early prophase where chromosome condensation is incomplete and the nuclear envelope is still present. Here the number of MTs is similar to that of the interphase cell. Fig. 12 *c* is a centriolar region from a cell at the stage of nuclear-envelope breakdown. The envelope is intact except at the region of the aster, where nuclear-envelope breakdown begins in vivo (31). The number of tubules associated with the centriole is greater than that of interphase or early prophase. In late prometaphase, when the nuclear envelope is completely absent, there is a further increase in the number of tubules which will form in the centriolar region (Fig. 12 *d*). This series of electron micrographs shows that the ability of the centriolar region to initiate MTs increases from early prophase through late prometaphase. We have also observed mitotic-center maturation in cells lysed in initiation-incompetent tubulin.

FIGURE 8 (*a*) Polarization optical images of a prometaphase cell incubated in 1×10^{-6} M colcemid for 25 min. There is no prominent pattern of BR. (*b*) Differential interference contrast image of the same cell in (*a*) taken 23 min after the addition of colcemid. The nuclear envelope has broken down, the cell is rounded up and the chromosomes are beginning to coalesce toward the center of the cell. (*c*) Polarization optical image of the same cell after lysis in 8 mg/ml tubulin brought to 37°C for 30 s before lysis after 27 min in colcemid and warmed to 37°C for 15 min. The arrows show the BR of two asters that have formed as a result of this treatment. (*d*) Differential interference contrast image of the same cell taken 15 min after lysis. Arrows mark the position of the two mitotic centers. Bar is 10 μm. All figures × 680.

FIGURE 9 Pair of centrioles from the same cell shown in Fig. 8, fixed 20 min after lysis. There is a large number of MTs associated with each centriolar region. Lower left centriole is seen in longitudinal section and upper right in cross section. The cell contents are well extracted under these lysis conditions. Bar is 0.2 μm. × 22,500.

FIGURE 10 (*a*) Kinetochore region of a chromosome from an early prometaphase cell treated for 20 min in colcemid and lysed into 6.5 mg/ml tubulin brought to 37°C for 30 s before lysis and fixed after 15 min. The outer layer of the kinetochore region is clearly visible and short tubule fragments are closely associated with it. Other tubules in the micrograph are associated with the centriolar region when tracked through serial sections. (*b*) Kinetochore region of a chromosome from a late prometaphase cell, treated for 25 min before lysis with 1×10^{-6} M colcemid, and lysed in 7 mg/ml tubulin brought to 37°C for 30 s before lysis. The kinetochore is more completely differentiated than in (*a*); the outer layer is very prominent in the lower kinetochore. The number and length of tubules in this region are greater than those seen in earlier stages of prometaphase under similar lysis conditions (*a*). Bar is 0.2 μm. × 25,400.


11
12a
b
c
d

DISCUSSION

We have demonstrated that the centriolar region and, to a lesser extent, the kinetochores, are competent to initiate the assembly of MTs in lysed cell preparations of mitotic mammalian cells when provided with high concentrations of exogenous tubulin. Treatment of cells with relatively high concentrations of colcemid before the onset of spindle formation seems to preclude the possibility that tubule fragments serve as seeds for MT assembly observed in the centriolar region. Addition of tubulin from high speed supernates, which will add on to tubule fragments but which cannot initiate MT assembly in vitro without an extended lag period, to lysed, colcemid-treated prometaphase cells also results in MT formation in the centriolar region. Thus, the mitotic center can initiate MT assembly in the absence of tubule fragments or normal in vitro initiators. This is the first direct indication that the MT-organizing capacity of a mitotic center is due to its ability to initiate tubule growth.

The time-dependent maturation of the mitotic centers during prophase-prometaphase focuses our attention on the question of what a cell does to its centriolar regions to initiate assembly of preformed tubulin in the right place at the right time. Kirschner (18) has presented evidence for the existence of two states of the 6s tubulin dimer, one called Y tubulin, which aggregates readily into a colchicine-insensitive 36s tubulin ring, a proposed intermediate in tubulin assembly, and another called X tubulin, which will not form this intermediate, but which can be incorporated into a tubule as it is formed. Tubulin that aggregates into the 36s form has been shown to be phosphorylated, while the nonaggregative form is not (41). More recently, Weingarten et al. (42) has isolated a protein that co-purifies with tubulin during assembly-disassembly. This factor, designated tau, is necessary for tubulin polymerization. A high molecular weight component has also been shown to co-isolate with tubulin and may also serve to control polymerization of tubulin (26). In this context, we can envision two satisfactory models for the maturation of the mitotic center: the centers serve as a locus for the aggregation during prophase-prometaphase of some colchicine-insensitive nontubulin molecule that is important for tubule assembly. Alternatively, the centers are the site of colchicine-insensitive oligomerization of tubulin; these oligomers serve as initiators for tubule growth after lysis, taking the place of the oligomers which were spun out of the tubulin preparation by the high speed centrifugation. These two possibilities are not mutually exclusive, since a nontubulin molecule could be important for polymerization, perhaps through the conversion of X tubulin to Y rather than some later step in the assembly pathway.

The concentration of unassembled tubulin in the lysis medium controls to a large extent the BR and tubule number that will develop at 37°C in a metaphase spindle. Depletion of the exogenous, subunit pool size takes place during the first minutes of polymerization at 37°C. Likewise, the

FIGURE 11 Centriolar region from a late prometaphase cell incubated in 1×10^{-6} M colcemid for 30 min and lysed in 1.4 mg/ml tubulin and fixed after 8 min at 37°C. The tubulin was prepared by high speed centrifugation and only showed the light scattering development after a 15 min lag period at 37°C. The arrangement of the tubules is similar to that found in similarly prepared cells lysed in initiation-competent tubulin. Bar is 0.2 μm. × 24,600.

FIGURE 12 *a–d* Centriolar region of colcemid incubated cells lysed at different stages of the cell cycle. The cells have been lysed in similar concentrations of initiation-competent tubulin ranging from 6.5 to 7.0 mg/ml. (*a*) Centriolar region of a cell lysed in interphase and fixed after 15 min at 37°C showing a small number of microtubules in the region of the centriole. (*b*) Centriolar region of a cell lysed in prophase. The chromatin is incompletely condensed and the nuclear envelope is intact. The number of tubules associated with the centriole is similar to that found in lysed interphase cells. (*c*) Centriolar region of a prometaphase cell lysed at the beginning of nuclear breakdown. The nuclear envelope remains intact except in the region of aster formation. The chromatin is more fully condensed and the number of tubules associated with the centriolar region is greater than that found in prophase cells. (*d*) Centriolar region of a late prometaphase cell lysed 7 min after nuclear envelope breakdown. The chromosomes are condensed and have coalesced toward the center of the cell. The number of tubules found in the centriolar region exceeds that seen in (*a*)-(*c*). Bar is 0.2 μm. All figures × 20,500.

gain in spindle BR and presumably spindle number occurs rapidly, usually reaching a maximum within 10 min after lysis. As the tubulin in the lysis mixture comes to equilibrium, the BR of the spindles also achieves a plateau. Lysis of metaphase cells with solutions of tubulin that have previously come to equilibrium will sometimes maintain spindle BR. This implies that, under our conditions, the tubules initiated by MTOCs have a similar equilibrium constant to the neurotubules initiated in vitro. There are instances where BR does fade slightly when cells are lysed with equilibrium tubulin solutions, and this may mean a slight difference in equilibrium monomer concentrations. However, in our lysed cell system it is impossible to know the precise concentration of molecules such as tubulin, GTP, and divalent cations in the spindle. To make an accurate comparison of the spindle tubule equilibrium with that formed by in vitro tubulin polymerization, a completely isolated spindle will be required.

It is not possible at present to account for the different tubule-organizing capabilities of the kinetochore and the centriolar region in lysed cells. The length of tubules that grow at kinetochores is substantially less than those that form at the mitotic centers when they are fully competent to initiate tubule formation. It may be that conditions for assembly are more stringent at the kinetochore than in the region of the centriole. The apparent lack of the complete kinetochore structure after colcemid treatment may suggest that the extracted substance is necessary for MT initiation. The greater stability of kMTs in metaphase spindles suggests other possibilities to account for the small MT growth at kinetochores. Differential stability could indicate a different tubulin which assembles to a different equilibrium or with different on and off rate constants. Further, a different type of tubule initiator could affect the stability of kMTs by altering the tubule surface lattice, or by requiring subunit addition to occur proximally, not distally, to the kinetochore.

Our inability to show tubule growth at kinetochores in metaphase spindles may be due to a time-dependent loss of initiation capacity which follows kinetochore maturation at late prometaphase. The cold treatment of metaphase spindles used to extract some kMTs may prevent the MTOC at the kinetochore from assembling tubules in a lysed system. Similar results have been noted by Weisenberg and Rosenfeld (46), who have shown that *Spisula* spindles homogenized in warm buffer show complete spindles with kMTs, but when homogenized in cold buffer and rewarmed show no kMTs—only asters are present. The images we have seen make it doubtful that kMTs arise by astral tubules terminating at kinetochores. A cold-depressed metaphase spindle lysed in high concentrations of tubulin and then rewarmed shows a several-fold increase in nkMTs; this should increase the probability of nkMTs terminating on kinetochores, thus making them kMTs. However, we have not noted this phenomenon in our system.

Further investigation of the conditions affecting tubule initiation at nucleating sites will require a more refined system. Complete isolation of mitotic centers would permit a characterization of the biochemical properties that allow a mature mitotic center to initiate growth of tubules from initiation-incompetent tubulin. Work toward this end is currently in progress in our laboratory

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